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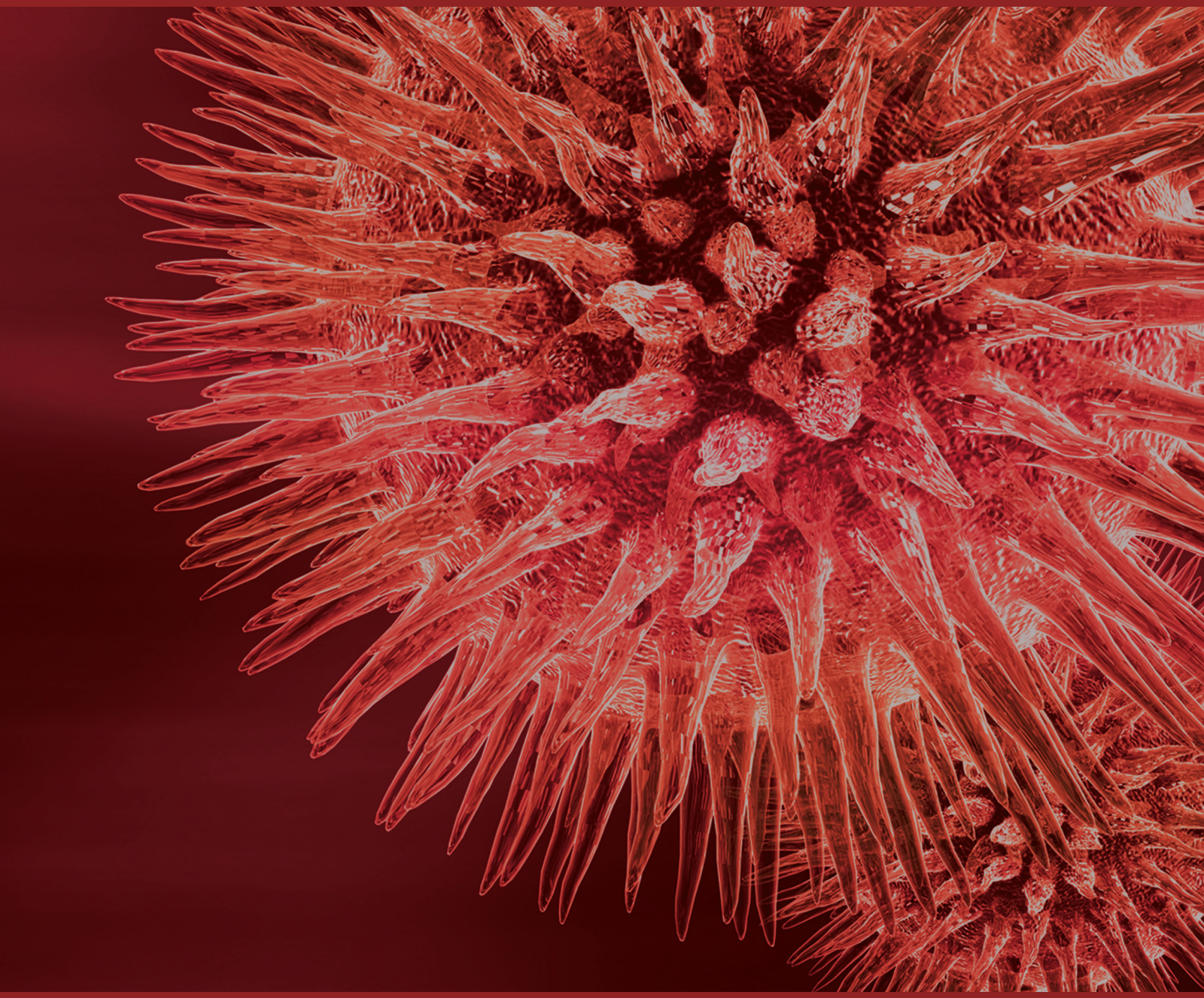
Guest Editors: Luis Sobrevia, Leslie Myatt, and Gregory Rice



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Editorial

Diseases of Pregnancy and Fetal Programming: Cell and Molecular Mechanisms

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A well-documented and accepted hypothesis of the origins of diseases that occur with a high frequency in adulthood is the causal relationship between an adverse intrauterine environment during fetal life and development of diseases including hypertension, diabetes, obesity, insulin resistance, and metabolic syndrome [1, 2]. Epidemiological studies provided the impetus for experimental studies to identify cell and molecular mechanisms that may underpin this relationship. The precise mechanisms, however, remain only partially described. It is anticipated that on-going studies will provide data that more rigorously test this hypothesis and provide insights into gestational age-dependent programming effects and the consequences of alterations in intrauterine life on risk profiles for adult diseases. To date, few studies have addressed whether the preconception period is also involved in this phenomenon [3]. Thus, it is clear that on-going investigative effort in this field for a better understanding of the cellular and molecular mechanisms underlying fetal programming of adult diseases is required. This special issue includes contributions over a wide area of expertise but particularly studies concerning the vascular physiology/pathophysiology of diseases of pregnancy that could result in programming of the fetus leading to adult diseases.

The articles included in the present issue include both clinical and basic science studies. The alterations caused by exposure to elevated D-glucose environment are discussed in chapters by J. B. Moreli et al. and D. C. Damasceno et al. These articles reported that hyperglycaemia causes damage to the maternal genetic material without a clear consensus regarding the impact of this adverse environmental condition on fetal cells. Since DNA repair mechanisms may be important to prevent the deleterious effects of hyperglycaemia in both the maternal and fetal DNA, it may be preventive of the development of diseases in adulthood. Studies in animal models support the targeting of the development of new therapeutics that minimise or prevent diabetes-induced DNA damage due to increased oxidative stress.

It is now clearer that maternal obesity is also a condition that impacts on fetal growth and development [4]. The prevalence of obesity, especially in women of childbearing age, and a supraphysiological increase in gestational weight gain during pregnancy is associated with adulthood diseases. This is clearly summarized in the chapter by S. A. Segovia et al. In addition, these authors highlight the fact that the predisposition of offspring to obesity and metabolic and cardiovascular disorders in later life occurs via poorly described mechanisms including programming of metabolic disorders. In this review, the authors discussed the possibility that maternal obesity-related inflammation may program insulin sensitivity of tissues in offspring. Interestingly, in the

retrospective cohort study of 3290 children (age ~11.5 years) from Chile (South America), F. Mardones et al. identify an association between prenatal growth and nutritional status, metabolic syndrome, and insulin resistance. The authors concluded that intrauterine growth is a factor influencing the manifestation of pathologies such as metabolic syndrome and insulin resistance. This study complements the review by F. Westermeier et al. regarding the same concept; that is, maternal obesity and neonatal insulin resistance associate with long-term development of obesity, diabetes mellitus, and increased global cardiovascular risk in the offspring. The mechanisms at a cellular level, however, are not understood. The authors summarize evidence showing that insulin resistance in the offspring of pregnancies with maternal obesity and/or supraphysiological gestational weight gain (many of these patients ending with obesity) may result from intrauterine activation of endoplasmic reticulum stress response in human pregnancies.

Several articles report the potential effect of insulin as a key factor to sustain human endothelial function via its capacity to stimulate synthesis of nitric oxide and uptake and metabolism of amino acids and other substrates [5]. Moreover, the biological actions of insulin appear to be dependent on the activation of adenosine receptors expressed in the endothelial cells from the human placenta [5, 6]. Indeed, adenosine may be a determinant in pregnancy diseases associated with altered umbilical blood flow, such as preeclampsia [7, 8]. Adenosine also increases human umbilical vein endothelial cell (HUVEC) proliferation and migration in preeclampsia, as reported by J. Acurio et al. The proposed mechanisms include increased protein abundance of A_{2A} adenosine receptors (A_{2A}AR), but impaired nitric oxide, and vascular endothelial growth factor signalling pathway following activation of these receptors. The involvement of other types of adenosine receptors, however, cannot be ruled out in these studies, as stated by the authors in their discussion. Indeed, A_{2A}AR and A_{2B}AR in endothelial cells from fetoplacental circulation are proteins that could be involved in similar mechanisms such as modulation of membrane transporters of adenosine in HUVECs [9] and in human microvascular endothelial cells [10]. Indeed, involvement of A_1AR in endothelial function has been recently regarded as a potential receptor required for beneficial actions of insulin in restoring functional alterations of HUVEC in gestational diabetes (E. Guzmán-Gutiérrez, L. Sobrevia, *unpublished*). Other types of membrane receptors are also involved in placental dysfunction in preeclampsia. For example, in the review by F. A. Zúñiga et al., high levels of lectin-like oxidized low-density lipoprotein receptor-1 are observed in the human placenta from pregnancies complicated by preeclampsia. How the activation of this type of receptors associates with higher production of reactive oxygen species leading to decreased intracellular NO bioavailability is described. It is in fact a mechanism that could associate with other pathological conditions, such as hypertension, hyperlipidaemia, and diabetes. Preeclampsia is a disease described as "deep placentation disorders" in the review by J. A. Carvajal. Since docosahexaenoic acid supplementation during pregnancy appears to prevent deep placentation disorders, the author proposes that docosahexaenoic acid supplementation early in pregnancy may help to reduce the incidence of deep placentation disorders. Thus, this compound could be a strategy for primary prevention of preeclampsia. More studies are required to confirm or refute this proposal.

D. I. Chiarello et al. report the effects of hypoxia on placental function, in particular, lipid peroxidation state of syncytiotrophoblast plasma membranes. The authors conclude that increased membrane calcium content interacts with phospholipids leading to exposure of hydrocarbon chains of fatty acids to free radicals, where magnesium might play a protective role. These findings could be key for the understanding of the abnormal function of the human placenta in states of hypoxia such as preeclampsia. In another set of articles in this issue the effect of hypoxia on pregnancy is reviewed by L. Wang et al. and A.-C. Peyter et al. Interestingly, studying rats exposed to hypoxia, L. Wang et al. described maternal hypoxia increased collagen (I and III) expression in the left ventricle of adult offspring. This phenomenon, however, is seen only with exposure during a critical window (days 10th to 14th of gestation) of cardiovascular development that results in pathological cardiac remodelling in the adult rat offspring. Another study regarding hypoxia by A.-C. Peyter et al. reported that inhaled nitric oxide administered simultaneously to perinatal hypoxia in mice has beneficial effects on the adult pulmonary circulation. Since inhaled nitric oxide is the therapy of choice in neonates with pulmonary hypertension, this article shows an interesting set of results documenting that relaxation of adult mouse pulmonary arteries to acetylcholine is restored following inhaled nitric oxide.

This series of articles and reviews identifies putative mechanisms that may impact on intrauterine life and predispose the newborn to adulthood diseases such as obesity, diabetes, preeclampsia, and metabolic syndrome. Further insights in the abnormal function of fetal cells, including endothelium, placenta cells, and circulating blood cells, is required to understand and to correlate these specific mechanisms with adult diseases.

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Review Article

The Possible Role of Extravillous Trophoblast-Derived Exosomes on the Uterine Spiral Arterial Remodeling under Both Normal and Pathological Conditions

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A tenet of contemporary obstetrics is that events that compromise placentation increase the risk of complications of pregnancy and contribute to poor pregnancy outcome. In particular, conditions that affect the invasion of placental cells and remodeling of uterine spiral arteries compromise placental function and the subsequent development of the fetus. Extravillous trophoblast cells (EVTs) proliferate and migrate from the cytotrophoblast in the anchoring villi of the placenta and invade the maternal decidua and myometrium. These cells are localised with uterine uterine spiral arteries and are thought to induce vascular remodeling. A newly identified pathway by which EVTs may regulate vascular remodeling within the uterus is via the release of exosomes. Trophoblast cells release exosomes that mediate aspects of cell-to-cell communication. The aim of this brief commentary is to review the putative role of exosomes released from extravillous trophoblast cells in uterine spiral artery remodeling and, in particular, their role in the aetiology of preeclampsia. Placental exosomes may engage in local cell-to-cell communication between the cell constituents of the placenta and contiguous maternal tissues and/or distal interactions, involving the release of placental exosomes into biological fluids and their transport to a remote site of action.

1. Introduction

A successful outcome to pregnancy is critically dependent upon events that affect implantation and early development of the placenta [1]. After implantation, trophoblast cells (CTs) that arise from blastocyst proliferate and differentiate into syncytiotrophoblasts (STs) and EVTs [2]. During first trimester, the placenta develops under low oxygen tension (~3% O_2) that, in part, is maintained by intravascular EVTs occluding uterine spiral arteries and preventing maternal blood from perfusing the placenta intervillous space. Remodeling of the uterine spiral arteries (SpA) into low resistance, high capacity vessels begins as EVTs invade the decidua during first trimester [3]. When EVTs "plugs" are lost between 9 and 11 weeks of gestation, maternal blood flows through the modified vessels to deliver nutrients and oxygen to support fetal growth and development [4]. EVTs continue to invade into the myometrium and remodel the SpA until mid-second trimester [5–8]. While the mechanisms by which EVTs remodel SpA remain to be fully elucidated, available data are consistent with the hypothesis that EVTs directly interact with vascular smooth muscle cells of uterine spiral arteries and affect their loss.

Over the past five years, our understanding of how cells communicate with each other, in health and disease, has undergone a paradigm shift with the recognition of the role of exosomes in intercellular signalling [9, 10]. Exosomes are small (40–100 nm), very stable [11], and lipid bilayer nanovesicles that are formed by the inward budding of multivesicular bodies. Although we know little about the mechanism by which exosomal packaging occurs, they contain a diverse array of signalling molecules and are released from the parent cell following the exocytotic fusion of multivesicular bodies with the cell membrane [12]. In this brief commentary, we develop the working hypothesis that exosomal signalling plays a critical role in normal

placentation and that disruption of exosomal pathways (and in particular the release of exosomes from EVTs) plays a key role in the pathogenesis of complications of pregnancy, including preeclampsia.

2. EVTs and Uterine Spiral Artery Remodeling

Remodeling of uterine spiral arteries by EVTs is fundamental for effective placentation and perfusion of the intervillous space. Approximately 100–150 uterine spiral arteries are transformed during placental development [13]. The main role of these vessels is to transport maternal blood to the placenta to support the growth and development of the fetus. This is achieved by converting arteries from high resistance low flow to high flow low resistance arteries [14]. The diameter of uterine spiral arteries during early pregnancy is 200 μm [8]. After remodeling, arteries have an average luminal size of 2 mm [15]. Dysfunctional remodeling of uterine spiral arteries is associated with complications of pregnancy, such as preeclampsia.

The principal placental cell type involved in uterine spiral artery remodeling is the EVT. EVTs invasion occurs through the interstitial pathway and endovascular pathway [16]. Interstitial EVTs migrate through the uterine stroma and endovascular EVTs through the distal end of the uterine spiral arteries [17]. By the eighth week of pregnancy, interstitial EVTs invade the decidua [18].

After week 10, endovascular EVTs cells invade decidua segment of uterine spiral arteries from the cytotrophoblastic shell [19]. Invasion by EVTs causes temporary artery plugging which decreases maternal blood flow that protects the fetus from oxidative stress [20]. When the plug disintegrates, endovascular EVT will further invade into the myometrium from week 14. These trophoblast cells will interact with the endothelium of the vessel and deposit fibrinoid material [5].

The initial steps of uterine spiral artery remodeling consist of vessel dilatation, vascular smooth muscle cell separation, endothelial cell swelling, EVTs infiltration, and fibrinoid deposition [17]. Vascular smooth muscle cells migrate or undergo apoptosis and are replaced by fibrinoid material, in which EVTs cells embed. The precise cellular mechanisms by which vascular smooth muscle cells are lost from the uterine spiral arteries are not known. Possible mechanisms include migration, apoptosis, and inhibition of proliferation and dedifferentiation [16]. Apoptosis of vascular smooth muscle cell is a process that occurs in normal pregnancy to maintain vessel homeostasis [21]. Vascular smooth muscle cell migration into decidual stroma and into the lumen of vessels is associated with several cytokines, growth factors, and breaking down of extracellular matrix [21].

3. Microenvironmental Factors

The functions of EVTs are affected by intrauterine microenvironmental factors, including oxygen tension and inflammatory mediators.

3.1. Oxygen Tension. Placentation is an oxygen sensitive process. The events that occur from the time of implantation to maternal perfusion of the placenta are influenced and directed by site-specific oxygen tensions [22]. An oxygen gradient exists between the placenta and endometrium during the first trimester. At the time of embryo implantation, the intrauterine oxygen tension is 3% [23] while the decidua and myometrium oxygen tension is 8–12% [24]. This standing oxygen gradient is thought to promote and direct the invasion of EVTs into the decidua and myometrium where they remodel maternal uterine spiral arteries [25]. Intraluminal EVTs occlude uterine spiral arterioles to maintain a low oxygen tension environment that is requisite for normal early placental and fetal development. Towards the end of the first trimester, low resistance, high capacity flow is achieved by the loss of intraluminal trophoblast plugs and the placental intravillous space is perfused with maternal blood, thus establishing effective maternofetal exchange. Dysfunctional placentation is associated with a failure to remodel uterine spiral arteries, abnormal placental perfusion, and oxygenation (similar to ischemia-reperfusion injury). After vascular remodeling of the SpA, the oxygen tension increase in the placenta [26]. These developmental changes in oxygen tension are thought to be an obligate regulator of cell function and phenotype. When perturbed, placentation and the subsequent perfusion of the placenta may be compromised. Activation of HIF-1α and inflammatory signalling pathways have been implicated in this process.

3.2. Inflammatory Mediators. Inflammation has a main role in supporting tissue homeostasis; indeed normal healthy pregnancy is characterised as a controlled, mild proinflammatory state [27]. The expression of inflammatory mediators is required to achieve a successful pregnancy that involves a series of intercellular interactions, particularly, at the site of implantation and placentation [28]. The inflammatory microenvironment is regulated by a balance between release of proinflammatory and anti-inflammatory cytokines [29]. These molecules have a critical and essential role in the maternal adaptation to the requirement of the different stages of gestation [30]. Complications of pregnancies such as fetal growth restriction and preeclampsia are frequently related to irregular maternal inflammation.

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine produced by different cells, such as fibroblast, macrophages, vascular cells, uterine NK cells, and placental cells that can promote trophoblasts growth and invasion [31–34]. It has been demonstrated that TNF-α have a key role in trophoblast migration into maternal decidua and spiral arterial remodeling [35, 36]; however, the mechanisms involving the transformation of uterine spiral arteries by EVTs cells have not been fully understood. TNF-α is a pleiotropic cytokine that has been found to be involved in many activities in preeclampsia [30]. In this instance, we believe that placental hypoxia is a consequence of arterial remodeling failure influenced by proinflammatory conditions in preeclampsia.

TNF-α was first detected in placental supernatants and amniotic fluid [37, 38]. Expression of TNF-α in placenta

changes during pregnancy and is responsive to changes in the extracellular milieu [39] suggesting that TNF-α has a specific function in developmental processes [40]. Expression of TNF-α mRNA in the first trimester of pregnancy has been found in all cell types belonging to the trophoblastic lineage. TNF-α expression, however, decreases in invasive cells at later stages of pregnancy [41]. TNF-α activates proapoptotic factors as well as antiapoptotic factors to maintain a microenvironment for successful arterial remodeling. It has been reported that trophoblast differentiation could be regulated by TNF-α [42].

The aetiological antecedents of preeclampsia are thought to be aberrant maternal-fetal immune tolerance that reduced trophoblast invasion. Recent studies have shown that immune maladaptation and overt activation of maternal immune system may be responsible in the pathogenesis of preeclampsia [43]. In the past decades, serum levels of TNF-α had elevated and increased expressions of TNF-α and TNF receptors were found in leukocytes and placenta of women with preeclampsia [40]. This rise can occur as early as 11–13 weeks of pregnancy, much earlier than detectable clinical manifestations [44]. TNF-α may inhibit EVT migration in first trimester placenta via activated macrophages. In early onset of preeclampsia, findings on TNF-α and interleukin-2 (an anti-inflammatory cytokine) suggested that there is an imbalance of proinflammatory and anti-inflammatory cytokines ratio [45]. Toll-like receptor which is the main danger signalling pathway involved in the pathophysiology of preeclampsia increases the production of TNF-α [46]. Another study performed by Hamai et al., [47] has shown an increase of TNF-α in early pregnancy of preeclampsia. In asymptomatic patients (patients who later developed preeclampsia in the second trimester), the level of TNF-α in the first trimester was 2-fold higher compared to healthy controls [47]. On the other hand, other authors have demonstrated that level of TNF-α increased significantly in women diagnosed with preeclampsia compared with healthy control [48–50].

Preeclampsia is characterised with reduced uteroplacental perfusion and incomplete uterine spiral arterial remodeling. Moreover, a high level of TNF-α has been found in plasma from patients with preeclampsia; however, the role of TNF-α in the failure of spiral artery remodeling and the mechanisms involved in this phenomenon still are not fully elucidated. In this regard, it has been established recently that small vesicles released by many cell types including human placental cells contain a membrane bound form of TNF-α [51]. Recent studies highlight the putative utility of tissue-specific nanovesicles (e.g., exosomes) in the diagnosis of disease onset and treatment monitoring [9, 52–56]. To date, there is a paucity of data defining changes in the release, role, and diagnostic utility of placenta-derived exosomes in pregnancies complicated by preeclampsia.

4. Exosomes: Definition and Characteristics

Exosomes are small (40–100 nm) and very stable membrane vesicles that are released when late endosomal bodies fuse with the cell membrane [57, 58]. Exosomes found in cell cultures and body fluids indicate that they can be released from different types of cells [59]. Exosomes are characterised by a buoyant density of 1.13–1.19 g/mL, an endosomal origin, and enrichment of late endosomal membrane markers (including Tsg101, CD63, CD9, and CD81), are released into extracellular compartments [60], and are identified in most biological fluids examined [61, 62]. Exosomes are generated by the inward budding of late endosomal structures, the multivesicular bodies (MVB). Moreover, the participation of Rab GTPases in the secretion of exosomes has been proposed [63]. Although we know little about the mechanism by which packaging occurs, exosomes contain a diverse array of signalling molecules and are released from the parent cell following the exocytosis fusion of multivesicular bodies with the cell membrane [12]. Signalling molecules, including miRNA; mRNA; and cytoplasmic proteins, are packaged into exosomes. Exosomal signalling occurs when released exosomes fuse with target cells and deploy their contents to alter cell function. In pathological pregnancies, exosomes secreted from the placenta may be involved in adaptive responses and different biological processes such as metabolism, development, cellular adhesion, and immune response of the mother and fetus. We have isolated and characterised exosomes released from placental cells and have demonstrated that trophoblast cells release exosomes that are bioactive and can regulate the biological function on cell target [58, 64, 65]. A representative standard size distribution graph and electron microscope image of the exosome samples isolated from placental cells are shown in Figure 1.

4.1. Exosomes and Cell-to-Cell Communication. Exosomes interact with target cells via multiple pathways, by directly activating target cell membrane receptors; by modifying the extracellular *milieu* of the target cell; and by fusing with the cell membrane and releasing their molecular cargo into the target cell [66]. Recently, it has been demonstrated that cells internalise exosomes through lipid raft-mediated endocytosis involving caveolin-1 protein and ERK1/2-heat shock protein 27 signaling in this process [67]. Their molecular cargo is cell specific [68], regulated by tissue physiology and cellular function, and fundamental to their bioactivity.

Exosomes may be assembled and secreted in response to instructions received from neighbouring cells, from distant tissues, or in response to local environmental factors (e.g., oxygen tension). Their molecular cargos, including mRNA [69], miRNA [68, 69], proteins [65], lipids [70], and membrane receptors, are transferred to adjacent cells and/or distal cells via biofluid transport (e.g., in blood, lymph, saliva, or ascites).

Currently, we have only a limited understanding of the role that exosomal signaling plays in normal physiology and pathophysiology and, in particular, in reproductive biology. This provides us with exciting opportunities to establish the role of exosomes in disease pathology and to advance diagnosis and treatment of clinically significant conditions.

Placental cells release exosomes *in vitro* and *in vivo* and have been identified in maternal blood [64, 71, 72]. They contain placenta-specific protein and miRNA and, as such,

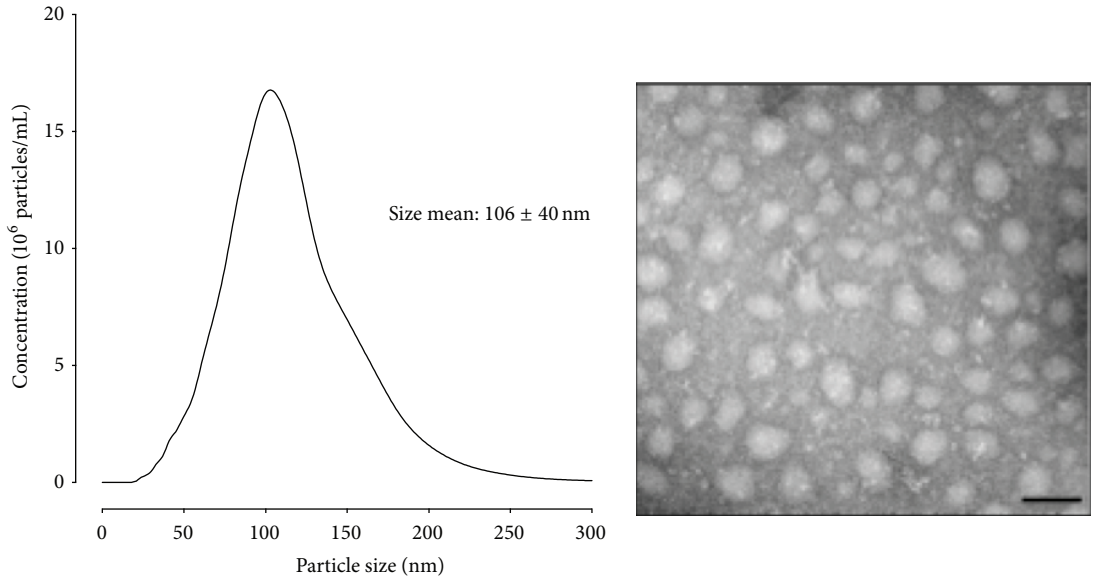

Figure 1: Placental exosomes characterisation. Exosomes vesicles were isolated from placental cells by differential and buoyant density centrifugation and purified using a sucrose continuous gradient as previously described [65]. (a) Representative graph size distribution of the exosome samples using a nanoparticle tracking analysis (NanoSight NS500). (b) Representative electron micrograph of enriched exosomes population. In (b), scale bar is 200 nm.

may be differentiated from maternally derived exosomes [53, 73]. The concentration of exosomes has been reported to increase in association with some complications of pregnancy (e.g., preeclampsia [72]). In this regard, complications of pregnancy are associated with a proinflammatory state (e.g., high TNF-α concentrations) and also with failure in the SpA remodeling where EVTs have been demonstrated to have an important role. Our group has isolated and characterised exosomes released from placental cells and has demonstrated that (i) first trimester cytotrophoblast (CT) cells release exosomes$^{CD63+,CD9+,CD81+,PLAP+}$ *in vitro* [65]; (ii) CT-exosome release and protein content are regulated by oxygen tension; and (iii) CT exosomes induce extravillous cytotrophoblast cell invasion and proliferation in a time- and dose-dependent manner [65]. In addition to direct effects on target cells, exosomes from nongestational tissues have been reported to remodel the extracellular matrix (ECM) surrounding target cells (i.e., cell fusion-independent effects). We have identified serine proteases (e.g., HtrA 4, which is expressed by CTs and syncytiotrophoblast (ST); present in maternal plasma; and increased in association with PE [74]) as well as metalloproteases (e.g., MMP 2, MMP 9, and MMP 12) in CT exosomes [65].

Recently, it has been proposed that MMP 12 secreted by trophoblast cells induces disruption of uterine vascular smooth muscle cell architecture favouring extravillous trophoblast invasion [75, 76]. The activity and capacity of trophoblast-derived exosomes to directly bind and remodel ECM in a cell fusion-independent manner have yet to be established. Exosomal remodeling of ECM may participate not only in cytotrophoblasts-extravillous trophoblasts interactions but also in the extravillous trophoblast-endothelial cells and extravillous trophoblast-vascular smooth muscle cell interactions. As we know that exosomes protect their content, we hypothesised that EVT-derived exosomes interact with vascular cells (i.e., smooth muscle and endothelial cells), delivering their specific cargo (e.g., MMPs) and contributing to the SpA remodeling.

4.2. Oxygen Tension Can Regulate the Effect of Placental Exosomes. Recently, we reported that changes in oxygen tension also regulate placental exosome release, content, and bioactivity [58, 65]. Hypoxia (1% O_2) increases the release of exosomes from CTs incubated *in vitro* when compared to CTs incubated under 3% or 8% O_2. The protein content of these "hypoxic" exosomes is also altered with increased enrichment of HIF-1α and IL-8 signalling molecules. In addition, the ability of these exosomes to induce cell migration is significantly enhanced. Oxygen tension also regulates the responsiveness of target cells to exosomes. This phenomenon has been demonstrated in other cell types (e.g., cancer cells), where exosomes content reflects the oxygenation status of cells [84]. These data provide new insights and understanding into how oxygen tension regulates cell function and, in particular, the role of oxygen tension in regulating exosomal signalling in the placenta. Our preliminary studies identify oxygen-dependent changes in the protein content of CT exosomes; however, effects on miRNA mediators remain to be established. Using nongestational tissue cell lines (epithelial ovarian cancer cells), we have also identified cell-specific packaging of miRNA into exosomes [68]. We will use this approach to identify cell- and treatment-specific effects on miRNA packaging into trophoblast exosomes. Human placenta and placental-derived exosomes express the

Table 1: Effects of exosomes vesicles on cell target.

Vesicles source	Isolation methods	Cell target	Biological function	Effect	References
Cytotrophoblast cells	UT + sucrose continuous gradient	EVT (HTR-8/Svneo)	Invasion and proliferation	Promote	[65]
pMSC	UT + 30% sucrose cushion	hPMEC	Migration and proliferation	Promote	[58]
Maternal plasma	UT + sucrose continuous gradient	HUVEC	Migration	Promote	[64]
Trophoblast (Swan 71)	UT	Monocytes	Migration	Promote	[54]
Chorionic villi explant	UT + sucrose continuous gradient	Jurkat T cells and PBMC	Apoptosis	Promote	[77]
Trophoblast cells	UT + 30% sucrose cushion	HUVEC	Viral infection	Resistance	[78]
Human macrophages	UT + sucrose continuous gradient	Endothelial cell	Migration	Decrease	[79]
CML cells	UT + 30% sucrose cushion	HUVEC	Migration	Promote	[80]
Dendritic cells	UT + 30% sucrose cushion	PBMC	Migration	Promote	[81]
Pancreatic adenocarcinoma cells	UT + sucrose continuous gradient	Endothelial cells	Migration	Promote	[82]
HUVEC	UT	SMCs	miRNAs expression	Transfer miRNAs	[83]

UT: ultracentrifugation (>100,000 ×g); EVT: extravillous trophoblast; pMSC: placental mesenchymal stem cells; hPMEC: human placental microvascular endothelial cells; HUVEC: human umbilical vein endothelial cells; PBMC: peripheral blood mononuclear cells; CML: chronic myelogenous leukemia; SMCs: smooth muscle cells.

chromosome 19 miRNA cluster (C19MC), which is regulated selectively by hypoxic stress [85]. Moreover, it has also been demonstrated that trophoblast cells utilise exosomes for the transfer of specific and unique miRNA (from cluster C19MC) to other cells (e.g., maternal and fetal cells) and confer them with viral resistance against infections [78]. Placental-derived exosomes under both normal and pathological conditions could perform a main role in the maternal adaptation to pregnancy (e.g., uterine vascular adaptation to pregnancy).

4.3. Exosomes Regulate Cell Migration on Cell Target. Exosomes mediate cell-to-cell communication and induce different effects on target cells depending on the cell origin and exosome content (e.g., miRNA and proteins). The function of placental-derived exosomes during normal or pathological pregnancy remains to be established. Several studies support the hypothesis that placental exosomes (i.e., release from cytotrophoblast, extravillous trophoblasts, and syncytiotrophoblast) are capable of promoting cell migration (Table 1). In addition, this phenomenon not only is restrictive to placental exosomes but also has been demonstrated in nonplacental exosomes [82]. We have previously reported that exosomes released from cytotrophoblast cells primary culture contain biologically active proteins [65] that can interact with the maternal endothelium and regulate their function (e.g., migration and angiogenesis). Furthermore, the release of exosome from placental mesenchymal stem cells and cytotrophoblast cells is regulated by the oxygen tension [58, 65]. Exosomes isolated from Swan 71 cells (trophoblastic cell lines) promote monocytes migration and increased the production of proinflammatory cytokines from these cells [86]. Primary human trophoblast cells are resistant to viral infection (e.g., human cytomegalovirus) and can transfer their viral resistance to nonplacental cells (i.e., endothelial cells) through exosomes, an effect completely abolished by sonication [78], highlighting that the exosome integrity is critical to mediate their effects on cell target.

We have recently demonstrated that exosomes isolated from peripheral plasma were biologically active, as assessed by their ability to increase endothelial cell migration *in vitro*. Moreover, the bioactivity of exosomes was greatest during the first trimester and gradually declined with advancing gestational age. These results suggest that, in normal pregnancy, exosomes isolated from plasma of pregnant healthy women in the first trimester may play a role in regulating the endothelium response to maternal adaptation to pregnancy. Exosomes are sensitive to environmental milieu (e.g., oxygen tension), changing their bioactivity and content; we propose that, under physiological conditions (e.g., normal pregnancy), placental exosomes promote vascular cell migration from the uterine spiral arteries; however, under pathological conditions (e.g., proinflammatory state and preeclampsia), the bioactivity of placental exosomes is reduced.

4.4. Preeclampsia Is Associated with Increased Release of Placenta-Derived Vesicles. Preeclampsia (PE) is a leading cause of maternal and fetal morbidity and mortality with an incidence rate of 3–5% of all pregnancies [88, 89]. One of the first events associated with development of PE is the failure in remodeling the uterine maternal arteries completely and consequently the inadequate placental blood flow. While the precise etiology of PE remains largely unknown, physiological, environmental, and immunological risk factors have been identified [89]. The hypothesis that trophoblast-derived vesicles and debris shed into maternal circulation promotes an inflammatory vascular response and causes endothelial damage that is correlated with the pathophysiology of PE that has been proposed by Redman

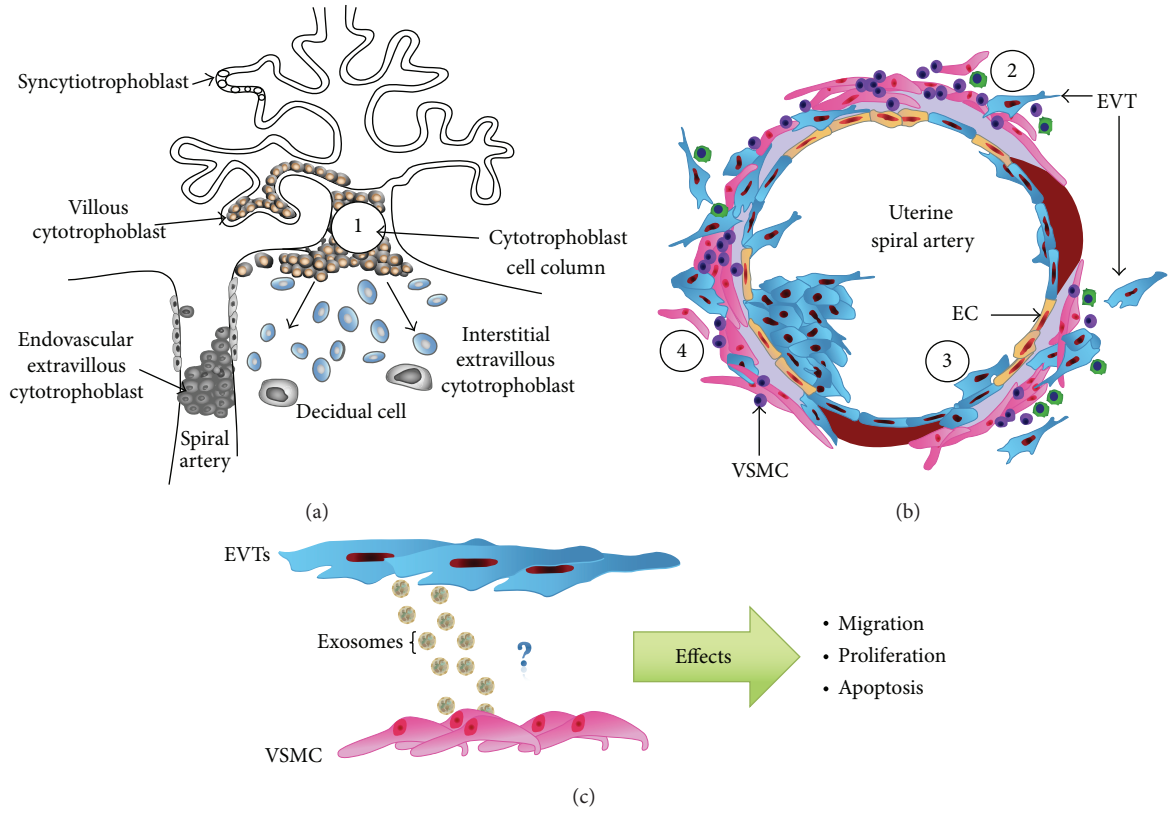

Figure 2: A hypothesis on the effect of EVTs-derived exosomes on SpA remodeling. Complications of pregnancy are thought to be clinical manifestations of a common developmental lesion inadequate invasion by extravillous trophoblast cells with a consequent failure to remodel the maternal uterine spiral arteries. EVTs migrate from the cytotrophoblast-anchoring villi ((a) [1]) of the placenta and invade the maternal decidua and myometrium. These cells are localised with uterine spiral arteries (b) and are thought to induce vascular remodeling (i.e., extracellular matrix remodeling [2]); the loss of vascular endothelial [3]; and smooth muscle [4] cells by apoptosis or migration out of the vessel wall. We propose that EVTs-derived exosome has participation on the SpA remodeling, specificity affecting process as migration, proliferation, and apoptosis of VSMC (c). In (b), cartoon is modified from Cartwright et al., 2010 [87].

et al.'s group [90]. The placental syncytiotrophoblast secretes a wide range of vesicles, including micro- and nanovesicles into the maternal circulation during normal pregnancy [64]. Using a flow cytometry approach and syncytiotrophoblast-specific antiplacental alkaline phosphatase (PLAP), significantly greater levels of placental-derived vesicles were found in both peripheral and uterine venous plasma from women with preeclampsia compared to normal pregnant women [91]. Moreover, similar results were observed using a dual placental perfusion system in placentae from preeclampsia pregnancy [92]. In contrast, a recent study using nanoparticles tracking analysis reported high level of placental-derived vesicles in pregnant women compared with nonpregnant women, without difference in the number of syncytiotrophoblast extracellular vesicles between normal pregnant women and plasma from patients with preeclampsia [93]. To our knowledge, wide variation between results can be attributed to methodological differences, while flow cytometer is still inadequate to detect single vesicles with size less 300 nm (without polystyrene beads) and the expression of PLAP is reduced in syncytiotrophoblast-derived vesicles (including micro- and nanovesicles) obtained from perfused placental from preeclamptic pregnancies [92].

The concentration of placenta-derived exosomes vesicles is also increased with the advancing gestational age [64]. The molecular composition and biological effects of these nanovesicles are determined by their cellular origin. Thus, events that impact on early trophoblast cell invasion and their interaction with maternal cells (including oxygen tension and glucose and fatty acid concentrations) may contribute to or predispose to complication of pregnancies [64]. It has been demonstrated that exosomal protein content is different in women with preeclampsia [94]. Moreover, the specific syncytiotrophoblast protein, syncytin-2, is markedly downregulated in exosomes derived from placenta of pregnant women with preeclampsia compared to healthy control (normal pregnancies) [95].

In contrast, high levels of syncytiotrophoblast-derived vesicles were found in plasma from women with early-onset preeclampsia [96]. Since trophoblast invasion and insufficient uterine vascular remodeling occur in early-onset preeclampsia, we, therefore, propose that the release and composition (i.e., exosomal proteins) of placenta-derived exosomes are altered in pregnancies that subsequently develop complications (e.g., preeclampsia) and that placental cell exosomes

derived from abnormal pregnancies differentially affect vascular smooth muscle cell function.

5. Summary

Uterine spiral arterial remodeling is an important physiological change during early pregnancy. EVTs migrate into maternal decidua and myometrium and interact with endothelial and vascular smooth muscle cells in uterine spiral arteries. Conversion of these arteries is associated with the loss of both endothelial cells and vascular smooth muscle cells from the vessel wall by apoptosis and/or migration out of the vessel. In this regard, communication between EVTs and vascular smooth muscle cells appears to be essential for successful arterial remodeling. The effect of exosomes released from EVTs on endothelial cells and vascular smooth muscle cells has not been established. We propose that in complicated pregnancies (e.g., preeclampsia), proinflammatory microenvironment regulates the release and bioactivity of EVT-derived exosomes. In normal pregnancy, EVT-derived exosomes may promote vascular smooth muscle cell migration favoring the spiral uterine arterial remodeling; however, high concentration of proinflammatory cytokines (e.g., TNF-α) may inhibit the effect of exosomes on vascular smooth muscle cell migration, triggering failure in arterial remodeling and stimulating the emergence of preeclampsia (Figure 2).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Early Onset Intrauterine Growth Restriction in a Mouse Model of Gestational Hypercholesterolemia and Atherosclerosis

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The susceptibility to develop atherosclerosis is increased by intrauterine growth restriction and prenatal exposure to maternal hypercholesterolemia. Here, we studied whether mouse gestational hypercholesterolemia and atherosclerosis affected fetal development and growth at different stages of gestation. Female LDLR KO mice fed a proatherogenic, high cholesterol (HC) diet for 3 weeks before conception and during pregnancy exhibited a significant increase in non-HDL cholesterol and developed atherosclerosis. At embryonic days 12.5 (E12.5), E15.5, and E18.5, maternal gestational hypercholesterolemia and atherosclerosis were associated to a 22–24% reduction in male and female fetal weight without alterations in fetal number/litter or morphology nor placental weight or structure. Feeding the HC diet exclusively at the periconceptional period did not alter fetal growth, suggesting that maternal hypercholesterolemia affected fetal weight only after implantation. Vitamin E supplementation (1,000 UI of α-tocopherol/kg) of HC-fed females did not change the mean weight of E18.5 fetuses but reduced the percentage of fetuses exhibiting body weights below the 10th percentile of weight (HC: 90% vs. HC/VitE: 68%). In conclusion, our results showed that maternal gestational hypercholesterolemia and atherosclerosis in mice were associated to early onset fetal growth restriction and that dietary vitamin E supplementation had a beneficial impact on this condition.

1. Introduction

In the last decades, the influence of the intrauterine development on the susceptibility to cardiovascular disease has been demonstrated both in humans and in experimental animal models. David Barker, pioneer in describing fetal early environmental programming, described how undernutrition *in utero* can alter human fetal growth and induce permanent changes in the body's structure, function, and metabolism that can lead to coronary heart disease later in life [1]. Additional studies showed that, beyond a caloric restriction, other intrauterine suboptimal conditions, such as excessive nutrient availability or exposure to pollutants, alcohol, or nicotine, can also increase the susceptibility of the adult offspring to cardiovascular disease (reviewed in [2]).

Maternal adverse conditions can affect embryos at different stages of gestation, even before implantation. In fact, mammalian preimplantation embryos from different animal models, ranging from mice to sheep, are particularly sensitive to environmental factors during the periconceptional period. Exposure of blastocysts to suboptimal conditions both *in vivo* (i.e., maternal undernutrition) and *in vitro* (i.e., inefficient

culture conditions) is associated with the onset of cardiovascular dysfunction in adult life [3].

The most common underlying cause of ischemic cardiovascular disease is atherosclerosis, a silent and progressive pathologic condition that originates very early in life, even during development in the womb, characterized by the accumulation of lipids, lipoperoxidation products, and macrophages in arterial vessels [4]. During pregnancy, maternal high plasma cholesterol levels may promote fetal atherosclerosis. In humans, fatty streaks and intimal thickening are higher in fetuses from hypercholesterolemic mothers [4, 5]. Although the human term placenta is impermeable to the atherogenic low density lipoproteins (LDL), the existing correlation between maternal and fetal plasma cholesterol levels before the sixth month of gestation suggests that maternal high levels of plasma cholesterol at early to midpregnancy may directly promote lesion formation in the fetus [4]. Hypercholesterolemia during pregnancy increases maternal lipid peroxidation, which is transmitted to the embryo via the placenta, suggesting that abnormal cholesterol metabolism can also have indirect effects on the placenta and fetus, that is, oxidative stress [6]. Indeed, maternal hypercholesterolemia associates with umbilical vein endothelial dysfunction due to arginase and eNOS signaling imbalance [7]. In humans, suboptimal fetal growth is one of the risk factors for atherosclerosis: weighing less than the 10th percentile of the population or being born preterm increases the development of endothelial dysfunction and preclinical atherosclerosis in young adults [8]. Also, fetal growth restriction in rats leads to the early appearance of atherosclerosis in adult offspring [9].

Progress in understanding fetal programming of human atherosclerosis has been slowed by the high genetic and environmental heterogeneity among individuals and by the difficulty of accessing noninvasively images from aortas of developing fetuses. In addition, the requirement of cholesterol to sustain appropriate fetal development [10] has hindered the use of pharmacological treatments to reduce cholesterol in hypercholesterolemic pregnant women due to their possible teratogenic effects. As in many other areas of biomedical research, animals have nevertheless proven valuable experimental models of fetal programming due to their genetic homogeneity, more controlled environment, accessibility to obtain fetuses at different developmental stages, and feasibility for interventions during gestation. A rabbit model established by the group of Napoli and Palinski provided the first straightforward evidence on the pivotal role of maternal hypercholesterolemia in atherosclerosis in the offspring [11]. This study also reported the involvement of oxidative stress in fetal atherosclerosis, as lesions were significantly reduced when pregnant females were fed with vitamin E-supplemented hypercholesterolemic diets. Other studies using rabbits showed that near term fetuses from high-cholesterol-fed dams exhibited 15–25% lower body weight than fetuses from chow-fed controls [12, 13].

LDL receptor deficient (LDLR KO) [14] and apolipoprotein E deficient (ApoE KO) [15] mice have been used to study fetal programming of atherosclerosis. LDLR KO mice fed with regular chow have a twofold increase in LDL cholesterol levels compared with wild-type mice, yet these mice only develop atherosclerosis when fed with a high-cholesterol diet that increases their plasma cholesterol above 300 mg/dl [16]. Recent studies using these mice have contributed to the understanding of some of the mechanisms implicated in the high susceptibility to atherosclerosis observed in the offspring of hypercholesterolemic pregnant females. Fetal exposure to maternal hypercholesterolemia induces changes in gene expression in different tissues (i.e., aorta and liver) that persist into adulthood [14, 17]. Interestingly, LDLR KO females fed with a western-type high fat/carbohydrate/cholesterol diet for 6 weeks before conception and during pregnancy give birth to smaller pups [18]. However, in both studies, using hyperlipidemic rabbits or mice, the onset of fetal growth abnormalities during intrauterine development has not been studied.

This work was aimed at analyzing the impact of periconceptional (until E5.5) versus gestational maternal hypercholesterolemia and atherosclerosis on fetal development at different stages of intrauterine development. This maternal condition was not associated with miscarriages or severe fetoplacental abnormalities. Fetal growth restriction was detected in mice from both sexes as a consequence of gestational but not periconceptional maternal hypercholesterolemia. Taking into account the low levels of vitamin E detected in mothers and newborns from human growth restricted pregnancies [19, 20] and the reduction of oxidative stress by vitamin E in placenta of hypercholesterolemic rabbits [21], we also analyzed whether dietary supplementation with vitamin E could prevent growth restriction in fetuses from HC-fed females.

2. Results

2.1. LDLR KO Fed with a Proatherogenic Diet Develop Hypercholesterolemia and Atherosclerosis in the Absence of Overweight or Infertility. LDLR female mice fed with a proatherogenic, high-cholesterol (HC) diet containing 0.625% cholesterol, fat, and cholic acid diet for 3 weeks were as fertile as females fed with a chow diet containing 0.02% cholesterol, as 4/5 (80%) of these hypercholesterolemic females with a vaginal plug became pregnant versus 5/6 (83%) in the control group. Feeding the HC diet before and during pregnancy induced severe hypercholesterolemia, essentially due to accumulation of non-HDL lipoproteins, in the endogenously hypercholesterolemic LDLR KO females [22] (7- and 8-fold at E15.5 and E18.5, resp.) (Figures 1(a) and 1(b)). This dyslipidemia led to the development of atherosclerotic lesions in the maternal aortic root as shown by immunohistochemical analyses (Figure 1(c)). No additional signs of adverse health conditions or body weight differences were detected in the diet-manipulated compared to the chow-fed LDLR KO group (Figure 1(d)).

2.2. LDLR KO Mice with Gestational Hypercholesterolemia and Atherosclerosis Exhibit Fetal Growth Restriction. Neither the litter size nor the mean number of resorptions differed between chow-fed and HC-fed pregnant LDLR KO females (Figure 2(a)). Placental gross morphology and size

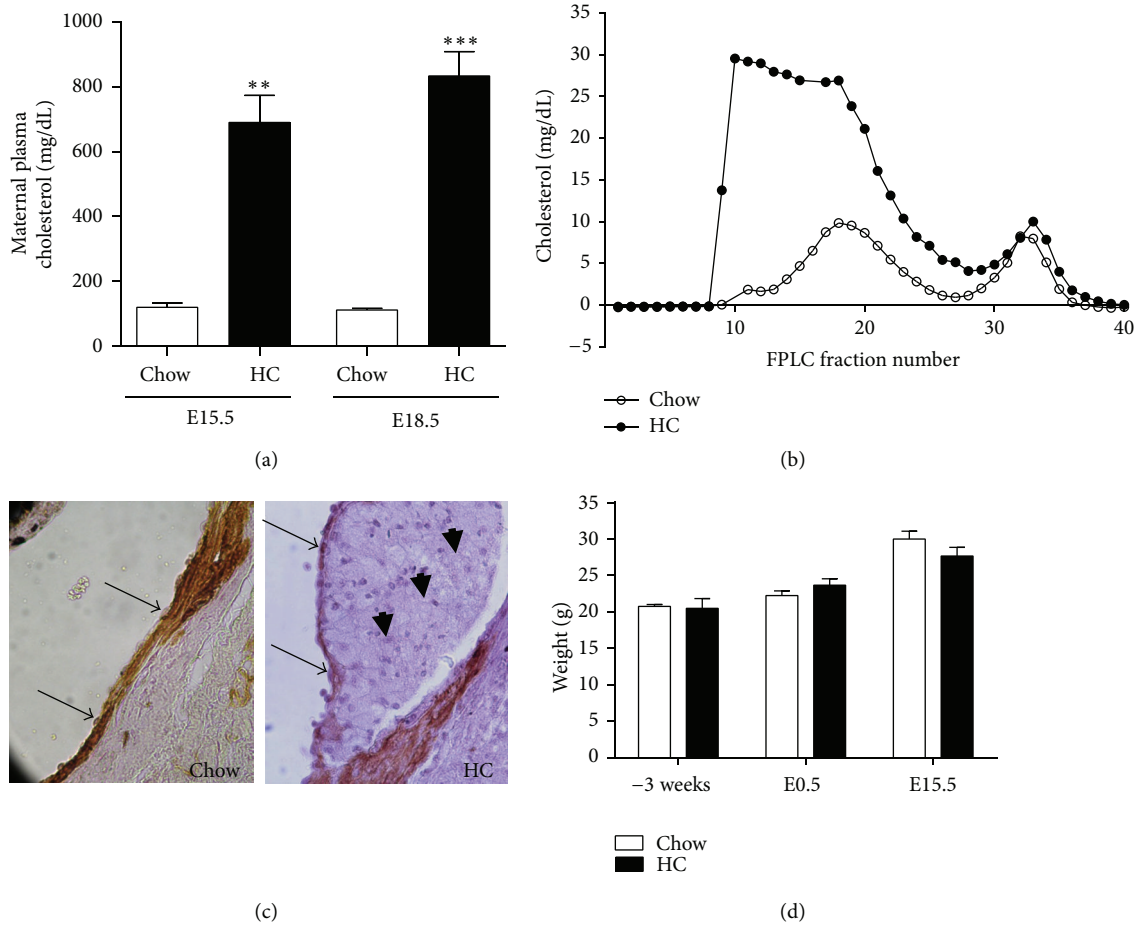

FIGURE 1: Gestational hypercholesterolemia and atherosclerosis in LDLR KO females fed with a HC diet. (a) Plasma cholesterol concentration was significantly higher in HC-fed females compared to chow-fed females at both E15.5 ($^{**}P < 0.05$, $n = 2$ and $n = 4$ females, resp., Student's t-test) and E18.5 ($^{***}P < 0.001$, $n = 5$ females in each group, Student's t-test). (b) Representative FPLC profiles of cholesterol content in lipoproteins in one chow- and one HC-fed pregnant female in E15.5. (c) Fatty streak in the aortic root of a HC-fed pregnant female in E15.5. Note the actin positive smooth cells (brown), the endothelial layer (arrows), and the foam cells in the subendothelial region (arrowheads) (magnification: 40x). (d) Feeding the HC diet did not induce overweight either before pregnancy or during pregnancy ($n = 3$ females in each group). Mean ± SEM.

(not shown) and placental weight (Figure 2(b)) were also similar in both groups at E15.5 and E18.5. The distribution of tissues conforming the three different layers of the mouse placenta, decidua, spongiotrophoblast, and labyrinth was normal in females from both groups at E15.5 (Figure 2(c)) and E18.5 (not shown). No defects in the labyrinth organization and the distribution of vascular exchange areas within placenta from hypercholesterolemic and atherosclerotic females were detected in histological sections from E15.5 placenta, as the mean area occupied by maternal sinuses and fetal capillaries was similar in placenta from chow- and HC-fed groups (Figure 2(d)).

Despite the normal placental growth and morphological parameters observed in pregnancies occurring in LDLR KO mice with hypercholesterolemia and atherosclerosis, the mean body weight of fetuses harvested from these females was lower than that of fetuses from chow-fed mice at the three gestational ages analyzed (Figures 3(a) and 3(b)). Fetal sex discrimination by PCR amplification of *smcx* and *smcy* alleles showed that fetal growth restriction was similar in both sexes in the HC-fed group (Figure 3(c)).

2.3. Periconceptional Exposure to Hypercholesterolemia and Atherosclerosis Does Not Affect Fetal Growth. To determine whether exposure to maternal hypercholesterolemia exclusively during the periconceptional period had an effect on fetal growth at later stages of intrauterine development, LDLR KO females were fed with the proatherogenic HC diet from 3 weeks previous to conception until implantation at E5.5 and then fed with regular chow diet. Plasma cholesterol levels from females exposed to the HC diet periconceptionally were already normalized at E15.5 [115 ± 25 mg/dl in periconceptional HC-fed LDLR KO females ($n = 2$ females) versus 108 ± 30 mg/dl in control chow-fed LDLR KO females ($n = 2$ females), $P = 0.88$ Student's t-test]. E15.5 fetuses retrieved from dams periconceptionally fed with the HC diet weighed

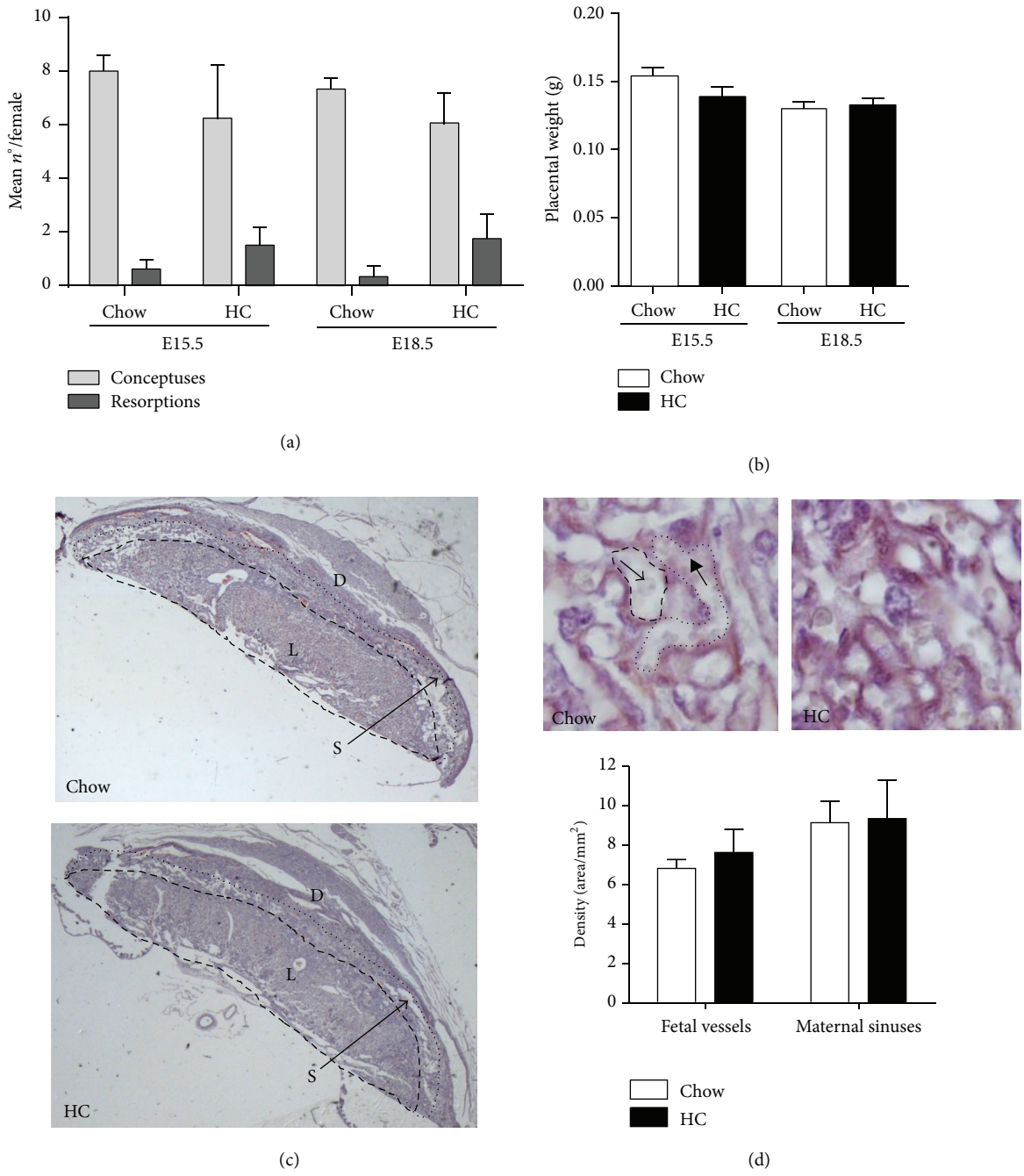

Figure 2: LDLR KO females fed with a HC diet have normal placental development. (a) The mean numbers of conceptuses and resorptions per female retrieved at E15.5 and E18.5 were similar in LDLR KO chow- and HC-fed pregnant females (n = 3 litters from each group). (b) Placenta from hypercholesterolemic pregnancies had similar weights compared to those from control pregnancies at E15.5 and E18.5. (c) Histological analyses of E15.5 placenta showed similar tissue organization and distribution of the different placental structures: labyrinth (L), spongiotrophoblast (S), and decidua (D) in control and hypercholesterolemic placenta (n = 3 per group) (magnification: 8x). (d) Analysis of the vascular organization in the labyrinth in control and HC pregnancies (n = 3 placentas from each group). Upper panel: representative immunohistochemistry showing anticytokeratin positive syncytiotrophoblasts marking the interface between maternal and fetal circulations (magnification: 1,000x). Lower panel: quantification of the mean areas per mm^2 labyrinth occupied by fetal vessels (surrounded by dashed line) containing large, immature red blood cells (arrow) and maternal sinuses (surrounded by dotted line) containing mature, enucleated red blood cells (arrowhead). Mean ± SEM.

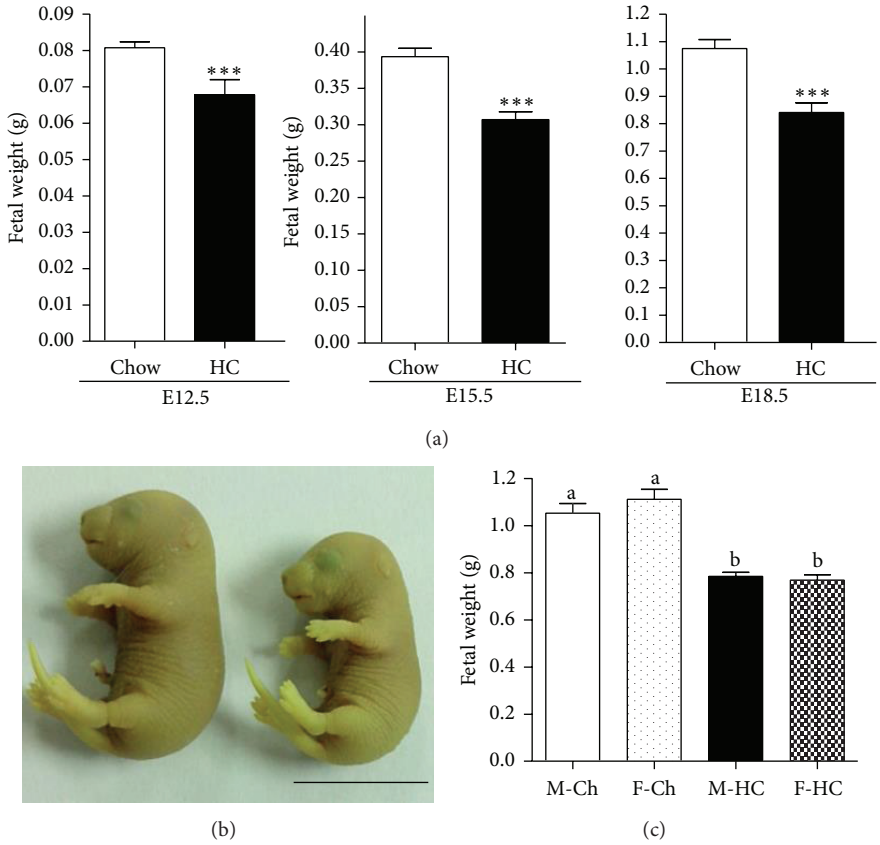

Figure 3: Fetal intrauterine growth restriction is observed in LDLR KO mice fed with a HC diet. (a) Fetuses retrieved from hypercholesterolemic pregnant females weighed significantly less than fetuses from control mice at the three stages analysed ($^{***}P < 0.001$) (E12.5, E15.5, and E18.5, $n = 43$, 24, and 10 fetuses in control females, and $n = 17$, 19, and 31 fetuses in HC-fed females, resp.). (b) Representative picture of a normal fetus obtained from a chow-fed female and one growth restricted fetus obtained from a HC-fed female fixed in Bouin. (c) Fetal weight in E18.5 male and female fetuses from control and HC groups ($n = 7$ to 10 fetuses in each group). Mean ± SEM.

similar to fetuses from chow-fed mice [382 ± 22 mg ($n = 11$ fetuses) versus 393 ± 22 mg ($n = 24$ fetuses), resp., $P = 0.58$ Student's t-test]. These results indicated that periconceptional maternal hypercholesterolemia in mice does not impact fetal growth.

2.4. Dietary Vitamin E Supplementation Reduces the Proportion of Growth Restricted Fetuses in Pregnancies with Gestational Hypercholesterolemia and Atherosclerosis. LDLR KO females were fed with chow, HC, or vitamin E-supplemented HC (HC/VitE) diets from 3 weeks before conception until E18.5, when fetuses were retrieved. Fetal weights displayed frequency distribution curves with the expected Gaussian distribution (Figure 4(a)) [23]. As observed previously (in Figure 3), maternal HC-feeding of LDLR KO females resulted in a significant reduction in the mean fetal weight (1.01 ± 0.02 g control ($n = 58$ fetuses) versus 0.79 ± 0.01 g HC ($n = 38$ fetuses), $P < 0.001$ Student's t-test). This defect can be appreciated in Figure 4(a) by the displacement of the frequency distribution curve to the left (see red arrow). In the HC-fed group, 90% of the fetuses exhibited weights that were under the 10th percentile of the control chow-fed population (Figure 4(b)). Vitamin E supplementation of HC-fed dams did not affect the mean weight of fetuses compared to the HC untreated group [0.82 ± 0.02 g HC/VitE ($n = 47$ fetuses), $P = 0.147$ Student's t-test] (small displacement of curves indicated by blue arrow) (Figure 4(a)). However, the percentage of fetuses weighing less than the 10th percentile of the control group was reduced from 90% in the untreated HC-fed group to 68% in the HC/VitE group (Figure 4(b)). The difference in the proportions of normal versus growth restricted fetuses in the HC-fed untreated (4 versus 34) and vitamin E- supplemented (15 versus 32) groups reached statistical significance ($P = 0.0207$, Fisher's exact test).

Vitamin E supplementation had an impact on plasma cholesterol levels in HC-fed dams, although this reduction did not reach statistical significance compared to HC-fed females [118 ± 6 mg/dl chow ($n = 3$ females), 844 ± 139 mg/dl HC ($n = 4$ females), and 527 ± 147 mg/dl HC/VitE ($n = 3$ females)] (Figure 4(c), left panel). Neither HC nor HC/VitE diets affected the levels of fetal plasma cholesterol at E18.5 [90 ± 5 mg/dl control ($n = 3$), 86 ± 16 mg/dl HC ($n = 4$), and 107 ± 18 mg/dl HC/VitE ($n = 3$); in this case each n corresponds to a pool of 2 fetuses] (Figure 4(c), right panel), which is consistent with the lack of association between maternal and fetal cholesterol levels in late pregnancy observed in humans [4].

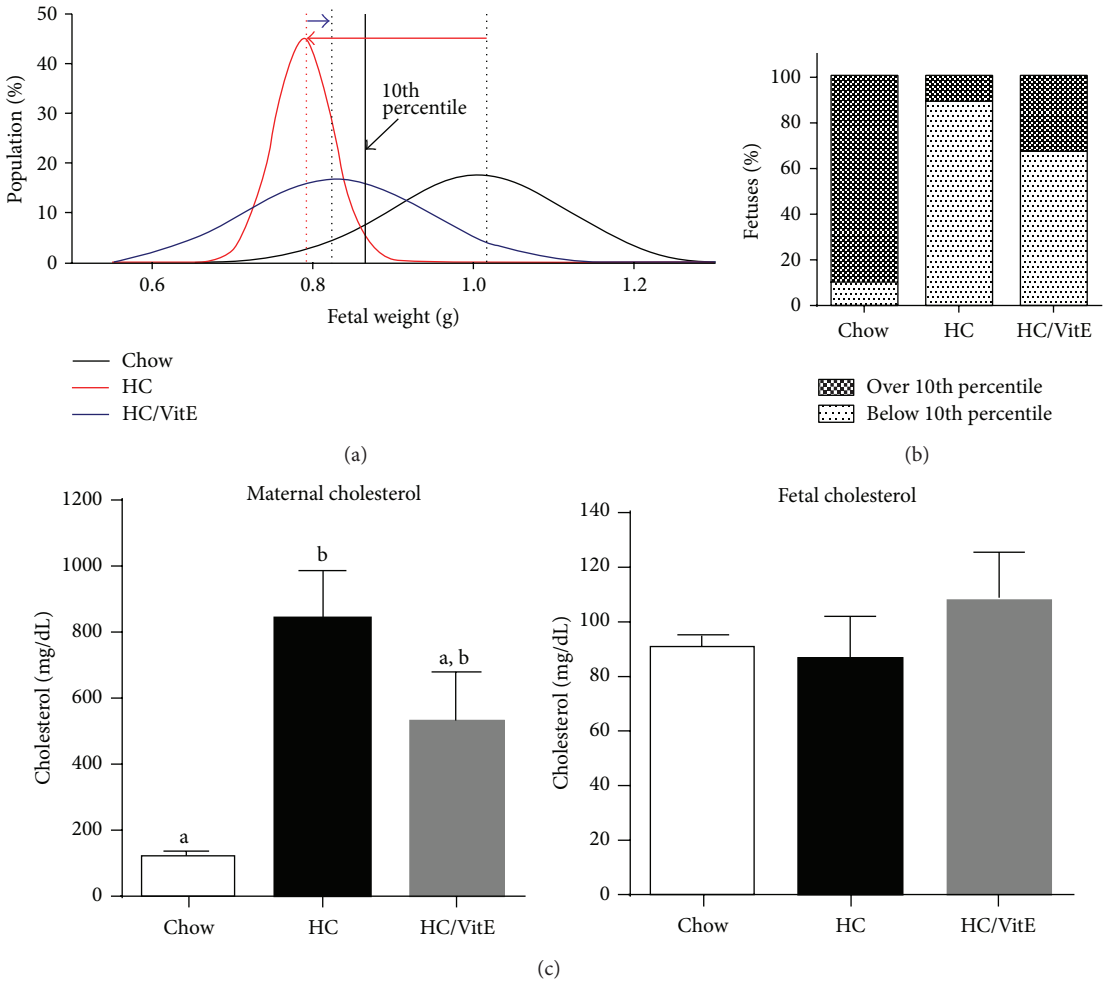

Figure 4: Effect of vitamin E supplementation on fetal growth restriction induced by maternal hypercholesterolemia in HC-fed LDLR KO mice. (a) Gauss normal curves for E18.5 fetal weights in females fed with chow (black, $n = 58$ fetuses, $r^2 = 0.86$), HC (red, $n = 47$ fetuses, $r^2 = 0.91$), or HC/VitE (blue, $n = 38$ fetuses, $r^2 = 0.89$) diets. The black continuous line represents the 10th percentile of WT fetal weight (865 mg); dashed lines indicate the mean weight for each group. The difference between weights from the HC- and chow-fed groups and HC- and HC/VitE-fed groups is represented by red and blue arrows, respectively. (b) Percentage of growth restricted fetuses showing an increase in fetuses over the 10th precentile in the HC/VitE group (32%) compared to the HC group (10%). (c) Left panel: high plasma cholesterol in HC-fed females was partially reduced by vitamin E supplementation (different lettering indicates significance at $P < 0.05$, one-way ANOVA, $n \geq 3$ females). Right panel: fetal plasma cholesterol was similar in females receiving chow, HC, or HC/VitE diets ($n \geq 3$ pools from 2 fetuses).

3. Materials and Methods

3.1. Animals. LDLR KO mice (B6.129S7-Ldlrtm1Her/J) were originally purchased from The Jackson Laboratory. Animals were housed in a temperature- and light-controlled room. Protocols were conducted in agreement with the National Research Council (NRC) Publication Guide for the Care and Use of Laboratory Animals (8th edition, 2011, National Academy of Sciences, USA). These studies were approved by the Ethics Committee for Animal Welfare from the School of Medicine of the Pontifical Catholic University of Chile. Six-week-old LDLR KO females were fed with a standard *chow diet* [0.02% cholesterol (Prolab RMH3000, Labdiet)], *high-cholesterol diet* [0.625% cholesterol 1 : 1 mix of chow diet and 57BB diet atherogenic diet], or a *high-cholesterol and vitamin E-supplemented diet* [containing 0.625% cholesterol and 1,000 UI of vitamin E/kg (1 : 1 mix of the 57BB atherogenic diet and Prolab 5P00 diet containing 2,000 IU vitamin E/kg, Labdiet)]. After 3 weeks receiving each diet, two females were housed with one male and the presence of vaginal plug was checked daily within the first hour of the light cycle. When the vaginal plug was detected, embryonic day 0.5 (E0.5) was recorded. All diets used before conception were continued during pregnancy until the collection of the embryos, except in the protocol where the impact of periconceptional maternal hypercholesterolemia was analyzed, in which females were fed with the HC diet until 5.5 and then received standard chow until embryo collection.

At E12.5, E15.5, or E18.5 pregnant dams were deeply anesthetized with a mix of ketamine: xylazine (100 : 10 mg per kg body weight) and blood was collected from the vena cava with a heparinized syringe. The dams were euthanized and

the embryos and extraembryonic tissues were retrieved and kept on ice. Each embryo and placenta were weighed before fixation in Bouin's fixative. Yolk sacs were used for sexing by PCR. At E18.5, fetal blood was collected after decapitation and blood pooled from 3 fetuses was used for cholesterol determinations.

3.2. Fetal Sex Determination by PCR. Individual sexing of embryos was performed by PCR as described elsewhere [24]. The following primers, F: 5′-CCGCTGCCAAATTCTTTGG-3′ and R: 5′-TGAAGCTTTTGGCTTTGAG-3′, were used to amplify bands of different sizes corresponding to the *smcx/y* gene in X and Y chromosomes. Samples were amplified using the GoTaq (Promega) PCR kit, following manufacturer instructions with the following PCR program: 2 minutes at 95°C, 35 cycles of 30 s at 95°C, 30 s at 58°C, 30 s at 72°C, and a final extension of 10 minutes at 72°C. Amplicons were resolved in a 2% agarose gel and the presence of one or two bands indicated female or male, respectively.

3.3. Cholesterol Determination. Plasma cholesterol concentration was determined using a standard enzymatic method reported previously [25]. Samples were incubated for 30 minutes at 37°C with 0.5 M Tris, pH 7.6, 50 mM phenol, 50 mM 4-chlorophenol, 1% Triton X-100, 0.37% sodium cholate, 0.04% 4-aminoantipyrine, 0.35 U/mL cholesterol esterase, 0.1 U/mL cholesterol oxidase, 1 U/mL peroxidase, and 490 nm absorbance.

3.4. Lipoprotein Chromatographic Separation. Plasma samples were subjected to chromatographic separation using a Superose-6 molecular exclusion column (GE Healthcare Life Sciences) and elution buffer (150 mM NaCl, 1 mM EDTA, pH 7.8) at a constant flux of 9 ml/h. Cholesterol content in each fraction was determined as described above.

3.5. Histological and Immunohistochemical Analyses. Bouin-fixed tissues were dehydrated and embedded in paraffin. Tissue sections (8 μm) were stained with hematoxylin and eosin or used for immunohistochemical procedures. For immunolocalization, sections were subjected to antigen retrieval using hot citrate and then incubated with commercial rabbit antibodies against smooth muscle actin (1 : 200, Sigma) or cytokeratin (1 : 200, Dako). After extensive washing, sections were incubated with appropriate secondary antibodies coupled to horseradish peroxidase (1 : 800, Sigma) and revealed with diaminobenzidine (Sigma). Negative controls included slides where the primary antibody was replaced by nonimmune rabbit serum.

3.6. Identification of Atherosclerotic Lesions. Whole hearts from pregnant dams were fixed and processed for immunohistochemical detection of smooth muscle actin as described above. Only sections that passed through the aortic root were considered, as turbulent flow promotes plaque formation in this part of the aorta. Foam cell infiltrates between the smooth muscle layer and the endothelium and positive staining of the endothelial cells indicated the presence of early atherosclerotic lesions.

3.7. Morphometric Analyses. Transversal sections of whole placenta containing the central arterial canal were immunostained with the anti-cytokeratin antibody to localize syncytiotrophoblast layers and identify maternal sinuses and fetal vessels. Fetal vessels were identified by the presence of an endothelium and enucleated red blood cells, whereas maternal sinuses were surrounded by syncytiotrophoblasts and contained mature, enucleated red blood cells. The area of all the fetal vessels or maternal sinuses in at least 3 different slides was quantified using ImageJ software (NIH, Bethesda). The occupancy of fetal and maternal areas in placenta was expressed as the mean of area of capillaries or sinuses per mm^2 in the slide.

3.8. Statistical Analyses. Analyses were performed using GraphPad Prism 6 software. Data are presented as mean ± SEM. The significance of the differences between means was evaluated using Student's t-test or ANOVA and Tukey post hoc test. The 10th percentile was calculated as $R = P * (n + 1)/100$, where P is 10 and n is the number of values in the chow group. The significance of the differences between proportions was analyzed using Fisher's exact test. Differences were considered significant at $P < 0.05$.

4. Discussion

In the present study we used a mouse model of pregnancy that exhibited high levels of plasma cholesterol and atherosclerosis, independent of overweight. Pregnancies in hypercholesterolemic and atherosclerotic females were characterized by normal numbers of fetuses and occasional resorptions, discarding a gross effect of this pathological metabolic condition on inhibition of implantation or abortion. The analysis of placental weight and morphology at two stages of late pregnancy, E15.5 and E18.5, indicated that the placenta grew and differentiated normally in HC-fed females. The labyrinth developed normally as well, as maternal and fetal vascular areas in this region were unaffected by maternal HC-feeding. Future studies will allow determining whether maternal hypercholesterolemia and atherosclerosis induce functional changes in placental nutrient and gas exchange.

A significant reduction in body weight was observed in fetuses from midpregnancy to preterm. This fetal growth restriction was already present as early as E12.5, a developmental stage when placenta achieves maturation in mice and when fetuses start growing exponentially. Whereas newborns or term fetuses from hypercholesterolemic rabbits and mice were previously shown to exhibit lower weights than control animals [13, 24], to our knowledge this is the first evidence describing fetal growth restriction associated with high maternal cholesterol levels during early stages of gestation.

As mentioned previously, preimplantation embryos can be susceptible to environmental stressors. Evidence from different experimental animal models showed that the exposure of embryos before implantation to adverse developmental

conditions (i.e., undernutrition, overnutrition, and inflammation), both *in vitro* and *in vivo*, can not only hinder the embryonic quality and implantation success but also exert more subtle effects that are expressed later during intrauterine development or even during adulthood [26–28]. Programming of disease can occur as early as in oocytes and zygotes, by mechanisms including the aberrant methylation of genes involved in body homeostasis [29, 30], defective mitochondrial function [28, 31], and the inefficient generation of appropriately sized stem-cell lineages due to abnormal proliferation [32]. Our studies showed that exposure to maternal hypercholesterolemia exclusively before implantation was harmless to embryo growth, as LDLR KO females fed with the proatherogenic HC diet periconceptionally did not exhibit fetal growth restriction. However, the existence of possible effects of periconceptional HC-feeding on other embryonic parameters besides embryonic growth cannot be ruled out from our analyses.

Epidemiological studies first described in the late 1990s showed that intrauterine growth is more affected by maternal undernutrition in boys than in girls [33]. It has been hypothesized that male and female human fetuses adopt different strategies to overcome low nutrient availability; for example, male fetuses trade off visceral development *in utero* to protect somatic and brain growth, an adaptive beneficial response which can promote chronic metabolic diseases during adulthood [34]. Several gender-specific differences in fetal responses to other adverse maternal conditions have been reported in animal models [35–37]. However, in our studies fetal growth restriction induced by HC-diet maternal hypercholesterolemia and atherosclerosis was not gender-specific, as both male and female fetuses were lighter than fetuses from control chow-fed females.

Both maternal hypercholesterolemia and intrauterine growth restriction in humans have been independently linked to the early onset of subclinical atherosclerosis and a higher predisposition to cardiovascular disease in their offspring [21, 38]. However, a direct link between these two conditions is not clear because, on the one hand, women carrying fetuses with intrauterine growth restriction have lower, and not higher, total cholesterol and HDL-cholesterol concentrations [39] and, on the other hand, no significant differences have been found in newborn weights from normal and hypercholesterolemic pregnancies [6, 40].

Oxidative stress is considered one of the potential pathological insults given by maternal hypercholesterolemia on placenta and fetuses. Indeed, plasma from hypercholesterolemic women shows an increase in markers in both placenta and fetus [6]. Interestingly, oxidized LDL particles accumulate within the human term placenta in pregnancies undergoing early onset intrauterine growth restriction [41]. In animal experimental models, maternal antioxidant treatment has shown to enhance fetal growth and/or prevent growth restriction [21, 42]. In our studies, vitamin E antioxidant supplementation was not sufficient to increase the mean fetal weight in HC-fed females. However, the fact that this vitamin significantly reduced the proportion of growth restricted fetuses in HC-fed dams suggested that oxidative stress could be one of the mechanisms implicated in the pathogeny of fetal growth restriction in this model. Nonantioxidant vitamin E activities may also be implicated in the beneficial effect of this vitamin on fetal growth in HC-fed females. The beneficial effect of vitamin E on fetal growth could be mediated by the lowering of cholesterol. It has been shown recently that vitamin E can reduce cholesterol biosynthesis *in vitro* [43]. As well, vitamin E supplementation of rats and hamsters fed with atherogenic diets can reduce their plasma cholesterol levels [44, 45]. Given the trend in lowering plasma cholesterol observed in vitamin E-supplemented HC-fed LDLR KO females, further studies will be pursued to determine the possible beneficial effects of this vitamin on the maternal cholesterol metabolism in hypercholesterolemic mouse pregnancies. On the other hand, vitamin E has been shown to regulate molecular pathways controlling cell proliferation and viability [46] and could promote fetal growth in pregnant mice with hypercholesterolemia and atherosclerosis. Vitamin E can also enhance the release of vasodilator prostanoids from human endothelial cells from the aorta [47] and umbilical cord [48] and could favor placenta-fetal blood flow, increasing nutrient absorption. Although vitamin E has not been successful in reducing the risk of major cardiovascular events in humans [49], its supplementation could still be useful to prevent the fetal programming of atherosclerosis, as observed in the offspring of HC-fed rabbits [21]. Our future studies will determine whether vitamin E maternal supplementation has an impact on fetal programming of atherosclerosis in mouse offspring from HC-fed LDLR KO.

The detection of low birth weight in rabbit and mouse newborns from hypercholesterolemic pregnancies [12, 24], together with the results of this work where early fetal growth was observed in HC-fed LDLR KO dams, is in contrast to studies showing that fetuses from human mothers with high-cholesterol levels exhibit normal birth weights [6, 40]. Species-specific differences in the response of fetal growth to maternal hypercholesterolemia could explain the divergent results in animals and humans. In this regard, although the mice have been a useful experimental model of fetal programming due to the high genetic homogeneity and the possibility to test dietary and pharmacological interventions during pregnancy, their lipoprotein metabolism has fundamental differences with humans [50]. In humans, cholesterol is mainly transported in LDLs whereas in mice cholesterol is carried almost exclusively in HDLs, mainly due to the fact that mice lack cholesterol ester transfer protein (CETP), which transfers cholesterol from HDLs to LDLs. Among the various genetically modified mice that have contributed greatly to progress in the field of dyslipidemia [51], the LDLR KO mice exhibit a lipoprotein profile that is very similar to the human normal profile [52]. However, due to the rapid clearance of LDL cholesterol through the apoE receptor, which is present in mouse and not in human livers, the accumulation of LDL in plasma in LDLR KO mice is mild, and high-cholesterol diets need to be used to induce significant hypercholesterolemia [53]. These differences need to be considered when trying to interpret the results obtained using mice as models of human dyslipidemia.

One limitation of this study is that the atherogenic diet used in the study contains cholic acid, a component commonly added to cholesterol-enriched mouse diets in order to increase cholesterol absorption and induce atherosclerosis. In a recent publication, mice fed with a chow diet supplemented with cholic acid were used to establish a model of intrahepatic cholestasis of pregnancy [54]. Strikingly, in that study cholic acid supplementation was shown to affect lipid biosynthesis and transport in the fetoplacental unit and increase the susceptibility of the offspring to metabolic disease. Thus, the fact that high-cholesterol diets containing cholic acid can induce more than one pathogenic condition in pregnant female mice (e.g., hypercholesterolemia and intrahepatic cholestasis of pregnancy) needs to be kept in mind when interpreting the results in this model, particularly when analyzing fetal programming. Regarding fetal weight, this parameter was not affected by maternal cholic acid supplementation [54], suggesting that the main effect on fetal growth in our study is not due to cholic acid itself.

In summary, this study shows that, in LDL KO mice, diet-induced maternal hypercholesterolemia and atherosclerosis during pregnancy can negatively impact fetal growth. Fetal growth restriction associated with this maternal condition is characterized by an early onset and similar prevalence in both sexes. Vitamin E dietary supplementation has a beneficial effect, preventing growth restriction in a significant proportion of fetuses from HC-fed mice. Further studies in this mouse model will allow understanding the mechanisms explaining fetal growth restriction and the possible prevention of atherosclerosis in the offspring by maternal vitamin E supplementation. This knowledge could be useful in designing strategies aimed at preventing or reducing cardiovascular disease prenatally.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

DNA Damage and Its Cellular Response in Mother and Fetus Exposed to Hyperglycemic Environment

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The increased production of reactive oxygen species (ROS) plays a key role in pathogenesis of diabetic complications. ROS are generated by exogenous and endogenous factors such as during hyperglycemia. When ROS production exceeds the detoxification and scavenging capacity of the cell, oxidative stress ensues. Oxidative stress induces DNA damage and when DNA damage exceeds the cellular capacity to repair it, the accumulation of errors can overwhelm the cell resulting in cell death or fixation of genome mutations that can be transmitted to future cell generations. These mutations can lead to and/or play a role in cancer development. This review aims at (i) understanding the types and consequences of DNA damage during hyperglycemic pregnancy; (ii) identifying the biological role of DNA repair during pregnancy, and (iii) proposing clinical interventions to maintain genome integrity. While hyperglycemia can damage the maternal genetic material, the impact of hyperglycemia on fetal cells is still unclear. DNA repair mechanisms may be important to prevent the deleterious effects of hyperglycemia both in mother and in fetus DNA and, as such, prevent the development of diseases in adulthood. Hence, in clinical practice, maternal glycemic control may represent an important point of intervention to prevent the deleterious effects of maternal hyperglycemia to DNA.

1. Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia resulting from a defect in insulin action and/or production [1]. In pregnancy, hyperglycemia poses a risk to maternal, fetal, and perinatal health [2–4]. Perinatal complications of a diabetic pregnancy include malformations, macrosomia, hypoxia, hypoglycemia, cardiomyopathy, hyperbilirubinemia, and hyperinsulinemia [3, 5–9]. The current literature acknowledges this adverse environment as associated with increased long-term risk for the development of diabetes, obesity, cardiovascular, and malignant diseases (Figure 1) [9–14].

Previous findings by our group have shown that maternal hyperglycemia is also adversely involved in fetal development by changing the placental production of proinflammatory cytokines, that is, TNF-α (tumor necrosis factor alpha) [15, 16]. The cellular redox status may be an important connection between inflammation and adverse perinatal outcomes in hyperglycemic pregnancies [17]. There is considerable evidence that hyperglycemia and inflammation results in the generation of reactive oxygen species (ROS), ultimately leading to increased oxidative stress. In the absence of an appropriate antioxidant response, the system becomes overwhelmed leading to production of reactive molecules that can cause cellular damage and are responsible for the

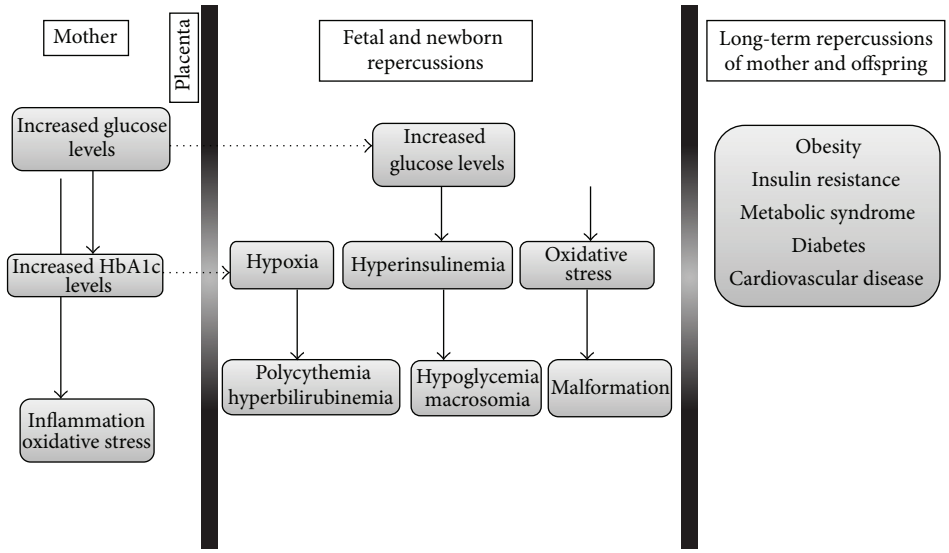

Figure 1: Schematic representation of outcomes classically associated with hyperglycemic pregnancies. The representation does not show all possible relationships between the characteristics that are depicted. Adapted from Metzger et al. [75], Negrato et al. [11], and Fraser and Lawlor [52].

late complications of diabetes [17, 18]. During pregnancy the placenta is an additional source of ROS generation, contributing to oxidative stress even in normal pregnancies. This is increased in pregnancies complicated by preeclampsia, intrauterine growth restriction, and pregestational diabetes where oxidative and nitrative stress have been clearly documented [19, 20].

Oxidative stress induces protein oxidation, lipid peroxidation, and DNA damage both in mitochondrial and nuclear DNA. Degradation processes can remove lipids and proteins but not DNA, which needs conversely to be repaired. When DNA damage exceeds the cellular capacity to repair it, the accumulation of errors can overwhelm the cell and result in cell death or the incorporation of genome mutations that can be transmitted to future cell generations if they occur in germ cells (Figure 2). In addition, mutations in somatic cells can promote genome instability and directly lead to various human diseases including cancer, neurological abnormalities, immunodeficiency, and premature aging [21–25].

Considering that hyperglycemia may alter genomic integrity and the consequences of this relationship to maternal and fetus genome is unclear, this review aims at (i) assessing the types and consequences of DNA damage during hyperglycemic pregnancy and lifelong risks, (ii) identifying the biological role of DNA repair during pregnancy, and (iii) proposing clinical interventions to maintain genome integrity.

2. Hyperglycemia-Induced Oxidative Stress and Its Effects on DNA Structure

Hyperglycemia causes many of the major complications of diabetes including nephropathy, retinopathy, neuropathy, and macro- and microvascular damage [1]. To date, there is emerging evidence that oxidative stress significantly contributes to the progression of diabetes and its complications and induces alterations in embryonic and fetal development during pregnancy [18, 26]. Li and collaborators [27] found that mothers with GDM and their newborns had higher levels of 8-Isoprostaglandin F2α (an oxidative stress marker) than control group. Hyperglycemia induces ROS production during such processes as nonenzymatic glycosylation, increased generation of superoxide anion radical by the mitochondrial respiratory chain and the overactivation of NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) [28, 29].

Overproduction of ROS is capable of altering the structure and function of all types of molecules including proteins, membrane lipids, and nucleic acids with serious consequences to cell viability [21, 30]. Different degradation processes can remove oxidized lipids and proteins. DNA, however, has to be repaired or in the case of mitochondrial DNA may even be removed. The latter is intrinsic to the various copies of mitochondrial genome present in each mitochondrion and the fact that many mitochondria populate a cell [21, 31]. ROS are able to induce DNA lesions as abasic sites (AP sites), single strand breaks, and double strand breaks and oxidize DNA bases. All four bases are susceptible to oxidative damage by ROS. However, due to the lower redox potential of guanine this base is more susceptible to oxidation [23, 30, 32]. The oxidized guanine (8-oxodG) has great biological importance as this is a mutagenic lesion that induces G-T transversions. It may also impair DNA replication and transcription and may be an intermediate for other types of lesions in DNA [23, 33].

Substantial evidence suggests that mitochondrial DNA may be more vulnerable than nuclear DNA to certain kinds of damage, in particular, ROS-mediated lesions [31, 34, 35].

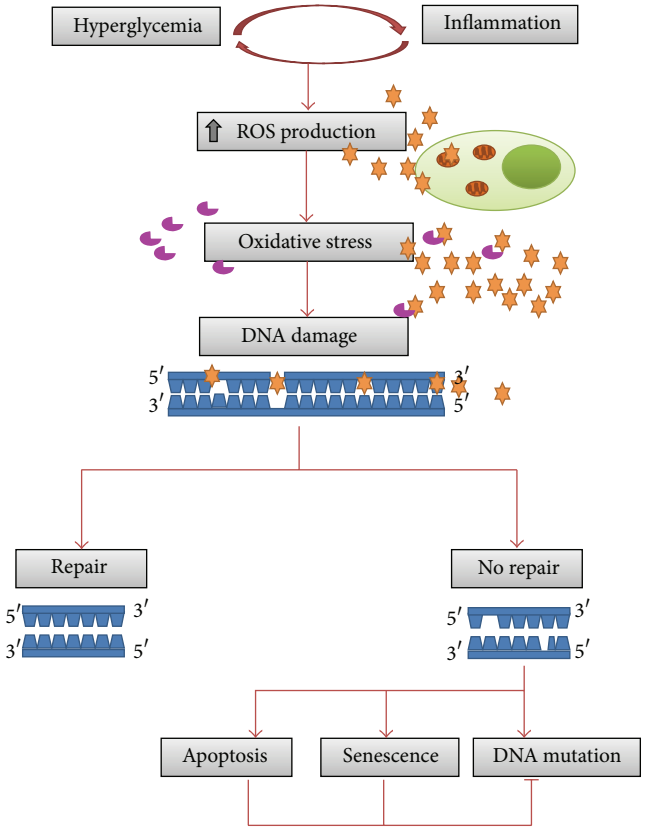

FIGURE 2: Hyperglycemia and inflammation are able to increased ROS production. When ROS production exceeds the detoxification and scavenging capacity of the cell, oxidative stress ensues. Oxidative stress induces DNA damage and when DNA damage exceeds the cellular capacity to repair it, the accumulation of errors can overwhelm the cell resulting in apoptosis, cell senescence, or fixation of genome mutations that will be transmitted to future cell generations. These mutations can lead to and/or play a role in cancer development.

Several reasons may underline this affirmation, including the immediate proximity of mitochondrial DNA to the electron transport chain in the inner mitochondrial membrane, which is the main source of endogenous ROS production. In addition, the repair of mitochondrial DNA lesions occurs only via base excision repair and unlike the nuclear genome, the mitochondrial DNA is not protected by histones [31, 34, 35].

It is important to remember that the genomes of all organisms are constantly being modified by reactive molecules that are produced endogenously, primarily via mitochondrial respiration or by environmental/exogenous physical, chemical, and biological agents including ultraviolet light, ionizing radiation, heavy metals, air pollutants, chemotherapeutic drugs, and inflammatory responses [25, 36].

3. Hyperglycemia, DNA Damage, and Pregnancy: Results of Experimental and Clinical Studies

In nonpregnant context, the relationship between type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), and DNA damage is well established [42–48]. Little is known about DNA damage in pregnancy, especially in pregnancy complicated by pregestational (T1DM or T2DM) or gestational diabetes mellitus (GDM) [7, 37–39, 41, 49].

Experimental studies conducted in our laboratory with streptozotocin-induced diabetic rats showed that the levels of basal DNA damage in leukocyte of mothers with severe diabetes (blood glucose ≥ 300 mg/dL) and their respective fetus was higher when compared with the control group [37, 38]. Subsequently, Lima et al. [7] demonstrated that rats with severe diabetes and their offspring showed higher oxidatively generated DNA damage in leukocyte detected by Fpg (formamidopyrimidine-DNA glycosylase) and endonuclease III-sensitive sites when compared to mild diabetes group (blood glucose levels between 120 and 299 mg/dL). Taken together, these experimental results suggest that the intensity of diabetes is related to the levels of oxidative DNA damage. Thus, hyperglycemia may have repercussions at the DNA level that go beyond the pregnant mother.

In a pilot study, Qiu and collaborators [39] evaluated, in early pregnancy, levels of urinary 8-oxodG trying to determine an association with the risk of GDM development. They observed that the risk for GDM was higher in overweight women with urine 8-oxodG concentrations ≥8.01 ng/creatinine mg (OR = 5.36; 95% Cl 1.33–21.55) when compared with lean women who had 8-oxodG concentrations <8.01 ng/creatinine mg. Interestingly, levels of 8-oxodG in umbilical vein plasma in pregestational and control groups were reported to be similar [40].

Evaluation of telomere length is another way to estimate the stability of the genetic material. Telomeric length and telomerase activity (a reverse transcriptase that limits telomere attrition) were studied in mononuclear cells isolated from umbilical cord blood of pregnant women with pregestational diabetes (T1DM and T2DM) and GDM. No difference was found in cord blood telomere length in pregnancies of women with diabetes compared with control subjects, but higher telomerase activity was observed in Type 1 and GDM groups. The upregulation of telomerase may be a compensatory response to *in utero* oxidatively generated DNA and telomere damage [41].

Previous study demonstrated that telomerase is found in nuclei and mitochondria. Telomerase is able to decrease mitochondrial levels of ROS, especially in mitochondria [34, 50]. Recently, Li and collaborators [27] evaluated the mitochondrial translocation of human telomerase reverse transcriptase (hTERT) in mononuclear cells isolated from umbilical cord blood during pregnancies complicated by GDM with confirmed oxidative stress. They found that the ratio of mitochondrial/nuclei hTERT was increased significantly in the newborn of GDM mothers, suggesting that mitochondrial hTERT in cord blood mononuclear cells may have a protective effect on neonatal mitochondrial DNA in GDM pregnancies. The authors concluded that this dynamic translocation could be an *in utero* adaptive response of a fetus that is suffering from elevated oxidative stress and could help our understanding of the roles of oxidative stress in fetal programming.

Table 1: Maternal and fetal DNA integrity in hyperglycemic environment.

Reference	Study type	Type of diabetes	Sample	Evaluation	Main results
[37]	Experimental	Severe	Maternal leukocytes	Comet assay	Basal DNA damage in severe diabetes
[38]	Experimental	Severe	Fetal leukocytes	Comet assay	Basal DNA damage in severe diabetes
[7]	Experimental	Mild and severe	Maternal and fetal leukocytes	Comet Assay with Fpg and Endo III enzymes*	Oxidative DNA damage in severe diabetes
[39]	Clinical	GDM	Maternal urine	8-oxodG levels	Elevated in early pregnancy that results in GDM
[40]	Clinical	Pregestational	Umbilical vein plasma	8-oxodG levels	No difference
[41]	Clinical	Pregestational and GDM	Cord blood mononuclear cell	Telomere length and telomerase activity	Telomerase activity higher in cord blood from T1DM and GDM
[27]	Clinical	GDM	Cord blood mononuclear cells	Mitochondrial translocation of hTERT	Increased mitochondrial hTERT levels in GDM

GDM: gestational diabetes mellitus; hTERT: human telomerase reverse transcriptase. *The endonuclease III (Endo III) and formamidopyrimidine-DNA glycosylase (FPG) are enzymes used to detect oxidative DNA damage.

A few years ago, epigenetic processes have been suggested as a link between maternal pregnancy diabetes and long-term offspring outcomes. Epigenetic modifications, such as DNA methylation, regulate gene expression without altering the DNA sequence. These alterations occur in response to environmental stimuli [51–54]. Recent studies compared the levels of global methylation in the placenta and umbilical cord blood among women with and without gestational diabetes, preeclampsia, and obesity. They found that the mother's metabolic problems during pregnancy may influence the epigenome in the offspring [51]. del Rosario et al. [54] found that epigenetic changes (DNA methylation) may increase the risk of type 2 diabetes; studies support this association but research in this area is still inconclusive [52].

In summary, the results found in the literature indicate that maternal and fetal cells, especially mononuclear cells of blood, respond differently to the hyperglycemic environment (Table 1). While it is clear that hyperglycemia can damage the maternal genetic material, the results in umbilical cord blood (fetal cells) remain unclear. It seems that umbilical cord blood cells have more efficient mechanisms working to protect the genome. Future investigations on the mechanisms involved in genome protection in fetal cells as well as the role of epigenetic changes may shed new light on the outcome on offspring born from women with gestational diabetes.

4. DNA Repair Mechanisms Are Important to Maintain the Genetic Stability

To maintain genetic stability organisms possess cellular mechanisms collectively termed the DNA damage response (DDR) to detect DNA lesions and signal their presence and promote their repair. Cells with DDR defects display higher sensitivity toward DNA damaging agents and many such defects lead to mutagenesis, cytotoxicity, cell death, and disease. In fact, genomic instability and defects in DDR are known to play a role in disease processes such as carcinogenesis, neurodegenerative disorders, immune deficiencies, infertility, aging, cardiovascular disease, and metabolic syndrome [30, 55]. In this session we will focus on DNA repair.

To repair different types of DNA lesions the cell counts on a variety of proteins that presumably undergo crosstalk to form a network for protection of the cellular genome. [25, 56–59].

Nucleotide excision repair (NER), mismatch repair (MMR), and base excision repair (BER) have been implicated in the repair of ROS-induced lesions in DNA. However, BER is the main mechanism involved in the removal of these lesions in nuclear DNA and is the unique mechanism demonstrated for mitochondria damaged DNA [31, 34, 35, 60]. BER predominantly repairs oxidized bases, AP sites, and single strand breaks. In general, BER initiates with the action of a DNA glycosylase that is able to remove the damaged base resulting in an AP site. The AP site is then cleaved by the AP-endonuclease, allowing the DNA polymerase (β in the nucleus or gamma in the mitochondria) to synthesize the repair patch. The latter is relegated based on DNA ligase III activity [60].

5. The Possible Role of DNA Repair during Pregnancy and Diabetes Disease

Studies have demonstrated the importance of DNA repair genes in pregnancy and perinatal development. Patients with mutations in XPD (Xeroderma pigmentosum D) and GTF2H5 (general transcription factor IIH, polypeptide 5), genes involved in the NER pathway and in transcription-couple repair, have the DNA repair diseases: trichothiodystrophy (TTD), xeroderma pigmentosum (XP), Cockayne syndrome (CS), cerebro-ocular facial syndrome (COFS), or a combination [24, 61, 62]. The pregnancies in which the fetus had TTD were at significantly increased risk of preeclampsia, HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome, and elevated mid-trimester maternal serum human chorionic gonadotropin levels. The

affected fetus had decreased fetal movement and preterm delivery with higher index of small for gestational age fetus [63]. The authors hypothesized that mutations observed in TTD patients affect placental development. Two years later, the same group revealed that only a specific subset of XPD mutations, which lead to TTD but are unrelated to XP, results in higher risk to develop preeclampsia and other gestational complications [64]. A functional polymorphism (199 Arg-399Gln) in XRCC1 (X-ray repair complementing defective repair in Chinese hamster cells 1), a gene involved in the BER pathway, showed higher frequency among patients with preeclampsia (OR 1.65; 95% CI 1.23–2.19) in an Iranian population [65]. However, this polymorphism was not associated with T2DM in a Polish population [66].

DNA repair was evaluated in lymphocytes of nonpregnant patients with T1DM and T2DM [45, 46]. The results of Blasiak et al. [45] suggest that T2DM may be associated not only with elevated levels of oxidative DNA damage but also with decreased efficacy of DNA repair. In an elegant study Pácal et al. [46] compared DNA damage and repair in lymphocytes of T1DM children, T1DM adults, and T2DM adults. The T2DM diabetics exhibited a significant increase in DNA damage and decreased DNA repair capacity when compared with T1DM (both children and adults). T1DM children displayed a significant decrease of DNA damage and increase in DNA repair when compared with diabetic adults (both T2DM and T1DM). These findings indicate significant age- and DM type-related changes of DNA damage and repair capacity in diabetic subjects.

In summary, the data available suggest that DNA repair mechanisms are involved in the long-term consequences of diabetes in T1DM and T2DM subjects. In pregnancy, DNA repair genes may affect the harmony of maternal-fetal interface resulting in adverse perinatal results.

6. Diabetes and Cancer

Epidemiologic evidence suggests that diabetic patients are at significantly higher risk for many types of cancer. T2DM, GDM, and cancer share many risks factors but potential biological links between the two diseases are unclear [67, 68]. Meta-analyses have reported an increased risk of liver, pancreatic, renal, endometrial, colorectal, bladder, and breast cancer as well as an increase in the incidence of non-Hodgkin lymphoma in T2DM subjects [68]. For those with T2DM compared with those without diabetes, the greatest increase in risk is for hepatocellular carcinoma (RR 2.5; 95% CI 1.8–3.5), with the relative risk for cancer at other sites being between 1.18 (95% CI 1.05–1.32) for breast cancer and 2.22 (95% CI 1.8–2.74) for endometrial cancer in those with diabetes [68, 69]. A prospective cohort study with 37.926 women in Jerusalem observed no cases of pancreatic cancer in the women with T1DM; however, women with a history of GDM showed a relative risk of pancreatic cancer of 7.1 (95% confidence interval 2.8–18.0) [70]. Similar results were observed with a late cohort in Israel [71]. In addition to the relationship between GDM and pancreatic cancer, the authors observed an increased risk of hematologic malignancies like non-Hodgkin's lymphoma, Hodgkin's lymphoma, and acute myeloid leukemia in the same population [71]. A relationship between GDM and breast cancer was found in a New Zealand population, but when studying the U.S. population this association was not observed [72, 73].

Experts assembled jointly by the American Diabetes Association (ADA) and the American Cancer Society (ACS) reviewed the possible biological links between diabetes and cancer risk. They suggested that diabetes may influence the neoplastic process by several mechanisms, including hyperinsulinemia, hyperglycemia, or chronic inflammation without reference to DNA damage and repair [67]. However, the increase in DNA damage and decrease in DNA repair observed in T2DM subjects may provide a new link between diabetes and cancer [45, 60, 74].

7. Proposed Clinical Intervention Strategy for Maintenance of Genomic Integrity

7.1. Control of Maternal Hyperglycemia. Maternal hyperglycemia is able to induce fetal hyperglycemia [1, 4] (Figure 1), increase the release of proinflammatory cytokines [15, 16], and ROS production [17, 18] (Figure 2). Thus, it appears that maternal glycemic control during hyperglycemic pregnancies is an old and safe strategy to assure maintenance of genomic integrity. Clinical studies have already demonstrated the benefits of maternal glycemic control during pregnancy and how to maintain optimal glucose levels without gestational risk [75, 76].

Nonpregnant adults with diabetes and pregnant women with GDM or pregestational diabetes (T1DM or T2DM) presented different glycemic recommendations [1]. During pregnancy, the glycemic limits are stricter than in nonpregnant state to prevent alteration in both maternal and fetal health [1, 75, 76]. Based on recommendations from the Fifth International Workshop-Conference on Gestational Diabetes Mellitus [77] and ADA's statement [1] it is important to maintain maternal capillary glucose concentrations at <95 mg/dL (<5.3 mmol/L) in the fasting state, <140 mg/dL (<7.8 mmol/L) at 1 h, and <120 mg/dL (<6.7 mmol/L) 2 h after starting the meal. For women with overt diabetes who become pregnant, the optimal glycemic goals are premeal, bedtime, and overnight glucose between 60 and 99 mg/dL (3.3–5.4 mmol/L) and peak postprandial glucose between 100 and 129 mg/dL (5.4–7.1 mmol/L) and HbA1C of 6.0% [78].

Diet therapy, control of weight gain, and increasing physical activity are the standard treatment of GDM [77]. Insulin administration is only performed for pregnant women who fail to maintain glycemic goals as well as to the ones who show signs of excessive fetal growth or overt diabetes. It is recommended that insulin administration be individualized to achieve the glycemic goals stated [77]. During the last decade, there was an increased interest in the use of oral antihyperglycemic agents as an alternative to insulin in achieving good glycemic control. However, the results are inconclusive [79, 80].

7.2. Antioxidant Supplementation during Pregnancy. Antioxidant supplementation is a questionable strategy during

pregnancy. The effects of vitamin C supplementation, alone or in combination with other supplements, have been evaluated on pregnancy outcomes. No difference was seen in the risk of stillbirth, perinatal death, birth weight, or intrauterine growth restriction between women supplemented with vitamin C alone or in combination with other supplements and placebo. In fact, women supplemented with vitamin C alone or combined with other supplements were at increased risk of giving preterm birth [81]. The same researchers also determined the effectiveness and safety of any vitamin supplementation on the risk of spontaneous miscarriage, maternal adverse outcomes, and fetal and infant adverse outcomes. The results indicated that vitamin supplements, alone or in combination, prior to pregnancy or in early pregnancy, did not prevent miscarriage or stillbirth. However, it was found that women taking vitamin supplements were less likely to develop preeclampsia while more likely to have multiple pregnancies [82, 83]. Mothers that have taken antioxidant supplementation during pregnancy had decreased frequency of micronucleus (a test used to quantify chromosomal damage) in peripheral blood mononuclear cells prior to and after hydrogen peroxide exposure. The additional antioxidants intake during pregnancy was also beneficial to reduce the frequency of micronucleus after hydrogen peroxide exposure in cord blood cells. The data demonstrated a positive effect of antioxidant supplementation on micronucleus frequency [84]. Experimental results in a model of diabetic pregnancy indicate that high doses of dietary antioxidants were need to normalize the development of offspring. However, treatment with such high doses may also have adverse effects in nondiabetic pregnancy [85].

It is clear based on the above findings that results about antioxidant supplementation during pregnancy are still inconclusive, and little is known about their impact at the DNA level. Despite this fact, taken together the data support the notion that maternal glycemic control is a good and safe plan to reduce the factors associated to genomic instability in hyperglycemic pregnancy.

8. Conclusions

Although it is clear that hyperglycemia can damage the maternal genetic material, the results obtained for cord blood are not yet clear. The data seem to support the hypothesis that umbilical cord blood cells have more efficient mechanisms to protect the genome than the mother's cells. DNA repair may be thus considered an important mechanism to prevent the deleterious effects of hyperglycemia in the genetic material. However, functional studies demonstrating the ability of DNA repair mechanisms in dealing with insults resulting from hyperglycemia during pregnancy need to be developed. For the time being, the control of maternal hyperglycemia seems a safe and important strategy to prevent the deleterious effects of hyperglycemia on maternal and potentially fetal DNA.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors equally participated in the development of this paper. All authors also read and approved the final paper.

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Research Article

Effect of Hypoxia on the Calcium and Magnesium Content, Lipid Peroxidation Level, and Ca^{2+}-ATPase Activity of Syncytiotrophoblast Plasma Membranes from Placental Explants

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In the current study the possible relationship between the Ca^{2+}/Mg^{2+} ratio of human syncytiotrophoblast plasma membranes and their lipid peroxidation and Ca^{2+}-ATPase activity was determined. Syncytiotrophoblast plasma membranes of placental explants cultured under hypoxia increased their lipid peroxidation and Ca^{2+} content, diminished their Ca^{2+}-ATPase activity, and kept their Mg^{2+} content unchanged. Membranes preincubated with different concentrations of Ca^{2+} increased their Ca^{2+} content without changes in their Mg^{2+} content. There is a direct relationship between Ca^{2+} content and lipid peroxidation of the membranes, as well as an inverse relationship between their Ca^{2+} content and Ca^{2+}-ATPase activity. On the contrary, preincubation of membranes with different concentrations of Mg^{2+} showed a higher Mg^{2+} content without changing their lipid peroxidation and Ca^{2+}-ATPase activity. Explants cultured under hypoxia in the presence of 4 mM $MgSO_4$ showed similar values of lipid peroxidation and Ca^{2+}-ATPase activity of their membranes compared to those of explants cultured under normoxia. Increased Ca^{2+} content of the membranes by interacting with negatively charged phospholipids could result in destabilizing effects of the membrane structure, exposing hydrocarbon chains of fatty acids to the action of free radicals. Mg^{2+} might exert a stabilizing effect of the membranes, avoiding their exposure to free radicals.

1. Introduction

Preeclampsia is a clinical syndrome characterized by vascular endothelial damage, hypertension, proteinuria, edema, generalized arteriolar vasospasm, and a state of oxidative stress [1]. One of the primary events in the pathophysiology of preeclampsia is reduced trophoblast invasion, which results in deficient conversion of the uterine spiral arteries during placentation [2]. This event, widely accepted as a key feature in the pathophysiology of preeclampsia, leads to a reduced placental perfusion and therefore to hypoxia, which has been linked to oxidative stress [3], a condition occurring when the body's antioxidant defenses are overwhelmed by the generation of reactive oxygen species (ROS). ROS can promote lipid peroxidation and vascular endothelial damage, which are commonly seen with preeclampsia [4, 5]. The placenta is considered to be the principal source of ROS in preeclamptic women, but maternal leukocytes and endothelium are also likely contributors [6].

Interaction of ROS with lipids, proteins, and carbohydrates of the plasma membranes can increase their level of lipid peroxidation, thus decreasing their fluidity and the activity of membrane enzymes [7]. Particularly, the plasma membrane Ca^{2+}-ATPase (PMCA) is dependent upon lipid-protein interactions, and its activity is greatly affected by the level of lipid peroxidation in its environment [8–12].

Incubation of placental tissue under hypoxic conditions, which occurs with preeclampsia, induces oxidative stress, the release of proinflammatory cytokines, and trophoblast cell death [13–16]. Consequently, incubation of placental villous fragments in hypoxia has been used as placental model of preeclampsia and also serves as a good source of placental lipid peroxides [17].

Interestingly, tissues maintained under periods of hypoxia increase their intracellular calcium [18], an effect that is worsened when the activity of the PMCA is decreased [17]. Higher intracellular calcium concentrations could increase the calcium content of the cell membranes, and then the ion, acting as an amplifier, could enhance their mechanisms of lipid peroxidation. It has been reported that calcium is able to alter the stability of macrophage plasma membranes, making them more sensitive to photoperoxidation by UV light [19].

Kisters and collaborators [20] observed a lower content of Mg^{2+} and a higher content of Ca^{2+} in membranes of red blood cells from preeclamptic pregnant women, as compared with the red blood cells of uncomplicated pregnant women. These alterations could lead to interactions of these ions with membrane components, resulting in modifications of the lipid microenvironment that interacts with membrane transporters. It is well known that the interaction of metal cations and lipids has a significant impact on membrane properties, such as the area per lipid or chain ordering [21].

In the current study, we incubated syncytiotrophoblast plasma membranes with different concentrations of $CaCl_2$ and $MgSO_4$, in order to modify their Ca^{2+}/Mg^{2+} ratio. In addition, syncytiotrophoblast plasma membranes were prepared from placental explants cultured under either normoxic or hypoxic conditions, in order to evaluate possible changes in the Ca^{2+}/Mg^{2+} ratio of their cell membranes. The results were used to try to establish a relationship between the Ca^{2+}/Mg^{2+} ratio, the lipid peroxidation, and the PMCA activity of the syncytiotrophoblast plasma membranes.

2. Materials and Methods

2.1. Placenta Collection. Term placentas obtained from uncomplicated (normal) pregnant women were collected immediately after delivery from the "Concepción Palacios" Maternity Hospital, Caracas, Venezuela, and transported to our laboratory on ice. All the women participated in this study in accordance with the ethical standards established by the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of "Concepción Palacios" Maternity and the Bioethics Committee of IVIC and all women gave signed informed consent. Patients were followed from the time of admission and then before and after delivery. All of the pregnant women enrolled in the study were nulliparous, gave birth by vaginal delivery, belonged to urban population of Caracas, and had similar demographic backgrounds. They had no history of hypertension or proteinuria during the pregnancy and gestational age was estimated from the date of the last menstrual period and confirmed by ultrasonography. The characteristic averages of the participating pregnant women are shown in Table 1.

Table 1: Clinical data from uncomplicated pregnant women.

	Averages
Number of pregnant women	10
Maternal age (yr)	21.6 ± 3.7
Percent nulliparous	100
Mean blood pressure at delivery (mmHg)	83.5 ± 3.3
Gestational weeks at delivery	39.9 ± 0.7
Birth weight (g)	2973 ± 156
Placental weight (g)	522 ± 37
Vaginal delivery (%)	100

Data are shown as mean ± S.E. Dichotomous variables are given as a percentage.

Criteria for exclusion from the study were a history of hypertension, diabetes, Ca^{2+} metabolism disorders or any other chronic medical illness, or over 1 g of supplemental Ca^{2+} per day during the pregnancy.

2.2. Explant Culture. After removal of the chorionic plate and about 0.25 cm of decidua, explants were prepared using only tissue from the intermediate region of the placenta. In brief, randomly sampled villous tissue fragments, of roughly 0.5 cm × 0.5 cm, were cleaned of large vessels and blood clots, rinsed 5 times in cold sterile saline, placed in 12-well plates (Nunclon TM Surface) containing 2 mL of DMEM-F12 medium and 10% fetal calf serum as culture medium, and enriched by addition of crystalline penicillin 100,000 UI/mL, gentamicin 48 μg/mL, and amphotericin B 3 μg/mL. The preparation of placental explants was carried out on ice at 4°C and completed in approximately 30 min. The explants prepared in this way, without further incubations, were identified as freshly prepared placental explants.

The tissue explants were incubated for 4 h at 37°C in 4 mL medium (DMEM-F12 with 10% FCS) in a sterile CO_2 incubator (Shel Lab Model IR2424) with a gas mixture that composed of 8 percent O_2, +87 percent N_2+5 percent CO_2 (normoxia), with constant gas pressure. The culture medium was then removed and replaced with new culture medium. The explants were divided in two groups: one group was kept under normoxia for 18 h at 37°C and the other group was cultured under hypoxia (2 percent O_2, +93 percent N_2+5 percent CO_2) for 18 h at 37°C. At the end of the culture period, the explants were carefully removed and rinsed 5 times in cold sterile saline. The explants were then used for the isolation of syncytiotrophoblast plasma membranes.

2.3. Preparation of Syncytiotrophoblast Plasma Membranes. The membranes were prepared from placental explants following a method previously described [22, 23]. In brief, the maternal decidua was removed, and the central portion between the maternal and fetal surfaces was used for the preparation. Placental villous tissue (80–100 g) was chopped into small pieces, washed with 0.9% NaCl to remove blood, and filtered through gauze. The purification method involved different steps: differential centrifugation, precipitation of nonmicrovillus membranes with magnesium ions, and a

sucrose gradient step. All solutions were buffered with 20 mM Tris-maleate, pH 7.4. Sucrose gradient preparation: a portion (2-3 mL) of the microvillus-enriched preparation and the basal membrane-enriched preparation was overlaid on the sucrose gradient. The band at the sucrose interface concentrations 37/45% (w/v) corresponds to the microvillus membrane fraction (MVM) and the band at the sucrose interface concentrations 47/52% (w/v) corresponds to the basal membrane fraction (BM). The two fractions were collected, diluted 10-fold with the buffer 20 mM Tris-maleate, pH 7.4, and centrifuged at 110,000 ×g for 30 min. The plasma membranes were resuspended in 300 mM sucrose, 20 mM Tris-Maleate, pH 7.4, and stored at −70°C (freezer). The purity and enrichment of the membrane fractions were determined routinely by assaying for adenylate cyclase/β-adrenergic receptor (by measuring ^{3}H-dihydroalprenolol binding), as well as cytochrome-c oxidase/succinate dehydrogenase and glucose-6-phosphatase and alkaline phosphatase activities [23, 24].

2.4. Preincubation of Syncytiotrophoblast Plasma Membranes with Ca^{2+} and Mg^{2+}. Aliquots of BM and MVM (1 mL of a 1 mg protein/mL suspension) were preincubated for 24 h at 0–4°C with a solution of 150 mM NaCl and, according to the experimental design, different concentrations of Ca^{2+} (as $CaCl_2$) and Mg^{2+} (as $MgSO_4$), in order to modify their Ca^{2+} and Mg^{2+} content. At the end of the preincubation period, the suspension was diluted with a solution containing 250 mM sucrose and 10 mM Tris-Hepes (pH 7.2) and washed 6 times by centrifugation-suspension at 47,500 ×g in order to remove traces of Ca^{2+} and Mg^{2+}. The membranes were resuspended with the same washing solution (0.8 mg protein/mL, final concentration) and kept at −70°C until use. The membranes were used to determine their PMCA activity, TBARS, and Ca^{2+} and Mg^{2+} content.

2.5. Determination of Ca^{2+} and Mg^{2+} Contents of Syncytiotrophoblast Plasma Membranes. Red blood cell ghosts were prepared with a modification of a previously described method [25]. Aliquots of BM and MVM were washed three times by centrifugation-resuspension at 47,500 ×g for 45 min. The membrane pellet was resuspended with a solution containing 250 mM sucrose and 10 mM Tris-Hepes (pH 7.2) and the protein concentration was adjusted to 1.5 mg/mL. A 200 μL aliquot of the membrane suspension was mixed with 500 μL of 70% nitric acid and placed in a heating block (Fisher Scientific Dry Bath incubator) at 80°C for 24 h. After 24 h digestion, 200 μL of 60% perchloric acid was added, and the digestion was continued for 24 h at 80°C. Finally, the whole mixture was suspended with 2 mL of MQ water. The Ca^{2+} and Mg^{2+} contents of the sample were measured by inductively coupled plasma spectroscopy (Perkin Elmer Optima 3000 DV ICP system).

2.6. PMCA Activity. The ATPase activity was determined by measuring the quantity of inorganic phosphate liberated from the hydrolysis of ATP, following a modification of the method described elsewhere [26]. Briefly, 180 μL of the incubation medium was preincubated for 2 min at 37°C, and the reaction was started by the addition of 20 μL of membrane suspension. After 10 min of incubation, the reaction was stopped by the addition of 300 μL of a cold solution containing 2.85% ascorbic acid, 1.76% HCl, 0.48% ammonium molybdate, and 2.85% SDS. The samples were shaken and kept at 0°C for 10 min. Then, 500 μL of a solution of 2% sodium citrate, 2% sodium arsenite, and 2% glacial acetic acid was added to each tube. The tubes were shaken and then incubated for 10 min at 37°C. The absorbance of each tube was determined in a Sunrise (Tecan) spectrophotometer at 705 nm. The PMCA activity was calculated as the difference in the phosphate liberated in a medium containing 250 mM sucrose, 5 mM ATP, 5 mM $MgCl_2$, 1 mM ouabain, 2 mM EGTA, 2 mM EDTA, 30 mM Tris-HCl (pH 7.2 at 37°C), 55 mM KCl, 2 μg/mL calmodulin, 1 mM thapsigargin, and 2 μM free calcium, minus the one liberated in the same medium, but in the absence of calcium. A blank control was run in parallel under the same conditions, except for the fact that the membrane suspension was added only after the inclusion of ascorbic acid solution. The ATPase activity was expressed as nmol Pi/mg protein per min, after subtraction of the value obtained with the blank. The protein concentrations were determined using the Bio-Rad microassay [27]. In order to avoid the presence of membrane vesicles, membrane fractions were pretreated with SDS before the assays, as previously described [28]. The optimal SDS/protein ratio was ~0.2 μg SDS/μg protein.

2.7. Lipid Peroxidation Measurements. The amount of lipid peroxidation of the plasma membranes was estimated by measuring the thiobarbituric acid-reactive substances (TBARS). The TBARS was determined according to the method described by Feix et al. [29]. The values are expressed as nmoles of malondialdehyde per mg of protein.

2.8. Statistical Analysis. Comparisons between the different conditions were assessed by one-way ANOVA with the post hoc analysis with the Student-Newman-Keuls test. All results are expressed as means ± standard error (S.E.) and n represents the number of experiments performed with different preparations. ATPase activities were calculated from paired data. A P value < 0.05 was accepted as statistically significant.

3. Results

Level of lipid peroxidation (determined as TBARS), PMCA activity, and Ca^{2+} and Mg^{2+} content of syncytiotrophoblast plasma membranes (BM and MVM) from freshly prepared placental explants are shown in Table 2, and those from placental explants cultured under either normoxic or hypoxic conditions are shown in Table 3. While the values of all the analyzed parameters for both BM and MVM from explants cultured in normoxic conditions are quite similar to those shown in Table 2 (freshly prepared explants), the membranes prepared from explants cultured under hypoxic conditions showed an increase in lipid peroxidation and Ca^{2+} content, as well as an important diminution in PMCA activity. Hypoxia did not produce any change in the Mg^{2+} content of either BM

Table 2: Lipid peroxidation (as TBARS), PMCA, and Ca^{2+} and Mg^{2+} content of syncytiotrophoblast plasma membranes from freshly prepared placental explants.

Measured parameters	BM	n	MVM	n
TBARS (nmol malondialdehyde/mg prot)	0.46 ± 0.11	(5)	0.61 ± 0.17	(5)
PMCA activity (nmol Pi/mg prot·min)	61 ± 4	(5)	110 ± 5	(5)
Membrane Ca^{2+} content (μmol/g prot)	67 ± 4	(5)	75 ± 5	(8)
Membrane Mg^{2+} content (μmol/g prot)	50 ± 3	(4)	55 ± 3	(8)

Values are expressed as the means ± S.E. for the indicated n between parentheses.

Table 3: Lipid peroxidation (as TBARS), PMCA, and Ca^{2+} and Mg^{2+} content of syncytiotrophoblast plasma membranes from placental explants cultured under either normoxia or hypoxia conditions.

Measured parameters	BM		MVM	
	Normoxia	Hypoxia	Normoxia	Hypoxia
TBARS nmol malondialdehyde/mg prot	0.72 ± 0.04 (4)	1.42 ± 0.09 (4)**	0.63 ± 0.03 (4)	1.48 ± 0.18 (4)**
PMCA activity nmol Pi/mg prot·min	51.0 ± 6.9 (5)	13.7 ± 1.9 (5)**	93 ± 5.6 (5)	34 ± 2.9 (5)**
Membrane Ca^{2+} content μmol/g prot	56 ± 3 (5)	82 ± 4 (6)**	66 ± 5 (6)	101 ± 8 (6)*
Membrane Mg^{2+} content μmol/g prot	54 ± 4 (6)	51 ± 2 (6)	57 ± 3 (6)	58 ± 4 (6)

Preincubations were carried out for 18 h at 37°C. Values are expressed as the means ± S.E. for the indicated n between parentheses.
*$P < 0.01$ versus normoxia.
**$P < 0.001$ versus normoxia.

or MVM. These results suggest that hypoxia, by increasing the Ca^{2+} content of the syncytiotrophoblast plasma membranes, stimulates lipid peroxidation, which in turn inhibits PMCA activity.

These effects of hypoxia were produced on plasma membranes prepared from placental explants cultured under hypoxic conditions with intact chorionic villi. However, the integrity of the cells might not be necessary to achieve these results. In order to rule out this possibility, BM and MVM were prepared from freshly obtained placental explants and then incubated with different concentrations of $CaCl_2$, in order to try to alter their Ca^{2+} content. The membranes were then analyzed for Ca^{2+} and Mg^{2+} content, level of lipid peroxidation, and PMCA activity. The results in Figures 1(a) and 1(b) show that incubation of both BM and MVM with different concentrations of $CaCl_2$ increased their Ca^{2+} content, without affecting their Mg^{2+} content. In addition, the level of lipid peroxidation of the membranes increased (Figure 1(c)) while the PMCA activity decreased (Figure 1(d)).

The variations shown in Figure 1 can be explained as the effect of increased Ca^{2+} on the membranes. However, as there were no changes in the Mg^{2+} content of the membranes, it is possible that the effects were due to an alteration of their Ca^{2+}/Mg^{2+} ratio, rather than to just their Ca^{2+} content. In order to study this possibility, syncytiotrophoblast plasma membranes were preincubated in the presence of different concentrations of Mg^{2+} (as $MgSO_4$) and then analyzed for Ca^{2+} and Mg^{2+} content, level of lipid peroxidation, and PMCA activity. The results of this experiment are shown in Figure 2.

Figures 2(a) and 2(b) indicate that while lower concentrations of $MgSO_4$ in the preincubation medium had a minimal effect on the Mg^{2+} content of the syncytiotrophoblast plasma membranes, a concentration of 4 mM $MgSO_4$ was able to significantly increase their Mg^{2+} content. None of the tested concentrations of $MgSO_4$ altered the Ca^{2+} content of the membranes (Figures 2(a) and 2(b)), the TBARS (Figure 2(c)), or the PMCA activity (Figure 2(d)).

If the molecular events occurring during culture of the placental explants under hypoxic conditions are similar to the response when the placental plasma membranes are incubated with Ca^{2+}, then it should be possible to circumvent the rise of the Ca^{2+} content of the membranes by adding Mg^{2+} to the preincubation medium. Therefore the hypoxia experiments were repeated but with Dulbecco's Modified Eagle culture medium (1.8 mM $CaCl_2$ and 0.8 mM $MgSO_4$) used for incubation of the placental explants adjusted to a final concentration of $MgSO_4$. The results of these experiments showed that the presence of 4 mM $MgSO_4$ prevented both the lipid peroxidation of syncytiotrophoblast plasma membranes (Figures 3(a) and 3(b)) and the decrease in the PMCA activity (Figures 3(c) and 3(d)).

4. Discussion

In this study it was found that syncytiotrophoblast plasma membranes (BM and MVM) of placental explants cultured under hypoxic conditions showed a significant increase in Ca^{2+} content and lipid peroxidation, as well as an important diminution in PMCA activity, when compared to plasma

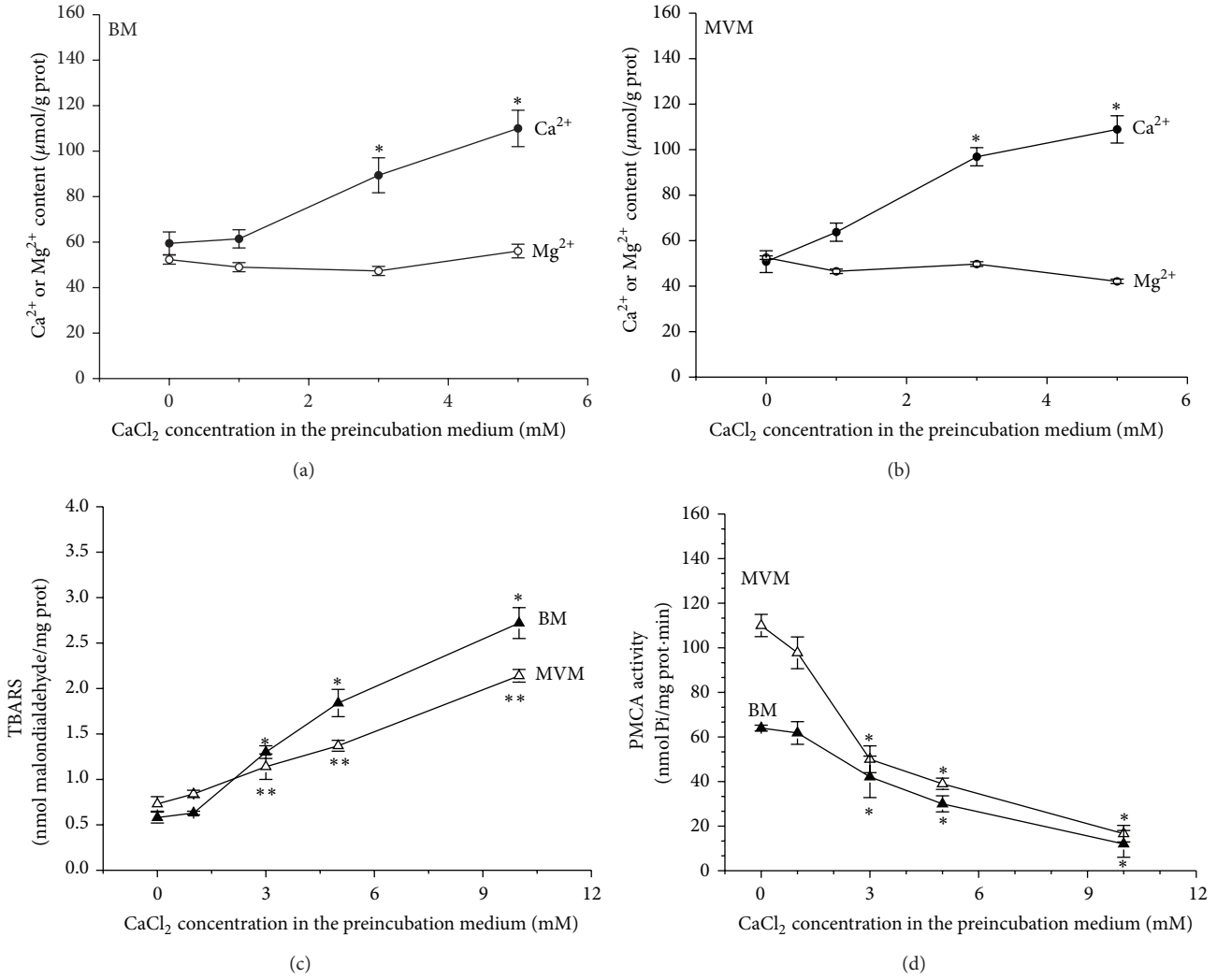

Figure 1: Panels (a) and (b). Ca^{2+} and Mg^{2+} content of syncytiotrophoblast plasma membranes (BM and MVM, resp.) prepared from freshly prepared placental explants and then incubated with different concentrations of Ca^{2+}. Values expressed as means ± S.E., for $n = 4$. Panels (c) and (d). Level of lipid peroxidation, determined as TBARS, and PMCA activity, respectively, of syncytiotrophoblast plasma membranes (BM and MVM) prepared from freshly prepared placental explants and then incubated with different concentrations of Ca^{2+}. Values expressed as means ± S.E., for $n = 4$. $^{*}P < 0.001$ versus 0 $CaCl_2$.

membranes either from freshly prepared placental explants or from placental explants cultured under normoxic conditions. In these experiments, the membrane Mg^{2+} content remained constant (Tables 2 and 3). Similar results were obtained by preincubating syncytiotrophoblast plasma membranes with different concentrations of Ca^{2+} (Figure 1). The Mg^{2+} content of the membranes increased when the preincubation media contained 4 mM $MgSO_4$, but it remained unchanged after preincubations with lower magnesium concentrations. However, varying the concentration of Mg^{2+} in the preincubation medium did not change the membrane Ca^{2+} content, level of lipid peroxidation, or PMCA activity.

These results may suggest that hypoxia, by increasing the Ca^{2+} content of the syncytiotrophoblast plasma membranes, stimulates their level of lipid peroxidation which, in turn, inhibits their PMCA activity. How can changes in the membrane Ca^{2+} content modify the degree of lipid peroxidation? Cations have been proposed to penetrate deeply into the head group region of the membrane, probably binding between the carbonyl oxygen and the phosphate group [30]. Consequently, there is the possibility of a direct interaction of calcium with negatively charged phospholipids of the cell membrane. This interaction could result in a destabilizing effect on the structure of the membrane, producing changes in the orientation of their phospholipids that expose hydrocarbon chains of fatty acids to the action of free radicals, thus making them more susceptible to peroxidation. It is widely accepted that Ca^{2+} ions interact with plasma membranes to produce important changes in their lipid profile; that is, phospholipid scrambling in the human erythrocyte membrane

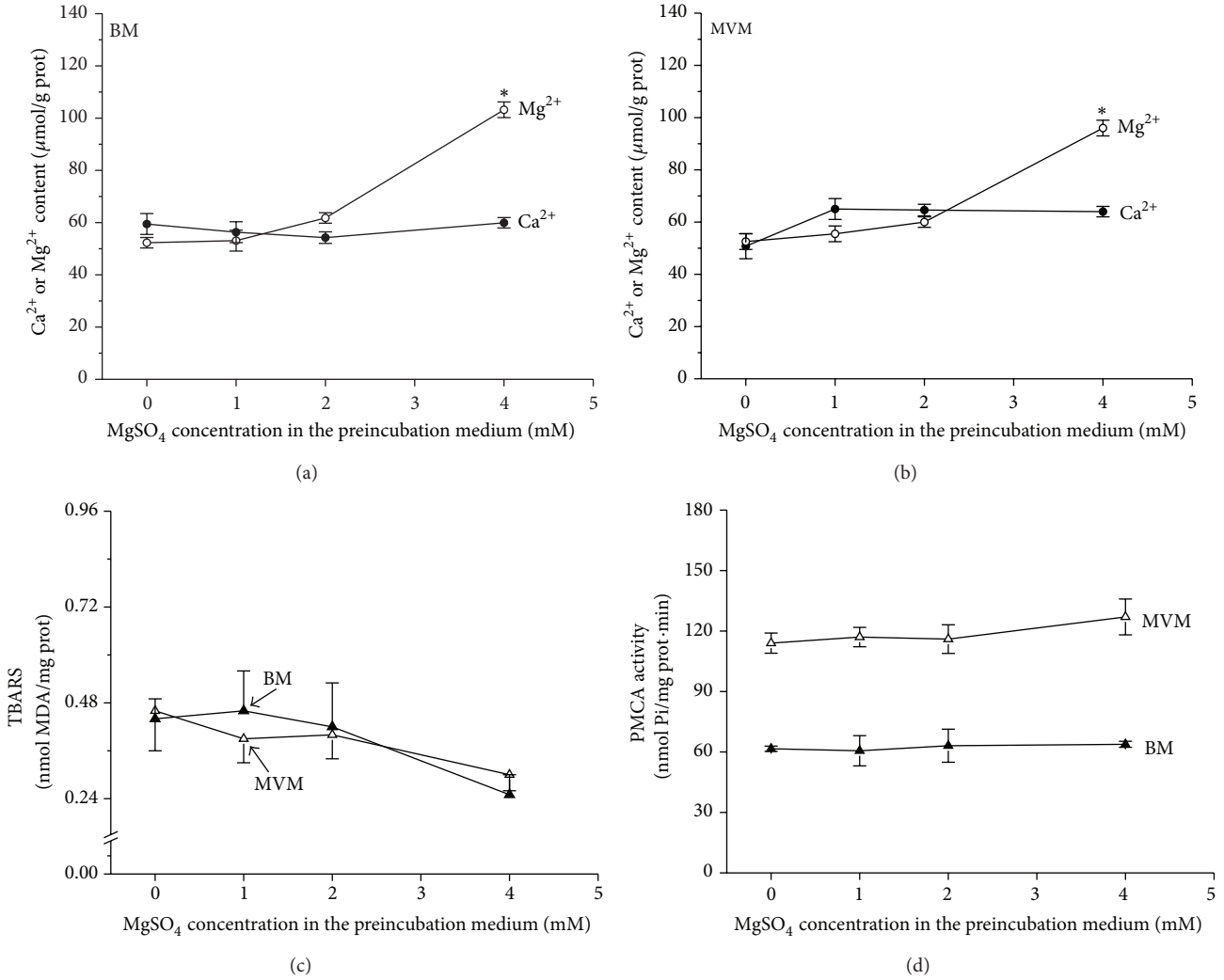

Figure 2: Panels (a) and (b). Ca^{2+} and Mg^{2+} content of syncytiotrophoblast plasma membranes (BM and MVM, resp.) prepared from freshly prepared placental explants and then incubated with different concentrations of Mg^{2+}. Values expressed as means ± S.E., for $n = 3$. Panels (c) and (d). Level of lipid peroxidation, determined as TBARS, and PMCA activity, respectively, of syncytiotrophoblast plasma membranes (BM and MVM) prepared from freshly prepared placental explants and then incubated with different concentrations of Mg^{2+}. Values expressed as means ± S.E., for $n = 3$. $^*P < 0.001$ versus 0 $MgSO_4$.

involves the synergistic action of phosphatidylinositol 4,5-bisphosphate and Ca^{2+} [31].

A relationship between lipid peroxidation and PMCA activity has been established for different cell membranes [9–12, 32]. Membrane ATPases have a strong dependence on lipid-protein interactions and consequently their activities are greatly affected by the degree of lipid peroxidation in the surrounding microenvironment [33]. Consistent with this, we found that with the increased level of lipid peroxidation there was diminution of the PMCA activity of syncytiotrophoblast plasma membranes (Table 3 and Figure 1). These results could be relevant for understanding the molecular basis of preeclampsia, as it has been found that red blood cell ghosts from preeclamptic women show high levels of membrane Ca^{2+} and low levels of membrane Mg^{2+} [20] and also high levels of lipid peroxidation and low PMCA activity [34].

Parenteral $MgSO_4$ administration is the medical treatment for preeclamptic women to prevent the recurrent seizures of eclampsia and for tocolysis in preterm labor [35]. In previous experiments, we have found that, twenty-four hours after the onset of $MgSO_4$ therapy, both the level of lipid peroxidation and PMCA activity of red blood cell membranes from preeclamptic women show values similar to those of normotensive pregnant women [34]. A similar effect was also seen when red blood cells from untreated preeclamptic women were incubated *in vitro* with 4 mM $MgSO_4$ for 24 h at 0°C [34]. In addition, the results shown in Figures 2(a) and 2(b) indicate that incubation of syncytiotrophoblast plasma membranes with sufficient $MgSO_4$ increases their Mg^{2+} content. Taken together, it may be concluded that the $MgSO_4$ treatment raises the Mg^{2+} content of the red cell ghosts and thereby reduces the level

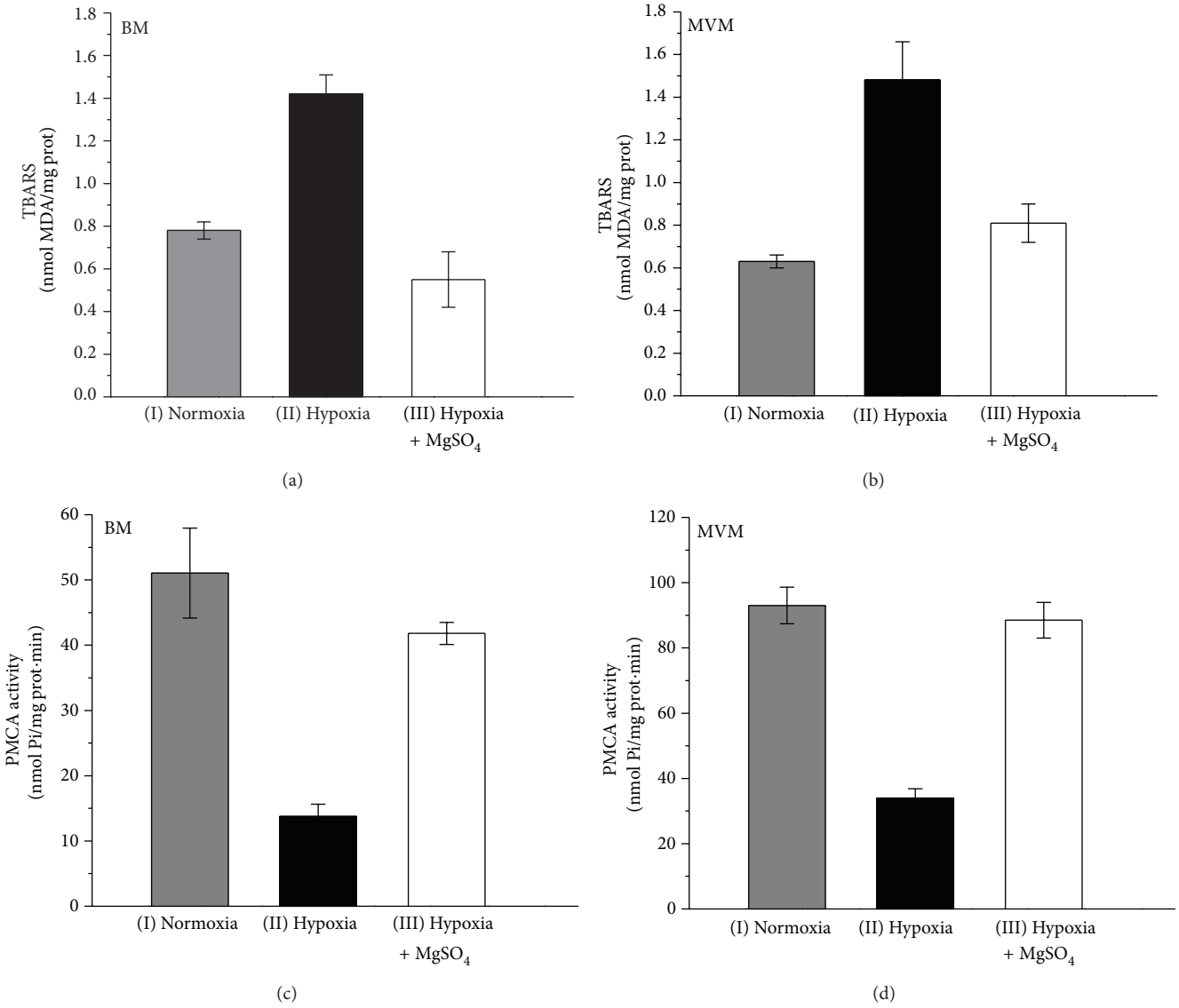

Figure 3: Panels (a) and (b). Lipid peroxidation (as TBARS) of basal (BM) and microvillus plasma membranes (MVM) prepared from normal human term placental explants, previously cultured under either normoxia, hypoxia, or hypoxia + 4 mM $MgSO_4$. Values expressed as means ± S.E., for $n = 5$. Panels (c) and (d). PMCA activity of basal (BM) and microvillus plasma membranes (MVM) prepared from normal human term placental explants, previously cultured under either normoxia, hypoxia, or hypoxia + 4 mM $MgSO_4$. Values expressed as means ± S.E., for $n = 5$. (II) versus (I) $P < 0.001$. (III) versus (I) n.s. (III) versus (II) $P < 0.001$.

of lipid peroxidation and increases the PMCA activity of these membranes. Furthermore, the results presented in Table 3 indicate that BM and MVM prepared from explants cultured under hypoxia in 1.8 mM $CaCl_2$ and 0.8 mM $MgSO_4$ (Dulbecco's Modified Eagle Medium) show a significant increase in their Ca^{2+} content, as compared to results from explants cultured under normoxia, with no variations in their Mg^{2+} content. However, increasing the concentration of the culture medium to 4 mM $MgSO_4$ protected the membranes against the effects of hypoxia (Figure 3). These results might be an indication that a minimum concentration of Mg^{2+} is required to protect the membranes and to circumvent the rise of lipid peroxidation and the inhibition of the PMCA activity of the syncytiotrophoblast plasma membranes seen during preeclampsia [34].

The exact molecular mechanisms explaining the interactions of Ca^{2+} and Mg^{2+} within the plasma membranes of preeclamptic women remain to be determined. However, our study points out the importance of these interactions for the control of the level of lipid peroxidation of the plasma membranes and for the activity of transporting proteins such as the PMCA during preeclampsia.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Reinaldo Marín and Fulgencio Proverbio are cosenior authors of this paper.

Acknowledgments

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Research Article

Perinatal Nitric Oxide Therapy Prevents Adverse Effects of Perinatal Hypoxia on the Adult Pulmonary Circulation

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Adverse events in utero are associated with the occurrence of chronic diseases in adulthood. We previously demonstrated in mice that perinatal hypoxia resulted in altered pulmonary circulation in adulthood, with a decreased endothelium-dependent relaxation of pulmonary arteries, associated with long-term alterations in the nitric oxide (NO)/cyclic GMP pathway. The present study investigated whether inhaled NO (iNO) administered simultaneously to perinatal hypoxia could have potential beneficial effects on the adult pulmonary circulation. Indeed, iNO is the therapy of choice in humans presenting neonatal pulmonary hypertension. Long-term effects of neonatal iNO therapy on adult pulmonary circulation have not yet been investigated. Pregnant mice were placed in hypoxia (13% O_2) with simultaneous administration of iNO 5 days before delivery until 5 days after birth. Pups were then raised in normoxia until adulthood. Perinatal iNO administration completely restored acetylcholine-induced relaxation, as well as endothelial nitric oxide synthase protein content, in isolated pulmonary arteries of adult mice born in hypoxia. Right ventricular hypertrophy observed in old mice born in hypoxia compared to controls was also prevented by perinatal iNO treatment. Therefore, simultaneous administration of iNO during perinatal hypoxic exposure seems able to prevent adverse effects of perinatal hypoxia on the adult pulmonary circulation.

1. Introduction

Numerous epidemiological studies have associated adverse events occurring in utero, like limitation of nutrients or oxygen supply, with an increased risk to develop chronic diseases in adulthood, including coronary artery disease, systemic hypertension, stroke, and noninsulin-dependent diabetes mellitus [1]. Therefore, an insult occurring in the perinatal period, namely, during late gestation and the first days of extrauterine life, may result in a definitive imprint predisposing to a pathological response later in life, leading to "programmed" diseases. However, cellular and molecular mechanisms of the programming process are not yet elucidated [2, 3]. In particular, chronic pulmonary vascular diseases and abnormal pulmonary vascular reactivity in adulthood may be associated with a hypoxic insult occurring around birth [4–8]. Humans and animals born in hypoxic conditions, or presenting neonatal pulmonary hypertension, show later in life an exaggerated pulmonary hypertensive response following a reexposure to hypoxia [7, 9–13]. The cellular and molecular mechanisms involved in the dysregulation of adult pulmonary vasomotor tone secondary to a transient perinatal insult remain incompletely understood.

The fetal pulmonary circulation is characterized by low perfusion and high vascular resistances, which need to rapidly fall at birth to allow pulmonary blood flow to increase nearly 10-fold [14, 15]. Pulmonary vascular tone is regulated by a complex and intricate group of mechanisms [16, 17], including the nitric oxide/cyclic guanosine monophosphate (NO/cGMP) signaling pathway, which plays a key role in establishing relaxation of the pulmonary vessels during neonatal transition as well as later in life [18, 19]. Sometimes, the normal decrease in pulmonary vascular resistances and increase in pulmonary blood flow do not occur, resulting in

persistent pulmonary hypertension of the newborn (PPHN). PPHN is a severe neonatal complication, associated with a high mortality and morbidity. Prevalence of PPHN is estimated at 1.9 per 1000 live births and mortality ranges from 4 to 33% [20]. This pathology represents 1 to 4% of the admissions in a neonatal intensive care unit [21, 22] and requires intensive therapy including supplemental oxygen and pulmonary vasodilators [23]. Inhaled NO (iNO) has been proposed as a "selective" pulmonary vasodilator since it acts directly on pulmonary vascular cells and is then inactivated in the bloodstream. Inhaled NO is now recognized as the therapy of choice for term newborns with a PPHN [24]. Most neonates who respond to this treatment will recover within a week and then develop normally. However, long-term effects of neonatal iNO therapy on adult lung vascular reactivity have not yet been investigated.

Adult pulmonary hypertension is also a severe disease of various origins, affecting life expectancy and quality of life [25], whose treatment is still a challenge and prognosis remains poor [26]. Pulmonary hypertension is related to an abnormal reactivity of the pulmonary vasculature resulting in lower perfusion of the lung and hypoxemia. Abnormal development of regulatory mechanisms of the pulmonary circulation in the perinatal period could be associated with an altered regulation of the adult pulmonary circulation.

We have previously established a murine experimental model in order to study the long-lasting effects of a transient exposure to hypoxia in the perinatal period [13, 27–29]. Pregnant mice were placed in hypoxic conditions (13% O_2) 5 days before delivery and left with their litter for 5 days after birth. Pups were then bred in normoxia until adulthood when studied. We first demonstrated that the relaxation induced by the endothelium-dependent agent acetylcholine (ACh) in the adult main pulmonary artery (PA) was completely mediated by endothelial nitric oxide synthase (eNOS) [28]. Our further studies showed that a transient hypoxic insult in the perinatal period resulted in altered regulation of pulmonary vascular tone in adulthood [13, 27, 29]. Adults born in hypoxia displayed an increase in right ventricular pressure, suggesting a higher resistance state in the pulmonary circulation, and an increased sensitivity to acute hypoxia compared to controls [13]. Moreover, perinatal hypoxia dramatically decreased endothelium-dependent relaxation induced by ACh in adult female pulmonary arteries [13]. This alteration of adult pulmonary circulation was associated with long-term alterations in the NO/cGMP signaling pathway, in particular muscarinic receptors and phosphodiesterases, and with a marked reduction in eNOS protein content in isolated pulmonary arteries from adult females born in hypoxic conditions compared to mice born in normoxia [13]. This could contribute to the observed decrease in ACh-mediated relaxation following perinatal hypoxia.

As iNO is actually the therapy of choice in humans presenting neonatal pulmonary hypertension, we decided to investigate in our murine experimental model whether iNO administered simultaneously to perinatal hypoxia could have potential beneficial effects on the adult pulmonary circulation.

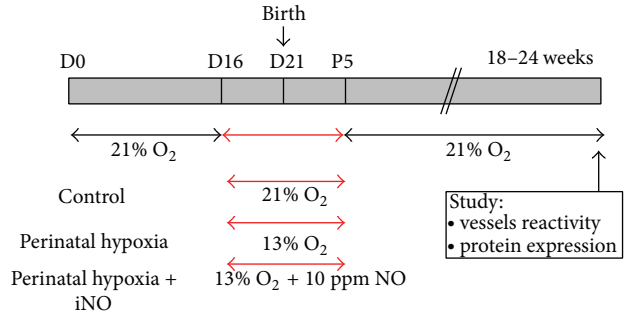

Figure 1: Experimental design. C57BL/6 pregnant mice were exposed from 5 days before delivery until 5 days after birth either to normoxia, hypoxia, or hypoxia with simultaneous administration of inhaled NO (iNO). Pups were then raised in normoxia until adulthood. Mice were studied as young adults (5-6 months) or old adults (12–15 months).

Effects of concomitant perinatal exposure to iNO and hypoxia were compared to those of perinatal hypoxia alone by investigation of ACh-induced relaxation and eNOS protein content in isolated pulmonary arteries, completed by anatomical data in young and old adult females.

In our murine experimental model, perinatal hypoxia triggered greater alterations in endothelium-dependent relaxation and the NO/cGMP pathway in adult females than adult males (unpublished data). Our previous manuscript [13] was therefore focused on alterations occurring in the pulmonary circulation of adult females. The present report was consequently also limited to the effects of iNO in females. The results obtained in mice exposed to perinatal hypoxia and iNO were directly compared to the corresponding data we previously published for mice born in normoxia or hypoxia alone [13], in particular for ACh-induced relaxation and anatomical data. Indeed, the present study was the direct continuation of our previous work in which we used exactly the same experimental model [13]. As all procedures were similar between both studies, we did not repeat the experiments performed in mice born in normoxia or hypoxia alone and therefore some data of the referred article [13] are shown in the present paper to better highlight the beneficial effects of iNO therapy compared to hypoxia alone.

2. Methods

2.1. Murine Model of Perinatal Hypoxia. All experimental procedures were approved and carried out in accordance with the Swiss Veterinarian Animal Care Office. C57BL/6 pregnant mice were purchased from Harlan (Horst, The Netherlands). They were all fed *ad libitum* and exposed to day-night cycles. Perinatal hypoxia was induced as previously described [13, 27] (Figure 1). Pregnant mice were placed in hypoxic conditions (13% O_2) 5 days before delivery and left under hypoxia with their litter for 5 days after birth. Pups were then raised in normoxia (21% O_2) until adulthood. Pups born and grown in normoxic conditions were used as

controls. Female mice were studied as adults before week 25 (young females) or after week 50 (old females).

2.2. Perinatal Exposure to iNO. Pregnant mice were placed in an atmosphere containing 13% O_2 and 10 ppm gaseous NO for 10 days, more exactly for 5 days before delivery and 5 days after birth (Figure 1). Pups were then raised in normoxia (21% O_2) until adulthood when studied.

Inhaled NO was administered using the SONIMIX apparatus (LNIndustries, Geneva, Switzerland), which has been adapted to allow injection of gaseous mixtures containing oxygen, nitrogen, and NO according to the preset relative concentrations. The applied concentration of gaseous NO was half the concentration currently used in clinical practice to treat human newborns with pulmonary hypertension. NO concentration in the box was monitored using a NO sensor (SensorNox, SensorMedics).

2.3. Anatomical Data. Body weight was measured 5 days after birth. Number of alive pups was recorded at the end of the perinatal exposure to normoxia, hypoxia, or hypoxia plus iNO.

In adults, the heart was dissected with removal of the auricles and separation of the right ventricle (RV) and the left ventricle plus septum (LV + S). The RV/(LV + S) ratio was calculated and used as an index of right ventricular hypertrophy, which is a direct consequence of increased pulmonary vascular resistances present in pulmonary vascular pathologies.

2.4. Vascular Reactivity Studies. The vascular reactivity of pulmonary arteries was investigated by isolated vessel tension studies, as previously described [13]. Briefly, adult mice were administered a lethal dose of pentobarbital (1 g/kg intraperitoneal) and the main pulmonary artery was immediately harvested. The vessel ring was suspended in organ chamber filled with 10 mL of modified Krebs-Ringer bicarbonate solution (mM: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, and 11.1 glucose) maintained at 37°C and aerated with 95% O_2-5% CO_2 (pH 7.4) [13, 27]. Vessels were brought to their optimal resting tension after 2 stretches of 0.5 g. After equilibration, indomethacin (10^{-5} M) was added in order to exclude possible interference of endogenous prostanoids. The vessels were then contracted with phenylephrine (10^{-5} M) and pharmacological response of isolated pulmonary arteries was evaluated in the presence of increasing concentrations of the endothelium-dependent relaxing agent ACh. Change in tension induced by the vasodilator was expressed as percent of the initial contraction induced by phenylephrine.

2.5. Western Blotting Analysis of Protein Expression. Specific protein content in pulmonary arteries was investigated by western blotting analysis as previously described [13]. Briefly, each flash-frozen main PA was crushed in a cryogenic mortar and homogenized in 26 μL of lysis buffer (50 mM HEPES, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM DTT, 5 μg/mL pepstatin, 3 μg/mL aprotinin, 10 μg/mL leupeptin, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 1 mM sodium vanadate, 50 mM NaF, and 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). After a 45 min incubation on ice, the homogenates were centrifuged for 10 min at 3000 ×g at 4°C. Five μL of each supernatant was diluted in Laemmli buffer and heated for 5 min at 95°C before loading on a 7.5% polyacrylamide gel. Proteins were fractioned by SDS-PAGE (35 min at 200 V) and transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA) during 2 h at 100V. Blots were blocked overnight at 4°C in Tris-buffered saline plus 0.05% Tween 20 (TBS-T) containing 5% nonfat dry milk. All washing steps were performed using TBS-T. Membranes were immunoblotted for 1 h at room temperature using specific antibodies targeted against eNOS (BD Transduction Laboratories, Franklin Lake, NJ, USA), or β-actin (Sigma), diluted 1 : 200 (for eNOS), or 1 : 250 (for β-actin) in TBS-T containing 5% nonfat dry milk. Blots were then incubated with ECL horseradish peroxidase-linked anti-mouse antibody (for eNOS detection) or anti-rabbit antibody (for β-actin) (Amersham Biosciences, Buckinghamshire, UK), diluted 1 : 10,000 in TBS-T. Finally, specific proteins were detected by chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposition to X-ray film. Expression of eNOS was quantified using the UN-SCAN-IT gel software (Silk Scientific, Orem, UT, USA) and normalized to β-actin content. Relative protein content in homogenates of mice born in hypoxia was reported to the content measured in controls.

2.6. Drugs. Unless otherwise specified, all drugs were purchased from Sigma (St. Louis, MO). Indomethacin was prepared in equal molar Na_2CO_3 [30]. The other drugs were prepared using distilled water.

2.7. Data Analyses. Statistical analyses were performed using InStat 3.0 or Prism 4.0 (GraphPad Software, San Diego, CA). Data are expressed as mean ± SEM, and n represents the number of animals per group. Unless otherwise mentioned, the influence of perinatal exposure to hypoxia and/or iNO on the studied parameters was analyzed using the Mann-Whitney test. Two-way ANOVA was performed to compare the dose-response curves in isolated vessel tension studies. The difference was considered statistically significant when $P < 0.05$.

3. Results

3.1. Neonatal Data. Table 1 summarizes body weight and number of pups per litter recorded at the end of the 10-day exposure to normoxia, hypoxia, or hypoxia plus iNO. Body weight was significantly reduced in 5-day-old pups born in hypoxia, with or without iNO, compared to controls. The number of alive pups per litter was also significantly lower after perinatal exposure to hypoxia, in the absence or presence of iNO, compared to normoxia. Although the number of alive pups appeared to be higher in litters exposed

Table 1: Body weight and number of alive pups per litter at 5 days after birth.

5-day-old mice	Control $n = 119$	Perinatal hypoxia $n = 46$	Perinatal hypoxia + iNO $n = 98$
Body weight (g)	2.90 ± 0.03	2.60 ± 0.07*	2.72 ± 0.04*
Pups per litter	7.0 (4–10)	4.5 (3–7)*	6.0 (3–7)*

Body weight and number of alive pups per litter were recorded at the end of the perinatal exposure to normoxia (control), hypoxia, or hypoxia and inhaled NO (iNO). Results are expressed as mean ± SEM for body weight and median (range) for the number of pups per litter; *n* corresponds to the number of animals studied in each group. *Significant difference compared to controls ($P < 0.05$, using the nonparametric Mann-Whitney test).

Table 2: Anatomical data.

5-6-month-old mice	Control $n = 25$	Perinatal hypoxia $n = 20$	Perinatal hypoxia + iNO $n = 13$
Body weight (g)	22.83 ± 0.39	22.63 ± 0.36	23.68 ± 0.41
Heart weight (g)	0.100 ± 0.002	0.097 ± 0.001	0.093 ± 0.002*†
Heart/body ratio	0.0044 ± 0.0001	0.0043 ± 0.0001	0.0039 ± 0.0001*†
RV weight (g)	0.0230 ± 0.0005	0.0227 ± 0.0006	0.0205 ± 0.0006*†
(LV + S) weight (g)	0.0771 ± 0.0015	0.0745 ± 0.0009	0.0721 ± 0.0013*
Ratio RV/(LV + S)	0.300 ± 0.006	0.304 ± 0.008	0.285 ± 0.010
12–15-month-old mice	Control $n = 11$	Perinatal hypoxia $n = 15$	Perinatal hypoxia + iNO $n = 17$
Body weight (g)	26.84 ± 1.10	28.15 ± 0.92	27.32 ± 0.64
Heart weight (g)	0.107 ± 0.003	0.107 ± 0.002	0.109 ± 0.002
Heart/body ratio	0.0040 ± 0.0002	0.0038 ± 0.0001	0.0040 ± 0.0001
RV weight (g)	0.0231 ± 0.0010	0.0252 ± 0.0004	0.0231 ± 0.0005†
(LV + S) weight (g)	0.0835 ± 0.0023	0.0821 ± 0.0018	0.0855 ± 0.0015
Ratio RV/(LV + S)	0.276 ± 0.009	0.309 ± 0.008*	0.271 ± 0.004†

Body weight was measured after sacrifice of adult mice. Heart weight corresponds to the sum of the wet weights of the right ventricle (RV) and of the left ventricle plus septum (LV + S). The RV/(LV + S) ratio is the ratio between the weights of RV and LV + S, used as an index of right ventricular hypertrophy. Results are expressed as mean ± SEM; *n* corresponds to the number of animals in each group. *Significant difference compared to controls and †significant difference between mice exposed to perinatal hypoxia plus iNO and mice with perinatal hypoxia alone ($P < 0.05$, using the Mann-Whitney test). Data related to 5-6-month-old mice born in normoxia (Ctr) or in hypoxia (PH) were previously published [13].

to perinatal hypoxia and iNO than in those with perinatal hypoxia alone, the difference was not statistically significant.

3.2. Anatomical Data in Adults. Table 2 summarizes anatomical data measured in adult females after sacrifice.

In young females (5-6-month-old mice), perinatal hypoxia did not influence the studied parameters, as previously published [13]. Simultaneous exposure to perinatal hypoxia and iNO, however, significantly reduced heart weight, the heart to body ratio, and RV weight compared to mice born in normoxia or hypoxia alone. The LV + S weight was also decreased in mice exposed to hypoxia plus iNO compared to controls. Finally, the RV/(LV + S) ratio seemed lower in mice exposed to hypoxia and iNO than in controls or mice with perinatal hypoxia, but the difference was not quite significant between mice born in hypoxia in the presence or absence of iNO ($P = 0.0677$, using the Mann-Whitney test), as well as between mice born in hypoxia plus iNO and controls ($P = 0.0967$, using the Mann-Whitney test).

In old females (12–15-month-old mice), anatomical data were similar between mice exposed to perinatal hypoxia and controls, except for the RV/(LV + S) ratio, which was significantly higher in mice born in hypoxia than in controls. Old females submitted to simultaneous exposure to perinatal hypoxia and iNO displayed similar anatomical data to controls. However, RV weight and the RV/(LV + S) ratio were significantly reduced compared to mice exposed to perinatal hypoxia alone.

3.3. Isolated Pulmonary Artery Reactivity. Pharmacological response to cumulative doses of the endothelium-dependent relaxing agent acetylcholine (ACh) was tested in isolated PA preconstricted with phenylephrine (Figure 2). We previously showed that ACh-induced relaxation was dramatically reduced in isolated PA from adult females born in hypoxia compared to controls [13]. In contrast, isolated PA from adult mice exposed simultaneously to hypoxia and iNO in the perinatal period displayed a dose-dependent relaxant response to ACh, which was similar to controls. Therefore, ACh-induced relaxation was significantly increased in mice exposed to perinatal hypoxia plus iNO compared to perinatal hypoxia alone (Figure 2).

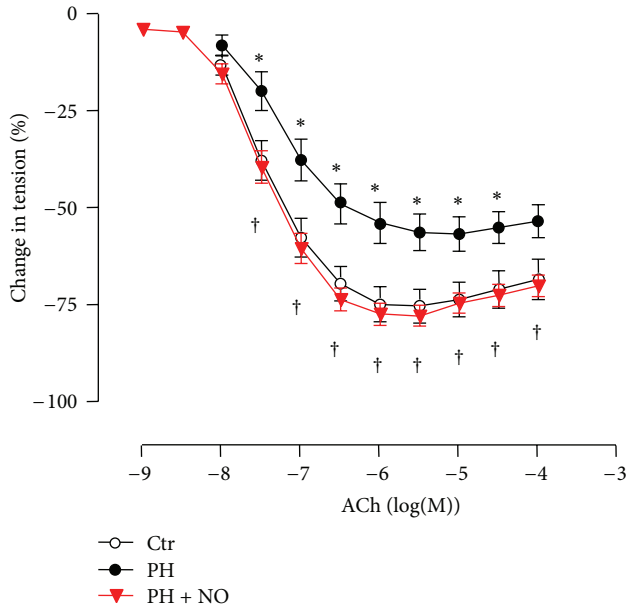

FIGURE 2: Pharmacological response of isolated pulmonary arteries to the endothelium-dependent agent acetylcholine. Dose-response to acetylcholine (ACh) was tested in pulmonary arteries preconstricted with phenylephrine (10^{-5} M), isolated from adult mice born in normoxia (Ctr), in hypoxia (PH), or in hypoxia with simultaneous administration of iNO (PH + NO). Results are expressed as mean ± SEM of percentage of change in tension induced by the vasodilator (n = 6–9 mice). *Significant difference compared to controls and †significant difference between mice exposed to perinatal hypoxia and iNO and mice with perinatal hypoxia alone ($P < 0.05$, two-way ANOVA). Results obtained with pulmonary arteries of mice born in normoxia (Ctr) or in hypoxia (PH) were previously published [13].

3.4. eNOS Protein Content. We previously showed that the eNOS protein relative content was significantly reduced in the main PA of adult females born in hypoxia compared to controls [13] (Figure 3(a)). In contrast, the eNOS protein relative content in PA of adult females exposed simultaneously to perinatal hypoxia and iNO was similar to controls (Figure 3(b)).

4. Discussion

We previously demonstrated, in a murine model of perinatal hypoxia, that a transient exposure to hypoxia during a critical period of development induced long-term adverse effects on the adult pulmonary circulation [13, 27, 29].

In the present study, we investigated in the same experimental model whether iNO administration during the whole exposure to perinatal hypoxia could have beneficial effects on the adult pulmonary circulation. Potential protective effects of iNO were tested by measurement of several parameters which were found to be altered in adult females exposed to perinatal hypoxia [13].

In our previous report [13], we demonstrated that perinatal hypoxia induced a marked decrease in endothelium-dependent ACh-induced relaxation of PA, whereas no difference was found in the relaxation induced by gaseous NO or NO donors. As the present study aimed to investigate potential protective effects of iNO against adverse effects of perinatal hypoxia, the functional experiments were therefore limited to the pharmacological response of isolated PA to ACh, which was altered in adult mice exposed to perinatal hypoxia alone compared to controls, whereas relaxation induced by the NO donor 2-(N,N-diethylamino)-diazenolate-2-oxide (DEA/NO) was similar in mice born in hypoxia and controls.

Vascular reactivity studies showed that although ACh-induced relaxation was significantly reduced in isolated PA from adult females exposed to perinatal hypoxia compared to controls, simultaneous administration of iNO during perinatal hypoxic exposure completely restored the vasorelaxant response to ACh.

Similarly, perinatal exposure to hypoxia with concomitant iNO treatment allowed a total recovery in eNOS protein content in the main PA of adult females, whereas eNOS protein expression was shown to be reduced by almost 40% in adult females born in hypoxia compared to controls [13].

Anatomical data recorded in young adult mice (5-6 months) were similar between females born in hypoxic conditions and controls. Although previous hemodynamic studies showed a higher right ventricular pressure in 5-month-old mice exposed to perinatal hypoxia compared to controls [13], strongly suggesting a higher resistance state in the pulmonary circulation, the RV/(LV + S) ratio was not significantly increased in young adult females. The RV/(LV + S) ratio was used as an index of right ventricular hypertrophy, which is a direct consequence of increased pulmonary vascular resistances accompanying pulmonary hypertension. Here we showed that, in old females (12–15 months), the RV/(LV + S) ratio was significantly higher in mice born in hypoxia than in controls, suggesting that perinatal hypoxia resulted in right ventricular hypertrophy later in life, probably as a result of increased pulmonary vascular resistances following perinatal exposure to hypoxia. Inhaled NO treatment during perinatal hypoxia significantly reduced RV weight compared to mice exposed to perinatal hypoxia alone, both in young and old females. In old females exposed to perinatal hypoxia and iNO, the RV/(LV + S) ratio was also significantly decreased compared to mice born in hypoxia alone and thus became similar to the RV/(LV + S) ratio observed in controls.

Taken together our results suggest that concomitant administration of iNO during the whole exposure to perinatal hypoxia exerts a protective effect on the pulmonary circulation. Such results argue in favor of a potential long-term beneficial effect of neonatal iNO therapy. It will be nevertheless necessary to investigate whether neonatal (instead of perinatal) treatment would confer the same protection and whether this protective effect could also be observed on additional parameters, like right ventricular pressure, sensitivity to acute hypoxia, and molecular components of the NO/cGMP pathway, such as muscarinic receptors and phosphodiesterases, which were found to be altered in adult females exposed to perinatal hypoxia [13].

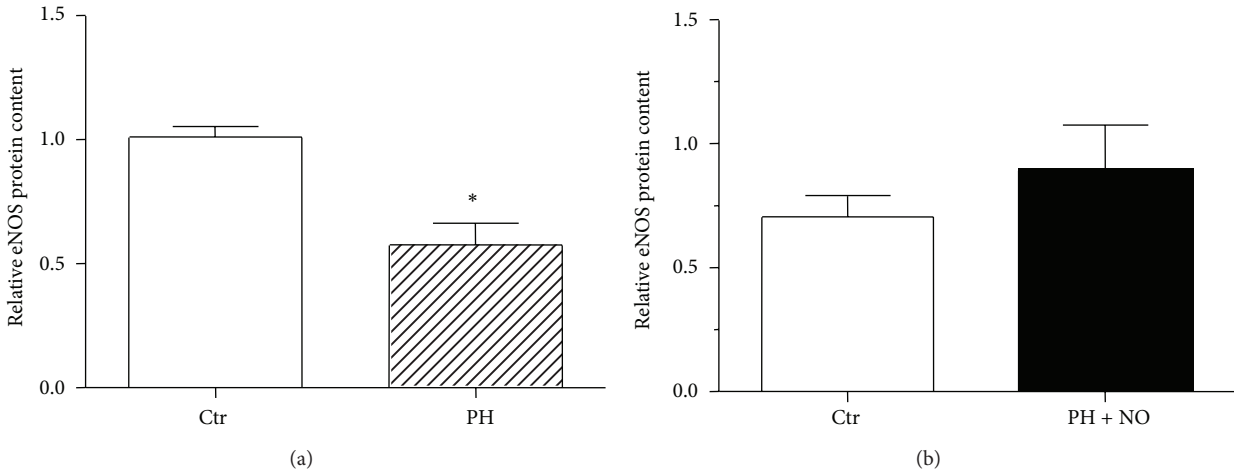

Figure 3: Western blotting analysis of eNOS protein expression in pulmonary arteries. (a) Western blotting analysis of eNOS protein relative content was performed in pulmonary arteries extracts ($n = 3$ pools of 10 PA) of adult females born in normoxia (Ctr) or in hypoxia (PH). These data were previously published in [13]. *Significant difference between Ctr and PH groups ($P < 0.05$ using unpaired t-test). (b) Relative eNOS protein content was analyzed in pulmonary arteries extracts of adult mice born in normoxia (Ctr) or exposed simultaneously to hypoxia and iNO in the perinatal period (PH + NO) ($n = 4$ mice). Results are expressed as mean ± SEM of the relative eNOS protein content after normalization by β-actin protein content.

Beneficial effects of iNO were also shown in other experimental models. For example, a 10-day treatment with iNO (10 ppm) during recovery from a 10-day exposure to neonatal hypoxia restored lung structure in eNOS-deficient mice, by stimulation of alveolar and vascular growth [31]. Similarly, concomitant exposure of 9-day-old rat pups to iNO (20 ppm) and hypoxia during 14 days attenuated pulmonary vascular structural changes, right ventricular hypertrophy, and growth retardation induced by exposure to neonatal hypoxia alone [32]. In a model of bleomycin-induced bronchopulmonary dysplasia in neonatal rats, iNO treatment improved lung structure and prevented right ventricular hypertrophy and pulmonary vascular remodeling [33]. In adult rats, continuous inhalation of NO (10 ppm) during a 2-week chronic exposure to hypoxia also reduced pulmonary vascular remodeling and right ventricular hypertrophy compared to rats exposed to hypoxia alone [34]. These reports pointed however mainly to protective effects of iNO against structural pulmonary alterations resulting from hypoxic exposure. More recently, inhalation of NO was found to have cardioprotective effects in murine models of cardiac ischemia-reperfusion injury [35] and to improve outcomes after cardiac arrest and successful cardiopulmonary resuscitation in adult mice [36], even in eNOS-deficient mice [37]. The latter protective effects required the presence of soluble guanylyl cyclase [35, 36]. However, all these studies showed short-term protective effects of iNO administration, whereas our data demonstrate long-lasting beneficial effects of perinatal iNO therapy on the pulmonary circulation throughout life. Indeed, in our experimental model, perinatal iNO administration was able to prevent not only the impairment of endothelium-dependent relaxation in young adults secondary to a perinatal exposure to hypoxia, but also progressive development of right ventricular hypertension in old mice.

The mechanisms implicated in the observed protective effects of iNO treatment during the whole exposure to perinatal hypoxia remain to be elucidated. It will be first necessary to distinguish whether protective effects are mainly due to prenatal or postnatal treatment with iNO.

Prenatal treatment with iNO probably mainly acts through reduction of pulmonary vascular resistances in pregnant mice. Indeed, closed-chest hemodynamic studies showed that acute exposure to iNO in hypoxic conditions was able to reverse hypoxia-induced increase in right ventricular pressure in anaesthetized adult mice (unpublished data). Due to the short half-life of gaseous NO, maternal exposure to iNO during hypoxia may not directly influence the fetal circulation, although there was recent evidence that extrapulmonary effects of iNO could be mediated, in addition to diffusion, by conversion of iNO into S-nitrosothiols further exported into the circulation by the type L amino acid transporter [38]. However, improvement of maternal pulmonary circulation, by reversing hypoxic pulmonary vasoconstriction, could enhance fetal oxygen delivery and perhaps also improve fetal growth. Indeed, placental insufficiency, a pathology associated with decreased nutrients and oxygen supply to the fetus, results in intrauterine growth restriction.

In our murine model of perinatal hypoxia, body weight measured in 5-day-old pups was decreased by about 10% after perinatal hypoxia compared to normoxia. Body weight in pups exposed to perinatal hypoxia plus iNO was also significantly reduced compared to controls and did not significantly differ from pups with perinatal hypoxia alone. This suggests that maternal exposure to iNO did not prevent growth restriction resulting from perinatal hypoxia. Therefore neonatal exposure to iNO during perinatal hypoxia probably contributes largely to the protective effects of iNO

against adverse effects of perinatal hypoxia on the adult pulmonary circulation.

Beneficial effects of neonatal iNO therapy could be explained by direct effect on the pulmonary circulation of pups exposed to hypoxia, thanks to the vasorelaxant properties of iNO allowing the counteraction of pulmonary vasoconstriction induced by hypoxia. Moreover, if prenatal exposure to hypoxia induced altered eNOS protein expression, exogenous NO could also act by compensation for endogenous NO production deficiency, allowing recovery of NO-mediated relaxation in pups, thus preventing further alterations in pulmonary vascular tone regulation. Such protective effects could be due to prevention of epigenetic modifications in pulmonary vessels leading to long-term alterations in the regulation of pulmonary circulation. In this line, it would be particularly of interest to investigate whether iNO could prevent the imbalance in the NO/cGMP pathway in adult pulmonary vasculature following perinatal hypoxia, with particular attention to muscarinic receptors and phosphodiesterases. Such a result would suggest that an appropriate intervention to counteract adverse effects of a hypoxic insult at a critical period of development could prevent long-term adverse effects of hypoxia on adult pulmonary circulation.

It remains therefore to test whether a neonatal iNO treatment, introduced from birth to the end of the 10-day perinatal exposure to hypoxia, will be able to exert similar protective effects against adverse effects of perinatal hypoxia on the adult pulmonary circulation in our murine experimental model.

It will be also necessary to confirm such observations in humans. Indeed, iNO therapy was introduced about 15 years ago to treat neonates with PPHN. However there is no data on the long-term effects of iNO on the regulation of adult pulmonary circulation in humans. For example, it was shown that young adults having recovered from PPHN before introduction of the iNO therapy (children admitted to the neonatal-care unit of the Lausanne University Hospital for Children between 1972 and 1979) displayed exaggerated pulmonary vasoconstrictive response following reexposure to hypoxia, namely, during high-altitude exposure [7]. It will be therefore interesting to investigate whether such augmented pulmonary hypertensive response to hypoxia could be prevented in young adults having suffered from PPHN treated with iNO in the neonatal period. Potential long-term beneficial effects, besides the short-term prognostic improvement, will be useful indications for neonatologists to assess the safety of the applied therapy.

In terms of basic research, our data may contribute to a better understanding of the regulatory mechanisms of the pulmonary circulation in the adult following a perinatal hypoxic exposure with or without iNO therapy. In terms of clinical implications, our studies may help to devise novel therapeutic strategies to prevent and treat adult pulmonary vascular disorders secondary to perinatal hypoxic events. More generally, such results should stimulate researchers to further investigate cellular and molecular mechanisms implicated in fetal programming of adult diseases in order to design novel targeted early therapeutic interventions to prevent long-term adverse effects of perinatal insults.

It should be noted that most results of the present study are related to the main PA. The overall reactivity differs between the large conduit PA and the more distal resistant pulmonary vessels. The total pulmonary vascular resistance depends on the combination of the reactivity of all these vessels, among which large pulmonary arteries have also been shown to play a role [39, 40]. However, the modifications observed in the main PA of mice born in hypoxia compared to controls were already significant. Therefore other alterations could be expected in more distal vessels. In our previous study [13], we displayed that alterations secondary to perinatal hypoxia were found both in the main PA and at the level of more distal vessels: molecular assays were performed in total lung homogenates as well as in PA extracts, to assess whether molecular changes observed in the main PA were consistent with changes occurring in total lung. Similarly, hemodynamic studies indirectly showed the influence of perinatal hypoxia on distal vessels, which contribute to the resulting pulmonary arterial pressure, as estimated by measurement of the right ventricular pressure. In the present paper, some data, like the prevention of right ventricular hypertrophy in old mice exposed to perinatal hypoxia, also suggest that iNO exerts a protective effect against alterations not only of the main pulmonary artery but also of more distal vessels.

Moreover, our experimental model was complex, because it combined pre- and postnatal stimuli. In our previous studies, the timing of exposure to hypoxia was chosen to cover the period of lung vasculogenesis, during which the functional units of gas exchange develop [41]. Further comparison between effects of prenatal or postnatal hypoxic insult would be of interest to better determine the susceptibility window of the lung vasculature. Moreover, although chronic hypoxia was found to modulate maternal milk composition [42], a postnatal exposure to hypoxia will mainly impact directly the pups, whereas the effects of a prenatal insult will also depend on the maternal metabolic environment and body composition. Exposure of pregnant rats or mice to chronic hypoxia is an established model of intrauterine growth restriction [43], although the effects vary depending on oxygen concentration and timing of exposure. Such experimental models led to reduced pups' body weight, with [44] or without [45–47] decrease in litter size compared to controls. In our murine model or perinatal hypoxia, we observed a significant reduction in both pups' body weight and litter size compared to controls. However, these data were recorded at the end of the perinatal exposure (at postnatal day 5), thus resulting from combined pre- and postnatal insult. Moreover, as food intake is sometimes [45, 47], but not always [46], reduced in pregnant animals exposed to hypoxia, such experimental models could introduce a component of undernutrition. Whether some malnutrition occurs in our murine model of perinatal hypoxia remains to be investigated, because maternal weight changes were not recorded.

Regarding the timing of iNO treatment, we aimed in the present study to show whether iNO administration during the whole exposure to hypoxia could reverse adverse effects of perinatal hypoxia on adult pulmonary circulation. Further investigation of potential beneficial effects of separate prenatal or postnatal treatment would be useful to determine the therapeutic window in which iNO administration could provide the greater protective effect. Such information could help to devise novel targeted therapeutic interventions. Particular attention will be paid to the postnatal treatment, which is closer to the current clinical practice.

However, despite several limitations, the present paper points to beneficial effects of perinatal iNO administration against some adverse effects of perinatal hypoxia on the adult pulmonary circulation. Further investigations will of course be necessary to better understand the involved mechanisms. However, although several studies displayed short-term protective effects of iNO administration, our data are the first, to our knowledge, to demonstrate long-lasting beneficial effects of perinatal iNO therapy on the pulmonary circulation throughout the life.

5. Conclusions

In the present report, we demonstrated that although a 10-day exposure to hypoxia in the perinatal period resulted in alterations of the adult pulmonary circulation, simultaneous administration of iNO during the whole exposure to perinatal hypoxia completely restored ACh-induced relaxation, as well as eNOS protein content, in isolated PA of adult females. Right ventricular hypertrophy observed in old mice born in hypoxia compared to controls was also prevented by perinatal iNO treatment. Therefore, concomitant administration of iNO during perinatal hypoxic exposure seems able to prevent adverse effects of perinatal hypoxia on the adult pulmonary circulation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Role of Lectin-Like Oxidized Low Density Lipoprotein-1 in Fetoplacental Vascular Dysfunction in Preeclampsia

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The bioavailability of nitric oxide (NO) represents a key marker in vascular health. A decrease in NO induces a pathological condition denominated endothelial dysfunction, syndrome observed in different pathologies, such as obesity, diabetes, kidney disease, cardiovascular disease, and preeclampsia (PE). PE is one of the major risks for maternal death and fetal loss. Recent studies suggest that the placenta of pregnant women with PE express high levels of lectin-like oxidized LDL receptor-1 (LOX-1), which induces endothelial dysfunction by increasing reactive oxygen species (ROS) and decreasing intracellular NO. Besides LOX-1 activation induces changes in migration and apoptosis of syncytiotrophoblast cells. However, the role of this receptor in placental tissue is still unknown. In this review we will describes the physiological roles of LOX-1 in normal placenta development and the potential involvement of this receptor in the pathophysiology of PE.

1. Introduction

Preeclampsia is a leading cause of maternal and neonatal morbidity and mortality. Despite the fact that this disease has been studied for more than hundreds of years, pathophysiology is still unclear. However, there is no doubt that one of the underling mechanisms associated with occurrence of preeclampsia is the alteration in the endothelial function, a phenomenon described as endothelial dysfunction. In turn, endothelial dysfunction is associated with unbalance between generation and activity of free radicals, including nitric oxide (NO) and superoxide anion (O_2^-), in favor of generation of nitrative and/or oxidative stress. Among several mechanisms related to generation of oxidative stress during preeclampsia, recent evidences suggest that expression of LOX-1, a scavenger receptor for oxidized low density lipoprotein (oxLDL), may be a keystone receptor that needs to be investigated, since it is involved in many processes related to pathophysiology of preeclampsia. Thus, the aim of this review is to describe the physiological and pathophysiological roles of LOX-1 in normal and preeclamptic pregnancies.

2. Vascular Endothelial Function and Nitric Oxide Generation

The endothelium is a monolayer of cells located in the inner wall of blood vessels and is the first physical and metabolic barrier between blood and tissues. The endothelium is involved in the regulation of hemodynamic function in physiological state, a phenomenon associated with synthesis and release of vasoactive molecules including nitric oxide (NO), prostaglandins, and thromboxanes [1]. In physiological conditions there is a tight balance between the generation of these different agents, and any disturbance in this equilibrium generates a pathological condition denominated

endothelial dysfunction. In general, endothelial dysfunction is a syndrome characterized by loss in antithrombotic, angiogenic, and inflammatory and vasodilator function. This syndrome has been observed in different pathologies, such as obesity, diabetes, kidney disease, cardiovascular disease, and preeclampsia [2–6]. Endothelial dysfunction is generally related to low bioactivity or bioavailability of NO, which in turn is associated with reduced vasodilator capacity and loss of vascular protection against harmful agents [7–9].

Nitric oxide is a potent vasodilator agent, inhibits platelet aggregation and leukocyte adhesion to the vascular wall, prevents proliferation of muscle cell, and reduces the expression of adhesion molecules and chemokines involved in monocyte infiltration [10]. Nitric oxide is derived from the conversion of L-arginine into L-citrulline (i.e., L-arginine/NO pathway) through a reaction catalyzed by NO synthase (NOS). There are at least three NOS isoenzymes coded by independent genes: neuronal (nNOS or NOS I, 12q24.2), inducible (iNOS or NOS II, 17cen-q11.2), and endothelial (eNOS or NOS III, 7q35-36) [11–13]. Bioavailability of NO is regulated by several mechanisms including reaction with reactive oxygen species (ROS) [14]. The interaction between NO and the superoxide anion (O_2^-) produces the relatively long-lived potent prooxidant peroxynitrite anion ($ONOO^-$), which is highly toxic, initiates lipid peroxidation, and nitrates tyrosine residues on proteins, thus inhibiting or promoting signal transduction pathways [15]. NO also modulates mitochondrial respiration and the redox state of mammalian cells [16]; it could react with sulfide-containing molecules (such as albumin) to form nitrosothiol compounds [17] and promotes vascular endothelial insulin transport [18]. These evidences show us the important role of NO in regulating vascular function and that is why abnormalities in their synthesis lead to alterations in vasodilation and changes in vascular function.

In 1997, Sawamura et al. [19] successfully identified the major endothelial receptor for oxidized LOX (oxLDL), a lectin-like oxidized LDL receptor-1 (LOX-1). LOX-1 is a key molecule in the generation of endothelial dysfunction [20, 21]. LOX-1 activation is associated with cell proliferation, apoptosis, and cell migration [22, 23]. Binding of oxLDL to LOX-1 rapidly activates NADPH oxidase, resulting in rapid increase of intracellular reactive oxygen species (ROS), including O_2^- and H_2O_2 [24], with concomitant decreased intracellular NO [25], and decline cytochrome P450 activity resulting in decrease of endothelium-derived hyperpolarizing factor [26] and endothelial cells dysfunction [27].

2.1. Receptor LOX-1. LOX-1 is an endothelial receptor for circulating oxLDL that has been studied extensively in pathological states, such as atherosclerosis, diabetes, coronary arterial heart disease, and hypertension [22, 23, 28]. LOX-1 is highly expressed in blood-vessel-abundant tissues such as placenta, lung, marrow, and spinal cord, is moderately expressed in hippocampi, testicle, and large arteries, and is slightly expressed in heart, skeleton muscle, and ovary [19]. At the cellular level, LOX-1 is expressed in macrophages, vascular smooth muscle cells, monocytes, and endothelial cells [27]. In vitro, the basal expression of LOX-1 is low, but the expression is highly induced by proinflammatory and prooxidative stimuli in endothelial cells, smooth muscle cells, and macrophages. The stimuli include TNF-α [29, 30], heparin-binding-EGF [31], oxLDL [32], oxidative stress [33], remnant-like lipoprotein particles (RLPs) [34], angiotensin II [35], D-glucose [36], and lysophosphatidylcholine [32].

Vascular LOX-1 gene expression is markedly enhanced in hypertensive rats [37–39], hyperlipidemic rabbits [40], and diabetic rats [28]. Ischemia reperfusion also increases the LOX-1 expression in myocardium and kidney [41–44]. Several clinical drugs can inhibit the expression of vascular LOX-1. These include antihypertensive (angiotensin II receptor agonist, calcium channel blockers, and angiotensin-converting enzyme inhibitors), antihyperlipidemics (statins) [45], antidiabetic (sulfonylurea, biguanide, and peroxisome proliferator-activated receptor-γ/PPARγ agonist) [46], antithrombotic (aspirin) agents [47], and dihydrotestosterone [48].

Although LOX-1 was initially characterized as a receptor for oxidized LDL, just like other scavenger receptors, LOX-1 exhibits binding activity for multiple ligands. The precise oxLDL epitope recognized by LOX-1 is not known but is thought to be peptide based [49]. Several studies have shown that LOX-1 can recognize other modified lipoproteins including hypochlorite modified high-density lipoprotein [45], but not native LDL. LOX-1 also binds anionic polymers such as polyinosinic acid and carrageenan [49], anionic phospholipids including phosphatidylserine [50], apoptotic bodies [51], activated platelets [52], AGEs (advanced glycation end-products) [53], and both gram-positive and gram-negative bacteria [54].

The human LOX-1 gene (OLR1; low density lipoprotein oxidized receptor 1, OMIM no. 602601) localizes within natural killer-gene complex (NKC) as a single-copy gene and is assigned to the p12.3-p13.2 region on the short arm of human chromosome 12 [55]. OLR1 gene has more than 7000 bp and is composed of 6 exons separated by 5 introns; introns 1 to 5 have a length that is in the range of 102–246 bp while exon 6 is longer extending up to 1722 bp. Exon 1 encodes the 5′-UTR region and the cytoplasmic domain of LOX-1; exon 2 encodes the remaining portion of the cytoplasmic domain and the transmembrane domain; exon 3 encodes the neck domain; exons 4, 5, and 6 encode the C-type lectin domain and the 3′-UTR region of the protein [56]. The LOX-1 promoter is constitutively active only at low levels but may rise in different pathological conditions, including hypertension, hyperlipidemia, diabetes, and atherosclerosis [22], and its expression can be induced by different ligands or activators, including oxLDL, shear stress, phorbol 12-myristate 13-acetate (PMA), advanced glycation end products (AGEs), and others [32, 56, 57]. LOX-1 protein has a molecular weight of 50 kDa and belongs to the family of the C-type lectin [19]. It is synthesized as a precursor protein of 40 kDa, which undergoes subsequent four glycosylation sites found in the extracellular domain C-terminus, being finally processed to the mature form of 48 kDa [58, 59].

The existence of certain SNP gene OLR1 is associated with an increased risk in developing acute infarction (AMI).

For instance, patients with allele T/T or C/T in the 3′-UTR region are at increased risk (OR 3.74) of developing AMI [60]. Another SNP related to cardiovascular disease is G501C polymorphism, which is found inversely proportional to the degree of stenosis and severity of coronary artery disease [61]. Furthermore, a polymorphism located in exon 4, which produces an amino acid change at position 167 (K167N) of the C-type lectin domain of the protein, causes a reduction in the binding and internalization of oxLDL [62, 63]. A total of 7 SNP in the OLR1 gene have been identified, six of them, located within introns 4, 5 and 3′-UTR, comprised a complete linkage disequilibrium block associated with the elevated risk for myocardial infarction [60]. Since SNPs are located in noncoding regions they do not produce changes in receptor expression; however, modulate the relative abundance of two transcripts generated by alternative splicing. One of these products corresponds to the entire shape of the receptor, while the other is a truncated version, called LOXIN.

LOXIN represents a variable isoform as a consequence of alternative splicing of LOX-1 receptor, which confers protection against proatherogenic effects, contributing to the formation of an inactive heterodimer with LOX-1 [64, 65]. LOXIN, lack of exon 5, lost 2/3 of the lectin-like domain and therefore is unable to bind oxLDL [64]. Interestingly, expression of LOXIN in COS-7 cells [64], endothelial cells, human endothelial progenitor cells (unpublished data), and mononuclear cells of peripheral blood [63, 65] results in a decrease in apoptosis mediated by oxLDL, revealing a potential cardioprotective effect of LOXIN. Currently, there are no studies that demonstrate the role of LOXIN in preeclampsia.

Through ROS generation, LOX-1 stimulates gene expression by activating two signal transduction pathways involving either p38MAPK or ERK1/2 and PI3K, both causing NF-κB activation [22, 23]. NF-κB regulates expression of vascular genes including P-selectin, VCAM-1, ICAM-1, MCP-1, and M-CFS, involved in the attachment and activation of monocytes [66–68]. Decrease of eNOS and Bcl-2 and increase of matrix metalloproteases (MMP1, 3, 9) and Fas expression cause cells injury and apoptosis of endothelial cells [69]. LOX-1 activation can also lead to cell proliferation that is blocked with anti-LOX-1 neutralizing antibody. Recent evidence has shown that a complex formed by LOX-1 and membrane type 1 matrix metalloproteinase (MT1-MMP) plays a crucial role in RhoA and Rac1 activation signaling pathways in Ox-LDL stimulation. Blockade of LOX-1 or MT1-MMP inhibits cell invasion, endothelial NO synthase protein downregulation, RhoA-dependent and NADPH oxidase activity, and reactive oxygen species generation, mediated by Rac-1 [70]. All these evidences suggest the close relationship between NO bioavailability and ROS generation during LOX-1 activation in human endothelial cells (Figure 1).

2.2. LOX-1 and Placenta. The placenta is the regulator of nutrient composition and supply from mother to fetus and the source of hormonal signals that affect maternal and fetal metabolism; appropriate development of the placenta is crucial to normal fetal development [71]. During pregnancy, cholesterol is an essential component for placental and fetal development, used by the placenta for the synthesis of steroid hormones [72]. Physiological adaptations of maternal lipoprotein metabolism occur throughout pregnancy, leading to an increase in lipoprotein concentrations from second trimester to term in preparation for the catabolic phase of late pregnancy, a period of rapid fetal growth.

The placenta exhibits a high expression of LOX-1 mRNA [19] and the maternal lipid profile is associated with placental protein expression of OLR1 [73] which suggest a crucial role of this receptor in the placental function. For instance, it has been suggested that LOX-1 might be involved in trophoblast invasion in early pregnancy [74, 75] and accelerated trophoblast apoptosis and endothelial dysfunction preeclampsia [76]. In addition, using a trophoblastic cell line, the choriocarcinoma JAR, it has been reported that LOX-1 is responsible for 40–50% of oxLDL uptake [77]. Developmental studies looking for expression of LOX-1 in murine and human placentas have described higher expression of LOX-1 during early to midgestational stages than late gestation [78]. In murine placenta, LOX-1-expressing cells were fibroblast-like stromal cells in metrial glands and decidua basalis and trophoblast cells in the junctional and labyrinth zones. In the human placenta, LOX-1 was detected in villous cytotrophoblasts in first trimester and term placentas. Other studies show that LOX-1 is localized in extravillous trophoblasts of first trimester placentas [74, 79] and in syncytiotrophoblast of normal and preeclamptic term placentas, the latter being higher than normal [76].

Despite the fact that LOX-1 is present in placenta, it is intriguing what would be its functions. From studies using LOX-1 deficient mice it is known that LOX-1 is not a lethal gene, since animals are fertile [80] and had no detectable abnormalities during pregnancy. However, cells that express LOX-1 in the placental or in the maternal-placental interphase may be involved in management and evolution of oxidative stress or inflammatory response during pregnancy. In this regard, studies of Satoh et al. [78] demonstrated that LOX-1 is localized in fibroblast-like cells, a cell type that is closely associated with uterine NK cells; then this receptor might be involved in regulation of trophoblast invasion and maternal vascular remodeling during implantation.

3. Preeclampsia Overview

Preeclampsia is a major cause of maternal and infant morbidity and mortality worldwide [81–83]. Stillbirth is more common in preeclamptic pregnancies while one-third of infants of preeclamptic women are growth restricted [84, 85]. The incidence of preeclampsia is variable, affects 7 to 10% of all pregnancies [81, 85–88], and depends on the demographic and sociocultural characteristics of the population, as well as the criteria used for diagnosis of the disease. According to the criteria of the International Society of the Study of Hypertension in Pregnancy, the pregnancy-induced hypertension is defined as "*diastolic blood pressure >90 mm Hg occurring after week 20 of gestation with proteinuria (either 300 mg protein per day or a urinary protein/creatinine ratio 30 mg/mmol)*" [89]. However, proteinuria is no longer an absolute requirement

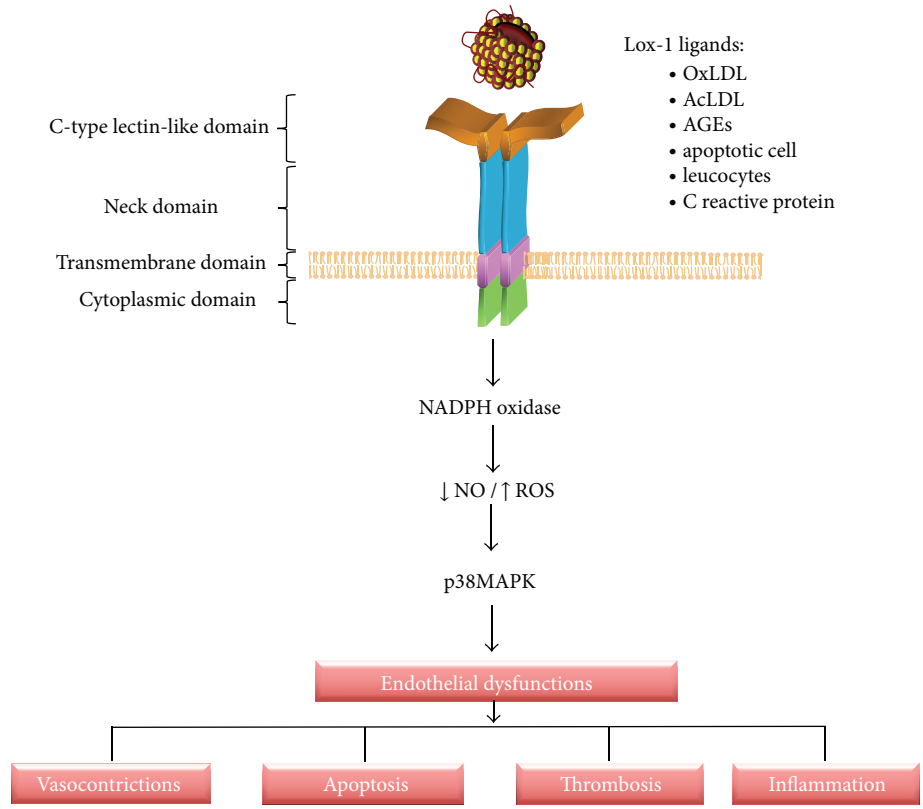

Figure 1: Structure and LOX-1 signaling. LOX-1 has four domains: cytosolic domain (1–34 amino acids), transmembrane domain (amino acids 35–61), neck domain (amino acids 62–143), and CTLD (amino acids 144–263). The LOX-1 activation by different ligand increases phosphorylation of p42/44MAPK and p38MAPK and the expression of gp91phox subunit of NADPH oxidase, causing an increase in reactive oxygen species and a decrease in NO and hence endothelial dysfunction manifested by cell apoptosis, thrombosis, inflammation, and vasoconstriction.

for the diagnosis of preeclampsia according to the 2013 guide of The American College of Obstetricians and Gynecologists [90]. The removal of proteinuria as a diagnostic requirement for preeclampsia/eclampsia reflects the recognition that many women (14%) with preeclampsia do not have proteinuria [91], and such women have historically experienced delays in diagnosis and treatment as a result. Alternatively, the diagnosis may be established by the presence of hypertension associated with thrombocytopenia (platelet count less than 100.000/μL), impaired liver functions (elevated blood concentrations of liver transaminases to twice the normal concentration), development of renal insufficiency (serum creatinine concentration greater than 1.1 mg/dL or a doubling of the serum creatinine concentration in the absence of other renal diseases), pulmonary edema, or new-onset cerebral or visual disturbances [92, 93].

The risk factor of developing preeclampsia is higher in women with diabetes, thrombophilia, and obesity with either preexisting vascular disease or conditions associated with increased cardiovascular risk, including renal disease, hypertension, and with previous preeclampsia [94]. Clinical manifestations are highly variable and may occur antepartum, intrapartum, or postpartum [95]. The preeclampsia can lead to problems in the liver, kidneys, brain, and the clotting system. Moreover, as stated above, preeclampsia is associated with low birthweight and perinatal deaths due to premature birth and intrauterine growth restriction [81, 96].

Although etiology of preeclampsia is still unknown, there is no doubt that this condition is associated with endothelial dysfunction, which leads to occurrence of classical clinical syndrome of hypertension, proteinuria, and edema [83, 97–100]. It is believed that the placenta has a key role in the pathophysiology of preeclampsia since the symptoms and complication of preeclampsia disappear after delivery of placenta [100, 101]. On the other hand, the uterus or fetus is probably not necessary for the development of this pathology, since there have been reports of preeclampsia with abdominal pregnancies [102] or hydatidiform moles [103].

Although endothelial dysfunction is well described in preeclampsia, the underling mechanism behind this alteration is not completely understood. Several studies have described the release of harmful molecules that may cause endothelial damage from the placenta toward maternal circulation during this disease [104–110]. For instance, the release of soluble receptor type 1 of vascular endothelial growth factor (sFlt-1) [88, 111], soluble endoglin [112], and lipid peroxides [113] has been described. Nowadays, many groups believe that trophoblastic microparticles/nanoparticles shedding toward maternal circulation are a keystone event in the generation of preeclampsia. The investigation is focused

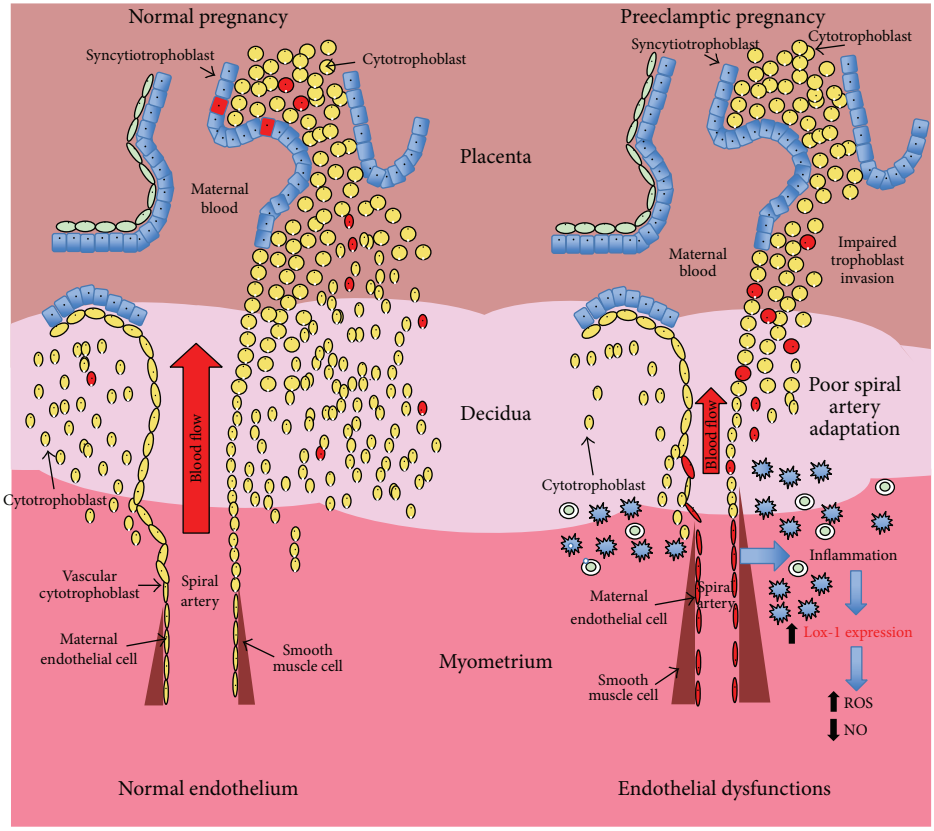

Figure 2: Preeclampsia and LOX-1. In a normal pregnancy adequate trophoblast invasion produces spiral arteries in saccular without a muscular layer, whereby the placental bed is a set of high flow and low resistance. However, in preeclampsia the uteroplacental blood flow was reduced due to an incomplete trophoblast invasion that generates a high strength and low blood flow. Under this condition, placentation arterial lesions were subsequently produced inducing an inflammatory condition, thus increasing perfusion deficit and oxidative stress. In normal pregnancy, lower levels of LOX-1 were found expressed in cells that are part of the trophoblast and syncytiotrophoblast. On the other hand, in preeclampsia LOX-1 levels increase significantly, which may be responsible for the generation of high levels of ROS and decreased levels of NO.

on trying to understand how these particles are generated, what would be their content, and what they are doing in the maternal physiology. Actual beliefs explain that incomplete or absent trophoblastic invasion to the uterine spiral arteries is related to reduced uteroplacental blood flow and placental ischemia [83, 105, 114, 115] (see Figure 2). In this scenario, hypoxia/reoxygenation of placental tissue leads to synthesis of free radicals [116] and in particular synthesis and release of reactive oxygen species (ROS), such as superoxide anion, which easily reach the maternal circulation directly or via modification of macromolecules where it can react with NO leading to reduction of NO bioavailability and impaired vascular response [117].

On the other hand, early placental development is characterised by rapid cell differentiation and migration, matrix remodelling, and angiogenesis. The enzyme NADPH oxidase is a major source of superoxide anions implicated in signalling pathways regulating these processes [118]. Normal pregnancy has an increased oxygen requirement by different organs, including the fetoplacental unit [119]; therefore it is necessary to maintain a tight control of the levels of oxidants and antioxidants during pregnancy. Diverse studies comparing biomarkers of oxidative stress in normal pregnant women and nonpregnant control subjects have shown that lipid peroxidation is enhanced during the second trimester of pregnancy as well as late in gestation [120], which is associated with decreased activity levels of SOD and GPX and decreased glutathione [121, 122]. The placenta is an important generator of lipid peroxides [120] but is also rich in antioxidant defense elements such as SOD, catalase, GPx, glutathione, and vitamins C and E [123]. ROS play important roles in normal placental development and may also play a role in influencing the growth trajectory of the placenta, and its component cell types, in contrast to the oxidative stress considered responsible for the pathophysiology of many diseases pathologies of pregnancy [124].

Growing evidence suggests that placental oxidative stress is involved in the etiopathogenesis of preeclampsia. Besides this, preeclampsia is characterized by a diminished antioxidant capacity [125]. Women with preeclampsia show increased biomarkers of oxygen radical damage and impairment of antioxidant defence. Placental tissue shows higher level of markers of lipid peroxidation such as F2-isoprostanes [126], nitrotyrosine and 4-hydroxynonenal [127]. Moreover,

the activities of SOD and GPX and the tissue levels of vitamin E were significantly lower in preeclamptic placentas compared to normal placentas, whereas the activity of catalase was significantly higher in preeclampsia. When analyzed at the mRNA level, expression of SOD and GPX was found significantly lower in preeclamptic versus normal placentas, but there was no change in the catalase expression [128]. In other studies, the tissue levels of endogenous antioxidant proteins such as superoxide dismutase, glutathione peroxidase, thioredoxin reductase, and thioredoxin were all reduced in preeclamptic tissue compared with normal tissue [129]. Glutathione is a major endogenous water-soluble antioxidant, and women with preeclampsia had lower glutathione concentrations in plasma [130] and in erythrocytes compared with normotensive control women [131]. Moreover, significantly lowered levels of vitamins E and C were observed in preeclamptic women as compared with controls [132]. Overall, the evidence suggests that increased oxidative stress and reduction in antioxidant defense mechanisms may contribute to the disease process in preeclampsia.

3.1. LOX-1 and Preeclampsia. Maternal hyperlipidemia is one of the striking changes to take place in lipid metabolism during even normal pregnancy [133]; however elevated maternal circulating cholesterol is a risk factor leading to fetal endothelial dysfunction, which could have serious consequences to the growing fetus [134]. There are several indirect evidences that suggest the participation of LOX-1 in the pathophysiology of preeclampsia. Thus, small dense LDL is also increased in the plasma of women with preeclampsia and LDL particles are more susceptible to oxidation, resulting in the generation of oxLDL [135]. Also it has been reported that levels of antibodies to oxLDL are elevated in women with established preeclampsia and in pregnant women with a history of repeated abortion [76]. Human placental cells express many lipoprotein receptors such as the LDL receptor (LDLR; [136]), the LDL receptor-related protein (LRP; [137]), the VLDL receptor (VLDL receptor; [138]), the scavenger receptors [139–141], and the LOX-1 [142].

More direct evidences have described high levels of LOX-1 in rat model of preeclampsia [143]. Moreover, the inhibition of LOX-1 prevents endothelial dysfunction in an in vitro model of preeclampsia [144]. On the other hand, it was found that upregulation of LOX-1 in HUVECs is exposed (24 h) to plasma from women with preeclampsia [135], a phenomenon that was also observed when plasma from preeclampsia was used in a rat model [145]. Indeed, in this animal model it has been demonstrated that exposure of plasma from women with preeclampsia with high content of oxLDL increased blood brain barrier permeability after acute exposition [145], suggesting that LOX-1 might be involved in occurrence of brain alterations associated with eclampsia. Other authors previously demonstrated an increase in the LOX-1 and arginase expressions in the maternal vasculature of women with preeclampsia when compared with normotensive pregnant women [135]. Contrasting to these results, but using placental tissue, Chigusa et al. [77] reported a decreased LOX-1 expression in preeclampsia.

Recently, Morton et al. [143] demonstrated in a rat model of preeclampsia a significant increase in the expression of both LOX-1 receptor and eNOS in the thoracic aorta, which was associated with increased superoxide anion generation and subsequent decreased endothelial function. LOX-1 activation has been shown to induce several intracellular signaling pathways, including increased expression of chemokines and adhesion molecules, triggering the CD40/CD40L pathway that activates the inflammatory cascade and increased generation of reactive oxygen species, such as superoxide anion in endothelial cells [146, 147]. Studying the soluble isoform of LOX-1 (sLOX-1), other groups have found no significant differences in sLOX-1 concentration in the plasma of patients with preeclampsia compared with matched control plasma [144].

Lee et al. [76] demonstrated that in vivo localization and the upregulated expression of LOX-1 are associated with reduced placental SOD-1 activity in preeclampsia, which fit perfectly in the well-described phenomenon of increased oxidative stress and reduced antioxidative defense in the preeclamptic placenta. Similar evidence suggests that oxLDL and LOX-1 could activate the NADPH oxidase enzyme system to generate superoxide anion. Sankaralingam et al. [135] demonstrated increased NADPH oxidase activity in cultured endothelial cells in response only to plasma from women with preeclampsia, which was significantly reduced by blocking with anti-LOX-1 or siRNA to LOX-1. Then, all these results suggest that LOX-1 is involved in the pathophysiology of preeclampsia.

4. Concluding Remark

LOX-1 is an important scavenger receptor of Ox-LDL and plays an important role in the pathogenesis of atherosclerosis. Ox-LDL causes endothelial dysfunction and its accumulation is the first step in the development of cardiovascular disease. LOX-1 activation via Ox-LDL is thought to be involved in the initiation and the development of other different pathological conditions, including hypertension, hyperlipidemia, diabetes, and preeclampsia.

The underlying participation in the initial development of normal placenta and the pathophysiology of preeclampsia seem far more complex than originally thought as LOX-1 plays a fundamental role in the process of trophoblast differentiation and syncytiotrophoblast formation. The activation of LOX-1 in early pregnancy contributes to inducing migration and invasion of the trophoblast and cell apoptosis, where this phenomenon seems to depend on the formation of reactive oxygen species. However, during preeclampsia there is an increased expression of LOX-1 receptor, suggesting that this receptor may contribute to the endothelial dysfunction observed in this pathology. The increased expression and activity of LOX-1 in preeclampsia would favor the formation of free radicals that alter cellular function. Free radicals have opposite effects depending on their concentration; high expression of LOX-1 in preeclampsia may be associated with high levels of ROS and endothelial dysfunction, whereas low levels of ROS promote cell proliferation and migration.

The evidence shown in this review suggests the important role of LOX-1 in normal and pathological conditions, and their identification could be useful for understanding the development of preeclampsia. However, more information is needed in order to better understand the role of LOX-1 in the development of the placenta and in some pregnancy complications, such as preeclampsia.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Programming of Fetal Insulin Resistance in Pregnancies with Maternal Obesity by ER Stress and Inflammation

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The global epidemics of obesity during pregnancy and excessive gestational weight gain (GWG) are major public health problems worldwide. Obesity and excessive GWG are related to several maternal and fetal complications, including diabetes (pregestational and gestational diabetes) and intrauterine programming of insulin resistance (IR). Maternal obesity (MO) and neonatal IR are associated with long-term development of obesity, diabetes mellitus, and increased global cardiovascular risk in the offspring. Multiple mechanisms of insulin signaling pathway impairment have been described in obese individuals, involving complex interactions of chronically elevated inflammatory mediators, adipokines, and the critical role of the endoplasmic reticulum (ER) stress-dependent unfolded protein response (UPR). However, the underlying cellular processes linking MO and IR in the offspring have not been fully elucidated. Here, we summarize the state-of-the-art evidence supporting the possibility that adverse metabolic postnatal outcomes such as IR in the offspring of pregnancies with MO and/or excessive GWG may be related to intrauterine activation of ER stress response.

1. Introduction

The global epidemic of overweight and obesity is defined by the World Health Organization (WHO) as abnormal or excessive body fat accumulation that presents a risk to health. WHO defines normal weight, overweight, and obesity as a body mass index (BMI, calculated as ratio of weight in kg/height in m^2) of 18.5–24.9, 25–29.9, and 30 or greater, respectively. Obesity is further categorized by BMI into class I (30–34.9), class II (35–39.9), and class III or extreme obesity (≥40) [1]. BMI data from the WHO show that 43% of countries with recent nutritional information reported that half or more of their adult population has a BMI ≥ 25 [2]. The increasing prevalence of this nutritional problem is associated with many diet-related chronic diseases, including diabetes mellitus, cardiovascular disease, stroke, hypertension, and certain cancers. In pregnancy, obesity is associated with various perinatal morbidities [3], including diabetes (pregestational and gestational), cesarean delivery, gestational hypertension and preeclampsia, congenital anomalies, macrosomia (birthweight > 4000 g), and maternal or fetal mortality [4, 5].

In addition to the perinatal complications of obesity during pregnancy, increasing epidemiological evidence suggests persistent and deleterious effects of maternal obesity (MO) on the offspring and through intrauterine programming [6, 7]. However, the underlying mechanisms that could explain a

potential link between MO and risk of problems such as insulin resistance (IR) in the offspring remain unclear. In overweight and obese individuals, nutrient excess is associated with a chronic inflammatory [8, 9] and cellular stress [10] signaling network involved in the adaptive response to persistent overload of glucose, amino acids, and free fatty acids (FFA) [11]. Adipose tissue produces circulating bioactive substances named adipokines (such as leptin, adiponectin, and resistin) and inflammatory markers (such as interleukin (IL) 6 and tumor necrosis factor α (TNF-α) [12]). These molecules are also implicated in the etiology of obesity-induced IR, based on common activation of stress-responsive proteins including c-Jun-NH2-terminal kinase (JNK), the inhibitor of nuclear factor kappa B (IKK), protein kinases C (PKC), and R (PKR) [12–14]. Growing evidence indicates that the cellular stress linking obesity and increased circulating and subcellular markers of IR implies a crucial role of the endoplasmic reticulum (ER) stress response [15–22]. The present review summarizes the findings supporting the hypothesis that adverse metabolic postnatal outcomes such as IR in the offspring of pregnancies with obesity and/or excessive gestational weight gain (GWG) are related to intrauterine programming and activation of the ER stress response.

2. Postnatal Effect of Maternal Obesity on the Offspring

Obesity and excessive GWG are recognized as independent risk factors for maternal and fetal complications [4, 5, 23]. Since the first publication by the Institute of Medicine in 1990 of GWG recommendations [24], there has been a 70% increase in the prevalence of prepregnancy obesity in the USA [4]. A large percentage of obese individuals will experience comorbidities during their life span, including the fertile age. Among the major general medical comorbidities are hypertension, cardiovascular disease, diabetes mellitus, hyperlipidemia, metabolic syndrome (a clinical condition associated with central obesity, hypertension, dyslipidemia, and IR), thromboembolic events, and cancer. In pregnancy, obesity is associated with various perinatal morbidities, including diabetes (pregestational and gestational), cesarean delivery, gestational hypertension and preeclampsia, congenital anomalies, macrosomia (birthweight > 4000 g), and maternal or fetal mortality [4, 5]. Prepregnancy obesity and excessive GWG have been implicated in an intergenerational "vicious cycle" of obesity [25]. Overweight or obese pregnant women have an increased probability of delivering macrosomic daughters, who are more likely to become obese themselves and deliver large neonates [26, 27]. In fact, GWG and birthweight are directly associated with BMI and risk of obesity in adolescence [28–30]. Based on these results, Oken et al. proposed that GWG guidelines should account for these influences of maternal nutrition on child weight [31]. The reported relationship was independent of other parental characteristics, potentially mediating peripartum factors, and abnormal dietary behaviors in the child, suggesting a role for the intrauterine environment in long-term offspring weight regulation. Interestingly, the association between maternal GWG and increased risk of adiposity in the offspring has been shown to emerge as early as 3 [31] or 7 years of age [32].

Considering the high prevalence of obesity in pregnancy and its association with gestational diabetes, there is an increasing interest in exploring the potentially negative influence of maternal overnutrition and elevated birthweight on the risk of disease in childhood and adulthood [25, 26, 41, 42]. Thus, it has been reported that children of obese women are more likely to develop IR later in life [43, 44]. Fraser et al. showed a detailed association of GWG and prepregnancy weight with offspring cardiovascular risk factors at the age of 9, in a large cohort of mother-offspring pairs from the Avon Longitudinal Study of Parents and Children (ALSPAC) [33]. In these studies, women who gained excessive weight during gestation were more likely to have offspring with greater BMI, waist size, fat mass, leptin, systolic blood pressure, C-reactive protein, and IL-6 levels and lower high-density lipoprotein cholesterol and apolipoprotein A levels (Table 1). Additional analysis demonstrated that excessive prepregnancy weight was also independently associated with greater offspring adiposity and adverse cardiovascular risk factors (Table 1) [34–38, 45, 46]. Epidemiological studies revealed that MO increases the incidence of metabolic syndrome in children [41, 47]. Moreover, the effect of MO on the susceptibility to obesity in offspring seems to be independent of gestational diabetes, as obese women with normal blood glucose have neonates with increased adiposity [39]. Interestingly, the same group has shown that MO is related to metabolic compromise already apparent at birth, characterized by reduced insulin sensitivity and increased serum inflammatory markers [26, 40]. Hence, maternal prepregnancy obesity and excessive GWG are independently related to an increased risk of obesity, IR, and very early markers of cardiovascular disease in the offspring. This evidence shifted our attention towards the gestational period as an extremely important intervention target in prevention of the obesity epidemic and its associated consequences such as IR and cardiovascular risk.

Ample evidence has indicated differential contributions of genetic and environmental factors in the development of noncommunicable diseases, such as obesity, diabetes mellitus, or cardiovascular diseases. In the case of obesity, the demographic shift of populations towards a fatty phenotype over a relatively short period of one or two generations argues against a major genetic contribution in favor of environmental or epigenetic mechanisms. In line with the concept of greater relevance of environmental factors, recent reports suggest that the prevention of childhood and adult obesity may need to begin even before conception [48–51]. Since pregnancy is a critical period of life, ethical considerations limit our ability to perform detailed mechanistic studies in humans. Therefore, animal models have been developed to address multiple questions in reproductive medicine.

Several animal models are used to study the mechanisms linking the altered prenatal environment in MO with the increased risk of obesity and other metabolic consequences in the developing offspring [52, 53]. Feeding animals a high fat diet (HFD) is a common model of overnutrition in pregnancy. Pups from rats on HFD during pregnancy and

Table 1: Cardiovascular risk factors in offspring of pregnancies with maternal obesity.

Parameters	Effect	Maternal obesity	Offspring age (years)	Reference
Blood pressure	Increased	eGWG	9, 21	[33, 34]
		ePPW	Neonates, 6, 17	[35–38]
Body fat	Increased	eGWG	9	[33]
		ePPW	Neonates, 6	[37–40]
BMI	Increased	eGWG	9, 21	[33, 34]
		ePPW	6, 17	[36, 37]
IL-6	Increased	eGWG	9	[33]
		ePPW	Neonates	[40]
CRP	Increased	eGWG	9	[33]
Abdominal fat	Increased	eGWG	9	[33]
		ePPW	6	[37]
Leptin	Increased	eGWG	9	[33]
		ePPW	Neonates	[40]
HDL	Decreased	eGWG	9	[33]
		ePPW	6	[37]
ApoA1	Decreased	eGWG	9	[33]
Insulin	Increased	ePPW	6	[37]
HOMA-IR	Increased	ePPW	Neonates	[40]

ePPW, excessive prepregnancy weight; eGWG, excessive gestational weight gain; BMI, body mass index; IL-6, interleukin 6; CRP; c-reactive protein; HDL: high-density lipoprotein; ApoA1: apolipoprotein A-I; HOMA-IR: homeostasis model assessment for insulin resistance.

lactation, for instance, were shown to be heavier, fatter, and more hyperglycemic and moreover had higher hepatic lipid content at weaning than pups from rats fed a control diet [54]. In a similar mouse model, chronic maternal overnutrition was associated with hyperphagic behavior, reduced locomotion, increased adiposity, nonalcoholic fatty liver disease, and IR in the offspring at 3 and 6 months of age [55, 56].

Rodent models genetically predisposed to obesity are also used to evaluate the effects of MO. Genetically normal offspring of obese Agouti mouse dams, for example, were heavier than offspring from controls [57]. Interestingly, although adult weight did not differ between groups, female offspring of obese Agouti mothers had reduced β-pancreatic cell function and altered glycemic homeostasis [57]. Another transgenic model of maternal obesity is the heterozygous leptin receptor-deficient mouse (Lepr (db/$^+$)) [58]. The pregnant Lepr (db/$^+$) female is characterized by overeating, increased weight gain during pregnancy, and spontaneous development of gestational diabetes. Moreover, wild-type offspring show increased fetal growth and postnatal markers of hepatic insulin resistance, suggesting the occurrence of fetal programming [58]. Several research groups have been working to understand the mechanisms by which intrauterine metabolic alterations lead to particular phenotypes and influence susceptibility to obesity and metabolic diseases. The molecular mediators and signaling pathways that could be related to offspring metabolic phenotype (such as obesity and IR) are not fully elucidated. However, multiple inflammatory cytokines, hormones such as leptin or adiponectin, and nutrients such as glucose, free fatty acids (FFA), and triglycerides could be implicated in a mechanistic explanation of the increased metabolic risk in the offspring of MO [6, 14].

During normal intrauterine life, maternal insulin [59] and human insulin analog lispro (Humalog) [60, 61] are unable to cross the placenta, whereas maternal glucose is actively transferred to the fetus [62]. The developing human fetal pancreas responds to a glucose load by producing insulin, which acts as a fetal growth hormone in addition to its hypoglycemic effects. This is the basic concept of the "Pedersen's hyperglycemia-hyperinsulinism hypothesis" [63] to explain why offspring of diabetic mothers exhibit relatively higher birthweight [64, 65]. Maternal overnutrition produces maternal hyperglycemia, which increases fetal insulin secretion in a way similar to that observed in gestational diabetes [64–66]. Therefore, secondary fetal hyperinsulinemia is believed to be involved in the intrauterine programming of obesity and diabetes [41]. Prospective studies indicate that, at 6 years of age, as at birth, the greatest increase in weight-to-height ratio (relative obesity) was seen in children who experienced the greatest exposures to insulin in the uterus (as judged by amniotic fluid insulin concentration) [59]. Furthermore, animal studies show that systematic insulin administration to rats during pregnancy produces increased fetal growth [67], hyperinsulinemia, and impaired glucose tolerance [68].

Leptin may be implicated in the metabolic impairment observed in the offspring of MO and diabetes, as elevated circulating levels of this hormone are found in maternal and neonatal serum in association with these conditions [69]. However, in spite of the fact that placental transfer of

leptin has been demonstrated *in vivo* [70], it is believed that umbilical levels of this circulating peptide are a marker of neonatal adiposity more than a modulator of fetal growth [69]. Several inflammatory cytokines are elevated in obese pregnant women [71] and have been postulated to be potential mediators of metabolic programming.

Consequently, the literature strongly suggests that altered metabolic phenotypes such as obesity and IR observed in the offspring of obese mothers could be partially explained by multiple mediators. It is likely that a model encompassing the multifactorial contributions of nutrient (such as glucose, fatty acid, and amino acid) and hormone (such as insulin and leptin) signals between the obese mother and the developing fetus would best describe the true mechanisms involved. The general question addressed in this review is how these factors induced by maternal obesity could modify insulin-dependent metabolic homeostasis in the offspring.

3. Insulin Resistance Mechanisms

Insulin is a key endocrine hormone that controls whole-body glucose, lipid, and protein homeostasis [72]. It also controls several other important processes such as cell growth, cell proliferation, survival, and differentiation [73]. Insulin mediates its biological effects via activation of insulin receptors A (IR-A) and B (IR-B) [74, 75] in major insulin target tissues [76], including human umbilical vein endothelial cells (HUVEC) [64] and human placenta microvascular endothelium (hPMEC) [65]. Subsequently, binding of insulin to IR-A and/or IR-B promotes its autophosphorylation and activation of the insulin receptor substrate family 1–4 (IRS1-4) [77, 78]. Phosphorylated IRS-1 (P-IRS-1) can bind adaptor proteins by linking its Src Homology 2 domain (SH2), such as p85 (regulatory subunit of phosphatidylinositol-3 or PI_3K) and growth factor receptor-bound protein 2 (Grb-2). Thus, when the SH2 domain of p85 binds to IRS-1-P, the catalytic subunit p110 of PI_3K is activated. In the same way, binding of SH2 domain of Grb-2 to P-IRS-1 activates the associated factor Son of sevenless (SOS). Next, activation of PI_3K generates lipid mediators such as inositol triphosphate (IP_3), which in turn initiates a cascade of signaling events dependent on protein kinases. These protein effectors include the IP_3-dependent kinase 1 (PDK-1) and protein kinase B (Akt) [79]. Depending on the cell type, the insulin signaling pathway culminates in a series of different effects, such as glucose transporter that mediates the uptake in mediated uptake in liver cells [79] or activation of nitric oxide (NO) production in HUVEC [64] and hPMEC [65] in normal pregnancies. In a similar phosphorylation pathway cascade, activation of Grb-2/SOS involves activation of GTP-binding proteins Ras/Raf and mitogen-activated protein kinases (MAPK) [77]. Because both MO [27] and gestational diabetes [80] have been associated with decreased insulin sensitivity and increased IR status in the offspring, we will analyze how those insulin signaling mechanisms could be impaired in pregnancy.

IR is defined as a pathophysiological condition of unresponsiveness to normal insulin concentrations in target tissues such as adipose, muscle, liver, or cardiovascular tissues. Impaired insulin action is caused by reduced expression and/or function of its complex cellular response machinery [78]. Postreceptor defects in the intracellular insulin signaling pathway at different levels (such as in the mitochondria) may explain IR [81]. Whereas the insulin pathway branch dependent on PI_3K has been thought of as being responsible for the metabolic and vasodilator effects in response to insulin stimulation, the Grb-2/SOS branch has been associated with the mitogen and vasoconstrictor actions of insulin [73, 78, 82]. Hence, abnormal predominance of the insulin derived from Grb-2/SOS signaling branch over the PI_3K pathway has been associated with altered insulin effects on multiple tissues (such as liver, muscle, fat, and blood vessels). Interestingly, stress-activated protein kinases that phosphorylate IR or IRS-1 in serine or threonine residues are associated with inhibition of PI_3K signaling and promotion of IR at metabolic and vascular levels [78].

In overweight and obese individuals, nutrient excess is associated with a chronic inflammatory and cellular stress signaling network involved in the adaptive response to persistent overload of glucose, amino acids, and FFA [11, 83, 84]. Circulating levels of adipokines (such as leptin, adiponectin, and resistin) and inflammatory mediators (such as IL-6 and TNF-α) are directly related to total body fat [12, 85]. These adipokines in turn are associated with autocrine and paracrine cell signaling alterations in response to obesity. All of these circulating products are implicated in the etiology of IR mediated by activation of stress-responsive proteins such us JNK, IKK, PKR, and PKC [12, 83, 85]. In fact, TNFα and FFA are potent activators of JNK, and increased concentrations of these mediators could explain the elevated function of this stress cascade in HFD and genetically obese mouse models [86]. Research which focused on the metabolic consequences of cellular stress in the context of IR development in obese individuals involves a crucial role for the ER stress response [15–20].

4. Endoplasmic Reticulum (ER) Stress Response

The ER is a complex intracellular membranous network that is essential for the synthesis and processing of secretory and membrane proteins [87]. It is highly sensitive to alterations in cellular environmental changes. It works as a quality control station that allows for transit of correctly folded proteins to the Golgi apparatus and retains unfolded or misfolded proteins [88]. Consequently, ER plays a key role in the general cellular response to altered environmental conditions, such as nutrient overload or deprivation, abnormal increase in synthesis of secretory proteins, expression of mutant or misfolded proteins, and microbial infections [88, 89]. All of these "stressor signals" can lead to disruption of ER homeostasis and accumulation of unfolded proteins in the lumen, a condition called ER stress. In order to adapt ER function to this stress, a highly conserved signaling pathway called the unfolded protein response (UPR), or the ER stress response, is activated [87, 89–91]. The activated UPR reduces the translocation of new proteins into the ER lumen and increases retrotranslocation and degradation of misfolded proteins, recovering the folding capacity of the ER. This integrated ER

stress response is characterized by transcriptional activation of multiple UPR-responsive genes mediated by inositol-requiring enzyme 1 alpha (IRE1α) and activating transcription factor 6 (ATF6), promoting a general decrease in translation initiation and a selective translation of several specific mRNAs mediated by PKR-like ER-associated kinase (PERK) [87]. IRE1α, PERK, and ATF6, transmembrane proteins localized on ER surface, are referred to as UPR sensors. These proteins are normally bound by the ER chaperone immunoglobulin binding protein BiP/GRP-78 at intraluminal domains. When immature proteins (also bound by BiP) exceed ER folding capacity, less BiP is available for binding to the UPR sensors. As a consequence, without BiP binding, PERK and IRE-1α autooligomerize and undergo autophosphorylation, leading to the activation of downstream signaling. A key mediator of the UPR is the mRNA encoding to X-box DNA binding protein 1 (XBP1), which is cleaved by the cytosolic endoribonuclease motif of activated IRE-1α, allowing for translation of its mRNA and consequently the generation of XBP1, a potent transcription factor. Moreover, activated PERK leads to an attenuation of general protein synthesis through inhibitory serine phosphorylation of eukaryotic translational initiation factor 2α (eIF2α). Interestingly, serine phosphorylation of eIF2α also results in specific translation of ATF4, another nuclear UPR mediator. Moreover, the release of ATF-6 from BiP binding frees this UPR sensor to be translocated to the Golgi, where it completes its activation as a functional transcription factor. All of these transcription factors (XBP1, ATF4, and ATF6) are translocated to the nucleus where they are able to stimulate the expression of multiple genes implicated in the final adaptive effects of UPR. In this context, it has been reported that transcriptional stimulation of adaptive genes depends on availability of specific ER stress response elements (ERSE), unfolded protein response elements (UPRE), or amino acid response elements (AARE) in the promoter region. Under normal conditions, the UPR pathway functions as a physiological adaptive mechanism. In contrast, when the primary stimulus is too persistent or severe, the ER stress response can lead to irreversible cell damage and programmed death through stimulation of proapoptotic transcription factor growth arrest and DNA damage-inducible gene 153 (GADD153, also called C/EBP homologous protein or CHOP) [87, 89–91].

The UPR is considered an efficient cellular mechanism of adaptation to multiple physiological conditions, but it has also been implicated in the physiopathology of various diseases [86, 88–90]. Despite the fact that first descriptions of UPR elements (such as BiP and IRE1) were associated with genes upregulated by glucose starvation, the ER stress response pathway is also evoked by the nutrient overload observed in diabetes mellitus and obesity. Currently, it is widely accepted that UPR plays a key role in the pathogenesis of diabetes due to its participation in pancreatic β-cell loss and peripheral IR [17, 18, 20, 92]. Moreover, the UPR stimulates the transcription of glucose-regulated proteins that may provide a protective function by increasing cellular capacity related to uptake and use of glucose. Nonetheless, during chronic hyperglycemia or nutrient excess, β-cells are exposed to high levels of immature insulin accumulated in the ER lumen, which may induce cell death through UPR-related mechanisms [17]. Hence, the ER stress response would play a dual role, acting as a beneficial regulator under physiological conditions or triggering β-cell dysfunction and apoptosis under a chronic stress environment.

Interestingly, it has been reported that HFD and obesity induce ER stress in the liver, which suppresses insulin signaling via JNK activation, establishing a potential link between obesity and IR [15, 88]. Moreover, liver cells exposed to pharmacological triggers of ER stress response show IR profiles characterized by serine phosphorylation of IRS-1 and suppression of insulin-induced Akt phosphorylation. Since these alterations in the insulin pathway are blocked by inhibition of JNK, ER stress may promote a JNK-dependent serine phosphorylation of IRS-1, which in turn inhibits insulin receptor signaling. Further experiments confirm crucial roles for IRE1 as a promoter and XBP1 as an inhibitor of ER stress-associated insulin resistance in obesity [15, 93]. In addition, it has been reported that preventing ER stress in obese and diabetic mice with chemical chaperones (such as 4-phenyl butyric acid and taurine-conjugated ursodeoxycholic acid, TUDCA) was associated with restoration of insulin sensitivity at systemic and tissue levels (liver, muscle, and fat) [15, 94]. All of these results suggest that treating individuals exposed to an obesity-related condition with ER stress-alleviating compounds could be used as a new therapeutic tool to prevent or reverse the deleterious effects of obesity, insulin resistance, and pro-inflammatory markers.

5. Link between Inflammation and ER Stress-Related Insulin Resistance

The classic function of the immune system is defense against infections by detecting pathogen-associated molecular patterns (PAMPs), such as bacterial and viral components. However, immune cells are also able to sense damage associated with damage-associated molecular patterns (DAMPs), such as extracellular nucleotides and cytoplasmic and nuclear components [95]. After activation, immune cells use different mechanisms for cell-to-cell communication, including cytokines, which are mainly soluble proteins that can promote pro- or anti-inflammatory responses [96]. Cytokines are produced not only by immune cells but also by almost all cells and activate immune response during injury or infection. Abnormal release of cytokines can promote development and progression of various pathological conditions with diverse etiologies [96]. Moreover, obese individuals exhibit high levels of several pro-inflammatory cytokines, which promote an inflammatory state related to tissue damage [97, 98]. Currently, there is a rising interest regarding the role of inflammation during obesity, especially in cases where exercise and dietary treatment are insufficient to restore the nutritional state [99]. This situation is likely due to a chronic pro-inflammatory response, mediated by various pro-inflammatory cytokines that promote modulation of T cell function toward the Th1 phenotype and macrophage differentiation toward a deleterious M1 phenotype. In contrast to this effect, the predominance of anti-inflammatory cytokines in healthy nonobese individuals shifts T cell and

macrophage polarization toward Th2 and M2 phenotypes, respectively [100]. After TNF-α was described as a major pro-inflammatory cytokine expressed in adipose tissue and with a relationship to IR in murine models of obesity [101], a rising number of studies showed that the immune system contributes to the sensing of metabolites and nutritional status in the whole body [84].

Inflammation has been related to ER stress development; nevertheless, controversy remains as to whether this cellular stress response promotes or prevents progression of several diseases [102]. Under obesity conditions, ER stress may have a deleterious effect associated with the pro-inflammatory state and induction of IR [20]. Interestingly, other pro-inflammatory cytokines directly affect both function and viability of β-pancreatic insulin-producing cells [103]. The adverse effects of TNF-α, IL-1β, and interferon γ (IFN-γ) are prevented when β-cells are treated with anti-inflammatory cytokines (IL-4, IL-10, and IL-13), showing that these molecules may modulate insulin serum levels, which in turn affect metabolic control at different levels. In addition, pro-inflammatory cytokines induce upregulation of ATF4 mRNA in β-pancreatic cells by disrupting Ca^{2+} signaling [104, 105]. Thus, because ATF4 is a classical effector of the PERK signaling cascade, a direct link between ER stress and inflammation has been proposed.

Indeed, ER stress is linked to cytokines because activation of ATF6 and cAMP-responsive element-binding protein hepatocyte specific (CREBH) is the main factor responsible for release of TNF-α, IL-1β, and IL-6 [102]. Adipose tissues from murine obesity models show increased mRNA levels of pro-inflammatory cytokines, which are restored to normal levels after treatment with TUDCA, a chemical chaperone that inhibits ER stress [106]. In addition, activation of ER stress-related protein PKR has been described in cells exposed to TNF-α [107], showing a direct induction of ER stress through this cytokine. In the same way, it has been suggested that IFN-γ may directly induce ER stress, because IFN-γ also activates PKR [108]. Furthermore, interferon regulatory factor 7 (IRF-7) was found to be a positive regulator of weight gain in a murine model [109], suggesting an obesity-related negative feedback cycle, depending on the interferon pathway. Moreover, PKR is involved in secretion of IL-1β and IL-18 [110], although the latter cytokine seems to prevent obesity and IR in mice [111]. We hypothesize that the final effect on insulin signaling may depend on the interaction among different pro- or anti-inflammatory cytokines and ER stress proteins at the systemic or microenvironmental level. Surprisingly, recent evidence has shown that insulin may increase ER stress markers in adipose tissue [112]. Therefore, this interesting new evidence suggests that ER stress may occur after development of IR, reinforcing the hypothesis regarding an obesity-inflammation-ER stress vicious cycle.

Unlike pro-inflammatory cytokines, anti-inflammatory molecules have been linked with prevention of ER stress development. Indeed, the major anti-inflammatory cytokine IL-10 has been related to impaired ATF6 nuclear translocation induced by both TNF-α [113] and tunicamycin [114], suggesting that mechanisms involved in ER stress inhibition by IL-10 may be independent of stress response. Thus, whether other anti-inflammatory cytokines, such as IL-4 or IL-13, are able to inhibit or prevent ER stress should be addressed. For example, it has been reported that IL-6 also inhibits obesity-induced ER stress in the rat hypothalamus [115]. Similarly, other anti-inflammatory agents, such as omega-3 fatty acids, may also produce insulin sensitization and antidiabetic effects (such as restoration of Akt signaling) through G protein-coupled receptor 120 (GPR120) [116]. Accordingly, it is possible that omega-3 fatty acids or other fatty acids may also inhibit ER stress.

Unexpectedly, a pro-inflammatory cytokine named resistin showed chaperone activity and was able to inhibit ER stress. Interestingly, this study demonstrated that resistin was retained inside the cell to inhibit ER stress, suggesting that soluble and cellular resistin may have different effects and cellular targets [117]. Klotho protein also promotes differential cellular effects in terms of insulin function and inflammation depending on the circulating or intracellular fraction of this aging suppressor protein [118, 119]. Intravenous administration of the soluble extracellular domain of Klotho, which is also found in the blood, binds to its putative receptor and inhibits the insulin pathway [120]. Furthermore, intracellular but not the secreted form of Klotho protein has an anti-inflammatory effect over retinoic acid inducible gene I (RIG-I) signaling and inhibits IL-6 and IL-8 release [121]. Recent evidence has also shown that overexpression of Klotho is able to inhibit chemically-induced ER stress [122]. The hypothesis of differential action depending on target location opens a new field for the study of cytokines, showing that soluble or intracellular cytokines may differentially modulate cellular responses in both physiological and pathophysiological conditions.

In the context of pregnancy, cytokines may significantly affect the metabolic state, which in turn promotes IR and a pro-inflammation condition associated with MO. Importantly, cytokine-induced fetal programming has been proposed in rats after maternal exposure to both TNF-α and IL-6 treatment, associated with increased fetal growth and IR in the offspring [123]. Moreover, IL-6 seems to play a pivotal role in the transference of a pro-inflammatory state from the mother to the fetus, as umbilical cord blood levels of IL-6 from obese mothers are higher than those from normal pregnancies [40]. This finding is also related to increased macrophage infiltration of placental tissue, associated with elevated pro-inflammatory markers in response to MO [124]. Interestingly, while pro-inflammatory cytokines can induce ER stress in placental tissue, an anti-inflammatory response may restore normal insulin sensitivity. Accordingly, administering an anti-inflammatory flavonoid named quercetin [125] during pregnancy and lactation significantly decreases ER stress activation in the offspring [126], suggesting that it may be possible to modulate the prenatal environment, preventing ER stress and its deleterious consequences.

Additional mechanisms related to cellular stress and/or inflammatory responses (such as maternal psychological environment, dietary behavior, and infections) could affect intrauterine development, highlighting the role of new players in obesity and immune system abnormalities associated with deleterious metabolic outcomes, both at the maternal

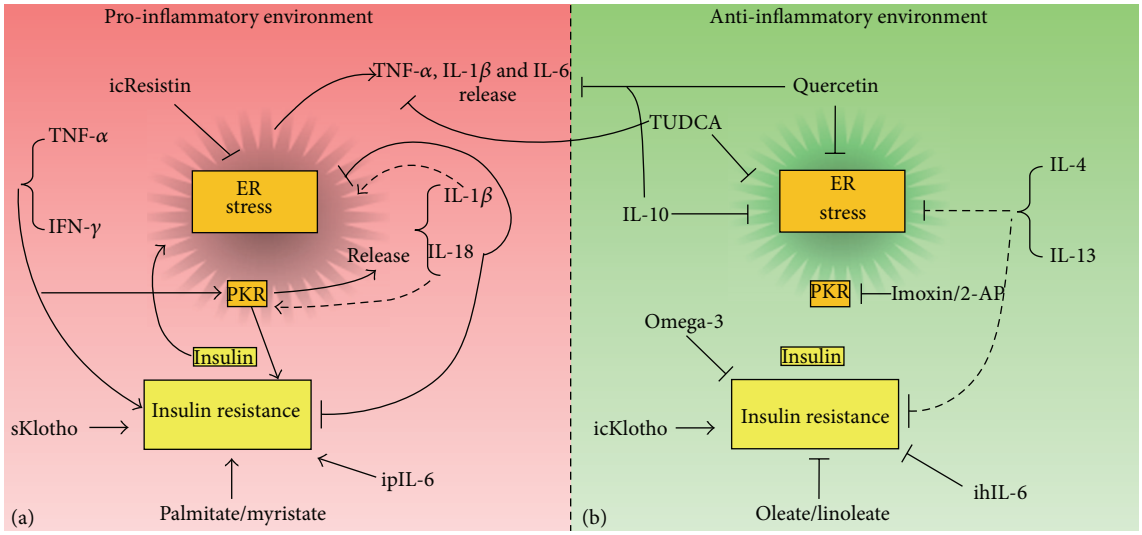

FIGURE 1: Balance of pro-inflammatory and anti-inflammatory cytokines in the development of ER stress and insulin resistance. In this scheme we highlight the role of several pro-inflammatory cytokines (a) and anti-inflammatory cytokines (b). These models integrate information from animal and cellular different models, which can be extrapolated to other systems. Cytokines or other molecules with pro-inflammatory effects have a tendency to induce the ER stress and produce insulin resistance. Interestingly, cytokines or other molecules with anti-inflammatory effects, such as TUDCA, omega 3 fatty acids, or quercetin, prevent the release of pro-inflammatory cytokines, inhibit the development of ER stress, and induce insulin sensitizing, improving glucose metabolism (icResistin = intracellular resistin; sKlotho = soluble Klotho; icKlotho = intracellular Klotho; ipIL-6 = intraperitoneally injected IL-6; ihIL-6 = intrahypothalamically injected IL-6).

and at the fetal levels. For example, PKR, which is an ER stress-dependent protein kinase, is also activated by viral infections and is characterized by inflammatory, IR, and ER stress responses [107]. In fact, inhibition of PKR has been associated with decreased expression of ER stress markers and improved insulin sensitivity in obese/diabetic mice, involving reduction of inflammation [127]. This finding suggests that PKR may play a key role as a pharmacological target in metabolic diseases under obesity conditions. Mental stress during pregnancy should also be considered as an initial risk factor related to obesity and IR development [128]. Neuroendocrine interactions with important roles in depression and sickness [129] are associated with impaired anorexigenic signaling and obesity tendencies in fetuses from mothers with MO [130]. Thus, it may be relevant to also consider inflammation-related processes, such as infection or mental stress during pregnancy, as potential risk factors contributing to fetal programming of metabolic diseases.

The balance between pro- and anti-inflammatory immune cell phenotypes may be modulated to avoid the deleterious immune imbalance that provokes metabolic alterations in pregnancies complicated by obesity. The complex interactions among multiple inflammatory mediators and the ER stress response should be considered in the study of fetal IR development attributable to MO (Figure 1).

6. Fetal Programming of Insulin Resistance by Maternal Obesity-Dependent ER Stress

As compared to normal pregnancy, MO is associated with an exaggerated lipid mobilization (increased plasma cholesterol and triacylglycerol) and abnormal accumulation of fat in the liver, pancreas, and placenta [40, 131]. In addition, obesity in pregnancy is related to increased IR [132], higher levels of inflammatory markers, and impaired endothelial function [71, 124, 133]. Moreover, maternal metabolic abnormalities associated with overnutrition during pregnancy may be transmitted to the fetal circulation, since fetal offspring from HFD-fed pregnant nonhuman primates showed increased markers of metabolic disorders associated with obesity, such as hepatic oxidative stress and nonalcoholic fatty liver disease (NAFLD) [134]. In this study, offspring of HFD pregnant animals also exhibited elevated hepatic expression of gluconeogenic enzymes and transcription factors, in addition to increased levels of plasma glycerol and liver triglycerides [134]. Consequently, these results suggest that stressor effects related to nutrient excess from maternal overfeeding are mimicked in fetal plasma and can produce fat-related liver disease in the offspring. Nevertheless, although ER stress has been implicated in conditions from hepatic steatosis to NAFLD [135], there is no evidence regarding the potential role of the endoplasmic reticulum in the pathological process described in the fetal liver from murine MO models. On the other hand, increasing epidemiological evidence has suggested intrauterine programming of IR in offspring from obese pregnant woman, evaluated both at an early neonatal stage and in young adulthood. Nevertheless, the mechanistic link between MO and offspring IR remains unclear. Since IR has been described as a keystone in physiopathology pathways associated with metabolic diseases such as diabetes and cardiovascular complications, the potential therapeutic target of ER stress during the early neonatal period or during pregnancy may be relevant to obstetric

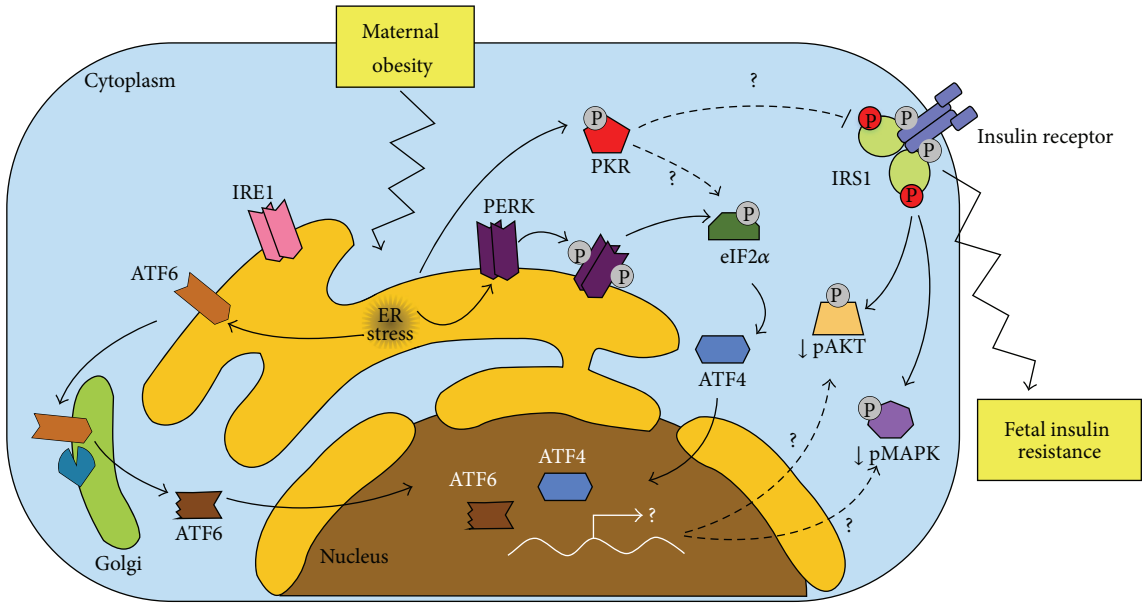

Figure 2: Proposed model of interaction among maternal obesity, ER stress, and insulin resistance. Maternal obesity is related to ER stress response in HUVEC, involving activation of ER stress proteins PERK and ATF6. ATF6 is released from ER membranes and then processed in the Golgi by proteolytic cleavage promoting its nuclear translocation. On the other hand, PERK is autophosphorylated (grey circles) and is able to phosphorylate eIF2α, leading to induction of ATF4. Moreover, eIF2α can also be phosphorylated by PKR, which is also an ER stress-dependent protein. Hence, both ATF6 and ATF4 nuclear translocations may be able to alter insulin signaling and lead to insulin resistance in HUVEC through reduction of AKT and MAPK phosphorylation. In parallel, PKR activation may cause insulin signaling inactivation through IRS-1 inhibitory phosphorylation (red circles). Solid lines represent previously established processes; dashed lines and question marks indicate hypothetical and unknown mechanisms in our model.

and postnatal outcomes. Accordingly, potential crosstalk between insulin signaling and ER stress pathways on human fetal cells exposed to maternal obesity is proposed (Figure 2).

Regarding the nutritional programming hypothesis, significant data have shown increased cardiometabolic risk in offspring from both under- and overnutrition in pregnancy. In overfed pregnant mouse models, fetal liver shows excessive lipid and fatty acid accumulation associated with activation of JNK, an oxidative stress, inflammatory, and apoptosis marker [134]. Hence, JNK activation and apoptosis are described as part of the ER stress pathway related to both IR and diabetes in response to obesity in various models. Therefore, it is possible that future interventions focused on preventing obesity-derived ER stress in pregnancy may target avoidance of IR development in fetal tissues. Although McCurdy et al. showed that prepregnancy diet normalization partially attenuated development of fatty liver disease in fetal offspring, there is no evidence regarding the potential beneficial effect of this nutritional intervention on fetal UPR or insulin sensitivity. Specific therapeutic interventions with chemical chaperones such as bile acids have shown improved hepatic insulin response in obese individuals [136]. However, although some bile acids are currently used in cases of icteric cholestasis of pregnancy [137], it remains unclear whether this treatment will be useful in preventing insulin resistance in the offspring of pregnancies with MO.

7. Conclusions

MO and neonatal IR are associated with long-term development of obesity, diabetes mellitus, and increased global cardiovascular risk in the offspring, involving deleterious mechanisms of intrauterine programming. Nevertheless, the entire signaling link among these conditions has not been fully elucidated. Recent evidence suggests that obesity-related ER stress may play an important role in the development of IR, associated with unfolded protein response (UPR) and inflammatory mediators. We propose a potential mechanism of MO-dependent ER stress response on human fetal cells, involving inflammatory cytokines such as TNF-α, IL-1β, IL-6, and/or IFN-γ, and activation of PERK, eIF2α, PKR, ATF4, and ATF6. Understanding this phenomenon may provide crucial information that would clarify the potential beneficial effects of new therapeutic tools to prevent the deleterious consequences associated with MO, inflammatory markers, and IR in the offspring.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Associations of Prenatal Growth with Metabolic Syndrome, Insulin Resistance, and Nutritional Status in Chilean Children

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Introduction. The association of prenatal growth with nutritional status, metabolic syndrome (MS), and insulin resistance (IR) was studied in school-age children. *Methods*. A retrospective cohort study was designed linking present data of children with perinatal records. 3325 subjects were enrolled. Anthropometry, blood pressure (BP), and pubertal status were assessed. Blood lipids, glucose, and insulin were measured. Linear associations were assessed using the Cochran-Armitage test. Odds ratios and nonlinear associations were computed. *Results*. 3290 children (52% females, mean age of 11.4 ± 1 years) were analyzed. Prevalence of obesity, stunting, MS, and IR was 16.0%, 3.6%, 7.3%, and 25.5%, respectively. The strongest positive association was between birth weight (BW) and obesity (OR 2.97 (95% CI 2.01–4.40) at BW ≥ 4,000 g compared to BW 2,500–2,999). The strongest inverse association was between birth length (BL) and stunting (OR 8.70 (95% CI 3.66–20.67) at BL < 48 cm compared to BL 52-53 cm). A U-shaped association between BL and BP ≥ 90th percentile was observed. Significant ORs were also found for MS and IR. Adjustments for present fat mass increased or maintained the most prenatal growth influences. *Conclusions*. Prenatal growth influences MS, IR, and nutritional status. Prenatal growth was more important than present body composition in determining these outcomes.

1. Introduction

Nutritional conditions during pregnancy, infancy, and childhood have been proposed to have a major impact on the development of obesity and related metabolic traits such as metabolic syndrome (MS) and insulin resistance (IR) [1–4].

Birth weight (BW), placental weight, and measures of size at birth have all been related to risk factors for chronic disease in adult life [2, 5]. Birth length (BL) has consistently been associated with adolescent and adult height [6–8]. Shorter than average adults are at a higher risk for obesity and are also more susceptible to diabetes and CVD, independent of BMI; in contrast, taller children have a higher risk for obesity [2, 9]. Obesity in childhood has consistently been related in young adults to blood pressure (BP), insulin resistance (IR), and lower high-density lipoprotein-cholesterol (HDL-C) concentration; cardiovascular risk profile may be especially unfavorable in children with increased waist circumference (WC) [2]. A recent report based upon the same sample of children as the present study has confirmed strong associations between obesity and BP, IR, HDL, WC, triglycerides (TG), and the MS [10]. Gestational age (GA) is an important factor and

premature births have been associated with increased BP and IR [11]; the incidence of prematurity is increasing worldwide [12].

The MS is a set of risk factors that may appear during childhood and have been closely linked to IR [13]. Obesity, especially of the abdominal type, and a sedentary lifestyle are important contributors to the development of IR.

We recently studied 2,152 Chilean schoolchildren aged 10–15 years, assessing possible associations between MS and IR to prenatal growth; however, nutritional status indicators were not included in those analyses [14]. The main results of that study showed that nonoptimal prenatal growth seemed to predispose to IR, high WC, and elevated BP in children.

This study aimed to investigate the associations of prenatal growth with MS and IR, including indicators of nutritional status, using a larger sample size than the previously reported.

2. Material and Methods

2.1. Design. A retrospective cohort study of school-aged children, from 20 public schools managed by the Municipality of Puente Alto, Santiago, Chile, was designed. The public schools reach nearly 40% of all primary schools in Chile [15]. All children attending 5th and 6th grade of primary education were asked to participate in the study during the years 2009–2011. The national individual identification number was used to link information gathered at school with perinatal data collected by the civil registry and subsequently published by the National Institute of Statistics [16].

2.2. Perinatal Variables. In addition to sex, perinatal data used in this study were birth weight (BW), birth length (BL), and GA. They were determined at the maternity hospitals immediately after delivery by trained personnel [17, 18]. Infants were weighed using a 1583 Tanita electronic scale (Tanita Corporation, Arlington Heights, IL) with an accuracy of 10 g or an electronic scale, Seca 345, the accuracy being 20 g (Secacorp, Hamburg, Germany). Crown-heel length was measured using an infantometer, to the nearest 1 mm. GA was estimated as completed weeks of gestation using the date of the last menstrual period; when this data was inaccurate an early obstetric ultrasound was used. This ultrasound examination is available for most women before 20 weeks of pregnancy in Chile and most of these scans are performed with a Voluson 730 ultrasound system (GE Healthcare, Chalfont St. Giles, UK) or a Acuson 120XP (Acuson Inc., Mountain View, CA) [17]. When ultrasound examinations were not available before 20 weeks of pregnancy, GA was usually estimated by a postnatal clinical examination of the newborns performed by the physician in charge. Preterm delivery was defined as GA ≤ 37 weeks [19].

2.3. Metabolic Syndrome. The criteria of Cook et al. were used to define MS in the children, when at least three out of five of its components were present, as defined by the following cut-off points [20]: WC ≥ 90th percentile [21], BP, either systolic (SBP) or diastolic (DBP) ≥ 90th percentile [22], low HDL-C ≤ 40 mg/dL, TG ≥ 110 mg/dL, and glucose (GLU) ≥ 100 mg/dL. Recently proposed local cut-off points of homeostasis model assessment index (HOMA), according to sex and pubertal maturation, were used to classify IR [23].

2.4. Anthropometry. The evaluations at each school were performed by a trained nurse and a nutritionist. Height and weight were measured using a stadiometer and a beam-scale Seca, with an accuracy of 50 g, while being barefoot and lightly clothed. The final height and weight were the respective averages of three measurements. We calculated body mass index (BMI = weight in kg/height^2 in m) expressed in percentiles and z-scores [24]. Nutritional status was classified according to BMI percentiles as normal: 5 to 84, overweight: 85 to 94, obese: ≥95, and underweight: <5; short stature, or stunting, was defined as stature-for-age <5th percentile [24]. WC was measured with an inextensible tape on the upper lateral border of the right ilium in the midaxillary line at the end of an exhalation [25]; two measurements were averaged and we used the ≥90th percentile as cut-off value [21]. The triceps and subscapular skin folds were measured with a Harpenden caliper using a standard technique [25]; both were used to calculate the percentage of fat mass (%FM) using Slaughter equations [26], previously validated in Chilean children [27, 28]. A Critikon Dinamap Pro 100 blood pressure monitor was used according to international norms and the averages of three measurements of SBP and DBP were obtained and classified as abnormal using the ≥90 percentile of the same reference [22]. A voluntary private self-report of pubertal status was requested by observation of standardized photos of breast development in girls and genitalia in boys, including the presence of pubic hair [29].

2.5. Blood Samples. Subjects were instructed to fast (water was allowed) for 12 hours prior to drawing of blood; non-compliers were asked to return another day for the blood sampling. A single laboratory at our university was used for all blood analyses. This laboratory has been credited by the standard ISO 15189 for medical laboratories [30].

Venous blood samples were collected for determination of glucose (Gluco-quant method, glucose/hexokinase, Roche Diagnostics GmbH, Mannheim, Germany) and insulin (immunoassay direct luminometer chemotherapy, ADVIA Centaur XP. Bayer HealthCare LLC, Kyowa Medex Co., Japan); this method measures concentrations of insulin from 0.5 to 300 mUI/mL (sensitivity of 0.5 mUI/mL) with a coefficient of variation of 3.48% and 6.17% for concentrations of 23.51 mUI/mL and 62.49 mUI/mL, respectively. The formula developed by Matthews et al. [31] was used to calculate HOMA = (glucose [mmol/liter] × insulin [mUI/mL]/22.5) and to estimate IR [23]. TG and HDL-C were analyzed on the Modular Analytics P-800 platform (Roche Diagnostics GmbH, Mannheim, Germany).

2.6. Socioeconomic Status. Socioeconomic status was estimated using as a proxy the number of maternal years of formal education as collected at birth by the civil registry [16]. This indicator has been considered closely related to the actual socioeconomic status and the access to health services in many studies [15, 32, 33]. Maternal level of education was classified in the following way: (a) basic: 0–8 years of

Table 1: Anthropometric and perinatal characteristics (mean ± SD) by sex in 3290 children from Puente Alto, Chile, 2009–2011.

Variable	Total ($n = 3290$)	Girls ($n = 1711$)	Boys ($n = 1579$)	P value
Age (years)	11.4 ± 1.0	11.4 ± 1.0	11.5 ± 1.0	<0.001
Weight (kg)	43.9 ± 11.2	44.4 ± 11.1	43.4 ± 11.3	0.018
Height (cm)	146.5 ± 8.1	146.8 ± 7.7	146.2 ± 8.5	0.042
BMI (kg/m^2)	20.3 ± 3.9	20.4 ± 3.9	20.1 ± 3.9	0.038
z-BMI	0.59 ± 1.1	0.59 ± 1.0	0.58 ± 1.1	ns
%FM	25.0 ± 11.4	27.0 ± 12.2	22.8 ± 10.1	<0.001
BW (g)	3350 ± 524	3292 ± 512	3412 ± 529	<0.001
BL (cm)	49.6 ± 2.4	49.2 ± 2.3	49.98 ± 2.4	<0.001
GA (w)	38.9 ± 1.8	38.9 ± 1.9	38.8 ± 1.7	ns

BMI, body mass index; %FM, percentage of fat mass; BW, birth weight; BL, birth length; GA, gestational age; ns, nonsignificant; SD, standard deviation.

education; (b) medium: 9–12 years of education; and (c) upper: 13 and more years of education.

2.7. Statistical Analysis. The prevalence of MS and its components were described as number of cases and percentage, while perinatal variables used mean and standard deviation (SD). To assess differences by sex, Pearson's chi-square test for proportions and independent samples Student's t-test for averages were used. Prevalence of MS and its components was computed at each category of the perinatal variables. The presences of linear trends in this prevalence were assessed using the Cochran-Armitage test, while nonlinear associations were evaluated allowing quadratic terms in a logistic regression. Multivariate analysis was performed using logistic regression allowing specific adjustments for sex, Tanner stage, and %FM; those factors could be confounders which potentially influence the dependent variables [13, 15, 23, 34]. Crude and adjusted odds ratios (ORs) were computed, with their 95% confidence intervals (95% CI).

All data analyses were performed using 2-sided P values and values ≤ 0.05 were considered statistically significant. SAS software version 9.1 was used to compute statistical analysis.

2.8. Ethics. Parents or their representatives signed an informed consent form and boys/girls an informed acceptance form. The study was approved by the Ethics Committees of the School of Medicine (Pontificia Universidad Católica de Chile) and the National Fund for Science and Technology (FONDECYT).

3. Results

Initially 5,614 subjects were eligible for the study, 2,616 females and 2,998 males. A total of 3,325 children and adolescents who presented the signed informed consent forms were evaluated and 2289 refused to participate; from the latter 34.5% were females and 47.4% males ($P < 0.001$). Complete information on studied perinatal variables was obtained for 3290 children (98.9%), of whom 52% were females. There were no significant differences between those who participated or those who did not in terms of age, years of maternal education, BW, and BL.

Based on maternal education attainment, the socioeconomic status distribution in the total group of eligible subjects was medium 60.1%, basic 33.0%, and superior 6.9%. Proportions in the actually studied subjects were similar: 60.9%, 32.5%, and 6.6%, respectively. There were no major associations observed with the study outcomes except for short stature which was slightly more frequent for basic education than the other categories of maternal education ($P = 0.039$) (data not presented).

In the studied sample, mean age was 11.4 ± 1 years (range 10–15 years) being higher in males than in females. Most anthropometric characteristics assessed were slightly higher among females; only BMI (z-score) did not differ by sex. Among boys, mean BW and BL were higher than in females (Table 1).

Prevalence of obesity, stunting, MS, and IR was 16.0%, 3.6%, 7.3%, and 25.5%, respectively (Table 2). Obesity was more frequent in males but stunting was more frequent in females. The prevalence of MS was higher in females; IR did not differ between the genders. In the study cohort TG ≥ 110 mg/dL was the most common component of the MS, followed by WC ≥ 90th percentile. Interestingly, 99% of cases having BP ≥ 90th percentile came from SBP ≥ 90th percentile.

Several associations were observed between perinatal variables and the outcome variables studied (Figure 1). Positive trends were observed between BW and obesity and between BW and WC ≥ 90th percentile; BL was also positively associated with obesity. Negative associations were observed between BW and stunting and between BW and BP ≥ 90th percentile. GA was negatively associated with MS, BP ≥ 90th percentile, and TG ≥ 110 mg/dL; two other negative associations were almost significant: GA with IR (P for trend of 0.0893) and GA with WC ≥ 90th percentile (P for trend of 0.0733). In addition, a U-shaped association between BL and BP ≥ 90th percentile was observed. Another association was found between BL and stunting (P for trend < 0.0001); this association was not included in Figure 1.

Table 3 presents crude ORs for the associations described above; bold numbers correspond to significant OR values. Adjusted ORs for %FM and adjusted ORs for %FM, sex, and Tanner stage are also presented.

Table 2: Anthropometric and perinatal characteristics [n (%)] by sex in 3290 children from Puente Alto, Chile, 2009–2011.

Variable	Total ($n = 3290$)	Girls ($n = 1711$)	Boys ($n = 1579$)	P value
Obesity (BMI ≥ 95 percentile)	527 (16.0)	227 (13.3)	300 (19.0)	0.002
Stunting (stature-for-age < 5th percentile)	120 (3.6)	63 (3.7)	57 (3.1)	0.049
MS (≥3 components)	239 (7.3)	150 (8.8)	89 (5.6)	0.001
HDL-C ≤ 40 mg/dL	555 (16.9)	324 (18.9)	231 (14.6)	0.001
TG ≥ 110 mg/dL	878 (26.7)	530 (31.0)	348 (22.0)	<0.001
SBP (mm Hg) ≥ 90th percentile	349 (10.6)	199 (11.6)	150 (9.5)	ns
DBP (mm Hg) ≥ 90th percentile	24 (0.73)	17 (1.0)	7 (0.4)	ns
BP (mm Hg) ≥ 90th percentile	354 (10.8)	201 (11.7)	153 (9.7)	ns
WC (cm) ≥ 90th percentile	697 (21.2)	405 (23.7)	292 (18.5)	<0.001
Glucose (mg/dL) ≥ 100 mg/dL	235 (7.1)	96 (5.6)	139 (8.8)	<0.001
IR (HOMA ≥ 90th percentile)	838 (25.5)	424 (25.2)	414 (26.4)	ns

BMI, body mass index; MS, metabolic syndrome; HDL-C, high-density lipoprotein; TG, triglycerides; SBP, systolic blood pressure; DBP, diastolic blood pressure; BP, blood pressure; WC, waist circumference; HOMA, homeostasis model assessment; IR, insulin resistance; ns, nonsignificant.

The lowest prevalence of obesity and WC ≥ 90th percentile at school-age was observed within the 2500–2999 g BW category and was therefore considered as the reference level. This prevalence increased with BW categories, almost tripling the odds for obesity and almost doubling the odds for WC ≥ 90th percentile at the highest category of BW. Adjustments for %FM increased most ORs. Adjustment for %FM, sex, and Tanner stage for obesity maintained similar values. Adjustments for %FM and for %FM, sex, and Tanner stage showed a slight increase of OR values for WC ≥ 90th percentile in the <2500 g BW category.

BW had an inverse association with the prevalence of stunting. Crude ORs in the 3500 g and over categories were protective; those values did not change after adjustments for %FM but disappeared after further adjustment for sex and Tanner stage.

BW did not show an influence on BP ≥ 90th percentile when assuming the 2500–2999 g BW category as reference (Table 3). However, when selecting the BW category with the lowest prevalence of BP ≥ 90th percentile as reference, that is, 3500–3999 g, the lowest BW category, that is, <2500 g, presented a higher risk for BP ≥ 90th percentile: OR 1.80 (95CI: 1.12–2.90); no changes were observed after adjustments.

BW had an inverse influence on IR prevalence. Crude ORs in the 3000 g and over categories were protective; those values were stable after adjustments.

52-53 cm BL was the category with the lowest prevalence of BP ≥ 90th percentile at school-age and was considered as the reference level. Both, the lowest and highest categories of BL doubled the risk of BP ≥ 90th percentile, depicting a U-shaped behavior. Adjustments for %FM and for %FM, sex, and Tanner stage maintained these ORs only for BL < 48 cm.

BL categories below 50 cm were protective for obesity. ORs declined and were more protective when adjusting for %FM. These associations disappeared when further adjusting for sex and Tanner stage.

52-53 cm BL was the category with the lowest prevalence of stunting at school-age; BL ≥ 54 cm category did not present stunted children. OR values < 50 cm showed a risk of at least 5 times the observed in the reference category. Adjustments for %FM and for %FM, sex, and Tanner stage maintained these ORs.

One BL category, that is, 48-49 cm, increased the risk for HOMA-IR just when adjusting for %FM, sex, and Tanner stage.

GA ≥ 41 weeks was the category with the lowest prevalence of BP ≥ 90th percentile, MS, and TG ≥ 110 mg/dL at school-age and was considered the baseline level. Both, crude and adjusted ORs of prematurity, defined as GA ≤ 37 weeks, behaved in a similar way showing the highest figures for BP ≥ 90th percentile, MS, and TG ≥ 110 mg/dL. No associations were found for GA and IR.

4. Discussion

This is the first study focusing upon associations between perinatal factors and nutritional status, MS, and IR in Chilean children. We observed that prenatal growth influences obesity characteristics, MS, IR, and stunting. In general, prenatal growth seemed to be more important in predicting these associations than present body composition because adjustments for present fat mass increased or maintained the most prenatal growth influences. Small body size at birth was associated with elevated BP, IR, and MS, while a large body size at birth was associated with measurements of obesity. The undeniable role of prenatal growth on metabolic outcomes appears, even in school-aged children, whose metabolic damages are only developing.

The finding of a U-shaped association between BL and BP ≥ 90th percentile is consistent with the well-documented associations between both low and high values of BW and BL with many perinatal health indicators in previous reports [19, 35].

The associations of BW with a higher prevalence of obesity or increased adiposity have been observed in previous studies [18, 36]. In our report there was also a nonsignificant tendency towards a J-shaped association which according to other studies may be stronger and significant at later ages.

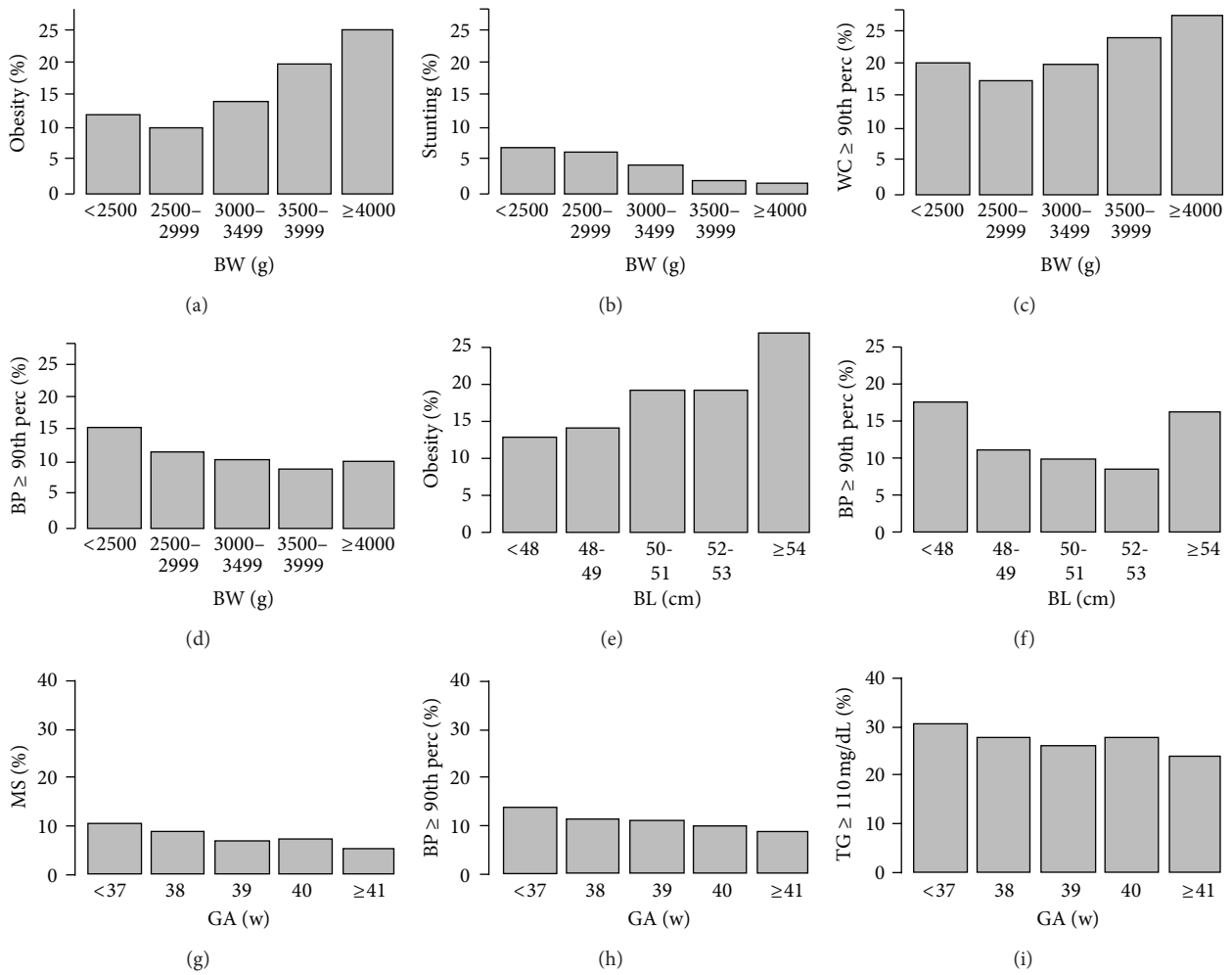

Figure 1: Association of perinatal variables and outcomes at 10–15 years of age, Puente Alto, Chile, 2009–2011. Association of BW with obesity ((a) P for trend <0.0001), stunting ((b) P for trend <0.0001), WC ≥ 90th percentile ((c) P for trend =0.0069), and BP ≥ 90th percentile ((d) P for trend =0.0176). Associations of BL with obesity ((e) P for trend =0.0027) and BP ≥ 90th percentile ((f) $P = 0.0002$ linear term, $P = 0.0029$ quadratic term). Associations of GA at birth with MS ((g) P for trend =0.0083), BP ≥ 90th percentile ((h) P for trend =0.0255), and TG > 110 mg/dL ((i) P for trend =0.0435). Panels (a), (c), and (e) show that, as the value of the perinatal variable increases, it also increases the prevalence of obesity or WC ≥ 90th percentile at 10–14 years of age. (b), (d), (g), (h), and (i) behave inversely; as the value of the perinatal variable increases, prevalence of stunting, BP ≥ 90th percentile, MS, or TG > 110 mg/dL decreases. Panel (f) shows a U-shaped behavior, denoting that the lowest and highest values of BL have the highest prevalence of BP ≥ 90th percentile, Puente Alto, Chile, 2009–2011. BW, birth weight; BL, birth length; GA, gestational age; WC, waist circumference; BP, blood pressure; MS: metabolic syndrome; TG, triglycerides; perc, percentile.

For example, the 1958 British birth cohort study showed that the association between BW and BMI was positive and weak becoming more J-shaped with increasing age; BMI was assessed at 7, 11, 16, 23, and 33 years in that study [37]. This inverse association between low BW and obesity and WC > 90th percentiles has been explained by Hales and Barker with the "thrifty phenotype" hypothesis. This proposes a greater susceptibility of the newborns with low BW to develop IR in order to survive intrauterine malnutrition and determine adaptive responses as diet preference to high-fat, hyperphagia, decreased muscle development, and large deposit of visceral adipose tissue [38].

BL was positively associated with obesity, probably reflecting the well-known previously reported finding that weight and height are positively correlated during adolescence but not during adulthood [9].

GA was inversely associated with important metabolic risk factors, BP ≥ 90th percentile, TG ≥ 110 mg/dL, WC ≥ 90th percentile, and MS as a whole, in these children. As previously mentioned prematurity was defined in this study as ≤37 weeks following recent national recommendations [35]. This fact leads to estimating a higher proportion of risky premature births at the national level; live births with 37 weeks, estimated to be in 6%, would add the proportion traditionally defined as premature births with GA < 37 w, which are now reaching 6.13% for 2011 [16, 35]. The incidence of preterm is increasing worldwide and, as previously mentioned, has been associated with increased BP and IR [11, 12]. A lower GA necessarily

Table 3: Trends of the main outcomes in school-age children with perinatal variables. Prevalence and crude ORs and ORs adjusted by %FM, Puente Alto, Chile, 2009–2011.

BW (g)	*n*	Obesity (%)	OR	95% CI	OR*	95% CI	OR**	95% CI
<2500	161	11.8	1.20	0.69–2.11	1.81	0.81–4.05	2.11	0.89–4.98
2500–2999	500	10.0	1.00	Baseline	1.00	Baseline	1.00	Baseline
3000–3499	1335	14.0	**1.47**	**1.05–2.04**	**2.00**	**1.28–3.13**	**2.06**	**1.27–3.35**
3500–3999	992	19.8	**2.22**	**1.59–3.09**	**3.55**	**2.26–5.58**	**3.07**	**1.87–5.04**
≥4000	302	24.8	**2.97**	**2.01–4.40**	**3.94**	**2.26–6.87**	**2.81**	**1.52–5.20**
BW (g)	*n*	WC ≥ 90th percentile (%)	OR	95% CI	OR*	95% CI	OR**	95% CI
<2500	161	19.9	1.18	0.75–1.85	**2.64**	**1.29–5.39**	**2.65**	**1.29–5.45**
2500–2999	500	17.4	1.00	Baseline	1.00	Baseline	1.00	Baseline
3000–3499	1335	19.7	1.17	0.89–1.52	**1.53**	**1.03–2.29**	1.49	0.99–2.23
3500–3999	992	23.5	**1.46**	**1.11–1.92**	**1.94**	**1.29–2.93**	**1.79**	**1.18–2.72**
≥4000	302	27.2	**1.77**	**1.26–2.49**	**1.75**	**1.03–2.98**	1.54	0.89–2.65
BW (g)	*n*	Stunting (%)	OR	95% CI	OR*	95% CI	OR**	95% CI
<2500	161	6.8	1.11	0.54–2.26	1.02	0.5–2.08	0.65	0.37–1.13
2500–2999	500	6.2	1.00	Baseline	1.00	Baseline	1.00	Baseline
3000–3499	1335	4.3	0.68	0.43–1.06	0.67	0.42–1.05	0.75	0.47–1.18
3500–3999	992	1.7	**0.26**	**0.15–0.48**	**0.27**	**0.15–0.50**	1.08	0.71–1.63
≥4000	302	1.3	**0.20**	**0.07–0.58**	**0.22**	**0.08–0.63**	**1.29**	**0.54–3.12**
BW (g)	*n*	BP ≥ 90th percentile (%)	OR	95% CI	OR*	95% CI	OR**	95% CI
<2500	161	15.5	1.35	0.81–2.23	1.41	0.85–2.34	1.42	0.85–2.35
2500–2999	500	12.0	1.00	Baseline	1.00	Baseline	1.00	Baseline
3000–3499	1335	10.9	0.89	0.65–1.23	0.90	0.65–1.24	0.90	0.65–1.25
3500–3999	992	9.3	0.75	0.53–1.06	0.73	0.52–1.03	0.74	0.52–1.05
≥4000	302	10.6	0.87	0.55–1.37	0.80	0.51–1.27	0.82	0.52–1.30
BW (g)	*n*	IR	OR	95% CI	OR*	95% CI	OR**	95% CI
<2500	161	24.8	0.75	0.5–1.12	0.83	0.53–1.30	0.82	0.52–1.29
2500–2999	500	30.6	1.00	Baseline	1.00	Baseline	1.00	Baseline
3000–3499	1335	24.5	**0.74**	**0.59–0.92**	**0.70**	**0.55–0.90**	**0.66**	**0.51–0.85**
3500–3999	992	25.3	**0.77**	**0.61–0.98**	**0.67**	**0.51–0.87**	**0.60**	**0.46–0.78**
≥4000	302	28.2	0.89	0.65–1.22	**0.67**	**0.47–0.95**	**0.57**	**0.40–0.82**
BL (cm)	*n*	BP ≥ 90th percentile (%)	OR	95% CI	OR*	95% CI	OR**	95% CI
<48	457	16.6	**2.22**	**1.49–3.31**	**2.25**	**1.51–3.35**	**2.22**	**1.48–3.33**
48-49	976	10.9	1.36	0.94–1.97	1.34	0.93–1.95	1.33	0.91–1.94
50-51	1264	9.3	1.15	0.80–1.65	1.12	0.78–1.62	1.12	0.77–1.61
52-53	522	8.2	1.00	Baseline	1.00	Baseline	1.00	Baseline
≥54	71	15.5	**2.04**	**1.00–4.17**	1.93	0.94–3.96	1.93	0.94–3.96
BL (cm)	*n*	Obesity (%)	OR	95% CI	OR*	95% CI	OR**	95% CI
<48	457	12.3	**0.64**	**0.45–0.92**	**0.38**	**0.23–0.62**	0.65	0.37–1.13
48-49	976	13.3	**0.71**	**0.53–0.95**	**0.43**	**0.29–0.66**	0.75	0.47–1.18
50-51	1264	18.2	1.03	0.79–1.34	0.81	0.56–1.18	1.08	0.71–1.63
52-53	522	17.8	1.00	Baseline	1.00	Baseline	1.00	Baseline
≥54	71	25.4	1.57	0.88–2.80	1.29	0.56–2.96	1.29	0.54–3.12
BL (cm)	*n*	Stunting (%)	OR	95% CI	OR*	95% CI	OR**	95% CI
<48	457	9.2	**8.70**	**3.66–20.67**	**8.64**	**3.63–20.57**	**8.74**	**3.65–20.94**
48-49	976	5.2	**4.74**	**2.02–11.12**	**4.86**	**2.07–11.42**	**4.98**	**2.11–11.75**
50-51	1264	1.2	1.45	0.58–3.62	1.51	0.6–3.76	1.52	0.61–3.79
52-53	522	1.2	1.00	Reference	1.00	Reference	1.00	Reference
≥54	71	0.0	—		—		—	

Table 3: Continued.

BL (cm)	*n*	IR	OR	95% CI	OR*	95% CI	OR**	95% CI
<48	457	25.8	1.12	0.84–1.49	1.15	0.83–1.58	1.38	0.99–1.91
48-49	976	27.4	1.21	0.95–1.55	1.19	0.91–1.56	**1.40**	**1.06–1.84**
50-51	1264	25.9	1.12	0.88–1.42	1.05	0.81–1.36	1.13	0.87–1.48
52-53	522	23.8	1.00	Baseline	1.00	Baseline	1.00	Baseline
≥54	71	28.2	1.26	0.72–2.19	1.04	0.56–1.94	1.00	0.53–1.85
GA (w)	*n*	BP ≥ 90th percentile	OR	95% CI	OR*	95% CI	OR**	95% CI
≤37	431	13.7	**1.73**	**1.09–2.75**	**1.74**	**1.09–2.78**	**1.77**	**1.11–2.82**
38	685	11.5	1.42	0.91–2.21	1.42	0.91–2.21	1.43	0.92–2.22
39	928	10.9	1.33	0.87–2.04	1.36	0.88–2.08	1.37	0.89–2.10
40	889	9.6	1.15	0.75–1.78	1.17	0.76–1.82	1.18	0.76–1.83
≥41	357	8.4	1.00	Baseline	1.00	Baseline	1.00	Baseline
GA (w)	*n*	MS (%)	OR	95% CI	OR*	95% CI	OR**	95% CI
≤37	431	10.2	**2.14**	**1.21–3.78**	**2.37**	**1.28–4.39**	**2.46**	**1.33–4.55**
38	685	8.2	1.68	0.97–2.90	1.76	0.97–3.17	1.78	0.99–3.22
39	928	6.5	1.30	0.76–2.24	1.51	0.84–2.70	1.56	0.87–2.79
40	889	6.9	1.39	0.81–2.38	1.55	0.86–2.77	1.58	0.88–2.84
≥41	357	5.0	1.00	Baseline	1.00	Baseline	1.00	Baseline
GA (w)	*n*	TG ≥ 110 mg/dL (%)	OR	95% CI	OR*	95% CI	OR**	95% CI
≤37	431	30.6	**1.41**	**1.03–1.94**	**1.48**	**1.06–2.07**	**1.52**	**1.09–2.13**
38	685	27.0	1.18	0.88–1.59	1.19	0.87–1.63	1.21	0.88–1.65
39	928	25.2	1.08	0.81–1.44	1.14	0.85–1.54	1.16	0.86–1.57
40	889	27.2	1.20	0.90–1.59	1.28	0.95–1.73	1.30	0.96–1.75
≥41	357	23.8	1.00	Baseline	1.00	Baseline	1.00	Baseline

*OR adjusted by %FM.
**OR adjusted by %FM, sex, and Tanner stage.
WC, waist circumference; BP, blood pressure; MS, metabolic syndrome; TG, triglycerides; BP, blood pressure; HOMA-IR: homeostasis model assessment index-insulin resistance; BW, birth weight; BL, birth length; GA, gestational age; OR, odds ratio; CI, confidence interval, %FM: percentage of fat mass.

implies a lower fetal growth as estimated by BW and BL; much more attention should be given to its prevention.

National proportions of BL < 50 cm and ≥54 cm are now reaching 44% and 2.5%, respectively, meanwhile national proportions of BW < 3000 g and ≥4000 g are now reaching 21.8% and 9%, respectively [16, 18, 19, 35]. It can be inferred that BL < 50 cm is the most frequent factor of risk in this study. Anyway, there is a clear need to avoid extreme values of perinatal variables.

Most of these results confirmed observations of a previous study [6]. However, this is the first Chilean sample that shows U- or J-shaped nature of some of the associations [39–46]. All previous studies had a lower sample size than the present study and also less power to detect J- or U-shaped associations between BL and BP. Besides, the other seven quoted reports apparently did not search for the possible U-shaped associations of BW with obesity or WC; anyway, in most of them infants or children were too young to show that kind of associations as discussed previously [37].

There are some weaknesses in our study that need to be addressed. The sample comes from all public schools in Puente Alto, the county with the highest number of inhabitants in Chile reaching more than 600,000 [16]. Cases that did not sign the informed consent were mostly boys. However the final study sample consisted of 52% females and 48% males, with a similar sex distribution to all live births at the national level [16]. No significant differences were found between the students who signed and did not sign the informed consent with respect to their prenatal and maternal characteristics. These findings suggest that there was not a biased selection of study subjects due to follow-up losses. Another limitation of this study is the lack of consensus on the diagnostic classifications of MS in children older than 6 years [18, 47, 48]; this difficulty is due to the possible influences of growth and puberty [49].

Body composition at school-age would need to be associated with the same information at birth and also during pregnancy [50]. Moreover, other maternal data could provide light on the role of nutritional status and certain pathologies on the observed results of this study. Although there is a known association between nutrition during pregnancy, infancy, and childhood with metabolic disorders, specific studies in different settings are needed [1–4].

5. Conclusions

Prenatal growth is related to MS, IR, and nutritional status in this large sample of 3290 Chilean children. Fetal growth seemed to be generally more important in these associations than in present body composition because adjustments for

present fat mass increased or maintained the most prenatal growth influences.

The most significant results may include association of anthropometry at birth with MS, obesity, BP ≥ 90th percentile, and stunting. Other results confirmed previous observations regarding high risk of obesity and high risk of WC ≥ 90th percentile in newborns who weighed ≥ 4000 g at delivery. BW had a strong negative association with BP ≥ 90th percentile as previously reported. Interestingly, BL < 50 cm was associated with at least ~30% reduction in the risk of obesity and at least five times increase in the OR of stunting. The lowest and highest categories of BL were associated with a 2-fold increase in the risk of high BP. On the other hand, children who were born ≤37 weeks exhibited higher risk for BP ≥ 90th percentile, hypertriglyceridemia, and MS.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Docosahexaenoic Acid Supplementation Early in Pregnancy May Prevent Deep Placentation Disorders

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Uteroplacental ischemia may cause preterm birth, either due to preterm labor, preterm premature rupture of membranes, or medical indication (in the presence of preeclampsia or fetal growth restriction). Uteroplacental ischemia is the product of defective deep placentation, a failure of invasion, and transformation of the spiral arteries by the trophoblast. The failure of normal placentation generates a series of clinical abnormalities nowadays called "deep placentation disorders"; they include preeclampsia, fetal growth restriction, preterm labor, preterm premature rupture of membranes, in utero fetal death, and placental abruption. Early reports suggested that a LC-PUFAs (long chain polyunsaturated fatty acids) rich diet reduces the incidence of deep placentation disorders. Recent randomized controlled trials are inconsistent to show the benefit of docosahexaenoic acid (DHA) supplementation during pregnancy to prevent deep placentation disorders, but most of them showed that DHA supplementation was associated with lower risk of early preterm birth. We postulate that DHA supplementation, early in pregnancy, may reduce the incidence of deep placentation disorders. If our hypothesis is correct, DHA supplementation, early in pregnancy, will become a safe and effective strategy for primary prevention of highly relevant pregnancy diseases, such as preterm birth, preeclampsia, and fetal growth restriction.

1. Introduction

Preterm birth is the one that occurs after 22 and before 37 weeks of gestation [1]. Its incidence ranges from 8 to 10% of all births, although with significant regional variations, with an incidence as high as 10–12% in the US, or as low as 5% in Chile and the European Union [2]. Excluding congenital malformations, 75% of perinatal deaths and 50% of childhood neurological disabilities are directly attributable to prematurity [2].

Preterm birth can be originated by (1) preterm labor, (2) preterm premature rupture of membranes, and (3) indicated preterm birth (premature medical termination of pregnancy due to maternal or fetal problems) [2–4]. Each of these three groups corresponds roughly to a third of all premature births; the data generated in our center confirms this universal trend [5]. Although many etiologies underlie any of these clinical groups, uteroplacental ischemia originated in disorders of deep placentation may be a common cause [6–13].

2. Uteroplacental Ischemia and Preterm Birth

An emerging hypothesis correlates by etiology idiopathic preterm labor with the phenomena of reduced blood flow to the uterus and placenta: uteroplacental ischemia. This hypothesis is supported by clinical and experimental evidence; for example, pregnant women at high altitude (>4,000 meters above sea level) have triple chance of premature delivery than women living at sea level (12 versus 4%) [14]. Clinical conditions that are a reflection of placental ischemia, such as preeclampsia and fetal growth restriction, are frequently associated with premature onset of labor [5, 15]. The anatomical-clinical correlation between preterm birth and

placental morphology (histology) indicating uteroplacental ischemia has also been demonstrated [7, 16–18].

Decreased uteroplacental blood flow can be estimated by studying vascular resistance in the uterine artery by Doppler. In pregnant women without risk factors for preterm labor, increased vascular resistance in the uterine artery increases by 5 times the risk of preterm birth [19]. Moreover, in women in spontaneous preterm labor, increased vascular resistance in the uterine arteries is associated with double risk of premature birth [20–22].

The first clinical series, investigating the different etiologies of preterm labor in 50 women, including placental histology and uterine artery Doppler velocimetry, concluded that 30% of patients with preterm labor show uteroplacental ischemia [17]. Our group conducted a controlled clinical trial in a group of 145 patients admitted to our hospital in preterm labor. In each of these patients and in a control group, we assessed the presence of uteroplacental ischemia (uterine artery Doppler, birth weight, placental histology, and placental weight) and infection (placental histology and amniotic fluid cultures). We reported that 30% of patients with idiopathic preterm labor have clinical and/or laboratory evidences of uteroplacental ischemia [7]. The group with uteroplacental ischemia as the etiology of preterm birth has larger neonatal morbidity than the group with neither infection nor ischemia [7].

Our group proposed that ischemia in any of the components of the uteroplacental unit (trophoblast, decidua, fetal membranes, or myometrium) generates paracrine mediators that trigger the premature onset of myometrial contractile activity. We have reported the role of fetal membranes derived B-type natriuretic peptide (BNP) in maintaining myometrial quiescence during pregnancy, and that the premature decrease of BNP production may cause preterm labor and preterm birth [23–25]. We postulate that uteroplacental ischemia, induced by abnormal placentation, may produce a premature decline in BNP production, being responsible for the premature activation of the myometrium. We studied the effect of hypoxia on BNP production by trophoblast explants; we found that hypoxia decreases by 50% the BNP production (unpublished).

To completely understand uteroplacental ischemia as a cause of premature birth, the series of events between ischemia and increased myometrial contractility remain to be determined. However what causes uteroplacental ischemia is already known: defective deep placentation.

3. Deep Placentation

Implantation and placentation establish a physical contact of the embryo/fetus and the mother, with two main objectives: (A) to establish a structural support of the embryo to the uterus and (B) to bring maternal and fetal circulation close enough to allow an adequate transfer of gases, nutrients, and waste products. Placentation begins with the implantation of the blastocyst; the outermost cells of the blastocyst (extra-embryonic cells) give rise to the trophoblast, a specialized epithelium that during implantation invades the decidua (maternal tissue originated in the endometrium prepared to receive the embryo) and the inner myometrium, developing the placenta. The embryoblast is surrounded by a "trophoblastic shell" of syncytiotrophoblast, through which columns of proliferating cytotrophoblast penetrate to invade the uterine stroma. Cytotrophoblast that penetrates beyond the syncytiotrophoblast shell assumes the extravillous cytotrophoblast lineage. The trophoblast forms two types of chorionic villi: floating and anchoring. The floating villi occupy the intervillous space ("gaps" filled with maternal blood) allowing the transport of gases and nutrients. Anchoring villi penetrate the uterine wall to provide physical support to the fetus and ensure adequate placental perfusion [26]. The columns of trophoblast are penetrated by extraembryonic mesoderm, in which fetoplacental blood vessels form by vasculogenesis, contributing to placental formation [27].

During the process of placentation, trophoblast cells attach to the basal membrane surrounding the stroma of these two types of villi. In the villi, the trophoblast cells fuse to create an external layer called syncytiotrophoblast; at the distal end of the anchoring villi, the trophoblast breaks the basal membrane and forms "trophoblast cell columns." The cell columns are formed by a subpopulation of cytotrophoblast cells called extravillous trophoblast that proliferates, invades the decidua and superficial layer of myometrium, and transforms the spiral arteries (a terminal branch of the uterine arteries that reach the endometrial surface) [26, 28]. Complete transformation of spiral arteries is required for a successful pregnancy since the transformed spiral arteries become low resistance vessels allowing a normal blood flow to the fetoplacental unit [10].

The mechanisms underlying extravillous trophoblast proliferation and invasion have not been fully established, but it is known that many molecular pathways are involved: (a) cellular interaction systems, whether cell-cell (cadherins) or cell-extracellular matrix (integrins), (b) proteolysis systems such as urokinase plasminogen activator (uPA)/plasminogen activator inhibitor type-2 (PAI-2) and matrix metalloproteinase type 9 (MMP-9)/tissue inhibitor of metalloproteinase-3 (TIMP-3), and (c) growth factors/vascular growth factors such as insulin growth factor II (IGF II) and its binding protein-1 (IGFBP-1), vascular endothelial growth factor (VEGF) and its receptors (Flt-1), and transforming growth factor beta (TGFβ) and its receptor (endoglin) among others [28–30].

The trophoblast-associated remodeling of the spiral arteries is a process of profound changes of the arterial wall of these vessels, mainly characterized by (a) replacement of the vessel wall (media and endothelium) by endovascular trophoblast and (b) replacement of the muscular and elastic arterial wall by interstitial trophoblast and fibrinoid material [31–34]. To transform the spiral arteries, the trophoblast invades the maternal tissue (decidua, myometrium, and arteries) via two different routes: interstitial and endovascular. In the interstitial invasion, the trophoblastic cells migrate towards the arterial wall replacing the musculoelastic wall [31, 32]. The interstitial trophoblast may also be originated from extravasation of the intraluminal trophoblast [34]. In the endovascular invasion the endovascular

trophoblastic cells infiltrate the lumens and walls of the arteries replacing the endothelium [31, 32, 34]; the endovascular trophoblast results from intravasation of interstitial trophoblast or by intraluminal migration of trophoblast [34].

The molecular basis of trophoblast-associated remodeling of the spiral arteries have not been fully established but integrins such as $\alpha v\beta 3$, $\alpha 1\beta 1$, and VE-cadherin (vascular endothelial cadherin); adhesion molecules like VCAM type-1 (vascular endothelial cell adhesion molecule 1), PECAM-1 (platelet cell adhesion molecule 1), and NCAM-1 (nerve cell adhesion molecule 1); and protease/anti-type protease systems such as MMP-9 and PAI-1/PAI-2 play a key role [35–40]. In knockout models for these factors, the more clearly linked to a severe abnormality of placentation is the αV-$\beta 3$ integrin knockout mouse; αV-$\beta 3$ integrin deficiency is associated with a 50% reduction in litter size and fetal mortality of 10% and is accompanied by a clear reduction in placental perfusion [35]. PAI-1 is produced by endothelial cells and is a marker of endothelial activation; PAI-1 level increases during pregnancy, but the increase is larger in the presence of endothelial dysfunction and abnormal placentation [41]. PAI-2 is produced by the cytotrophoblast; its production increases in normal pregnancy but is reduced in abnormal placentation [42]. In normal pregnancy PAI-1/PAI-2 reason is progressively reduced with increasing placental mass, but in the presence of abnormal placentation the PAI-1/PAI-2 ratio has been used as a marker of placental insufficiency and high risk of developing preeclampsia or fetal growth restriction [43–47].

A key question is how the trophoblast invasion is directed to the blood vessel to proceed to its transformation. Attention has been focused on two areas: (a) biological effects of oxygen partial pressure on the trophoblast and (b) maternal endothelium-derived factors. Oxygen partial pressure: it has been suggested that extravillous trophoblast proliferation and invasion is modulated by the interstitial partial pressure of oxygen [34]. At low partial pressures (2% O_2), as is the case at the start of the trophoblast cell column, the trophoblast proliferates intensively but does not express the integrins $\alpha v\beta 3$ and $\alpha 1\beta 1$, needed for invasion. In the presence of higher oxygen partial pressures (8% O_2, closer to the blood vessel) the trophoblast exhibits an invasive phenotype, showing that oxygen modifies cell proliferation and differentiation [48]. The underlying mechanism involves the transcription factor HIF-1α (hypoxia inducible factor-1α) that modulates the secretion of transforming growth factor $\beta 3$ (TGF$\beta 3$), $\alpha 1\beta 1$ integrin expression, and production of MMP-9 [49]. Endothelium-derived factors: they play a primary role in (a) trophoblast chemotaxis, (b) trophoblast primary interaction with the endothelium (adhesion molecules), and (c) maintenance of arteriolar vasodilation [50–53]. It is known that in vitro VEGF plays a chemotactic role in trophoblast migration [53]; VEGF and PGF (placental growth factor) are produced by the cyto- and syncytiotrophoblast and their secretion is modulated by hypoxia (VEGF increase and PGF decrease in response to hypoxia) [52]. VEGF upregulates the production by endothelial cells of proinvasive integrins ($\alpha v\beta 3$ and $\alpha 1\beta 1$) and upregulates the expression of the intracellular adhesion molecule-1 (ICAM-1) that participates in the process of trophoblast-endothelium interaction, a key event in vascular transformation [50, 51].

4. Timing of Trophoblast Invasion and Spiral Arteries Transformation

The classical view is that this process occurs in two stages: (1) the transformation of the decidual segment of the spiral arteries by a "first wave" of endovascular trophoblast migration in the first trimester; and (2) the transformation of the myometrial segments of the spiral arteries in the second trimester by a "second wave" of trophoblast [54]. However, more recent studies have suggested that trophoblast invasion of the spiral arteries is a continuous process [55]. Anyway, deep transformation of the spiral arteries, reaching the myometrial segment of the spiral arteries, is required for successful placentation and normal pregnancy progression [10] while deep placentation failure may cause clinically relevant pregnancy disorders [6]. The earlier in pregnancy impaired placentation starts, the larger deep placentation defect and the greater clinical consequences will be observed. Similarly, those interventions to improve placentation will be more successful the earlier in pregnancy they are started [56, 57].

5. Defective Deep Placentation and Pregnancy Diseases

The failure of normal placentation generates a series of clinical abnormalities nowadays called "deep placentation disorders" [8, 10, 31]. Originally deep placentation disorders were considered preeclampsia (PE) and fetal growth restriction (FGR) [6]; however, it has been shown that deep placentation disorders also included preterm labor (PL) and preterm premature rupture of membranes (PPROM), in utero fetal death and placental abruption [9, 11, 12]. Considering that all these deep placentation disorders are the main problems of modern obstetrics, generating enormous adverse impact on maternal and perinatal health, great efforts have been invested in strategies to prevent deep placentation disorders [58–64]. We should observe that most of these strategies are based on secondary prevention, that is, prevention of recurrence, guiding prediction strategies, and/or treatments to high risk pregnancies.

Some of the strategies to prevent deep placentation disorders have shown benefit: aspirin for prevention of PE and FGR [65, 66] or the use of progesterone for prevention of PL and PPROM [67]. It is important to observe that the preventive effect is stronger when the intervention starts earlier in pregnancy [68, 69]. Probably the need of using these drugs early in pregnancy reflects the effect they have on improving placentation, avoiding deep placentation disorders [6]. Since these strategies to prevent deep placentation disorders are aimed to high risk pregnancies, we observe two problems: an early identification of population at risk is required (to start treatment) and the opportunity for primary prevention is lost

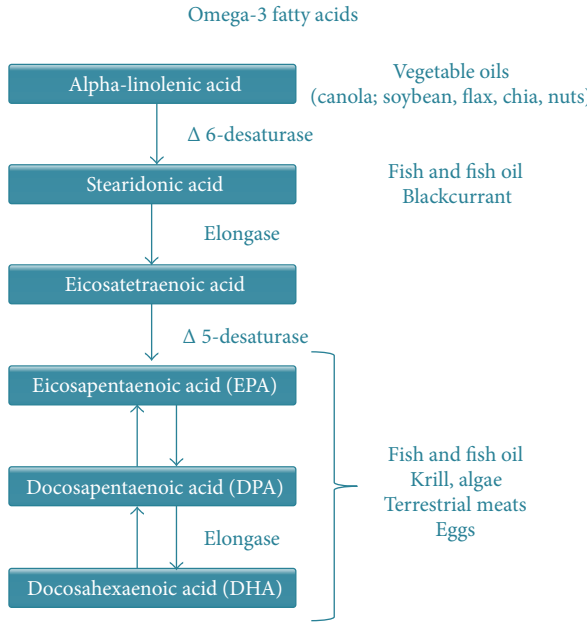

Figure 1: Docosahexaenoic acid (DHA) is an essential fatty acid of the family of long chain polyunsaturated fatty acids (LC-PUFAs) or omega-3 fatty acids. In humans, DHA can be consumed (mainly fish or fish oil) or converted from EPA.

(low-risk population is not considered). Strategies of deep placentation disorders prevention are required to implement at the population level, that is, drugs or supplements that can be administered to the general population to prevent the occurrence of deep placentation disorders (primary prevention); unfortunately studies of primary prevention have failed to be effective [70–72].

6. Polyunsaturated Fatty Acids

Docosahexaenoic acid (DHA) is an essential fatty acid of the family of long chain polyunsaturated fatty acids (LC-PUFAs) or omega-3 fatty acids [73]. To this family eicosapentaenoic acid (EPA) and alpha-linolenic acid (α-LA) also belong (Figure 1). These fatty acids are considered essential because our body cannot synthesize them; thus the LC-PUFAs must be acquired through food [74, 75]. The LC-PUFAs are essential components of phospholipids present in all our tissues and actively involved in the functional regulation of cellular and subcellular membranes [74–76]. Early reports, mainly observational, suggested that LC-PUFAs rich diet reduces the incidence of deep placentation disorders (preeclampsia, intrauterine growth restriction, and preterm delivery) [77–79]. We recently submitted a critical review of the safety and efficacy of DHA supplementation during pregnancy to improve pregnancy outcome in general population of pregnant women; we conclude that the evidence currently available does not support or completely rule out this intervention during pregnancy [80].

7. Effect of DHA Supplementation on Pregnancy Duration

Three recent randomized studies have reported the effect of DHA supplementation on pregnancy duration in general population of pregnant women: one of them showed a significant reduction in the proportion of preterm birth before 34 weeks (1.09% versus 2.25%, DHA versus placebo, resp., RR 0.49, 95% CI 0.25–0.94, $P = 0.03$) [81]. The second study did not find differences in gestational age at delivery (39.1 ± 1.7 versus 39.0 ± 1.9 weeks, DHA versus placebo, resp., mean ± standard deviation, $P > 0.05$) or the proportion birth before 37 weeks (10.1% versus 8.3%, DHA versus placebo, resp., RR 1.2, 95% CI 0.8–1.8, $P = 0.33$) [82]. Finally, a more recent study demonstrated a significant prolongation of pregnancy and a reduction in the rate of delivery before 34 weeks (0.6% versus 4.8%, $P < 0.05$, DHA versus placebo, resp.) [83]. A randomized controlled trial in high risk population (women with a history of prior spontaneous singleton preterm birth) failed to demonstrated benefit of DHA supplementation to prevent preterm birth (37.8% versus 41.6%; RR 0.91, 95% CI 0.77–1.07, DHA versus placebo, resp.) [84]. The systematic Cochrane review studying the effect of marine oil (70% DHA; 30% EPA) supplementation during pregnancy failed to demonstrate a significant reduction of preterm birth but suggested a significant reduction of birth before 34 weeks (RR 0.69, 95% CI 0.49–0.99), including two trials [85]. A recent meta-analysis of LC-PUFAs supplementation during pregnancy shows that women receiving LC-PUFA had a 26% lower risk of early preterm delivery (<34 weeks) (RR = 0.74; 95% CI 0.58–0.94) and no difference in the risk of preterm

delivery (<37 weeks) (RR = 0.91; 95% CI 0.82–1.01) [86]. Because of the heterogeneity of the studies and the great variability in the observational studies, they conclude that more studies are needed to confirm the findings, especially in undeveloped countries [86].

8. Effect of DHA Supplementation on Birth Weight

The newborn weight was presented as a secondary analysis of two randomized studies [81, 82] showing that average birth weight significantly increased in women who received DHA. The first study reported a lower proportion of infants weighing less than 2500 g in the group of women receiving DHA supplementation compared to the control group (3.41% versus 5.27%, resp., RR 0.65, 95% CI 0.44–0.96, $P = 0.03$) [81]. The other study showed that DHA supplementation reduced the risk of intrauterine growth restriction (weight below the 10th percentile for gestational age), only in primiparous women (7.1% versus 14%, DHA versus placebo, resp., RR 0.5, 95% CI 0.3–1.0, $P = 0.03$) [82]. The meta-analysis failed to demonstrate a reduction in the rate of low birth weight in response to LC-PUFAs supplementation [85–87].

9. Effect of DHA Supplementation on the Incidence of Preeclampsia

Observational studies have suggested that increased intake of polyunsaturated fatty acids reduces the risk of preeclampsia [88–90]. Furthermore lower concentration of polyunsaturated fatty acids has been shown in placentas of women with PE compared with normotensive pregnant women [91], in addition to an inverse relationship between LC-PUFAs and sFLT-1 (a marker of defective vasculogenesis) in patients with PE [92, 93]. However, two meta-analyses failed to demonstrate reduced risk of PE associated with higher intake of LC-PUFAs [86, 87] and the same goes for the systematic review of marine oil [85]; these two meta-analyses do not differentiate between early or late PE.

10. Polyunsaturated Fatty Acids and Placentation

The effect of fatty acids in extravillous trophoblast cell lines (HTR8/SVneo cells) has been studied, showing that DHA, EPA, and AA (arachidonic acid) have a proangiogenic effect, expressed by an increase in the development of capillary sprouts [94]. In the same model, it has been demonstrated that linoleic acid supplementation in vitro stimulates the production of proangiogenic factors and the formation of capillary sprouts [95]. The comparative analysis showed that DHA is more powerful in stimulating extravillous trophoblast vasculogenesis [94, 96].

Another line of research using immortalized trophoblast cells of neoplastic origin (BeWo cells) demonstrated that DHA reduces oxidative stress [97]. Similarly, DHA supplementation during pregnancy in the rat reduces placental oxidative stress [98]. This is important since placental oxidative stress may play a key role in the pathophysiology of deep placentation disorders [99]. Also, defective deep placentation induces oxidative stress that may cause the clinical disorders related to defective placentation [100, 101].

Additionally, DHA supplementation is associated with elevated markers of trophoblast proliferation, measured in placentas obtained at term of pregnancy [102]. The direct effect of DHA supplementation during pregnancy on placentation, for example, using placental bed biopsies, has not been studied. However, in the rat it was shown that LC-PUFAs supplementation early in pregnancy increased fetal and placental size [98].

11. Conclusions

Considering these antecedents we propose that maternal supplementation with LC-PUFAs, especially DHA, reduces deep placentation disorders, by improving deep placentation. We consider that more information is needed to get a final conclusion about the therapeutic role of DHA supplementation during pregnancy; we have recently obtained ethical approval and financial support for a randomized placebo controlled trial of DHA supplementation from early pregnancy to prevent deep placentation disorders.

We need such a new trial, since there are still doubts about the efficacy of DHA to improve perinatal outcome. The difference among studies may be explained, at least partially, by the timing of supplementation and the main outcome measured. Regarding the timing of supplementation we postulate that the effect of DHA, which improves the clinical outcome, is to prevent defective deep placentation. Therefore, the more early in pregnancy DHA starts, the greater the effect on improving placentation is, making it possible to achieve a significant improvement in clinical outcomes. In fact, many of the studies failing to demonstrate the effect of DHA started supplementation after 16 weeks of pregnancy [82, 84]. Concerning the main outcome of the study, we must observe that early preterm (<34 weeks) and late preterm (>36 weeks) are probably two different diseases with different etiologies; the same happens for early and late preeclampsia and early or late fetal growth restriction [103–105]. Current evidence suggests that early prematurity (preterm labor or preterm premature rupture of membranes), early fetal growth restriction, and early preeclampsia share deep placentation defects as common etiology [6]. Thus, deep placentation disorders are the main outcome perhaps clinically modifiable with DHA supplementation early in pregnancy.

LC-PUFAs, including DHA, supplementation in pregnant women, breastfeeding mothers, and infants, have been well tolerated (more than 90% adherence to the protocol) and did not generate any serious adverse events [81, 83, 85–87]. Minor potential side effects are nausea, intestinal gas, bruising, and prolonged bleeding, fishy taste, belching, nosebleeds, and loose stools. Taking DHA with meals can often decrease these side effects [81, 83, 106]. Consuming large amounts of fish oil from some dietary sources is possibly unsafe. Some fish meats (especially shark, king mackerel,

and farm-raised salmon) can be contaminated with mercury and other industrial and environmental chemicals, but fish oil supplements typically do not contain these contaminants [107, 108]. The maternal intake of LC-PUFA during pregnancy and lactation is considered very important, since the LC-PUFAs are provided during perinatal development through placental transfer and maternal milk [80, 109]; thus DHA or LC-PUFAs intake during pregnancy is encouraged [110].

Although a precise DHA intake level for pregnant women has not been fully established [111], the worldwide recommendation for DHA intake during pregnancy is 200 mg/day [112]. In Chile there is not a local recommendation, but powdered milk supplied to pregnant women in the public health system (*Purita Mamá*) contains DHA 240 mg + EPA 76 mg per 100 g; this taken to 200 mL provides 60 mg DHA and 19 mg of EPA per day. Beyond the nutritional recommendation (eating fish), and the contribution of milk, in Chile there is no national standard for the indication of DHA in capsules during pregnancy. While there are products on the market containing DHA with other vitamins, for pregnant women, their use is not a regular practice among obstetricians in our country. Thus, it seems possible and ethically plausible to conduct a randomized study in which one arm of the study received a placebo containing no DHA.

Most of the intervention trials have included few participants and provided fish oil supplements that contain large and varying doses of DHA (100 mg to 5 gr/day) [106]. The two trials that demonstrate benefit of DHA supplementation to prevent preterm birth use either 600 [83] or 800 mg DHA per day [81]; thus a new trial must use a dose of 600 or 800 mg DHA per day.

Studies have also measured cognitive development or visual accuracy of children in the long term [113–118]. Thus, children monitoring, about visual or neurocognitive development, would be also important in a clinical DHA trial.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Influence of Hypoxia during Different Pregnancy Stages on Cardiac Collagen Accumulation in the Adult Offspring

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We evaluated whether the timing of maternal hypoxia during pregnancy influenced cardiac extracellular matrix accumulation in the adult offspring. Rats in different periods of pregnancy were assigned to maternal hypoxia or control groups. Maternal hypoxia from day 3 to 21 of pregnancy or day 9 to 21 of pregnancy increased collagen I and collagen III expression in the left ventricle of adult offspring (both $P < 0.05$). Maternal hypoxia from day 15 to 21 of pregnancy had no effect on adult collagen levels. Our results indicate that maternal hypoxia at critical windows of cardiovascular development can induce pathological cardiac remodeling in the adult rat offspring.

1. Introduction

Cardiovascular disease is a leading cause of death in developed countries [1]. Several risk factors, including smoking, hypertension, and high body mass index have been identified. These risk factors, however, are not sufficient to explain the prevalence of disease. Recently, the concept of fetal origins of adult disease has emerged as a possibility to explain the high incidence of cardiovascular disease. This concept hypothesizes that an adverse intrauterine environment during a critical window of fetal or infant development can exert long-term effects on adult tissue structure or function [2–4].

Maternal hypoxia during pregnancy in rats leads to changes in cardiac structure and function in adult male offspring [5]. Other animal models indicate that timing and severity of the fetal insult are critical to the programming of adult disease [6, 7]. In this report, we elucidate the influence of maternal hypoxia during different periods of pregnancy on cardiac collagen accumulation in adult offspring.

2. Methods

2.1. Animals and Hypoxic Protocol. All experimental procedures were in accordance with the National Institutes of Health guidelines and were approved by the Standing Committee on Ethics and Animal Experimentation at the Fujian Medical University (China). Virgin female Sprague-Dawley (SD) rats (250–280 g in body weight) were obtained from the Shanghai Experimental Animal Center, Shanghai, China. They were housed individually in standard rat cages under controlled temperature (22 ± 1°C) with lights on from 07.00 to 19.00 h. Food and water were provided *ad libitum*. The female rats were mated within the animal facility, and the vaginal smears were checked every morning. Pregnancy was confirmed by the presence of sperm-positive vaginal smears (day 0). The 24 pregnant rats were randomized to one of four groups: a hypoxia group from day 3 to day 21 of pregnancy (G3–21; $n = 6$), a hypoxia group from day 9 to day 21 of pregnancy (G9–21; $n = 6$), a hypoxia group from day 15 to day 21 of pregnancy (G15–21; $n = 6$), and a control group (G0; $n = 6$).

Maternal hypoxia was induced by the method described by Wang and colleagues [8]. Briefly, the pregnant rats assigned to the hypoxia groups were placed inside a plexiglass chamber (140 L) for 3 h per day during the light cycle. Nitrogen gas and compressed air were continuously infused into the chamber to maintain an oxygen concentration of 10 ± 1%. The oxygen concentration in the chamber was monitored

using a portable gas analyzer that was calibrated daily (S-450; IST-AIM).

After birth, litters were reduced to 8 pups per dam to standardize the nutrient supply. Offspring were weaned at 3 weeks and housed in the animal facilities of Fujian Medical University (China). Experimental models have shown that male offspring are more susceptible to the adverse fetal environment [9, 10]; accordingly, only male offspring (2 or 3 rats per litter) were evaluated in this experiment.

2.2. Blood Pressure Measurements. The systolic blood pressure of the male offspring was determined 3 months (n = 12 in each group) and 5 months (n = 6 in each group) of age using the tail cuff method (RBP-1B, Clinical Medical Instrument Institute of Beijing Sino-Japan Friendship Hospital, Beijing, China). For the rats that were evaluated at 5 months of age, blood pressure was also measured at 3 months of age. The male offspring were trained for one week before the blood pressure measurements were taken. The average measurements from each animal on three separate days were taken to determine the individual mean blood pressure for that animal.

2.3. Tissue Collection. After being anesthetized with an intraperitoneal injection of pelltobarbitalum natricum (20 mg/kg), hearts were removed from male offspring at the age of 3 months (n = 6) and 5 months (n = 6). After determination of heart weight (HW), the left ventricle was carefully separated from the atria and the right ventricle and weighed. The wet weight of the left ventricle (LV) was recorded, normalized for body weight (BW), and expressed as the ventricular mass index. The LV tissue was divided into 2 sections. One section was fixed in 4% paraformaldehyde for histological analysis and the other section was snap-frozen in liquid nitrogen for protein analysis.

2.4. Histopathology. Tissues were dehydrated through graded alcohols and xylene and then embedded in paraffin. Sections were cut at 5 μm thickness and stained with haematoxylin and eosin. The diameter of cardiomyocytes was determined by measuring the shortest transverse diameter of myocytes that were cut transversely with an intact membrane and a centrally located nucleus. The measurements were performed using ImageJ software.

2.5. Immunoblot Analyses. Immunoblotting was performed as described previously [5]. Briefly, LV tissue was homogenized in ice-cold lysis buffer. Crude homogenates were centrifuged for 5 min at 10000 g, and protein concentration of the supernatants was determined by the BCA method (Pierce Chemical Co). Supernatants were added to loading buffer and heat denatured by boiling for 3 min. Protein (25 ug) was electrophoresed in separate lanes on 8% SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blocked in a 5% nonfat dried milk solution at room temperature. The blot was incubated with antibodies against collagen I or collagen III (1 : 100 dilution; Beijing Biosynthesis Biotechnology Company, China). A peroxidase-conjugated avidin secondary antibody was applied for visualization (1 : 5000 dilution; Santa Cruz Biotechnology, USA). The blots were stripped and reprobed with a β-actin antibody (1 : 2000; Beijing Biosynthesis Biotechnology Company, China) to normalize for protein loading. The integrative grayscale pixel area-density was captured with a CCD camera and quantified using Quantity One software.

Table 1: Systolic blood pressure of male offspring.

Group	3 months (n = 12)	5 months (n = 6)
G3–21	122 ± 3*	129 ± 3*
G9–21	116 ± 4	117 ± 3
G15–21	112 ± 3	117 ± 3
G0	108 ± 3	115 ± 4

Values are expressed as mean ± SEM mm Hg, *$P < 0.05$ compared with the control group. At 3 months of age, n = 12 for each group; at 5 months of age, n = 6 for each group.

2.6. Data Analysis. Data were expressed as mean ± SEM. A one-way ANOVA, followed by an SNK post hoc test, was performed to compare groups. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Systolic Blood Pressure of Male Offspring. At the age of 3 months and 5 months, we found significantly higher systolic blood pressure in male offspring from the G3–21 group compared with the G0 group (three months: G3–21 122 ± 3 mmHg versus G0 108 ± 3 mmHg, $P < 0.05$; five months: G3–21 129 ± 3 mmHg versus G0 115 ± 4 mmHg, $P < 0.05$). There were no significant differences in systolic blood pressure between the G9–21, G15–21, and G0 groups (three months: G9–21 116 ± 4 mmHg, G15–21 112 ± 3 mmHg, n = 12; five months: G9–21 117 ± 3 mmHg, G15–21 117 ± 3 mmHg; Table 1).

3.2. Body Weight and Left Ventricular Weight of Male Offspring. At 3 months of age, the HW/BW ratio and the LVW/BW ratio were significantly increased in the G9–21 male offspring compared with the G0 male offspring (HW/BW: G9–21 2.92 ± 0.06 versus control 2.67 ± 0.06, $P < 0.05$; LVW/BW: G9–21 2.15 ± 0.05 versus control 1.98 ± 0.02, $P < 0.05$, n = 6), while there was no significant difference between the G3–21, G15–21 and G0 groups (HW/BW: G3–21 2.86 ± 0.08, G15–21 2.69 ± 0.05, n = 6; LVW/BW: G3–21 2.07 ± 0.02, G15–21 2.06 ± 0.04, n = 6; Figure 1(a)). At 5 months, the HW/BW ratio and LVW/BW ratio remained higher in the G9–21 male offspring compared with G0 rats (HW/BW: G9–21 2.64 ± 0.06 versus control 2.39 ± 0.03, $P < 0.05$, n = 6; LVW/BW: G9–21 1.96 ± 0.05 versus control 1.80 ± 0.02, $P < 0.05$, n = 6). In addition, there was a significantly increased HW/BW ratio and LVW/BW ratio in male offspring from the G3–21 group compared with the G0 and G15–21 group (HW/BW: G3–21 2.68 ± 0.05 versus control 2.39 ± 0.03 or

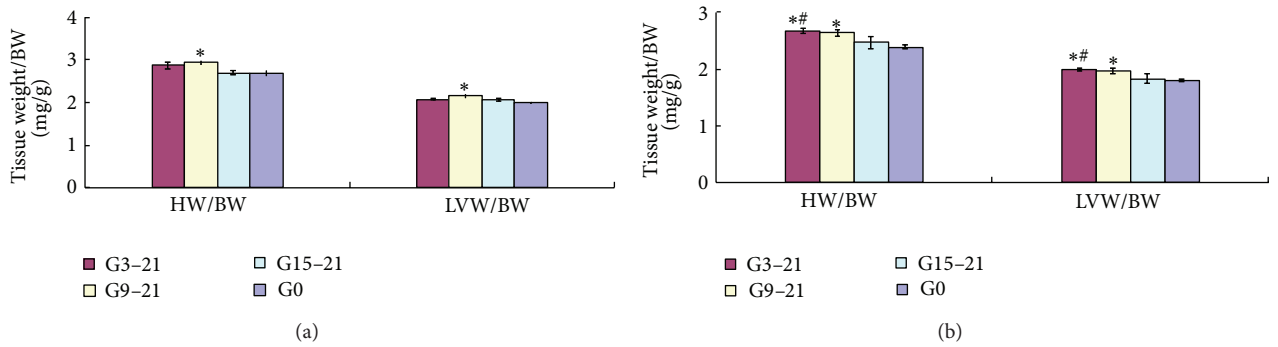

Figure 1: HW/BW ratios and LVW/BW ratios of rat male offspring at 3 months of age (a) and 5 months of age (b). All data are expressed as mean ± SEM, $n = 6$. HW, heart weight; BW, body weight; LVW, left ventricle weight. G3–21, maternal hypoxia group from day 3 to day 21 of pregnancy; G9–21, maternal hypoxia group from day 9 to day 21 of pregnancy; G15–21, maternal hypoxia group from day 15 to day 21 of pregnancy; G0, control group. $^{*}P < 0.05$ compared to control group, $^{\#}P < 0.05$ compared to G15–21.

G15–21 2.47 ± 0.11, $P < 0.05$, $n = 6$; LVW/BW: G3–21 1.99 ± 0.03 versus control 1.80 ± 0.02 or G15–21 1.83 ± 0.08, $P < 0.05$, $n = 6$). The HW/BW ratio and the LVW/BW ratio in offspring from G15–21 group were not significantly higher compared with the G0 group (HW/BW: G15–21 2.47 ± 0.11, $n = 6$; LVW/BW: G15–21 1.83 ± 0.08, $n = 6$; Figure 1(b)).

3.3. Diameter of Cardiomyocytes from Male Offspring. The transverse diameter of the cardiomyocytes from the male offspring was not significantly different in the G3–21, G9–21, G15–21, and G0 groups at either 3 or 5 months (three months: G3–21 13.1 ± 0.4 μm, G9–21 12.5 ± 0.3 μm, G15–21 12.2 ± 0.3 μm, G0 12.2 ± 0.1 μm, $n = 6$; five months: G3–21 15.1 ± 0.2 μm, G9–21 14.8 ± 0.3 μm, G15–21 14.6 ± 0.3 μm, G0 14.3 ± 0.2 μm, $n = 6$; Figure 2).

3.4. Expression of Collagen I and Collagen III Protein. Collagen I protein expression was significantly greater in the LV tissue from the G9–21 male offspring at 3 months compared with the G0 male offspring (collagen I/β-actin: G9–21 0.98 ± 0.02 versus G0 0.87 ± 0.02, $P < 0.05$). The collagen III/β-actin ratio was also significantly higher in the G9–21 male offspring compared to either the G0 or G15–21 offspring (collagen III/β-actin: G9–21 0.87 ± 0.01 versus G0 0.82 ± 0.01 or G15–21 0.79 ± 0.02, $P < 0.05$, $n = 6$). There was no significant difference in the expression of collagen I and collagen III between the G3–21, G15–21, and G0 groups (collagen I/β-actin: G3–21 0.94 ± 0.04, G15–21 0.93 ± 0.03; collagen III/β-actin: G3–21 0.85 ± 0.02, $n = 6$; Figures 3(a) and 3(c)).

In the 5-month-old offspring, expression of collagen I and collagen III was significantly increased in the LV tissue from both the G3–21 and G9–21 male offspring compared with the G0 or G15–21 male offspring (collagen I/β-actin: G3–21 1.76 ± 0.07 or G9–21 1.61 ± 0.05 versus G0 0.48 ± 0.04 or G15–21 0.63 ± 0.07, $P < 0.05$, $n = 6$; collagen III/β-actin: G3–21 0.68 ± 0.05 or G9–21 0.61 ± 0.04 versus G0 0.34 ± 0.03 or G15–21 0.41 ± 0.03, $P < 0.05$, $n = 6$), but there was no significant difference in collagen expression between the G15–21 and G0 groups (Figures 3(b) and 3(d)).

4. Discussion

Our previous studies have shown that maternal hypoxia with an oxygen concentration of 10 ± 1% induces significant hypoxemia without CO_2 retention and acidosis, and a hypoxic duration of 3 h per day reduces neonatal size and organ weight but does not reduce maternal food intake [8, 11]. Our present study demonstrated that maternal hypoxia during different periods of pregnancy differentially altered collagen accumulation in the adult male offspring.

The LVW/BW ratio is an established index of cardiac hypertrophy [12]. Our study showed that maternal hypoxia from days 3 to 21 and from days 9 to 21 of pregnancy, but not from days 15 to 21, resulted in cardiac hypertrophy in the male offspring. Pathologic cardiac remodeling might be characterized by the abnormal accumulation of collagen [13]. In our study, there was no difference in the diameter of cardiomyocytes from the male offspring, and the expression of both collagen I and III dramatically increased in the hypertrophic ventricle. This suggests that the accumulation of collagen may be the primary mechanism for the increased wall thickness. One limitation of this study was that only one index of cardiac hypertrophy was measured, namely, the HW or LV mass to BW ratios. Additional markers of hypertrophy would have confirmed this result.

Not only does cardiac collagen provide a structural framework for cardiomyocytes and the coronary blood vessels, but also collagen determines the physiological performance by modifying myocardial stiffness and tolerance to deformation [14]. The cardiac collagen matrix remodels with age or pathology [15]. This remodeling often includes changes in the quality and quantity of the collagen matrix. The adverse accumulation of collagen fibers will increase cardiac stiffness and affect diastolic function. With further deposition of collagen, the systolic motion, coronary flow reserve, and electrical activity might then be affected and contribute to pathology [16, 17].

Collagen I and collagen III are the major collagen fibers in adult hearts, and pathological remodeling is associated with increased collagen I and collagen III. For these reasons, we focused on detecting the two types of collagen and

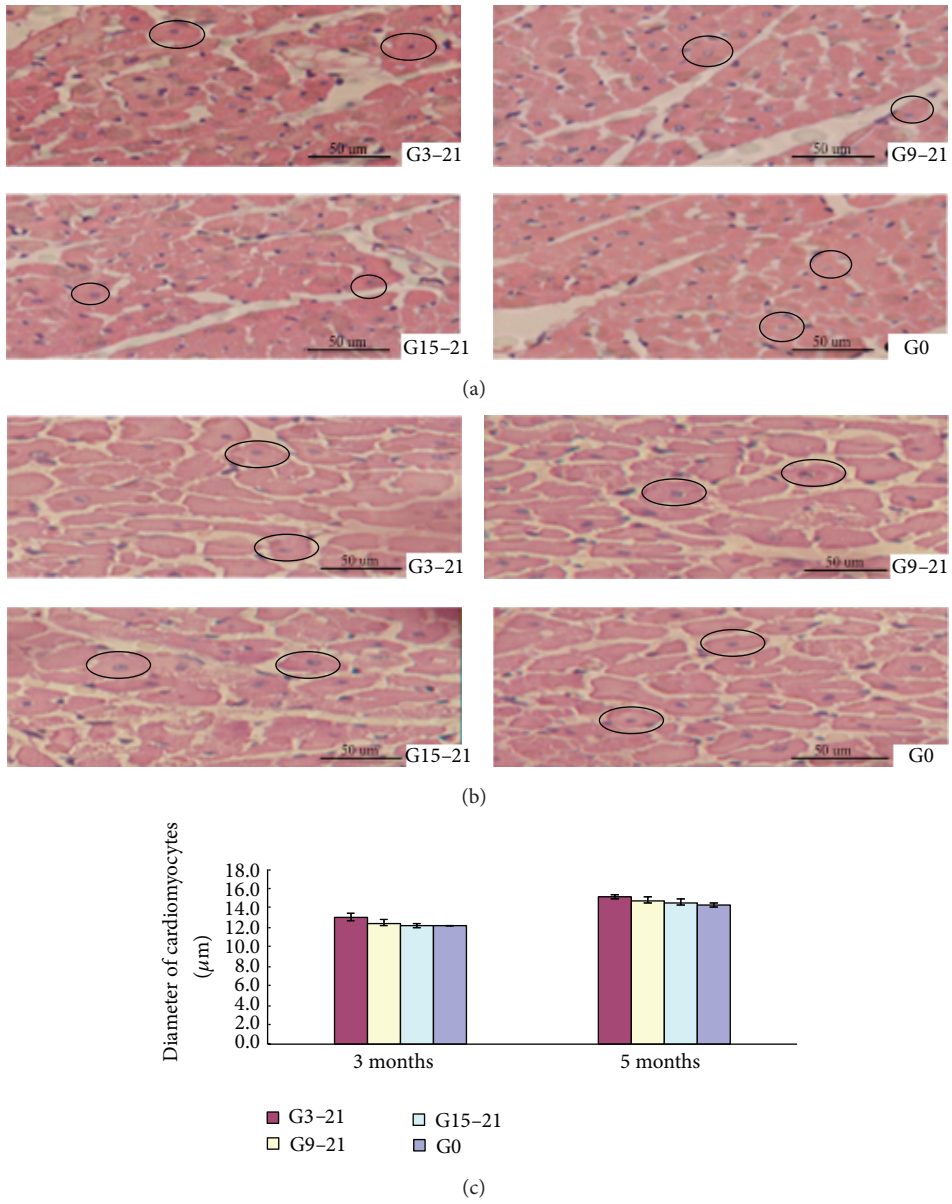

Figure 2: Representative light micrographs of cardiomyocytes stained with haematoxylin-eosin from male offspring at 3 months (a) and 5 months (b) of age. Examples of myocytes that met the criteria for evaluation are circled. Quantification of myocyte diameter from male offspring (c). All data are expressed as mean ± SEM, $n = 6$. No significant differences in the transverse diameter of cardiomyocytes from male offspring were seen among the groups. G3–21, maternal hypoxia group from days 3 to 21 of pregnancy; G9–21, maternal hypoxia group from days 9 to 21 of pregnancy; G15–21, maternal hypoxia group from days 15 to 21 of pregnancy; G0, control group. The circles in the picture indicate myocytes which met the criteria. Magnification for all photomicrographs is ×400.

did not detect cross-linked versus noncross-linked collagen. Our study demonstrated that collagen I and collagen III were increased in the hearts exposed to maternal hypoxia started in early or midpregnancy times, compared with the control group. Studies have suggested that prenatal deficiencies of metabolic substrates including oxygen or nutrients will affect postnatal cardiac structure. In our study, maternal hypoxia might have led to abnormal regulation of cardiac collagen by directly influencing metabolism [5, 18]. Moreover, previous studies have shown that the rat cardiovascular system differentiates and develops most from the 9th to 13th embryonic day and that this time period is regarded as a critical window for cardiovascular development in rat embryos [19, 20]. It is interesting to speculate that the changes of internal environment around the critical window of cardiovascular development will have the most influence on the cardiac structure of the rat offspring. That would explain why there was no increase of collagen deposits in the hearts of the G15–21 hypoxia group that was exposed outside the critical development window, from day 15 to day 21. Further studies are necessary to test this hypothesis.

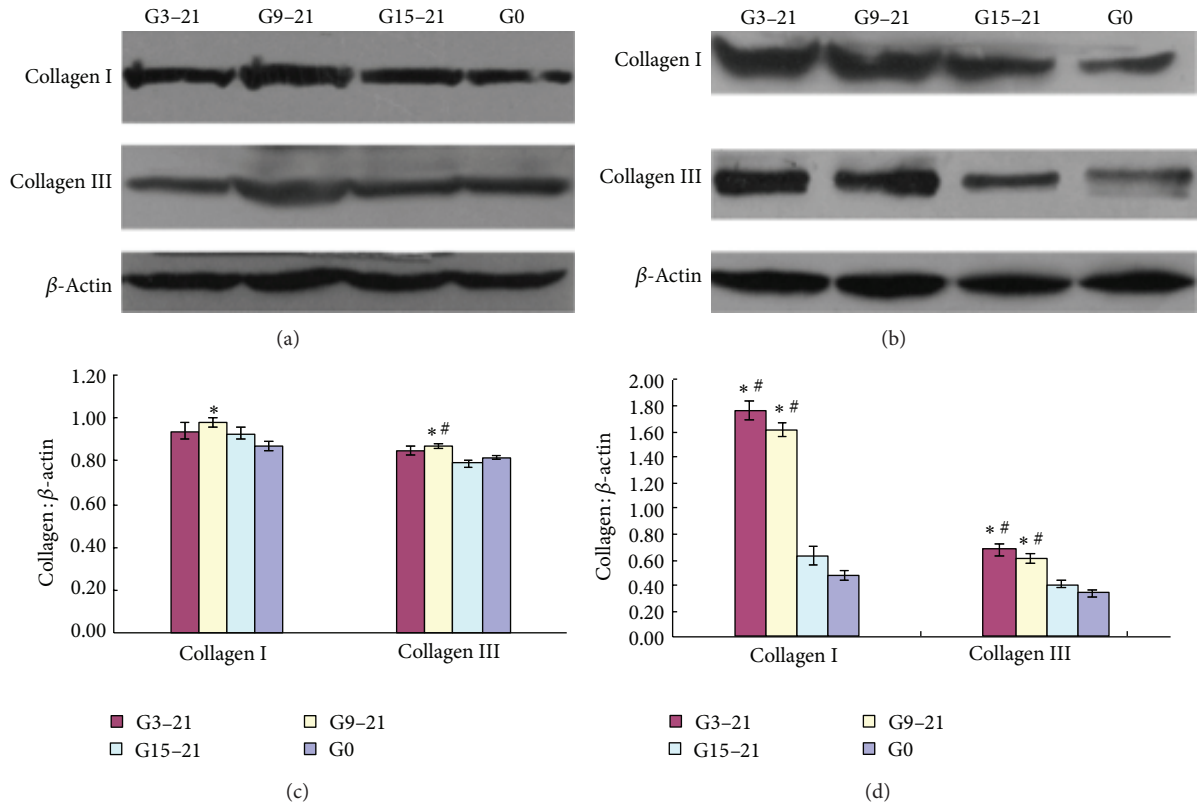

FIGURE 3: Representative immunoblots of collagen I and collagen III in the left ventricle of male offspring from the four experimental groups at 3 months (a) and 5 months (b) of age. Quantification of collagen I and collagen III is depicted for offspring at 3 (c) and 5 months of age (d). All data are expressed as mean ± SEM, $n = 6$. G3–21, maternal hypoxia group from days 3 to 21 of pregnancy; G9–21, maternal hypoxia group from days 9 to 21 of pregnancy; G15–21, maternal hypoxia group from days 15 to 21 of pregnancy; G0, control group. $^{*}P < 0.05$ compared to control group and $^{\#}P < 0.05$ compared to the G15–21 group.

Abnormal collagen accumulation is also associated with hypertensive heart disease, but in the G9–21 maternal hypoxia group there was collagen deposition in the hearts of offspring in the absence of hypertension. This result indicates that collagen deposition caused by maternal hypoxia might be independent of hypertension, which is consistent with previous studies [5, 21].

5. Conclusions

Maternal hypoxia around a critical window of cardiovascular development might have the most important influence on cardiac collagen deposition of the adult rat offspring. Pathology occurs without cardiomyocytes hypertrophy and it appears to be independent of hypertension.

Abbreviations

LV: Left ventricle
LVW: Left ventricular weight
BW: Body weight
HW: Heart weight.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Streptozotocin-Induced Diabetes Models: Pathophysiological Mechanisms and Fetal Outcomes

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Glucose homeostasis is controlled by endocrine pancreatic cells, and any pancreatic disturbance can result in diabetes. Because 8% to 12% of diabetic pregnant women present with malformed fetuses, there is great interest in understanding the etiology, pathophysiological mechanisms, and treatment of gestational diabetes. Hyperglycemia enhances the production of reactive oxygen species, leading to oxidative stress, which is involved in diabetic teratogenesis. It has also been suggested that maternal diabetes alters embryonic gene expression, which might cause malformations. Due to ethical issues involving human studies that sometimes have invasive aspects and the multiplicity of uncontrolled variables that can alter the uterine environment during clinical studies, it is necessary to use animal models to better understand diabetic pathophysiology. This review aimed to gather information about pathophysiological mechanisms and fetal outcomes in streptozotocin-induced diabetic rats. To understand the pathophysiological mechanisms and factors involved in diabetes, the use of pancreatic regeneration studies is increasing in an attempt to understand the behavior of pancreatic beta cells. In addition, these studies suggest a new preventive concept as a treatment basis for diabetes, introducing therapeutic efforts to minimize or prevent diabetes-induced oxidative stress, DNA damage, and teratogenesis.

1. Introduction

Diabetes mellitus (DM) is a chronic disease characterized by hyperglycemia resulting in insulin resistance and/or insulin secondary deficiency caused by the failure of beta- (β-) pancreatic cells. Diabetes can be classified into four clinical categories, type 1 diabetes (due to autoimmune destruction of the β cells, usually leading to absolute insulin deficiency), type 2 diabetes (due to a progressive insulin secretory defect in the background of insulin resistance), gestational *Diabetes mellitus* (GDM) (diabetes diagnosed during pregnancy that is not clearly overt diabetes), and other specific types of diabetes due to other causes, for example, genetic defects in β cell function or insulin action, drug- or chemical-induced alterations (such as in the treatment of HIV/AIDS or after organ transplantation), and any diseases of the exocrine pancreas characterized by a process that diffusely injures the pancreas can cause diabetes. Diabetes is usually diagnosed based on plasma glucose criteria, either the fasting plasma glucose (FPG) or the 2 h plasma glucose (2 h PG) value after a 75 g oral glucose tolerance test (OGTT). Besides, recently, an International Expert Committee added the A1C (threshold $\geq$ 6.5%) as a third option to diagnose diabetes. In type 1 diabetes, patients often present acute symptoms of diabetes and markedly increased glucose levels and in some cases ketoacidosis. Type 2 diabetes is frequently not diagnosed until complications appear. ADA for the first time recommended that all pregnant women not known to have prior diabetes undergo a 75 g OGTT at 24–28 weeks of gestation based on an International Association of Diabetes and Pregnancy Study Groups (IADPSG) consensus meeting. In U.S. approximately one-fourth of the population may have undiagnosed diabetes.

Acquired processes include pancreatitis, trauma, infection, pancreatectomy, and pancreatic carcinoma (such as cystic fibrosis). However, for the clinician and patient, it is less important to label the particular type of diabetes than it is to understand the pathogenesis of hyperglycemia and to treat it effectively [1].

Research in health has been improving the quality of medical care, influencing health policies and ensuring patient safety. Translational research is an important tool that allows researchers in clinical practices to establish knowledge and implement the results [2]. Translational research covers two areas. One is the process of applying discoveries generated during research in the laboratory and in preclinical studies to the development of trials and studies in humans. The second area of translation concerns research aimed at enhancing the adoption of best practices in the community. The cost-effectiveness of prevention and treatment strategies is also an important part of translational science [3]. According to this definition, translational research is part of a unidirectional continuum in which research findings move from the researcher's bench to the patient's bedside and the community. In the continuum, the first stage of translational research (T1) transfers knowledge from basic research to clinical research, while the second stage (T2) transfers findings from clinical studies or clinical trials to practice settings and communities where the findings improve health [4]. Due to ethical issues involving human studies that can require invasive aspects and the multiplicity of uncontrolled variables that can alter the uterine environment during clinical studies [5], it is necessary to use animal models to better understand diabetic pathophysiology [6]. Thus, this review aimed to gather information about pathophysiological mechanisms and fetal outcomes in streptozotocin-induced diabetic rats.

2. Pancreatic Islets: Structure and Function

The pancreas is a complex organ that consists of two functionally and morphologically distinct cell populations derived from the endoderm. The exocrine pancreas consists of acinar cells that secret digestive enzymes, such as amylases, lipases, proteases, and nucleases, which are emptied into the pancreatic duct through an elaborately branched network of tubules composed of epithelial cells. Acinar cells also produce bicarbonate ions and electrolytes, which, together with exocrine enzymes, are transported through the main duct into the duodenum, where they contribute to food processing [7, 8].

Groups of endocrine cells called pancreatic islets represent the endocrine portion, which composes only approximately 2% of the pancreas. Each islet is composed of at least five types of cells, including insulin-producing β cells (65–80%) [9], glucagon-releasing α cells (15–20%) [10], somatostatin-producing δ cells (3–10%) [11], pancreatic polypeptide-containing PP cells (1%) [12], and ghrelin-containing ε cells [13]. All of these hormones are involved in the regulation of nutrient metabolism and glucose homeostasis [14]. The cytoarchitecture of rodent and human islets presents notable differences [15, 16]. In islets from mice and other rodents, the β cells are predominately located in the central core with α and δ cells localized in the periphery forming a mantle [17–21]. In human and monkey islets, α cells are not localized in the periphery but rather are dispersed throughout the islet [15, 16, 19, 21, 22].

There are several lines of evidence that pancreatic islets cannot be considered aggregates of cells. It was well established more than 20 years ago that the integrated secretory responses of isolated islets are greater than those of dispersed islet cells, suggesting that cell-to-cell interactions are necessary for the normal secretory function of the endocrine pancreas [23–27].

However, there are no reports regarding the effect endocrine hormones in the diabetic environment. Several studies have emphasized the importance of interactions among the different cells [28, 29] and between cells of the same type [29, 30] in metabolic control. In normal islets, the insulin-producing cells are under the influence of other hormone-secreting islet cells. Glucagon is known to enhance both insulin and somatostatin secretion, while somatostatin exerts an inhibitory effect on insulin and glucagon secretion [31, 32]. Communication among these hormones is important to maintain physiological balance, and any disorder in this system would result in excessive blood glucose levels, for example, damaging the organism.

Hyperglycemia during pregnancy impairs the intrauterine environment, affecting normal fetal development and resulting in long-term effects on the function and structure of fetal pancreatic islets [33, 34]. This status increases the offspring's risk of obesity/adiposity, glucose intolerance, and type 2 diabetes later in life [1, 35–37]. Animal studies have shown that the offspring of diabetic rats can be insulin resistant [38, 39] and diabetic [39, 40]. Studies support the concept that developing organs have critical periods of intense structural and functional reorganization. In the case of the pancreas, this circumstance may render it vulnerable to environmental stimuli [41, 42], which may lead to consequences for the next generation [43] and future studies should consider the hormone interactions involved for this glucose control.

The literature shows that the administration of pancreatic hormones analogs (insulin, glucagon, and somatostatin) in in vitro studies is important to investigate the mechanisms of hormonal synthesis and secretion in an isolated manner [44–46]. In addition, insulin is the most studied hormone in the maternal and fetal organism in an attempt to understand the repercussions of hyperglycemia [47–54]. In our laboratory, we hypothesized that glucoregulatory hormones such as glucagon and somatostatin, in addition to insulin, are relevant for embryo-fetal development and diabetes-derived alterations. We performed an experimental study in rats to evaluate the importance of the endocrine pancreatic hormonal triad in maternal, fetal, and neonatal organisms exposed to a hyperglycemic intrauterine environment. According to our results, somatostatin levels were altered in all developmental points studied, showing that pancreatic alteration in maternal and fetal organisms persisted in the neonatal period. These results suggest that somatostatin might be a predictor of adverse effects in adulthood. In fact, our data show the importance of studying hormonal interactions in the endocrine pancreas to understand the pathophysiological mechanisms

related to glycemic control in maternal and fetal organisms [55].

3. Pathophysiological Mechanisms of Diabetic Disease

The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels [1]. Type 1 *Diabetes mellitus* results from the cell-mediated autoimmune destruction of the β-cells of the pancreas. Markers of the immune destruction of the β-cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to GAD (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2b [1]. Autoimmune destruction of β-cells has multiple genetic predispositions and is also related to environmental factors that are still poorly defined. Individuals who present Type 2 *Diabetes mellitus* have insulin resistance and usually develop relative (rather than absolute) insulin deficiency. Although the specific etiologies are not known, autoimmune destruction of β-cells does not occur. Most patients with this form of diabetes are obese, and obesity itself causes some degree of insulin resistance [1]. Elevations in plasma glucose and free fatty acids are thought to increase reactive oxygen species (ROS) levels [56, 57], which in turn activate inflammation signaling pathways such as mitogen-activated protein kinases [58] and nuclear factor-kB [59]. The activation of these inflammation cascades is thought to cause insulin resistance [60].

Gestational *Diabetes mellitus* (GDM) has been defined as any degree of glucose intolerance with onset or first recognition during pregnancy (ADA). Glucose intolerance was first introduced in 1979 to replace "borderline" diabetes and other categories of hyperglycemia that did not appear to carry a risk of microvascular complications [61, 62]. It was only in the most recent reports that the category of nondiabetic fasting hyperglycemia was defined and given the name impaired fasting glycemia (IFG) [63, 64]. This indicates glucose concentrations that are clearly above normal but fall short of the diagnostic value for diabetes [65].

4. Diabetes and Pregnancy: Experimental Models

Experimentally induced diabetes through the administration of β-cytotoxic drugs such as streptozotocin (STZ) is well characterized [66]. Streptozotocin is an antimicrobial agent and has also been used as a chemotherapeutic alkylating agent [67]. "Streptozotocin diabetes" [68] is caused by the specific necrosis of the pancreatic β-cells, and this agent is the first choice for diabetes induction in animals [69, 70]. Depending on the animal strain, dose, route of drug administration, and the life period in which STZ is administered in rats, severe diabetes (blood glucose superior to 200/300 mg/dL) [71–77] or mild diabetes (glycemia between 120 and 200/300 mg/dL) are generated [68, 78–81]. For severe diabetes induction, STZ is administered at 40–50 mg/kg body weight intravenously or intraperitoneally during adulthood. After approximately three days, these animals present blood glucose levels greater than 300 mg/dL [79, 82–86]. In our laboratory, to induce mild diabetes, which is characterized by low glycemic intensity, the rats received a STZ injection (dose of 100 mg/kg body weight) subcutaneously at birth. Approximately three days after STZ administration, these animals developed hyperglycemia (>300 mg/dL) and presented low blood glucose levels (120–200 mg/dL) at adulthood [83, 85–93]. This fact might be explained by the high regenerative capacity of β-cell during the neonatal period [94, 95].

The literature has shown that several organs are able to undergo catch up growth when necessary [96]. In case of severe cell loss or physiological conditions, the pancreatic β-cells of rodents can regenerate in the early life period [97]. Cell regeneration can occur through different mechanisms such as neogenesis, proliferation [98, 99], and transdifferentiation [100]. Scaglia et al. [101] showed that during the neonatal period, the pancreas suffers physiological changes, events that can also be identified in other organs, for example, liver, kidneys, and central nervous system [102–105]. This pancreatic remodeling is due to increased replication and apoptosis rates of β-cells between days 13 and 17. These data show that in physiological conditions the organism has a dynamic β-cell mass, maintaining glucose homeostasis [95, 106].

Because β-cells are able to regenerate in physiological conditions, the next step was to develop an experimental model to induce islet injury to study the mechanisms involved in cell regeneration process. Bonner-Weir et al. [97] published some of the first data about pancreatic islet regeneration, administrating STZ on the second postnatal day. Two days after the induction of diabetes, the animals presented high blood glucose levels (>300 mg/dL) and reduced β-cell numbers compared to the control group. At postnatal day 10, the animals became euglycemic, and partial regeneration of pancreatic β-cells was evidenced. The authors suggested cellular proliferation as the mechanism of cell regeneration. After STZ administration, cells that were not affected by STZ-induced necrosis showed increased mitotic characteristics. Bonner-Weir et al. [107] showed increased mitosis, apoptosis, and hypertrophic cells and suggested that hypertrophy might be related to increased β-cell mass given that cell death is a mechanism of regulation related to the rate of mitosis, which could maintain an appropriate number of islet cells. Therefore, according to these authors, the increased β-cell mass could be due to replication, individual cell hypertrophy, or islet neogenesis by ductal cell differentiation [108]. Regarding the regeneration mechanisms of β-cells, some authors also suggest cell transdifferentiation from non-β-cells to insulin-producing cells. In contrast, Scaglia et al. [101] concluded that, once cells do not present hormonal co-expression, there is no transdifferentiation, suggesting that non-β-cells are not differentiating into β-cells.

In contrast, other authors defend the idea that α-cells are able to differentiate into β-cells by direct conversion of transcription factors [100, 109–112]. Some of the essential transcription factors involved in pancreatic regeneration have been investigated, such as neurogenin 3 (Ngn3), paired domain homeobox gene 4 (Pax4), and homeobox-containing

gene (Arx). Ngn3 is expressed by precursors of the endocrine pancreas, Pax4 has selective expression throughout the pancreatic islet during its development and is then restricted to β-cells [113, 114], and Arx is expressed by pancreatic α-cells [100].

Liang et al. [95] found that β-cells were damaged four days after neonatal STZ induction. At day 8, these authors verified that there was β-cell recuperation, but 20 days after STZ injection the β-cell mass was still reduced, even though blood glucose levels reverted to normal. This study focused on transcription factors, and the authors used double immunofluorescence to stain Ngn3 and insulin or glucagon. By analyzing the coexpression of Ngn3 and glucagon, they observed abundant expression of Ngn3 in the α-cells of STZ-treated rats 8 and 12 days after STZ injection. However, in the control rats, few α-cells expressed Ngn3. The results of the diabetic group indicated that α-cells dedifferentiated into precursor cells and may be candidates for β-cell formation. The coexpression of Pax4 and insulin or glucagon was also studied and indicated a relationship between insulin and Pax4 in both the control and STZ groups. However, the coexpression of Pax4 and glucagon was verified 12 and 20 days after STZ administration. Ngn3 expression is necessary for transdifferentiation from α- to β-cells. In addition, Pax4 is an important transcription factor that specifies the β-cell lineage. The same authors observed Pax4 expression in α-cells of both control and STZ-treated rats, but this coexpression increased in the α-cells at day 20. These results demonstrate that α-cells are sources of β-cell regeneration and can undergo transdifferentiation. Another study using STZ showed that Arx inactivation in α-cells results in pancreatic islet hypertrophy and increased number of cells that are phenotypically β-cells. The authors suggest that when α-cells are subjected to Arx inactivation, they undergo transdifferentiation to β-cells. These results show that strategies aiming at inhibiting the expression of Arx may offer new avenues for diabetes treatment [115].

Therefore, the literature includes several mechanisms to explain β-cell regeneration, and most studies suggest that the proliferation of the remaining β-cells is the primary source of regeneration. Nevertheless, current studies have demonstrated the direct participation of α-cells in β-cell regeneration. Thus, we conclude that the regeneration of pancreatic β-cells may be due to different mechanisms, but further studies are needed to precisely elucidate each mechanism and its contribution to the regeneration as a whole.

5. Diabetes-Induced Teratogenesis

Diabetes has been recognized as a disease that increases the risk of birth defects in offspring by 3 to 5 times [116]. A significant improvement has been observed in the evolution of the diabetic pregnancy after the discovery of insulin, reducing the incidence of ketoacidosis, spontaneous abortions, stillbirths, and congenital malformations [117]. A total of 25% of offspring have been reported presenting these complications, and early detection and subsequent strict metabolic control of pregnant women with diabetes should decrease the frequency and severity of some of these complications in offspring [118]. Studies have shown that spontaneous abortions can result from the malformation of structures required for fetal viability, such as the cardiovascular system or the placenta, but could also be attributable to maternal effects, such as endocrinopathies or vascular complications affecting uterine perfusion [119].

The following are the two principal advances that have improved the offspring survival rate during diabetic pregnancy: (1) a good maternal glycemic control to reduce the morbidity and mortality of both the mother and fetus/neonate [120]; (2) availability of surfactant to reduce perinatal mortality from respiratory distress syndrome (RDS) [121]. Uncontrolled diabetic status throughout the pregnancy has been associated with a spectrum of disorders involving neural tube defects (NTDs), including spina bifida, anencephaly, encephalocele, holoprosencephaly, and cardiovascular [122–124] kidney, and skeletal system defects in addition to growth delay and miscarriage [116]. In fact, any organ can be affected, and 8% to 12% of diabetic pregnant women presented malformed fetuses [125].

Experimental studies have also been performed to understand diabetic teratogenesis. Damasceno et al. [126] and Volpato et al. [75] administered STZ (40 mg/kg) to adult virgin female Wistar rats before mating. During pregnancy, these rats presented hyperglycemic levels higher than 200 mg/dL. At term, the fetuses from diabetic dams presented skeletal (nonossified sternebrae and cleft palate) and visceral malformations (microphthalmia and hydronephrosis). Gäreskog et al. [127], using a similar diabetes model, recovered embryos from diabetic rats at day 10 or 11 of pregnancy. These embryos were cultured within their intact visceral yolk sac for 24 or 48 h and presented decreased Bcl-2 levels and increased Bax levels and increased activation of caspase 3. Thus, exposure to diabetes during organogenesis increased cellular apoptosis and embryonic dysmorphogenesis. However, some skeletal defects that can occur in human diabetic pregnancy, particularly caudal regression syndrome, are rarely observed in animal models, making it difficult to study their molecular etiology [119].

Several studies have tried to identify biochemical disturbances associated with malformations in animal models of diabetic pregnancy. Teratogenic processes in embryonic tissues include alterations of metabolic and signaling systems [118] such as metabolism of inositol [128], the polyol pathway [129], arachidonic acid/prostaglandins [130, 131], and reactive oxygen species (ROS) [132]. In the polyol pathway, the aldose reductase enzyme is responsible for catalyzing excess glucose into sorbitol. Sorbitol accumulation has been demonstrated to negatively affect cell function in glucose-permeable tissues. However, aldose reductase inhibitors (ARIs) can diminish some diabetes-related changes in affected tissues without modifying the hyperglycemia. It has been proposed that polyol pathway overactivity is responsible for diabetic nephropathy, neuropathy, and retinopathy due to the depletion of myoinositol, and this could be applied to diabetic congenital malformations [118].

Another theory centers on linoleic acid. It is the precursor of arachidonic acid, an essential fatty acid required

throughout gestation [133]. The literature shows that arachidonic acid release from plasma membranes by phospholipase A2 is lower in diabetic rodents. The formation of the palate, the neural tube, the heart, and external genitalia involve the folding and fusion of opposing layers and require phosphatidylinositol turnover and arachidonic acid signaling [131]. Several studies have identified PGE2 as a prostaglandin (PG) derived from arachidonic acid involved in the prevention of malformations in experimental diabetic models [134, 135]. Supporting this idea, one study showed that the concentration of PGE2 decreased during neurulation in embryos from a diabetic mouse [136]. In vitro as well as in vivo results demonstrated that a high glucose concentration causes decreased cyclooxygenase (enzyme catalyzing the synthesis of PGE2 from arachidonic acid) gene expression [137], suggesting that diabetes causes decreased prostaglandin biosynthesis and that the inhibition of the arachidonic cascade may be a cause of diabetic embryopathy [138].

Another hypothesis is that increased glucose metabolism enhances the production of ROS, causing oxidative stress [139]. In the embryo, the energy metabolism is characterized by a high rate of glycolysis and lactic acid production (anaerobic glycolysis) with minimal activity of the Krebs cycle-electron transport system [138]. In accordance with the low activity of the mitochondrial oxidative pathway, scavenging enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) seem to be immature during the period of early organogenesis [140]. A study performed on cultured rat embryos in high glucose (25 and 50 mM glucose) showed increased activity of the free radical scavenging enzyme superoxide dismutase (SOD) providing evidence of enhanced ROS production in a hyperglycemic environment [141]. Kinalski et al. [142] verified an increase in malondialdehyde (MDA) levels and reduced glutathione (GSH) and decreased activity of cytoplasmic Cu/Zn superoxide dismutase (Cu/Zn SOD) in the infants of mothers with pregestational and gestational diabetes. These data show increased oxidative stress and lipid peroxidation in these fetuses, which serve as indicators of fetal distress caused by maternal hyperglycemia [143].

Wentzel and Eriksson [144] evaluated embryoneural crest cells recovered from inbred Sprague-Dawley rat exposed to 5.5 or 30 mmol/L glucose for 48 hr on gestational day 10. Cells exposed to 30 mmol glucose/L presented decreased mRNA levels of catalase, Cu/Zn SOD, manganese superoxide dismutase, and extracellular superoxide dismutase. This altered gene expression induced by glucose may be the etiology of malformations in diabetic pregnancy.

6. Diabetes-Induced Oxidative Stress and DNA Damage

In addition to reactive oxygen and nitrogen species, the products of free radicals, which are dependent on fatty acid oxidation, can induce chromosome breaks [145, 146]. Therefore, these products can interact with the embryo chromatin, resulting in congenital malformations [147]. Free radicals can also react with DNA bases, impairing their structure [148, 149] and potentially leading to mutations [148–150]. DNA oxidation is the most common type of damage [151], although the methods used to assess this damage are still controversial. One marker used to study oxidative DNA damage [152, 153] is 8-OHdG or 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodGuo or 8-oxoGua). It is a product of the deoxyguanosine nucleoside oxidation that is directly excreted in urine. Qiu et al. [154] demonstrated that the 8-OHdG urine concentration might be related to increased risk for gestational diabetes mellitus.

To evaluate DNA damage levels, the comet assay presents advantages compared to other methods to detect genotoxic substances. This test is not useful for detecting mutations but can detect genomic lesions, which can result in mutation. In contrast to mutations, the genomic lesions detected by the comet assay can be repaired. The comet assay is fast and sensitive; moreover, it can detect oxidized DNA bases using endonucleases such as endonuclease III (Endo III) and formamidopyrimidine DNA glycosidase (Fpg). Use of Fpg and Endo III allows the identification of both oxidized purine and pyrimidine bases, respectively [155, 156].

Although streptozotocin is an alkylating agent, it is also useful in genotoxicity studies. Some authors have suggested that STZ can irreversibly damage β-cell DNA. To investigate this hypothesis, Mossman et al. [157] performed an in vitro study showing that STZ induces single-strand DNA breaks in rodent cells (RINr 38), and these lesions are repaired 24 hours after STZ exposition. Studying the same cell lineage, Pettepher et al. [158] demonstrated that STZ also induces alkali-labile site breaks in mitochondrial DNA, and even though the formation of this lesion is dose-dependent, it can be repaired as well. After 8 hours of STZ exposition, 55% of the mitochondrial DNA lesions were repaired, rising to 70% in 24 hours. These data confirm that STZ by itself is not responsible for the high levels of DNA damage.

In regard to experimental studies with mild and severe diabetes, Lima et al. [86] evaluated oxidative damage in the lymphocytes of pregnant diabetic rats and whole blood samples of their offspring by the comet assay using repair enzymes (Endo III and Fpg). These authors found that mildly diabetic rats and their offspring presented more sensitive sites to Fpg, reflecting damage related to hyperglycemia. Tats with severe diabetes and their offspring showed oxidative DNA damage detected by Fpg as well as by Endo III, typical general diabetes outcomes. The enzymatic indication of DNA damage suggests that the repercussions of maternal diabetes are associated with oxidative lesions in maternal and fetal DNA. Damasceno et al. [159] demonstrated that severely diabetic rats presented higher DNA damage levels at term pregnancy compared to control rats. In addition, their offspring also showed higher DNA damage levels and increased rate of congenital malformations at term, confirming the interaction between hyperglycemia-induced genotoxicity and teratogenesis. These studies show the relationship among diabetes, oxidative stress, and oxidative DNA damage.

Although many studies have been performed to understand the congenital malformations induced by diabetes, additional research is necessary to identify new markers

involved in the regulation of embryogenesis and occurrence of congenital malformations. It is necessary to comprehend DNA damage trigger factors in diabetes to reduce the impairment of gene expression, avoid fetal congenital malformation, and contribute to the normal development of organs during organogenesis.

7. Conclusion

Pancreatic islet loss and reduction of insulin-producing beta cell mass are relevant aspects to diabetic pathogenesis. It is important to study the regeneration of pancreatic beta cells not only to understand the mechanisms and factors involved in this process but also to provide new preventive concepts as a basis for the treatment of diabetes. Thus, these therapeutic efforts might minimize or prevent diabetes-induced oxidative stress, DNA damage, and teratogenesis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Maternal Obesity, Inflammation, and Developmental Programming

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The prevalence of obesity, especially in women of child-bearing age, is a global health concern. In addition to increasing the immediate risk of gestational complications, there is accumulating evidence that maternal obesity also has long-term consequences for the offspring. The concept of developmental programming describes the process in which an environmental stimulus, including altered nutrition, during critical periods of development can program alterations in organogenesis, tissue development, and metabolism, predisposing offspring to obesity and metabolic and cardiovascular disorders in later life. Although the mechanisms underpinning programming of metabolic disorders remain poorly defined, it has become increasingly clear that low-grade inflammation is associated with obesity and its comorbidities. This review will discuss maternal metainflammation as a mediator of programming in insulin sensitive tissues in offspring. Use of nutritional anti-inflammatories in pregnancy including omega 3 fatty acids, resveratrol, curcumin, and taurine may provide beneficial intervention strategies to ameliorate maternal obesity-induced programming.

1. Introduction

The prevalence of obesity in both developed and developing countries has been steadily increasing over the past 40 years [1–3]. Consequently, obesity and its associated comorbidities are a significant concern, in terms of global public health and public health spending. Depending on the population, the prevalence of obesity (body mass index $\geq 30\,kg/m^2$) in women of reproductive age can be as high as 34% [3]. Obesity during pregnancy is of major concern due to the well-characterized risk factors to both the mother and her offspring. These can include, but are not limited to, maternal and fetal death, preeclampsia, gestational diabetes, and congenital abnormalities [4]. In addition, epidemiological evidence and data derived from animal models have demonstrated that maternal obesity has long-term consequences for offspring, predisposing or "programming" them to the development of metabolic disease in adulthood [5]. It has become increasingly clear that metabolic disease is associated with a state of chronic low-grade inflammation [6]. Inflammation has received extensive attention recently because of its association with several diseases, including cancer, diabetes, and obesity—it is a tightly regulated process—deviations from this process present a significant health risk because unresolved inflammation can compromise tissue function. In human pregnancies, maternal obesity is associated with metabolic inflammation, characterized by elevated adipose tissue and systemic proinflammatory cytokine levels and adipose tissue macrophage accumulation [7, 8]. Additionally, these changes extend to the placenta, suggesting that maternal obesity exposes the fetus to an inflammatory environment during development [9]. Thus, in the context of developmental programming, early life exposure to metabolic inflammation may represent a key mechanism by which developmentally programmed phenotypes may manifest later in life. For example, in animal models, maternal obesity has been shown to induce fetal inflammation which can result in promotion of adipogenesis and increased adiposity in offspring [10]. The critical windows of innate immune vulnerability during prenatal and neonatal maturation are when developmental programming and the trajectory for childhood and adult inflammatory responses are largely established. Clearly, there

is a need for targeted intervention strategies to ameliorate and reduce the adverse effects of maternal obesity on offspring health outcomes during later life. This review will discuss maternal obesity related inflammation as a mechanism of developmental programming of metabolic disorders in offspring and the potential of intervention strategies.

2. The Developmental Origins of Health and Disease

Barker first suggested that the fetal environment may have an effect on the development of disease in adulthood, known as the fetal origins of adult disease (FOAD) hypothesis [11]. Epidemiological evidence from UK birth records indicated a geographical correlation between high rates of infant mortality and adult ischaemic heart disease [12]. They hypothesized that maternal undernutrition resulted in fetal programming which caused permanent alterations in the structure, function, homeostatic pathways, and/or metabolism of the developing offspring, predisposing them to disease later in life. Since these initial observations, the concept has evolved into the developmental origins of health and disease (DOHaD) hypothesis, which describes the process by which an environmental stimulus, including altered nutrition, during a critical period of development can program alterations in organogenesis, tissue development, and metabolism, predisposing offspring to metabolic and cardiovascular dysfunction during adulthood [13–15]. These effects can be amplified in the setting of a poor postnatal diet [16].

In today's society, maternal obesity is a more prevalent and emerging cause for concern. Considerable epidemiological evidence demonstrates that maternal obesity is a predictor for development of obesity, type 2 diabetes, and cardiovascular disease in offspring [17–19]. Mechanistic studies in human cohorts are challenging due to the number of potential postnatal confounders and the time course required for prospective studies and thus remain largely observational. Therefore, animal studies have become the primary tool for investigating the myriad of potential mechanisms underlying the developmental programming paradigm. Maternal obesity-induced developmental programming has been validated in mouse, rat, sheep, and nonhuman primate models and has been shown to affect numerous metabolic pathways culminating in a metabolic syndrome like phenotype [20–23].

There is an increasing body of evidence demonstrating the capability to ameliorate or reverse programming by targeted interventions during specific periods of developmental plasticity [24–26]. A particular focus has been on the adipokine leptin (a proinflammatory signal in adipose tissue) as a mediator of programmed changes in the regulation of appetite and metabolism. Obese individuals exhibit higher circulating levels of leptin, contributing to a state of leptin resistance, which further perpetuates obesity, inflammation, and metabolic disease [27]. Leptin levels are known to be elevated in pregnancies complicated with enhanced inflammatory processes in the placentae [9, 28]. Of note, maintenance of a critical leptin level during early development facilitates the normal maturation of tissues and signaling pathways involved in metabolic homeostasis. In rats, maternal undernutrition results in neonatal hypoleptinemia—leptin administration to these neonates reverses maternal undernutrition-induced metabolic programming in adult female offspring [29]. We have also shown that preweaning growth hormone treatment in a rat model of undernutrition reverses programmed hypertension, obesity, and inflammatory profiles in adult offspring [26, 30]. In rats, supplementation with docosahexaenoic acid (DHA) in the setting of maternal undernutrition has also been shown to protect offspring against later metabolic dysfunction [31], but data in the setting of maternal obesity are less clear. In rat models of protein restriction, dietary cofactors, including folate and glycine, have also been shown to reverse postnatal metabolic and cardiovascular abnormalities in offspring [32–34].

Taken together, these studies suggest that programming effects can be prevented by early intervention strategies. To date, the majority of developmental programming studies are primarily descriptive and the underlying mechanisms, particularly as regards the inflammasome, of how maternal obesity impacts upon early life development and subsequent adult disease phenotypes are not well understood. Elucidating the mechanisms of maternal obesity-induced developmental programming is of utmost importance and may allow for application of therapeutic and/or nutritional interventions to minimize adverse programming effects in offspring.

3. Adipose Tissue Dysfunction in Obesity

Historically, white adipose tissue (WAT) was viewed as an inert energy storage depot. However, it is now appreciated as a major endocrine organ which contributes to metabolic homeostasis. Adipose tissue is composed of not only multiple cell types, mainly adipocytes (fat cells), but also the stromal vascular fraction (SVF), which includes preadipocytes, fibroblasts, endothelial cells, and immune cells [35]. Adipose tissue secretes a broad range of bioactive factors, collectively referred to as adipokines [36]. Adipokines have a range of essential physiological roles, including adipocyte differentiation, glucose and lipid metabolism, satiety, immune regulation, cardiovascular function, and neuroendocrine function [37]. Aberrant regulation of adipokine secretion has been shown to mediate cross talk with other organs and contribute to the development of obesity-induced comorbidities such as insulin resistance and metabolic syndrome [38–40]. The complex adipokine profile is still not fully understood, with novel adipokines still being identified [41, 42]. Additionally, it is important to note that the study of adipose tissue dysfunction is confounded by the wide range of etiologies which include genetics, environment, and now early life stressors such as maternal obesity.

In healthy individuals, the adipose tissue is composed of mainly preadipocytes and adipocytes, with few inflammatory leukocytes. With obesity, the composition, phenotype, and function of adipose tissue are disrupted [43–45]. Persistent

excess energy intake causes adipocytes to undergo hypertrophy (increased adipocyte volume) in attempt to meet the increased energy storage needs [46]. Adipocyte hypertrophy can contribute to further complications including hypoxia, adipocyte necrosis, chemokine secretion, and compromised regulation of fatty acid flux [47]. Hypertrophied cells alter the balance of adipose tissue-derived cytokines and adipokines to a proinflammatory state, acting as a critical factor linking obesity to the pathogenesis of metabolic disease in both mother and offspring [48, 49]. Inflammatory mediators, including C-reactive protein (CRP), interleukin-6 (IL-6), IL-1β, and tumour necrosis factor α (TNFα), are systemically elevated in obesity in animal and human models [50–52]. Additionally, as the adipose tissue expands, the blood supply becomes inadequate and hypoxia occurs [53]. This contributes to further cellular dysfunction in adipocytes, including downregulation of adiponectin mRNA expression and induction of endoplasmic reticulum stress, which can further exacerbate the inflammatory state [35, 53].

A hallmark of adipose tissue inflammation is the infiltration of immune cells including monocytes/macrophages, neutrophils, B lymphocytes, and T lymphocytes [54–56]. Macrophages are phagocytic cells, which act to engulf and digest pathogens and cellular debris. Adipose tissue macrophage infiltration has recently emerged as a major contributor of inflammatory mediators contributing to dysfunction in obesity after seminal publications by Xu et al. and Weisburg et al. in 2003. Xu et al. showed that macrophage-specific genes including monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), CD11b, F4/80, and CD68 were upregulated in adipose tissue of diet-induced obese mice. Interestingly, this preceded development of hyperinsulinemia and treatment with the insulin sensitive drug rosiglitazone caused downregulation of these genes. Weisburg et al. analyzed the profile of 1304 body mass related transcripts, finding that 30% of the 100 most significantly correlated genes encoded genes which were characteristic of macrophages. Immunohistochemical analysis of multiple adipose depots showed a significant correlation between the percentage of F4/80 expression and adipocyte size and body mass. These results have since been corroborated in a number of studies [54, 57]. Surgical or diet-induced weight loss in obese individuals results in decreased MCP-1 gene expression and reductions in macrophage infiltration and inflammation [58, 59]. Additionally, macrophage activation appears to shift towards M2 (alternatively activated) over M1 (classically activated) status postgastric bypass surgery in morbidly obese individuals, contributing to a less inflammatory phenotype [60].

4. Metabolic Inflammation as a Programming Mechanism

Interest in the developmental origins of obesity and its associated metabolic sequelae has grown in recent years. There is evidence to support a number of potential mechanisms, including programming of offspring appetite, gene expression, and functional changes to adipose tissue [61]. These conditions are linked by the activation of a number of inflammatory pathways, including the NLR family, pyrin domain containing 3 (NLRP3) inflammasome, peroxisome proliferator-activated receptors (PPAR) signaling, and nuclear factor-κB (NF-κB) pathway [62–64].

Classical inflammation is the body's process of responding to injury or infection to restore homeostasis [65]. However, in obesity, the inflammatory response, which has been coined "metainflammation," is chronic and is on a lower scale than the typical classic inflammatory response. The persistent state of chronic low-grade inflammation induced by obesity is characterized by abnormal cytokine production, an altered adipokine profile, and activation of inflammatory pathways [6]. The role of chronic low-grade inflammation in obese mothers has become an emerging focus in the developmental programming field. Our group have demonstrated that unbalanced maternal nutrition results in metainflammation in the mother and programs inflammation in offspring tissues [30, 66, 67]. However, current understanding of how these pathways are activated in the context of developmental programming remains poorly defined. Of note, most studies have characterised the programmed offspring as adults when the phenotype is already manifested. There is now strong evidence that early changes in inflammatory markers can be predictors of later metabolic and cardiovascular disease; thus evaluation of offspring inflammatory profiles at early stages of development may provide a useful biomarker for later life metabolic adversity [68].

In rodents, maternal immune activation during pregnancy with an immunostimulant such as lipopolysaccharide (LPS) has been shown to modify the immune response of offspring [69, 70]. These offspring exhibit a more proinflammatory macrophage (M1) phenotype and enhanced IL-1β production upon immune challenge in adulthood. Similarly, a state of maternal obesity is linked to an enhanced inflammatory response in offspring. Challier et al. observed macrophage accumulation and increased expression of proinflammatory cytokine expression in placenta from obese women compared to those from lean women [9]. Infiltrating macrophages have the capability to secrete inflammatory cytokines into the maternal or fetal systemic circulation. It is speculated that this is a contributing mechanism for the programmed alterations in offspring metabolism associated with increased adiposity and insulin resistance. The placenta transports free fatty acids from the maternal circulation and transports them for uptake by the fetal liver, where they are esterified and released as triglycerides into the circulation [71]. This has implications for fetal growth in humans, where associations between increased maternal triglycerides and macrosomia (large for gestational age) in offspring have been reported [72]. Zhu et al. observed elevated free fatty acids, cholesterol, and triglycerides in fetal circulation from obese ewes which were accompanied by upregulation of toll-like receptor 4 (TLR4), NF-κB, and JNK signalling in cotyledonary tissue [73]. These findings suggest greater fatty acid uptake by the placenta, which can cause activation of inflammatory pathways in the placenta. Therefore, the

state of chronic low-grade inflammation in pregravid obesity persists in pregnancy and contributes to an inflammatory environment for the developing fetus.

5. Programmed Effects of Maternal Obesity on Metabolic Function in Offspring

In animal models of diet-induced obesity, high fat feeding during pregnancy programs features of the metabolic syndrome, independent of environmental factors and postnatal diet [74, 75]. In humans, a high BMI before pregnancy and during early pregnancy is predictive for having a high birth weight baby, with these babies being at higher risk of developing the features of metabolic syndrome [76, 77]. In contrast, maternal obesity is also found to be associated with an increased incidence of intrauterine growth restriction (IUGR) in humans, with supporting evidence from animal work, underscoring the complexity of the maternal obesity paradigm [78, 79]. Our group and the work of others have shown that rodent models provide strong evidence for tissue specific impairments in offspring from obese mothers [80–84]. Impairments to metabolically critical or insulin sensitive tissues, especially adipose tissue, pancreas, liver, and skeletal muscle, may have profound effects on the development of insulin resistance and type 2 diabetes in offspring.

5.1. Adipose Tissue. Adipogenesis is the process of the development of stem cell precursors into adipocytes and largely occurs during the late gestation and early postnatal life in humans [85]. This process is sensitive to *in utero* conditions, such as a deficient or excess nutrient supply. Turnover of adipose cells in adulthood is low, with adipocyte number leveling off in adulthood [86]. This underscores the importance of the *in utero* environment and early postnatal life in a predisposition to adult onset of obesity. Perturbation of adipogenesis, and therefore the development of the adipose tissue as a whole, can alter its functional metabolic properties. Maternal obesity can promote excess accumulation of body fat in offspring and predispose them to obesity during later life.

In a mouse model of maternal diet-induced obesity, 3-month-old offspring from obese dams exhibited adipocyte hypertrophy, reduced mRNA expression of β2- and β3-adrenoreceptors, and increased mRNA expression of PPAR-γ2, a key mediator of adipogenesis [21]. In a similar model in rats, offspring displayed increased adiposity in later life despite a normal birth weight, as well as a high percentage of large adipocytes in concomitance with enhanced PPAR-γ expression [20]. In sheep models, maternal overnutrition during the late gestational period programmed increased mRNA expression of PPAR-γ, lipoprotein lipase (LPL), adiponectin, and leptin in fetal perirenal fat [87]. These findings suggest maternal overnutrition and subsequent obesity may increase the lipogenic capacity of adipose tissue, promoting a shift from a thermogenic to lipid storage function, which could be a contributing cause of increased adiposity in offspring. While an increased fat mass in offspring may be acting as a compensatory mechanism to promote lipid storage rather than ectopic fat deposition, excessive adiposity causes aberrant inflammatory cytokine and adipokine regulation of the tissue and subsequently a metabolic syndrome-like phenotype. A recent study in mice by Murabayashi et al. demonstrated that offspring of mothers exposed to a high fat diet displayed increases in expression of TNFα, CD68, and MCP-1 and decreased GLUT4 mRNA expression, suggesting that maternal obesity may affect fetal insulin sensitivity by altering inflammatory processes [88].

5.2. Liver. In humans, the liver is the most metabolically complex organ, playing pivotal roles in whole body metabolism including regulation of glucose homeostasis, lipogenesis, detoxification, protein metabolism, cholesterol production, and bile production [89]. WAT is critical for storage of excess lipids, but in humans WAT development does not occur until the third trimester of pregnancy [90]. Therefore, it is postulated that maternal obesity results in excess exposure of the fetal liver to triglycerides, lipids, adipokines, and other factors, causing alterations in gene expression which upregulate lipogenesis and downregulate lipolysis, contributing to hepatic lipid accumulation and inflammation. In a number of animal models, maternal overnutrition is found to elevate triglyceride levels, increase inflammatory markers, and cause fatty livers in offspring [21, 91, 92]. Although the etiology of liver disease can vary, nonalcoholic fatty liver disease (NAFLD) linked to obesity and metabolic syndrome is currently one of the most common causes of adult chronic liver disease [93]. NAFLD refers to a progressive range of stages of pathologies caused by fat buildup within hepatocytes—simple fatty liver, nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis [89]. In humans, NASH is associated with increased gene expression of inflammatory factors both locally in the liver and systemically [94, 95].

McCurdy et al. demonstrated the programming effects in response to a maternal high fat diet in nonhuman primates [23]. Interestingly, not all mothers receiving the high fat diet developed obesity and insulin resistance. However, when examined during the early third trimester of gestation, all offspring of high fat fed mothers demonstrated signs of NAFLD such as hepatic inflammation, triglyceride accumulation, and premature gluconeogenic gene activation. Elevated triglyceride levels were also observed in P30 and P180 offspring, and in addition, offspring had a 2-fold increase in body fat percentage. Collectively, these observations suggest that consumption of a chronic high fat diet can independently increase risk of offspring developing NAFLD. Similar results have been replicated in mice fed high fat diets during gestation and lactation. Increased fat depot weight, increased serum insulin, triglycerides, proinflammatory cytokines, and hepatic IκB kinase phosphorylation were observed [96, 97]. In offspring of mice fed a high fat diet during only gestation (G), only lactation (L), or both (GL), hepatic steatosis was observed [98]. Expression of sterol regulatory element-binding protein-lc (SREBP-1c) expression was higher in G and GL offspring, indicating a stimulation of lipogenic gene transcription and fatty acid synthesis. Expression of GLUT-2

was reduced in G offspring, indicating impaired carbohydrate metabolism.

5.3. Skeletal Muscle. Comprising about 40–50% of body mass, skeletal muscle is the chief peripheral insulin responsive tissue, responsible for glucose and fatty acid uptake in response to insulin. Similar to adipose tissue, skeletal muscle displays enhanced inflammation in response to high fat feeding, including increases in proinflammatory macrophages and inflammatory gene expression [99, 100]. Chronic inflammation also occurs in insulin resistant skeletal muscle, displayed by increased macrophage infiltration and increased inflammatory cytokines [101, 102].

Alterations to the development of skeletal muscle can have physiological consequences for the offspring. Proper skeletal muscle development in the fetal period is of critical importance as there is no net increase in muscle fibre number after birth [103]. Skeletal muscle is also sensitive to an adverse *in utero* environment during development as it has a lower priority in nutrient partitioning compared to other organs including the brain, heart, and liver [104]. Additionally, changes to adipogenesis in the fetal skeletal muscle can induce increased number and size of intramuscular adipocytes, which can act in a paracrine fashion to contribute to insulin resistance later in life [64, 105].

In dams fed a cafeteria diet (palatable processed food with high fat and high sugar) during gestation lactation, offspring displayed increased adiposity at weaning, reduced muscle cross-sectional area, fewer muscle fibres, muscle atrophy, and fibre hypoplasia [106]. Functional impairments to muscle included intramuscular fat deposition and preferential fat accretion in muscle fibres. This was accompanied by an increase in muscle PPAR-γ expression, which was suggested as a compensatory response to maintain insulin sensitivity.

Work by Du et al. showed that maternal obesity resulted in low-grade inflammation which altered the commitment of mesenchymal stem cells in fetal muscle through mechanisms including inhibition of AMP-activated protein kinase (which promotes adipogenesis) and inflammation-induced epigenetic modifications via polycomb group proteins [107]. In the offspring of high fat fed ewes Akt phosphorylation (the main downstream insulin signalling pathway) and insulin receptor mRNA expression were reduced [64]. Additionally, inflammation was observed in skeletal muscle with upregulation of TLR2 and TLR4 expression, NF-κB pathway (IKK phosphorylation), JNK pathway, and increased TNFα expression.

6. Anti-Inflammatory Strategies to Reverse Programming

Despite the evidence demonstrating maternal obesity and effects of inflammation in offspring, knowledge on the effectiveness of anti-inflammatory agents during pregnancy is minimal. Although health care professionals highly recommend weight loss to reduce the risk factors associated with obesity during pregnancy, women are likely to maintain prepregnancy lifestyle habits throughout pregnancy. Therefore, this avenue is of utmost importance as rates of obesity continue to increase and the long-term effects negative to offspring become more apparent.

6.1. Omega 3 Fatty Acids: Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA). Polyunsaturated fatty acids (PUFAs) are a group of lipids which can modulate the immune system, alter the regulation of pro- and anti-inflammatory cells, and affect transcriptional regulation [108]. The two main families of PUFAs are omega 6 (n-6; linoleic acid (LA)) and omega 3 (n-3; alpha linolenic acid (ALA), eicosapentanoic acid (EPA), and docosahexanoic acid (DHA)) fatty acids [109]. EPA and DHA are highly associated with brain function [110]. In general, eicosanoids derived from n-6 PUFAs are more proinflammatory and immunoactive; eicosanoids derived from n-3 PUFAs are therefore considered more anti-inflammatory [108]. PPARs, a group of nuclear receptor proteins that act as transcription factors and are responsible for regulating the expression of genes involved in adipogenesis, inflammation, and lipid metabolism, can be activated by a diverse range of ligands, including omega 3 and 6 fatty acids [111]. The Western diet, which has also become increasingly predominant in developing countries, contains a significantly higher proportion of n-6 PUFA compared to n-3 PUFA [112]. Associations between low n-3 PUFA intake and increases in the incidence of obesity, cardiovascular disease, inflammatory diseases, and cancer have been an area of active research [113]. EPA and DHA have an antiobesogenic effect and can both reduce existing adiposity and prevent high fat induced obesity [114, 115]. The n-3 PUFAs have been documented to exert anti-inflammatory effects in the context of obesity by modulating adipose tissue, skeletal muscle, and hepatic function [116]. *In vitro*, EPA stimulates glucose and fatty acid uptake in skeletal muscle cells by increasing expression of the transporters GLUT1 and CD36/FAT (fatty acid translocase) and increasing glucose oxidation [117]. In the adipose tissue of obese rats, n-3 PUFA modulates the secretion profile of adipokines and cytokines, decreasing secretion of proinflammatory cytokines including TNFα and IL-6 and reducing MCP-1 levels and adipose tissue macrophage infiltration, contributing to anti-inflammatory and insulin sensitizing effects [118]. Upon high fat feeding in rats, n-3 PUFAs increase fatty acid oxidation and inhibit lipogenesis in the liver, causing fatty acids to be preferentially oxidized rather than being stored [119]. The PPAR signalling pathway is implicated as a mechanism for the insulin sensitizing effects of EPA [120]. Neschen et al. conducted a study in which wildtype mice and PPAR-α knockout mice were fed an isocaloric high fat diet with or without additional fish oil [121]. Within wildtype mice, the fish oil supplemented group had improved hepatic insulin sensitivity. These effects were not seen within PPAR-α knockout mice, suggesting the insulin sensitizing effects are attributed to PPAR signalling. Despite the strong support of improvements by EPA and DHA to obesity and related insulin resistance in animal models, results in human clinical trials have been less consistent, as human trials are complicated by composition of n-3

PUFAs used, dosage, duration of administration, dietary and lifestyle habits, and other confounders [122]. Furthermore, the amnion, which surrounds the developing embryo, is sensitive to inflammatory modulation by EPA and DHA, likely partially mediated by PPAR-γ [123]. Explants treated with EPA, DHA, or a mixture had reduced IL-8 and IL-6 concentrations compared to untreated controls. When treated with a PPAR-γ agonist, IL-8 secretion was significantly decreased, yet this effect was partially reversed when treated with a PPAR-γ antagonist. Short-term supplementation with pioglitazone, an insulin sensitizing agent that stimulates PPAR-γ, to offspring from obese mothers attenuated the programmed obesity and insulin resistance associated with maternal obesity [124]. A study by Heerwagen et al., using Fat-1 transgenic mice (capable of converting endogenous n-6 PUFA to n-3 PUFA), demonstrated the potential to reduce inflammation associated with diet-induced obesity and improve metabolic outcomes in offspring [125]. Fat-1 mice were protected from adverse effects of a high fat diet, including adipose tissue macrophage accumulation and systemic increases in TNFα, IL-1β, IL-6, and MCP-1. Although there were no observed changes in inflammatory markers in the placenta, fetuses from high fat diet mothers showed minor growth restriction compared to mothers on a control diet, which has also been previously reported [79]. Additionally, although high fat fed mothers did not display hyperlipidemia when measured in late pregnancy, their offspring had increased lipid deposition in the fetal liver, which was reduced in offspring from Fat-1 high fat diet mothers. This underscores that birth weight may not be an accurate measure of fetal health, but rather other measures (e.g., hepatic lipid accumulation) may be more accurate. Adult wildtype male offspring from Fat-1 high fat diet mothers displayed less adiposity, hepatic lipid accumulation, adipose tissue macrophages, and insulin resistance, compared to offspring from high fat mothers. Collectively, these findings suggest that targeting inflammatory processes involved in maternal overnutrition and obesity may be beneficial in reversing or mitigating harmful programming effects on offspring in later life.

6.2. Resveratrol. Resveratrol is a stilbenoid (natural phenol) and phytoalexin naturally produced by some plants, such as Japanese knotweed and the skin of red grapes [126]. Resveratrol gained significant interest when it was proposed to be responsible for the beneficial cardiovascular effects of red wine, described as the French paradox [127]. Subsequent studies have shown resveratrol to have a multitude of health benefits, including cancer chemopreventive, antioxidant, antiplatelet, and estrogen modulatory and caloric restriction mimetic activities [128–131]. Resveratrol increases the expression of sirtuin 1 (SIRT1), a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase [132]. SIRT1 has been shown to modulate genes which regulate a number of biological processes including cell proliferation, apoptosis, gluconeogenesis, lipolysis, adipogenesis, and inflammation [133–136]. Resveratrol treatment results in activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) by SIRT1, which prevents development of diet-induced obesity and insulin resistance [137]. Resveratrol has been shown to attenuate TNFα induced MCP-1 gene expression and secretion in a dose-dependent manner in 3T3-L1 adipocytes [138]. These effects were not observed when adipocytes were treated with a NF-κB inhibitor prior to resveratrol exposure, suggesting this effect was mediated by NF-κB.

Resveratrol's antidiabetic activity, including improving insulin sensitivity and decreasing glucose, dyslipidemia, and adiposity, has been well documented in diet-induced and genetic diabetic animal models [139, 140]. Resveratrol's antidiabetic activity is at least partially mediated by AMP-activated protein kinase (AMPK), which is involved in regulating mitochondrial biogenesis, inducing fatty acid oxidation in the liver and muscle, increasing muscle glucose uptake, and inhibiting lipogenic activity, collectively resulting in increased insulin sensitivity [141]. In mice deficient in either the a1 or a2 catalytic subunit of AMPK, resveratrol does not significantly affect insulin sensitivity or glucose tolerance, implicating AMPK as a mechanism for resveratrol's effects [142]. Long-term intracerebrovascular infusion in high fat fed obese/diabetic mice has been shown to normalize hyperglycemia and improve hypoinsulinemia associated with NF-κB activation [143]. These effects were independent of changes to body weight and food intake, suggesting a potential role of the central nervous system in resveratrol's antidiabetic activity.

The hypoglycemic effects of resveratrol are critical in avoiding diabetic neuropathies and damaging effects to organs. A consequence of diabetes during pregnancy is diabetic embryopathy, which is associated with oxidative stress and can disrupt normal organogenesis [144]. Embryos from diabetic dams had increased apoptosis and oxidative stress markers, but resveratrol administration to diabetic dams during pregnancy protected against these effects and also improved measures of embryonic development including weight, crown rump length, and somite number [145].

In rats, doses of up to 750 mg/kg/d during gestation did not result in fetal abnormalities or have adverse effects on placenta weight or litter size [146]. A recent study conducted by Roberts et al. assessed the role of resveratrol supplementation during pregnancy in nonhuman primates. Mothers were fed a Western-style diet (36% fat) with or without resveratrol supplementation. Resveratrol improved placental inflammatory markers (IL-1β and macrophage migration inhibitory factor), maternal and fetal hepatic triglyceride accumulation, uterine blood flow, and insulin sensitivity [147]. Resveratrol was detected in maternal plasma, demonstrating an ability to cross the placental barrier and exert effects on the fetus. Although resveratrol supplementation did not alter fetal body mass, there was a 42% increase in pancreas mass in the fetus, which was confirmed by immunohistochemistry. Furthermore, resveratrol was shown to increase uterine artery blood flow thereby increasing fetal weight in a murine model of fetal growth restriction [148]. There is also evidence that resveratrol improves the metabolic profile of offspring born growth restricted by reversing Akt mediated insulin resistance in liver and skeletal muscle [149]. Taken together, these findings suggest that resveratrol may

improve the maternal and offspring metabolic profile in maternal overnutrition, obesity, and diabetic pregnancies. However, as the consequences of resveratrol treatment have significant effects on parameters such as pancreatic mass in offspring with unknown long-term effects, further studies in humans to determine adequate and safe therapeutic dosage and routes and frequency of administration are required.

6.3. Curcumin. Curcumin is a polyphenol responsible for the yellow pigment present in turmeric. Its use in traditional medicine is well known, but research now supports anti-inflammatory, antidiabetic, antioxidant, chemopreventive, and cardiovascular protective properties [150, 151]. Curcumin is pleiotropic, with the ability to interact with various targets and exert its effects through several mechanisms of action. Curcumin has been shown to reduce the inflammatory response through NF-κB, suppressing its activation by inhibiting IκKα kinase (IKK) activation [152, 153]. Curcumin decreases inflammation by acting as an agonist of PPAR-γ. In a rodent model of sepsis, intravenous administration of curcumin resulted in downregulation of TNFα and decreased markers of tissue damage. Administration of a PPAR-γ antagonist reversed these effects, confirming the decreased inflammation to be mediated via PPAR-γ [154]. Studies have also shown that curcumin may have beneficial anti-inflammatory effects for treatment of postoperative inflammation, acute respiratory distress syndrome, and inflammatory bowel disease [155–157]. In mouse models of obesity-induced insulin resistance, oral administration of curcumin has beneficial effects on the inflammatory response and decreased insulin sensitivity associated with high fat feeding [158, 159]. Increased adiponectin, decreased TNFα and MCP-1, reduced macrophage infiltration, attenuation of NF-κB activation, and inhibition of lipogenic gene expression were also observed.

Although studies of the use of curcumin in pregnancy are lacking, its safety in humans has been demonstrated, with doses of up to 12 g/day being well tolerated and having low toxicity [160–162]. In a two-generation reproductive toxicity study in Wistar rats, there was no observed adverse effect level on reproductive performance in two successive generations, even in high doses of 10000 ppm (equivalent to 847.4 mg/kg body weight in F0 males) [163]. However, *in vitro* exposure of curcumin to mouse blastocysts during the early postimplantation stages had adverse effects in a dose-dependent manner [164]. Administration of pegylated curcumin (increased solubility) in mice had negative effects to reproductive functions, attributed to estrogen-mimicking or androgen-antagonizing properties [165]. These discrepancies are likely attributed variations in the route of administration. Curcumin is lipophilic and oral administration involves digestion, absorption, and metabolism in the liver, therefore reducing bioavailability at the target organ. Direct administration of curcumin *in vitro* does not accurately reflect physiologic conditions.

However the anti-inflammatory effects of curcumin have been shown to reverse ethanol-induced cognitive impairments in rat offspring by dampening NF-κB signalling and proinflammatory cytokine expression [166]. There is further evidence to suggest that the anti-inflammatory properties of curcumin may have cardioprotective effects in cardiac progenitor cells [167] and augment lung maturation in fetal rats via blockade of TGF-β [168]. Therefore, curcumin appears to be an effective anti-inflammatory strategy in the context of obesity; despite its safety, optimal therapeutic dose and benefits in the context of obesity during pregnancy have yet to be validated *in vivo* in animals and humans.

6.4. Taurine. Taurine is a nonessential sulfated amino acid with a range of physiological benefits in heart function, hypertension, neuromodulation of the central nervous system, and retina function [169, 170]. Taurine is found in high amounts in mammalian plasma and cells, with a particularly high concentration in human neutrophils, where it can react with myeloperoxidase and form taurine chloramine (TauCl), which has reported anti-inflammatory effects [171]. In inflammatory conditions, taurine has been shown to decrease inflammation by downregulating NF-κB [172, 173]. TauCl has been shown to oxidize IκB-α, preventing the activation of NF-κB [174].

In a human double-blind placebo controlled study, obese individuals had 41% lower plasma taurine levels compared to matched controls at baseline [175]. Eight weeks of taurine supplementation improved inflammation indices, increasing adiponectin and decreasing CRP in obese individuals. In 14 weeks of high fat feeding in mice, treatment with taurine prevented weight gain and hyperglycemia and resulted in decreased TNFα and IL-10 [176]. Additionally, taurine reduced macrophage infiltration and promoted shift in macrophages to an M2-like phenotype in the adipose tissue.

In models of gestational protein restriction in rodents, supplementation of taurine had protective effects on the programmed impairments to the pancreas, liver, and skeletal muscle associated with protein restriction [177–179]. Additionally, taurine was shown to normalize the changes in gene expression associated with protein restriction [177–179]. However, in the context of obesity, less is known of taurine's effect on developmental programming. A study by Li et al. showed conflicting results of taurine supplementation as a potential strategy to reverse maternal obesity-induced developmental programming effects on offspring [67]. Dams were fed an obesogenic diet (high fat: high fructose diet), which led to increased weight, hyperglycemia, insulin resistance, hepatic steatosis, and systemic inflammation. Taurine supplementation attenuated systemic inflammation, yet exacerbated impairments to lipid metabolism and inflammatory markers in the liver. In contrast, the neonates of taurine supplemented obesogenic diet dams demonstrated normalization of the detrimental hepatic proinflammatory effects of maternal obesogenic diet. In control pregnancies, taurine increased neonatal mortality and resulted in significantly lower birth weights in female pup birth weight. Although taurine supplementation did have beneficial effects in reversing programming in some conditions, further investigation is required to elucidate mechanisms of how taurine functions in the context of maternal obesity.

7. Other Avenues for Intervention

There is also accumulating support for the role of epigenetic regulation of gene expression as a mediator of the programming of adult-onset metabolic disease. Epigenetic regulation describes stable and heritable DNA alterations that do not involve DNA mutation including DNA methylation, post-translational histone modifications, and chromatin remodeling [180]. Understanding how these epigenetic changes alter the postnatal phenotype could allow identification of biomarkers to enable early detection of children at risk of developing adult disease from developmental programming.

A cross-sectional study in healthy pregnant women found a positive correlation between maternal BMI and the degree of PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) methylation in the umbilical cord of offspring, highlighting a potential role of DNA methylation as a mediator for the programming effects of maternal obesity [181]. Maternal obesity has been shown to induce epigenetic modifications in offspring. For example, offspring from obese mice have enhanced expression of Zfp423, accompanied by reduced methylation in the Zfp423 promoter [182]. Zfp423 is a transcription factor that plays roles in cell commitment to the adipogenic lineage; therefore these changes are likely to contribute to enhanced adipogenic differentiation during fetal development and predisposition to obesity. In a multigenerational mouse model, Ding et al. found that high fat feeding caused a "feed-forward cycle" exacerbating adipose tissue inflammatory processes via DNA hypomethylation, resulting in epigenetic changes to expression of Tlr1 and Tlr2 [183]. Maternal supplementation with methyl donors has been shown to protect offspring against the adverse effects of a maternal obesogenic diet, but whether these changes are mediated in part by alterations in inflammatory profiles is not known [184, 185]. DNA methyltransferase (DNMT3b) plays an important role in regulation of macrophage polarization through epigenetic processes. In obesity, elevations in saturated fatty acids increase DNMT3b expression, leading to DNA methylation at the PPAR-γ1 promoter; this may contribute to deregulated adipose tissue macrophage polarisation, inflammation, and insulin resistance [186]. Collectively, these studies demonstrate the potential of epigenetic regulation as another target for intervention to prevent or treat maternal obesity programming effects.

8. Discussion and Conclusion

In recent years, understanding of the developmental programming effects of maternal obesity on offspring metabolic health has expanded. However, deciphering the complex interactions and mechanistic pathways involved in the process still remains a challenge. Studies range in duration, model of obesity (cafeteria diet, high fat, and high salt/high fat), and stage of development of the intervention (i.e., periconception, gestation, lactation, and weaning). The maternal-fetal obesity paradigm is extremely complex, with factors related to overnutrition, obesity, and inflammatory processes likely impacting the development of the fetus. Additionally, a majority of studies investigating the programming effects of maternal obesity observe more pronounced impairments in male offspring, and it is not well understood why these sex-specific differences occur. Nutritional intervention remains a promising therapeutic target to minimize complications to fetal development in a poor maternal environment. However, it is unclear whether these compounds are beneficial by directly affecting offspring, or rather improving the metabolic profile in the mother. However, what is clear is that weight loss and specific dietary interventions such as decreased intake of saturated fat in women who intend to become pregnant are the most effective and safe way to improve metabolic outcomes for offspring. In conclusion, evidence from animal and clinical studies provides strong evidence for the developmental origins of obesity and metabolic disorders. Intervention strategies to ameliorate the negative outcomes of maternal obesity on offspring are greatly needed as they present an easy cost effective way of decreasing potential noncommunicable disease risk for future generations to come.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Potential Role of A_{2B} Adenosine Receptors on Proliferation/Migration of Fetal Endothelium Derived from Preeclamptic Pregnancies

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To investigate the functionality of A_{2B} adenosine receptor (A_{2B}AR) and the nitric oxide (NO) and vascular endothelial growth factor (VEGF) signaling pathway in the endothelial cell proliferation/migration during preeclampsia, we used human umbilical vein endothelial cells (HUVECs) isolated from normal pregnancies ($n = 15$) or pregnancies with preeclampsia ($n = 15$). Experiments were performed in presence or absence of the nonselective adenosine receptor agonist NECA, the A_{2B}AR selective antagonist MRS-1754, and the nitric oxide synthase (NOS) inhibitor L-NAME. Results indicated that cells from preeclampsia exhibited a significant higher protein level of A_{2B}AR and $\log EC_{50}$ for NECA-mediated proliferation than normotensive pregnancies. The stimulatory effect of NECA (10 μM, 24 h) on cell proliferation was prevented by MRS-1754 (5 nM) coincubation only in cells from normotensive pregnancies. Nevertheless, L-NAME (100 μM, 24 h) reduced the NECA-induced cell proliferation/migration in HUVEC from normal pregnancy; however in preeclampsia only NECA-induced cell proliferation was reduced by L-NAME. Moreover, NECA increased protein nitration and abundance of VEGF in cells from normal pregnancy and effect prevented by MRS-1754 coincubation. Nevertheless, in preeclampsia NECA did not affect the protein level of VEGF. In conclusion HUVECs from preeclampsia exhibit elevated protein level of A_{2B}AR and impairment of A_{2B}AR-mediated NO/VEGF signaling pathway.

1. Introduction

Preeclampsia is the leading cause for maternal and neonatal morbidity and mortality worldwide [1]. The pathophysiology of the fetal complications in preeclampsia is still unclear, but it has been associated with placental alterations compatible with under perfusion [2, 3] and reduced placental blood flow [4], which in turn may limit the delivery of nutrients to the fetus. Since placental and proximal umbilical vessels lack innervation [5], the regulation of vascular blood flow depends on the synthesis and release of endothelial vasoactive molecules, such as nitric oxide (NO) and adenosine [6–8]. In this regard, several evidences suggest that preeclampsia is characterized by endothelial dysfunction in both maternal [9, 10] and fetoplacental circulation [11–14], which may impair not only vascular tone regulation but also angiogenesis.

Adenosine is a naturally occurring nucleoside, which controls several physiological processes including vascular

tone regulation and angiogenesis [15, 16], via activation of G-protein-coupled adenosine receptors (AR) [16]. Four types of AR have been identified: A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR [16, 17]. At the physiological nanomolar range, adenosine mainly activates A_1AR, A_{2A}AR, and A_3AR, whereas A_{2B}AR requires micromolar concentrations [18]. Nevertheless, exposure of any AR to agonists for shorter or longer times generally leads to the attenuation of the agonist response. In this regard, desensitization of A_{2B} receptor has been described in different cell lines (see details in [18]), but little is known regarding A_{2B}AR desensitization during human diseases.

In the fetoplacental circulation from preeclampsia, elevated level of adenosine has been reported in umbilical vein (1.7 versus 0.5 μmol/L, preeclampsia versus normal pregnancy, resp.) [19, 20] and in culture medium of human placental microvascular endothelial cells (hPMEC) (2.7 versus 0.6 μmol/L, preeclampsia versus normal pregnancy, resp.) [7], suggesting that under this pathological condition all subtypes of adenosine receptor would be activated [18]. However, little is known regarding the potential role of adenosine receptors in the regulation of placental homeostasis during preeclamptic pregnancies. In particular, regarding A_{2B}AR, high levels of this receptor have been reported in total placental homogenate [21], but no changes in hPMEC [7] derived from preeclampsia compared to normal pregnancy. Interestingly, elevated adenosine uptake seen in hPMEC from preeclampsia reverted by A_{2A}AR/A_{2B}AR inhibitors, suggest a tonic activation of adenosine transport by these receptors in preeclamptic pregnancies [7]. Nevertheless, other groups have reported that activation of A_{2B}AR in HUVEC exposed to hypoxia [22] or not is associated with upregulation of proangiogenic factors such as vascular endothelial growth factor (VEGF) [23–26] and NO [27]. Stimulation of A_{2B}AR increases proliferation of several cell types including porcine, rat endothelium [28], and human retinal endothelial cell [15]. Notwithstanding, in HUVEC from preeclampsia occurring before 34 weeks of gestation, we have described reduced cell proliferation associated with decreased activation of A_{2A}AR/NO signaling pathway compared to controls [29]. Despite these evidences, it is unknown whether preeclampsia may affect the proangiogenic role of A_{2B}AR in fetal endothelium.

We propose that HUVECs isolated from preeclamptic pregnancies exhibit reduced activation of A_{2B}AR compared to normal pregnancy.

2. Patients and Methods

2.1. Reagents. Unless otherwise indicated, all reagents were purchased form Sigma-Aldrich (MO, USA): nonselective agonist for adenosine receptors, NECA, 5′-(N-ethylcarboxamido) adenosine; selective A_{2B}AR antagonist, MRS-1754, 8-{4-{((4-cyanophenyl) carbamoylmethyl)oxy}phenyl}-1,3-di(n-propyl) xanthine hydrate; nonselective inhibitor of nitric oxide synthase, L-NAME, N_ω-nitro-L-arginine methyl ester hydrochloride; antibodies: anti-A_{2B}AR (Santa Cruz Biotechnology, CA, USA); antinitrotyrosine (Merck Millipore, MA, USA); anti-eNOS (Transduction Laboratories, NJ, USA), anti-VEGF (Cell Signaling, MA, USA), and anti-β-actin (Sigma Aldrich, MO, USA).

2.2. Patients. The Ethical Committee from the Universidad del Bío-Bío approved this cohort study and the informed consent was obtained from each participant. Pregnant women who attended to the Obstetrics and Gynecology Department of the Herminda Martin Clinical Hospital, Chillan, Chile, for their delivery were included in this study. Exclusion criteria included chronic hypertension, altered renal function, diabetes, chronic diseases, twin pregnancies, recurrent miscarriages, and abruption placenta. Women were classified into normal pregnancy (maternal blood pressure < 140/90 mmHg, absence of proteinuria, and no medical complications) and preeclampsia (new onset hypertension defined as blood pressure ≥ 140/90 mmHg, with at least 2 measurements 6 h apart, and proteinuria > 300 mg/24 hours after 20 weeks of gestation). The gestational age was defined as the period of time from the first day of the mother's last menstrual period and the delivery date, confirming this age by a first trimester ultrasound. Moreover, according to the standard protocol, all patients with preeclampsia received antihypertensive treatment and magnesium sulfate to prevent convulsions in case of severe preeclampsia. Diagnosis of small gestational age was performed when the newborn weight was <10th percentile to Chilean population [30].

2.3. HUVEC Culture. Endothelial cells were isolated from the human umbilical vein by digestion with collagenase (0.25 mg/mL) and then cultured (37°C, 5% CO_2) in medium 199 (M199) as previously described [31]. All experiments were performed in duplicate, after overnight serum deprivation and in presence of adenosine deaminase (ADA 1 IU/mL) (Merck Millipore, Darmstadt, Germany). Cells were used in passage 2.

2.4. Cell Proliferation and Migration. Cell proliferation was analyzed after treatment (24 h) with NECA (10 μM) and/or MRS-1754 (5 nM) or L-NAME (100 μM) by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay following the manufacture instruction (Promega, WI, USA) as described previously [29]. Moreover, in parallel experiments, cell migration was analyzed by transwell chambers assay as described elsewhere. In brief, HUVECs were trypsinized and seeded in the upper compartment of the transwell (Corning, NY, USA) at a density of 150×10^3 cells/well in M199. The lower compartments were loaded with culture medium (control) or AR agonist and/or antagonist. After 24 hours, cells that had migrated to the bottom of the transwell membrane (8 μm) were stained using hematoxylin (Winkler, Santiago, Chile). Membranes were observed at 40x magnification using a light microscope (Olympus, Tokyo, Japan) and 3 photos were taken from each preparation [29]. In addition, both cell proliferation and migration were measured in a concentration-response curve in presence of NECA.

2.5. Immunocytochemistry. The presence of A_{2B}AR in HUVECs was evidenced by immunocytology following the manufacturers protocol (Vector Laboratories, CA, USA), as described previously [29]. Briefly, HUVECs were fixed in 4% paraformaldehyde prepared in phosphate buffer (PBS (mM): NaCl 13.7, KCl 2.7, Na_2HPO_4 0.9, KH_2PO_4 1.8, pH 7.4, 4°C) for 20 minutes. After blocking unspecific binding, cell preparations were incubated overnight with primary A_{2B}AR antibody (1 : 200 v/v) followed by incubation with respective secondary antibody (1 : 500 v/v) diluted in PBS with 5% serum. Antigen-antibody reaction was further revealed by diaminobenzidine reaction (DAB). Analysis was blinded and performed using a bright field microscope (Olympus, Tokyo, Japan). For densitometric analysis three random pictures from each preparation were taken using a digital camera (Guangzhou Micro-shot Technology Co., Ltd, Guangdong, China). Estimation of the intensity of staining in the pictures was analyzed using ImageJ software (NIH, MD, USA) after light calibration and color deconvolution as previously described [32]. Values are expressed as the ratio of the area of positive brown stain divided by the total area of the reference field.

2.6. Western Blot. Cell, protein extracts (70 μg) were separated by SDS-PAGE (10%) transferred to nitrocellulose membranes and probed with primary anti-A_{2B}AR (1 : 2000 v/v), antinitrotyrosine (1 : 3000 v/v), anti-eNOS (1 : 2000 v/v), anti-VEGF (1 : 3000 v/v), and anti-β-actin (1 : 10000 v/v) antibodies diluted in PBS with 0.1% Tween (pH 7.4) and 5% milk. Then, a horseradish peroxidase-conjugated secondary antibody was used for visualization [29]. Secondary antibody was selected according to respective primary antibody. Dilution range of secondary antibody was 1 : 2000 to 1 : 150000 v/v in PBS/Tween buffer (pH 7.4) with 5% milk.

2.7. Nitrite Measurement. Nitrite levels were measured by the Griess reaction using a commercially available kit (Promega, WI, USA). In brief, confluent cells were incubated (30 min) in the presence or absence of NECA (10 μM) and/or MRS-1754 (5 nM); and then, 100 μL of M199 was collected for nitrite quantification in a spectrophotometer (Autobio, Zhengzhou, China) using a 540 nm filter [29].

2.8. Statistical Analysis. Comparisons between groups were performed by the Mann-Whitney test. We used X^2 test to analyze proportions. Values are mean ± S.E.M., where n indicates number of different cell cultures (in duplicate). $P < 0.05$ is considered statistically significant. The statistical software GraphPad Instat 3.01 and GraphPad Prism 5.00 (GraphPad Software Inc., California, USA) were used for data analysis.

3. Results

3.1. Clinical Characteristics of Patients. Thirty women were included in the study divided into normal pregnancies (n = 15) and preeclampsia (n = 15). Compatible with diagnostic criteria, women with preeclampsia exhibited higher systolic and diastolic blood pressure than normal pregnancy (Table 1). Gestational age at delivery, newborn weight, and height were lower in preeclampsia than normal pregnancy. 27% of pre-eclamptic women had small for gestational age babies. Despite the trend to lower placental weight in preeclampsia, it was not statistically significant compared to controls (Table 1).

Table 1: Characteristics of the included women.

	Normal (n = 15)	Preeclampsia (n = 15)
Age (years)	25.1 ± 1.6	26.5 ± 1.7
BMI at delivery (kg/m^2)	31.4 ± 1.3	33.5 ± 1.4
GA at delivery (wk)	39.4 ± 0.3	37.4 ± 0.3*
SBP (mmHg)	115.2 ± 1.5	161.9 ± 4.8*
DBP (mmHg)	72.7 ± 1.7	102.1 ± 2.3*
Proteinuria, g/24 h	0	2.2 ± 1.0*
Newborn		
Male/female	7/8	7/8
Weight (g)	3333 ± 111.3	2726 ± 178.8*
Height (cm)	48.8 ± 0.4	46.3 ± 0.7*
SGA, n (%)	0	4 (27)*
Cephalic perimeter (cm)	34.1 ± 0.2	33.3 ± 0.6
Placenta		
Weight (g)	542.7 ± 33.5	490.0 ± 34.6
Area (m^2)	3.3 ± 0.3	2.8 ± 0.2
NBW/PlW (g/g)	6.3 ± 0.3	5.7 ± 0.2

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; SGA: small for gestational age (<10th percentile according to gestational age); NBW: newborn weight; GA: gestational age; PlW: placental weight. *$P < 0.05$ versus normal pregnancy. Values are mean ± SEM.

3.2. Functional Characterization of $A_{2B}AR$. Western blot and immunocytology showed significantly higher (1.6 and 1.7, resp.) A_{2B}AR protein levels in HUVEC from preeclampsia than those from normal pregnancy (Figure 1). In the presence of NECA (10 μM, 24 h), it was observed a significant elevation in both proliferation (Figure 2(a)) and migration (Figure 2(b)) of HUVEC from normal and preeclamptic pregnancies. Moreover, NECA-induced cell proliferation was not observed when cells derived from normal pregnancies were coincubated with MRS-1754. However, in cells from preeclampsia, coincubation with NECA and MRS-1754 exhibited similar response to NECA alone. In addition, MRS-1754 did not affect cell proliferation in normal or preeclamptic pregnancies.

The effect of NECA on cell proliferation was also tested in a concentration-response curve in both normal and preeclamptic derived cells (Figure 3). The calculated logarithmic EC_{50} ($LogEC_{50}$) for NECA was significantly higher (−7.7 ± 0.5 and −9.6 ± 0.7 M, resp.; $P < 0.05$) in preeclampsia compared to normal pregnancy.

Regarding cell migration, despite the stimulatory effect observed after NECA incubation was similar in both normal and preeclamptic cells compared to its respective basal condition (1.5- and 1.4-fold, resp.), it was observed that cells

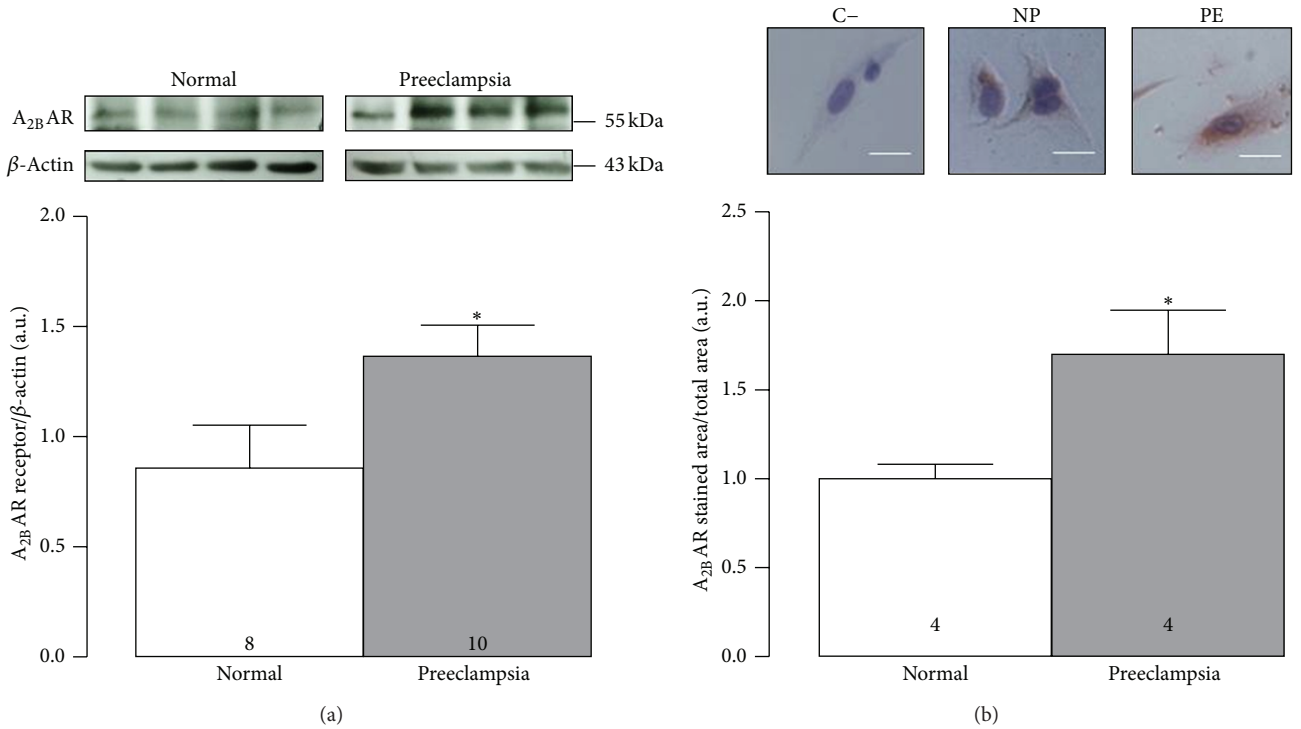

FIGURE 1: Protein levels of A_{2B}AR in both normal and preeclamptic cells. (a) Cells derived from normal (NP) or preeclamptic pregnancies (PE) were used for estimating protein abundance of A_{2B}AR by western blot. Upper panel shows representative images of A_{2B}AR protein (55 kDa) and β-actin (43 kDa). Bottom panel shows densitometry of A_{2B}AR/β-actin ratio. (b) Upper panel shows representative images of immunocytochemistry for A_{2B}AR. Bottom panel included the ratio of the digital analysis of the stained area by A_{2B}AR divided by total area as described in Section 2. Line in the photos represents 5 μM. $^{*}P < 0.05$ versus respective value in normal pregnancy. C− is negative control without primary antibody. Values are mean ± SEM. Respective n is indicated in each bar. All experiments were performed in duplicate.

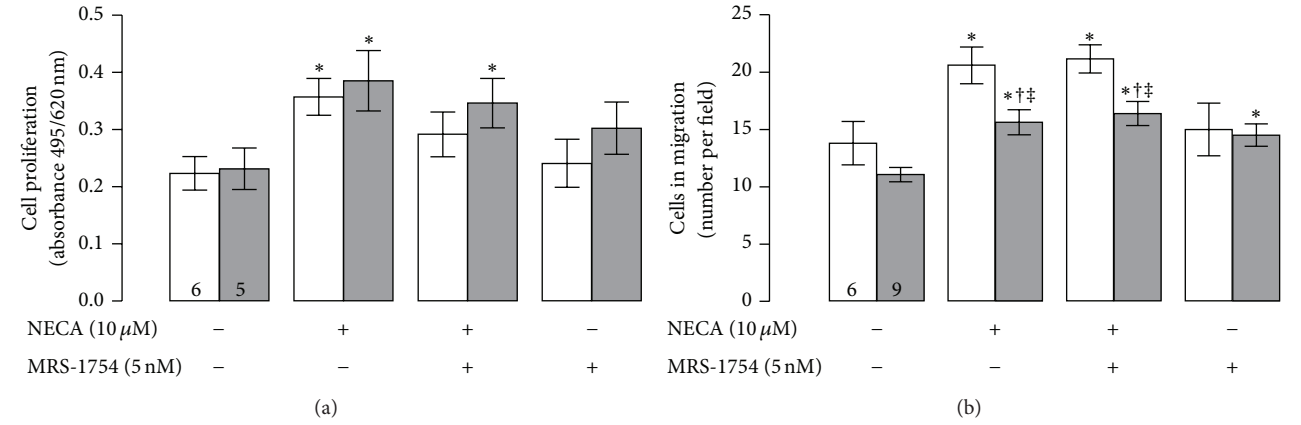

FIGURE 2: Cell proliferation and migration induced by A_{2B}AR. HUVECs were isolated from normal (white bars) and preeclamptic pregnancies (grey bars) and used for (a) analysis of cell proliferation by MTS assay in presence (+) or absence (−) of NECA (10 μM, 24 h) and/or MRS-1754 (5 nM, 24 h) or (b) cell migration as described in (a). $^{*}P < 0.05$ versus basal condition in normal pregnancy. $^{\dagger}P < 0.05$ versus basal condition in preeclampsia. $^{\ddagger}P < 0.05$ versus respective value in normal pregnancy. Values are mean ± SEM. Values in respective control column indicate n. All experiments were performed in duplicate.

from preeclampsia do not reach similar response compared to normal pregnancy (Figure 2(b)). In fact, migratory response was 29 ± 3% less in cells from preeclampsia compared to normal pregnancy. NECA-mediated cell migration was not affected by MRS-1754 co-incubation in cell from normal or pre-eclamptic pregnancies. Yet, cells from preeclampsia exposed to MRS-1754 alone exhibit a significant increase (1.3-fold) in cell migration compared to its basal condition without any treatment, whereas no effect was observed in cells from normal pregnancy incubated with this antagonist.

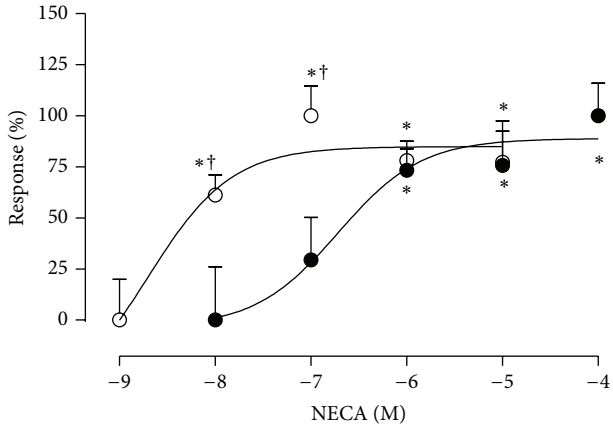

Figure 3: Cell proliferation induced by NECA in a concentration-response curve. HUVEC was isolated from normal (open circles, $n = 6$) and preeclamptic pregnancies (closed circles, $n = 3$) and used for cell proliferation in presence of NECA (10^{-9} to 10^{-4} M, 24 h). $^{*}P < 0.05$ versus respective low NECA concentration. Values are mean ± SEM. All experiments were performed in duplicate.

3.3. A_{2B}AR Stimulation and NO. There were no differences in the protein abundance of endothelial nitric oxide synthase (eNOS) between preeclampsia and normal pregnancies (Figures 4(a) and 4(b)). Moreover, neither NECA (10 μM) nor MRS-1754 incubation (5 nM, 12 h) changed the protein abundance of eNOS in either preeclampsia or normal pregnancy.

Nevertheless, during short incubations (30 min), NECA induce nonstatistically significant reduction in the nitrite levels measured in culture medium (Figure 4(c)), as well as a significant elevation in the intracellular nitrotyrosine formation (Figure 4(d)) in HUVEC from both preeclampsia and normal pregnancy. Moreover, coincubation of NECA + MRS-1754 was associated with reduction in the nitrite level only in preeclamptic cells. Furthermore, NECA-mediated nitrotyrosine formation observed in cells from normal or pre-eclamptic pregnancies was not blocked by MRS-1754.

In addition, increase in cell proliferation induced by NECA in normal or pre-eclamptic pregnancies (1.9 and 1.7 fold, resp.) (Figure 5(a)) was partially reduced by coincubation with L-NAME (100 μM). Furthermore, when the percentage of reduction mediated by L-NAME coincubation was calculated, it was observed that normal pregnancy derived cells exhibited a higher percentage (38 ± 2%) than preeclampsia (18 ± 1%) ($\chi^2 = 8.9$, $P < 0.05$). Nevertheless, combination of L-NAME and MRS-1754 reduced in 43±1% and 29±1% the NECA-mediated augmentation in cell proliferation in normal and preeclamptic HUVEC, respectively (data not shown). On the other hand, L-NAME induces a partial reduction (27±4%) in the stimulatory effect of NECA on cell migration, whereas no affect was observed in preeclampsia (Figure 5(b)).

3.4. A_{2B}AR Stimulation and VEGF. There were no significant differences in the protein level of VEGF in HUVEC derived from normal and preeclampsia. After stimulation with NECA (10 μM, 12 h), cells from normal pregnancies exhibited an increase (1.7-fold) in the VEGF protein abundance compared to corresponding controls without any treatment. The latter effect of NECA was not observed in cells coincubated with MRS-1754 (5 nM). Contrarily, neither NECA nor MRS-1754 alone or in combination affected the VEGF protein level in preeclampsia (Figures 6(a) and 6(b)).

3.5. NO and VEGF Expression. The stimulatory effect of NECA (10 μM, 12 h) on VEGF protein levels was partially blocked by L-NAME or MRS-1754 (Figures 6(c) and 6(d)). Interestingly, the reductive effect of these last two inhibitors upon NECA effect was more than additive since cell coincubated with L-NAME, MRS-1754, and NECA exhibited reduced VEGF levels even below control condition without any treatment.

4. Discussion

Results presented in this work describe that HUVEC from preeclampsia exhibits elevated protein level of A_{2B}AR compared to normal pregnancy. However, functionality of this receptor may be reduced in preeclampsia. This is supported by results showing a shift in the concentration-response curve to NECA during cell proliferation, where $\log EC_{50}$ is higher in preeclampsia than normotensive pregnancies. In addition, a reduced migratory response to NECA was seen in preeclampsia compared to normal pregnancy; and contrary to cell from normal pregnancy, in pre-eclampsia the expression of VEGF associated with A_{2B}AR stimulation was absent. Results from coincubation of agonist and antagonists suggest that activation of A_{2B}AR triggers intracellular pathways involving protein nitration, which may participate in cell proliferation/migration and VEGF protein expression in HUVEC from normal pregnancies. However, in preeclampsia only cell proliferation seems to be associated with protein nitration induced by A_{2B}AR. In conclusion, HUVECs from preeclampsia exhibit elevated protein level of A_{2B}AR. Moreover, tyrosine nitration and VEGF protein expression mediated by A_{2B} are associated with cell proliferation/migration in normal cells, but this cell signaling is impaired in preeclampsia.

4.1. Clinical Context. Preeclamptic placenta exhibits reduced fetoplacental blood flow [4], generated by placental under perfusion [2, 3] associated with limited placental invasion into maternal spiral arteries [33] and impaired remodeling process of those vessels [34]. Furthermore, hemodynamic changes generated by those alterations in the maternal-placental interphase may generate a turbulent blood flow that hits the placenta and leads to impaired placental villi architecture [35], then compromising the fetoplacental circulation in preeclampsia [4]. All these alterations have been associated with generation of oxidative and nitrative stress within the placenta [36, 37], leading to endothelial dysfunction in the fetoplacental circulation [11–14]. Others and we believe that part of this endothelial dysfunction present in the preeclamptic placenta may include alteration in the angiogenesis process [3, 4, 14, 29, 38], which in turn

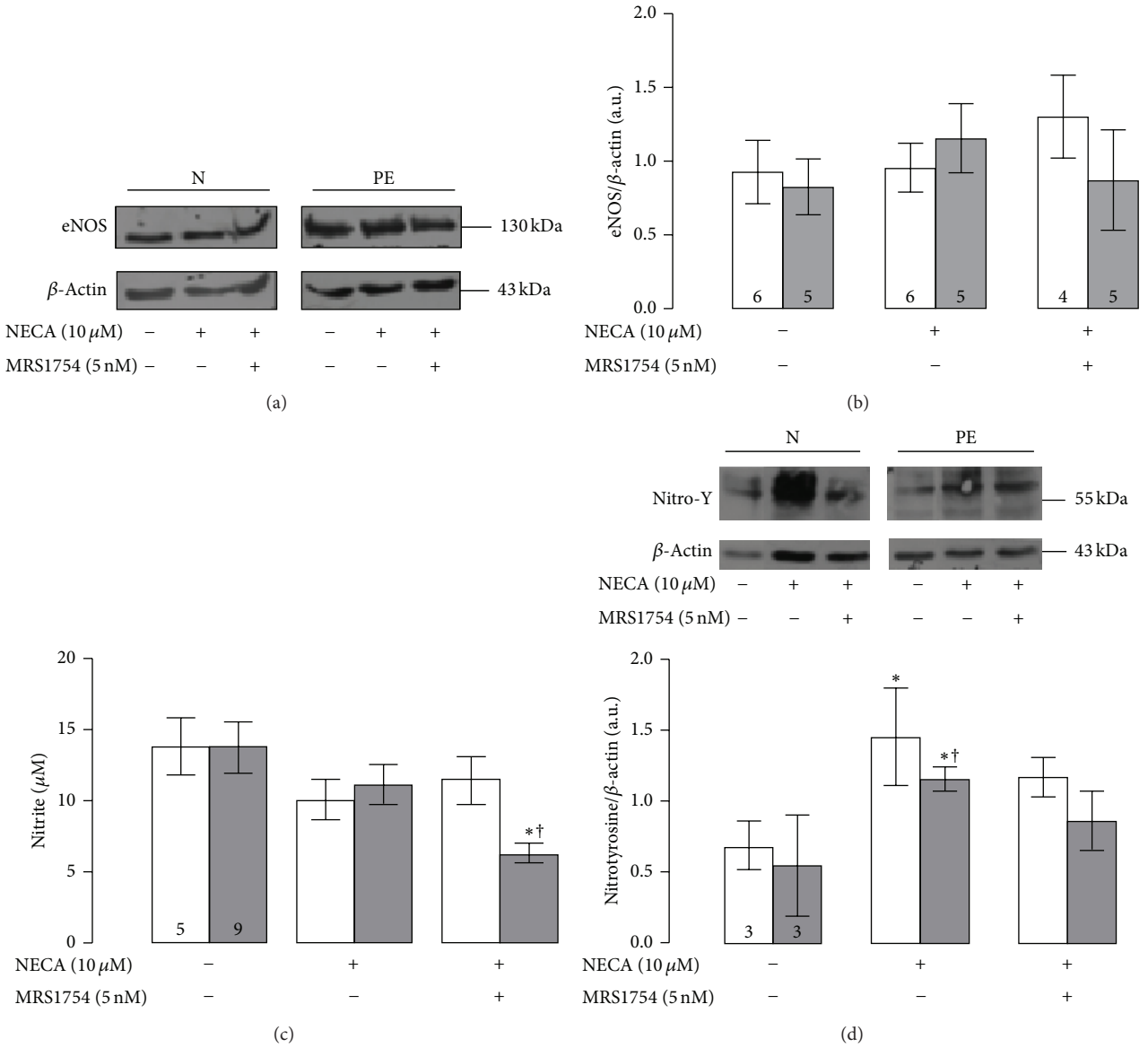

FIGURE 4: Stimulation of $A_{2B}AR$ generates nitrotyrosine formation. (a) Representative images of western blots for endothelial nitric oxide synthase (eNOS, 130 kDa) and β-actin (43 kDa) in absence (–) or presence (+) of NECA (10 μM) and/or MRS-1754 (5 nM) during 12 hours in cells from normal (N) or preeclamptic pregnancies (PE). (b) Densitometry of the eNOS/β-actin ratio as showed in (a). (c) Nitrite concentrations in homogenate of cell after incubation (30 min) with NECA (10 μM) and/or MRS-1754 (5 nM). (d) Upper panel, representative images of western blots for nitration of tyrosine residues (Nitro-Y) in protein(s) at 55 kDa and β-actin (43 kDa). Bottom panel presents the densitometry of the nitrotyrosine/β-actin ratio in the analyzed groups as showed in the upper panel. In (c) and (d), $^{*}P < 0.05$ and $^{\dagger}P < 0.05$ versus value in basal condition (i.e., without any treatment) of normal pregnancy or preeclampsia, respectively. Values are mean ± SEM. Values in respective column indicate *n*. All experiments were performed in duplicate.

could explain the elevated risk for short and long term complications observed in children exposed to preeclampsia intrauterine. Regarding short-term complication associated to preeclampsia, clinical data presented in this study, showing that preeclamptic women exhibited high blood pressure (mean value 161/102 mmHg), associated with reduced gestational age and 4 of 15 preeclamptic women had a baby small for gestation age. Although we did not analyze the data according to the severity of the disease or gestation age of presentation, our study describes major differences in the $A_{2B}AR$/NO/VEGF signaling pathway when cells from preeclampsia are compared with those derived from normotensive pregnancies. However, the impact of confounding variables including degree of severity of the disease, gestational age at onset, treatment received, and children sex should be addressed in future studies.

4.2. Adenosine and HUVEC Proliferation/Migration. Adenosine promoted angiogenesis. The underling mechanisms are

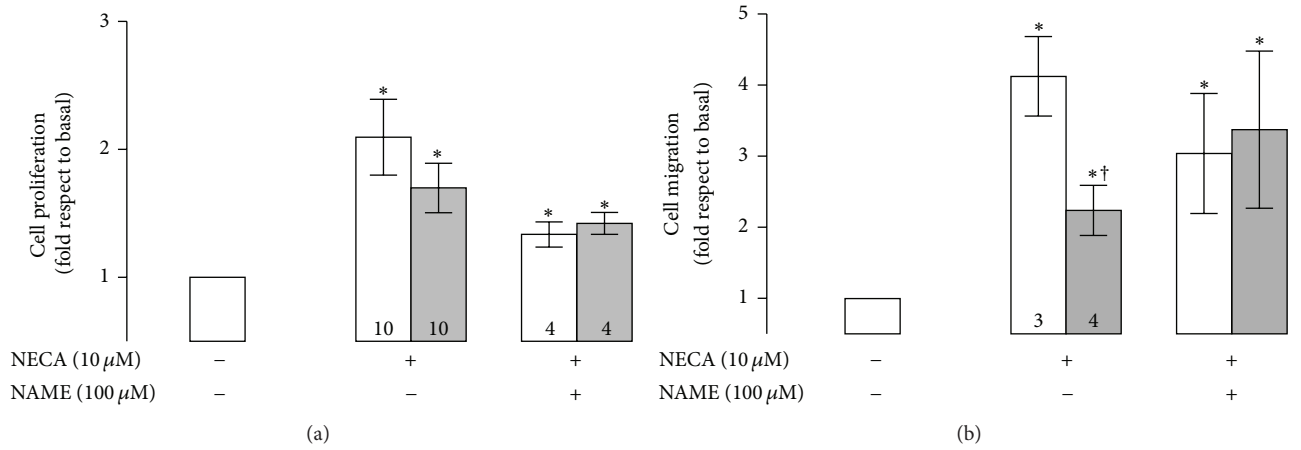

Figure 5: Effects of NOS inhibitor on NECA-mediated cell proliferation/migration. (a) Cell proliferation in presence (+) or absence (−) of NECA (10 μM) and/or L-NAME (100 μM) during 24 hours in normal (white bars) or preeclamptic pregnancies (grey bars). (b) Cell migration as in (a). $^{*}P < 0.05$ versus respective value in basal conditions. $^{\dagger}P < 0.05$ versus respective value in normal pregnancy. Values are expressed in fold of change considering respective basal condition (i.e., without any treatment) in normal or preeclamptic cells. Values in mean ± SEM. Values in respective control column indicate *n*. All experiments were performed in duplicate.

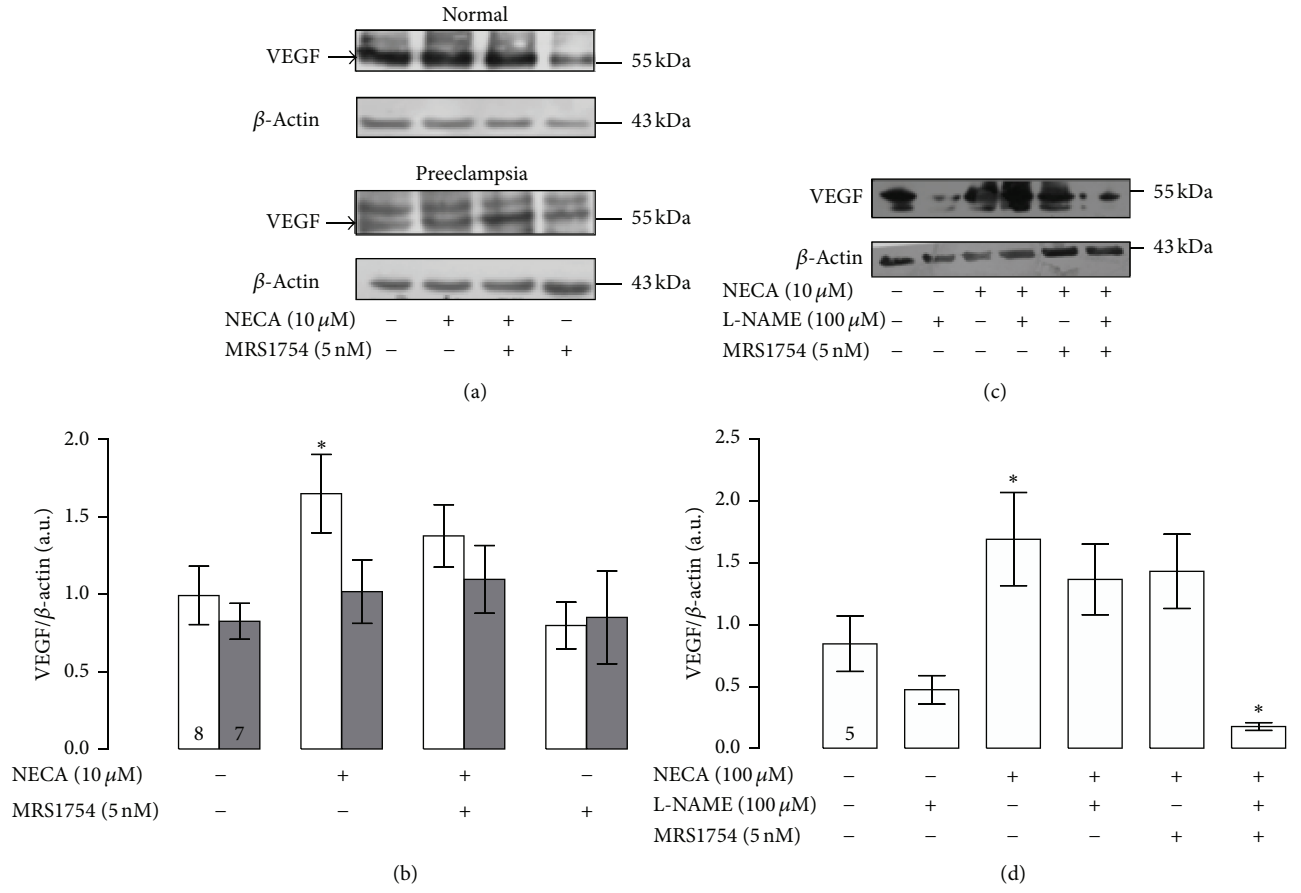

Figure 6: Effect of A_{2B}AR stimulation in the VEGF protein level. (a) Representative images of western blot for VEGF (55 kDa) and β-actin (43 kDa) in HUVEC from normal or preeclamptic pregnancies incubated in absence (−) or presence of NECA (10 μM) and/or MRS-1754 (5 nM) during 12 hours. (b) Densitometry of the VEGF/β-actin ratio as showed in (a). (c) Representative images of VEGF (55 kDa) and β-actin (43 kDa) in HUVEC from normal pregnancies treated with NECA (10 μM) and/or MRS-1754 (5 nM) and/or L-NAME (100 μM) during 12 hours. (d) Densitometry of the VEGF/β-actin ratio as showed in (c). $^{*}P < 0.05$ versus basal condition in normal pregnancy. Values are mean ± SEM. Values in respective control column indicate *n*. All experiments were performed in duplicate.

under investigation and include direct and indirect actions on several different cell types and practically all adenosine receptors may be involved [23, 39]. Focusing on endothelial cells, either A_{2A}AR or A_{2B}AR has been shown to mediate the proliferative actions of adenosine in human retinal microvascular endothelial cells [15, 40], HUVEC [22, 29], or porcine coronary artery and rat aortic endothelial cells [28], while it remains still unclear if A_1AR and A_3AR are functionally expressed, and what role, if any, they play in endothelial cells.

Particularly, expression of A_{2B}AR in HUVEC and its participation in angiogenesis have been well characterized in the literature [22, 27, 39, 41–43], which is also supported by our results. Moreover, functional presence of A_{2B}AR leading to activation of NO/VEGF signaling pathway in cells derived from normal pregnancy is suggested because the NECA-mediated augmentation in cell proliferation, nitrotyrosine formation, and VEGF protein abundance is inhibited by MRS-1754 coincubation. These results agree with previous observations regarding induction of NO production after A_{2A}AR/A_{2B}AR stimulation in porcine carotid artery endothelial cells (PCAEC) [44], human iliac arterial endothelial cells (HIAEC) [45], and HUVEC [27, 29, 46, 47], as well as with others indicating adenosine mediated augmentation in the VEGF expression in many endothelial cells [29, 40, 41, 48–53].

Moreover, our results in HUVEC derived from normal pregnancy suggest that A_{2B}AR-mediated activation of NO synthesis would not be the unique pathway involved in the cell proliferation/migration in HUVEC. This is because NECA-mediated increase in cell proliferation/migration and VEGF protein abundance was only partially reverted by the NOS inhibitor, L-NAME, whereas coincubation with both inhibitors MRS-1754 (A_{2B}AR antagonist) and L-NAME has additive effects upon these two NECA-mediated effects. It is unknown how NO/protein nitration may control cell proliferation and VEGF expression in HUVEC. However, potential mechanism might include activation of hypoxia inducible factor mediated by nitration [54].

In our study, we used the unspecific inhibitor of NOS, L-NAME, in a concentration (100 μM) that should be inhibiting all the isoforms of NOS according to the estimated value of K*i* (neuronal NOS (nNOS, 15 nM), eNOS (39 nM), and inducible NOS (iNOS, 65 μM)). Although our cell model expresses constitutively eNOS, we could not roll out the participation of other isoforms in our experimental data. Another limitation regarding importance of NO in our study is the use of nitrite measurements and nitrotyrosine formation as indirect markers for NO synthesis. Although both techniques have several advantages and disadvantages for estimating NO synthesis, it is clear that more direct measurement is required in order to elucidate participation of NO in the A_{2B}AR signaling pathway. Therefore, future studies should consider the use of more specific inhibitors for eNOS, or molecular biology techniques for suppressing or overexpressing eNOS.

4.3. Preeclampsia, Adenosine, and HUVEC Proliferation/Migration. In the preeclampsia field, elevated level of plasma adenosine has been reported in the fetoplacental circulation [7, 19, 20], which reaches the micromolar range in umbilical vein; therefore it is feasible that under these conditions all adenosine receptors would be stimulated [18, 55]. Moreover, elevation of adenosine in the fetal circulation of preeclamptic pregnancies seems to be a phenomenon depending on the severity of the disease, since only children with alteration in the Doppler velocimetry of umbilical artery exhibit elevation in the adenosine level compared to normal pregnancy [20]. Causes and consequences of elevated extracellular adenosine concentration in preeclampsia are unclear.

We have previously documented that HUVECs isolated from late-onset preeclampsia exhibit high cell proliferation/migration, while in early onset preeclampsia these parameters were reduced in relation to women with normal pregnancy [29]. Interestingly, activation of A_{2A}AR was heterogeneous between late-onset preeclampsia and early onset preeclampsia. Thus, whereas late-onset preeclampsia was associated with a "basal" activation of the adenosine/NO/VEGF pathway, early onset preeclampsia exhibited a downregulation of this particularly via [29], suggesting that changes in cell proliferation/migration observed between late-onset preeclampsia and early onset preeclampsia may be explained by changes in the A_{2A}AR/NO/VEGF signaling pathway activation.

In the actual report, although we did not classify the women in late-onset or early onset preeclampsia, due to sample size, most of the preeclamptic women belong to group with early onset preeclampsia, as indicated by reduced gestational age at delivery. In addition, in order to avoid potential "basal" effect of high extracellular level of adenosine, we have performed all experiments in presence of adenosine deaminase. Under these experimental conditions, an increased protein abundance of A_{2B}AR associated with reduction in its function is present in HUVEC from preeclampsia. These results allow speculating the following. (1) Elevation in the A_{2B}AR observed in preeclampsia may be associated with activation of hypoxia inducible factor 1 alpha (HIF-1α) [56], since the A_{2B}AR promoter contains a functional binding site for HIF-1 α [57], which promotes A_{2B}AR expression in HUVEC [22]. (2) The reduced activation of A_{2B}AR observed in preeclampsia may be related to reduced availability of the receptor in the cell membrane. Indirect evidences for this hypothesis are elevated logEC_{50} for NECA-mediated proliferation observed in HUVEC from preeclampsia compared to normal pregnancy. Clearly, more studies are required to elucidate the mechanisms linked with A_{2B}AR expression and activation in preeclampsia.

Result in this study suggests that A_{2B}AR/NO/VEGF pathway observed in normal pregnancy would be dysfunctional in HUVEC from preeclampsia. This idea is supported by the fact that although activation of A_{2B}AR is associated with nitration and cell proliferation, it was not related to VEGF protein expression. In this regard, Feoktistov and colleagues [22] have reported that hypoxia-mediated upregulation of A_{2B}AR in HUVEC has a functional impact, since only under this condition the A_{2B}AR was coupled to upregulation of VEGF. Our results partially agree with this concept, with respect to upregulation of A_{2B}AR in preeclampsia, a condition characterized by placental under perfusion [2, 3]

and HIF-1 α activation [56]. Contrarily, we did not find an A_{2B}AR-mediated VEGF expression in preeclampsia, whereas it was evidenced in cell from normal pregnancy. Apparent discrepancy may be related to experimental condition, since in the Feokstitov's report [22] they use a HUVEC cell line, rather than primary culture as we reported; and hypoxia was defined as 4.6% oxygen, which is considered normoxia for primary culture of HUVEC [58].

There are some intriguing results in our study that we would like to discuss. For instance, A_{2B}AR activation might not be associated with cell migration in either normal pregnancy or preeclampsia, because MRS-1754 was unable to block the stimulatory effect of NECA upon cell migration. Although we did not analyze the participation of other adenosine receptors in this particular study, it has been described previously that activation of A_{2A}AR increases cell migration in HUVEC from either normal or preeclamptic pregnancies [29]. Then, A_{2A}AR, rather than A_{2B}AR, may be involved in the cell migration during normal pregnancy and preeclampsia. Another intriguing result is the reduction in the nitrite levels (i.e., NO metabolites) in the culture medium of preeclamptic cells induced by coincubation with NECA and MRS-1754 (Figure 4(c)). As stated above, we could not roll out the participation of other adenosine receptors (except A_{2B}AR) in this response.

5. Conclusion

HUVECs from normal pregnancies exhibit a functional presence of A_{2B}AR, whose activation is associated with cell proliferation, mediated at least partially, via intracellular protein nitration and VEGF synthesis. On the other hand, cells from preeclampsia are characterized by upregulation in the A_{2B}AR protein expression, but its activity is diminished and might not be involved in the control of VEGF expression.

Abbreviations

NO:	Nitric oxide
NOS:	Nitric oxide synthase
VEGF:	Vascular endothelial growth factor
A_{2A}AR:	A_{2A} adenosine receptor
A_{2B}AR:	A_{2B} adenosine receptor
L-NAME:	NG-nitro-L-arginine methyl ester
NO:	Nitric oxide
VEGF:	Vascular endothelial growth factor
HUVECs:	Human umbilical vein endothelial cells
hPMEC:	Human placental microvascular endothelial cells.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

The work was carried out in collaboration between all authors. Jesenia Acurio and Carlos Escudero defined the research topic. Patricio Bertoglia defined diagnosis and included patients. Jesenia Acurio, Felipe Troncoso, and Carlos Escudero designed methods and performed experiments. Jesenia Acurio, Felipe Troncoso, and Carlos Escudero analyzed the data; Jesenia Acurio, Claudio Aguayo, Carlos Salomon, Luis Sobrevia, and Carlos Escudero interpreted the results and co-wrote the paper.

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