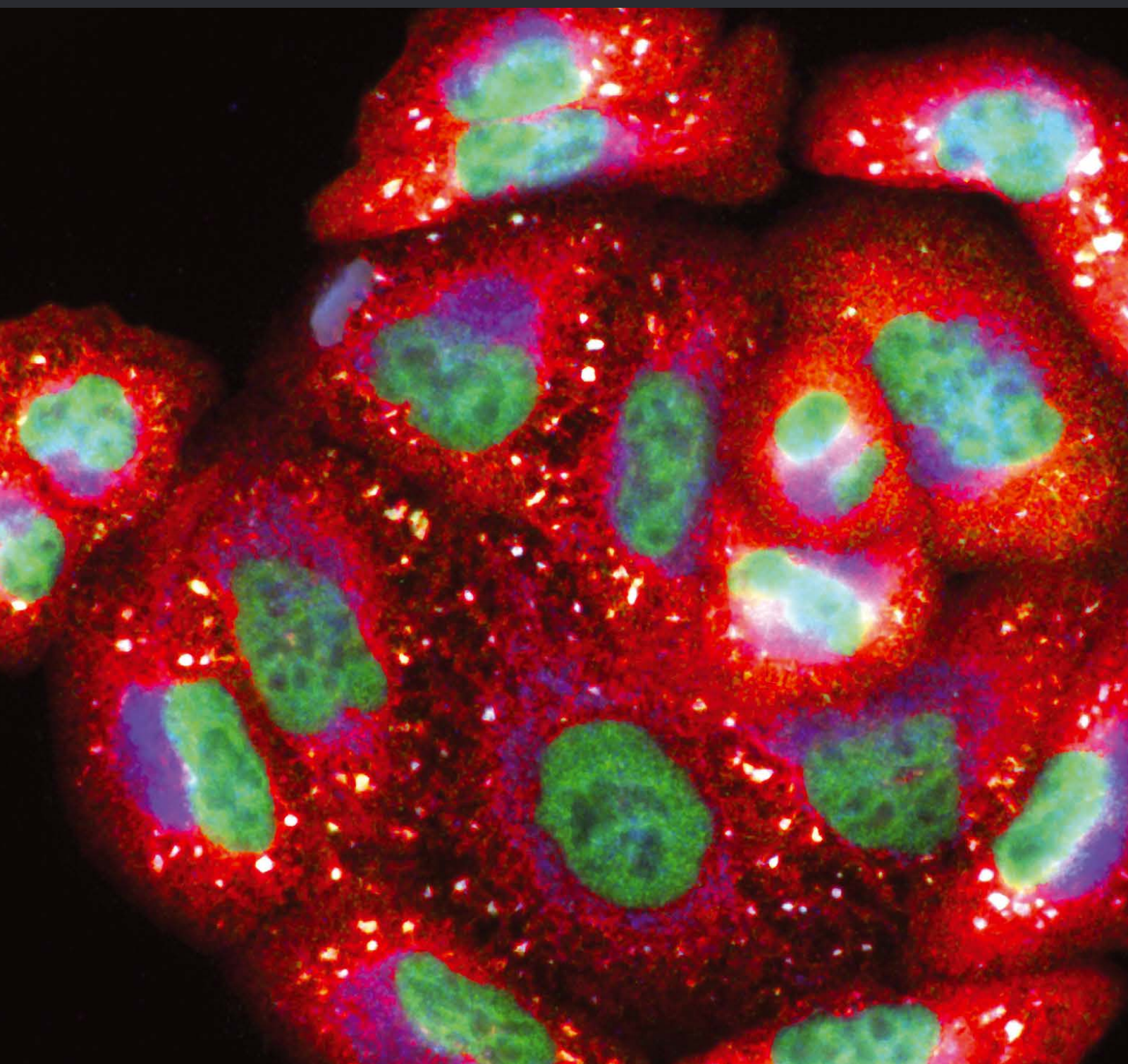
Oxidative Medicine and Cellular Longevity

Oxidative Stress in the Newborn

Guest Editors: Giuseppe Buonocore, Serafina Perrone, and Maria L. Tataranno



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Editorial Board

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 1094247, 2 pages
http://dx.doi.org/10.1155/2017/1094247

Editorial

Oxidative Stress in the Newborn

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Received 11 January 2017; Accepted 15 January 2017; Published 31 January 2017

Oxidative stress (OS) occurs when there is an unbalance between free radicals (FR) production and antioxidant capacity. OS can be a risk factor for fetal programming, representing a key process linking adverse fetal growth, impaired fetal well-being or preterm birth, and later increased risks of diseases in adolescence and adulthood. Adverse outcome to the offspring can extend beyond the neonatal period and includes neurodevelopmental disorders (motor and cognitive problems, attention deficit hyperactivity, and psychotic disorders), asthma, insulin resistance, diabetes mellitus, hypertension, coronary heart disease, and stroke. Free radicals can alter gene expression or damage lipids, proteins, and DNA at a critical developmental point leading to a higher susceptibility to many disorders.

The present special issue aims to stimulate the continuing efforts to understand the pathophysiology underlying OS damage in neonates. Furthermore we aimed to highlight new possible strategies to treat or prevent OS-mediated diseases and to evaluate neonatal outcomes.

This issue includes papers on (i) recent developments in OS-mediated diseases in fetus and newborns in both human and animal models; (ii) advances in identification of prenatal and neonatal characteristics of the infant/fetus at increased risk of OS-mediated damage; (iii) mechanisms of OS-mediated tissue damage using model systems; (iv) recent advances in antioxidant and other protective strategies.

Each manuscript was reviewed by at least 2 external reviewers and one guest editor. We received about 20 papers and only the present 8 papers were selected for the final draft. All the papers were selected on the basis of relevance and novelty for the reader.

The review by Y. Ozsurekci and K. Aykac is an overview on the complex and wide world of oxidative stress pathophysiology and its link to neonatal diseases.

M. Chełchowska and colleagues examined the relationship between selected adipokines and markers of oxidative stress/antioxidant defense in the umbilical cord of neonates exposed and nonexposed in utero to tobacco smoke. Maternal smoking is considered as a source of oxidative stress, which has been implicated to disrupted adipokines expression in adipose tissue. To support this thesis, they found that cord serum visfatin, oxidized low density lipoproteins, and total oxidant capacity were significantly higher, while adiponectin and total antioxidant capacity were lower in smoking group than in nonsmoking group, demonstrating that maternal smoke enhances oxidative status and depletes antioxidant potential in otherwise "healthy" term newborns.

The research group from Italy showed, in the paper authored by S. Perrone et al., a relationship between pain degree and OS in healthy full-term newborns. They found that the amount of OS is gender related, being higher in males. Furthermore nonpharmacological approaches were able to reduce pain score together with pain-related OS in healthy term newborns.

The previous paper links to the paper by J. Wilhelm and colleagues, with the first focused on term healthy newborns and the latter on the effect of OS on fetuses and developing rat brains. In this study authors were able to localize reactive oxygen and nitrogen species (RONS) production in the developing rat brain. In particular, the fetuses showed moderate RONS production, which changed cyclically during further development. The periods and sites of peak production of individual RONS differed, suggesting independent generation. They showed dramatic changes in the amount and the sites of RONS production on day 4 while the adult animals did not produce increased OS markers.

The subsequent topic highlighted in this issue is centered on OS after heart surgery in the neonatal population.

Heart surgery is associated with increased inflammation and the production of reactive oxygen species. The use of the extracorporeal cardiopulmonary bypass during correction of congenital heart defects generates reactive oxygen species through different pathways. They underlined the fact that the immature myocardium is more vulnerable to reactive oxygen species because of developmental differences compared to the adult heart but also because of associated congenital heart diseases that can deplete its antioxidant reserve, thus the importance of preventative strategies such as exogenous antioxidants, use of steroids, cardioplegia, blood prime strategies, or miniaturisation of the cardiopulmonary bypass circuit.

D. Hoeber and collaborators made an interesting overview on cerebral white and grey matter injury as the leading cause of an adverse neurodevelopmental outcome in prematurely born infants and on the role of erythropoietin as a promising antioxidant drug. They focused on motor-cognitive outcome up to the adolescent and adult age in an experimental rat model of preterm brain injury. Oligodendrocyte degeneration, myelination, and modulation of synaptic plasticity-related molecules were evaluated in rats exposed to hyperoxia. The analysis of white matter structures revealed a reduction of acute oligodendrocyte degeneration in erythropoietin treated animals. A single erythropoietin administration reverted hyperoxia-induced reduction of neuronal plasticity-related mRNA expression up to four months after injury, highlighting the importance of erythropoietin as a neuroregenerative treatment option in neonatal brain injury.

An intriguing research coming from United Kingdom, authored by E. Rocha-Ferreira and colleagues, deals with protective strategies against OS after asphyxia. The authors found that immediate remote ischemic postconditioning reduced brain nitrotyrosine formation in a piglet asphyxia model. Thus remote ischemic postconditioning (RIPostC) is a promising therapeutic intervention that could be administered as an alternative to cooling in cases of perinatal hypoxia-ischemia. The RIPostC beneficial effect seemed to be mediated by modulation of nitrosative stress, despite glial activation.

Another paper, by S. Manti and colleagues, focuses on causes of atopy. Extensive epidemiological and laboratory studies have been performed to identify the environmental and immunological causes of atopy. Till now, genetic predisposition seemed to be the biggest risk factor for allergic diseases. However recent findings suggest that the establishment of a peculiar epigenetic pattern may also be generated by OS and perpetuated by the activation of OS-related genes. The paper is centered on analyzing the role of maternal and neonatal oxidative stress and oxidative stress inducible genes in order to review the current knowledge about the relationship between maternal and neonatal OS-related genes and the development of atopic diseases.

The wide spectrum of review and research articles presented in this issue provides new inputs and new insight into the pathophysiology, diagnosis, and treatment of OS injury in neonates both for clinical practice and for neonatal research. We call for further investigation on OS in the perinatal period in order to find new tailored strategies to treat or prevent OS-mediated diseases, with the aim of ameliorating neonatal outcomes.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 3759287, 7 pages
http://dx.doi.org/10.1155/2017/3759287

Research Article

Oxidative Stress as a Physiological Pain Response in Full-Term Newborns

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Received 1 August 2016; Revised 29 November 2016; Accepted 7 December 2016; Published 4 January 2017

Academic Editor: Francisco J. Romero

This research paper aims to investigate if oxidative stress biomarkers increase after a painful procedure in term newborns and if nonpharmacological approaches, or sex, influence pain degree, and the subsequent OS. 83 healthy term newborns were enrolled to receive 10% oral glucose or sensorial saturation (SS) for analgesia during heel prick (HP). The ABC scale was used to score the pain. Advanced oxidation protein products (AOPP) and total hydroperoxides (TH) as biomarkers of OS were measured at the beginning (early-sample) and at the end (late-sample) of HP. The early-sample/late-sample ratio for AOPP and TH was used to evaluate the increase in OS biomarkers after HP. Higher levels of both AOPP and TH ratio were observed in high degree pain (4–6) compared with low degree pain score (0–3) (AOPP: $p = 0.049$; TH: $p = 0.001$). Newborns receiving SS showed a significantly lower pain score ($p = 0.000$) and AOPP ratio levels ($p = 0.021$) than those without. Males showed higher TH levels at the end of HP ($p = 0.005$) compared to females. The current study demonstrates that a relationship between pain degree and OS exists in healthy full-term newborns. The amount of OS is gender related, being higher in males. SS reduces pain score together with pain-related OS in the newborns.

1. Introduction

Nociceptive pathways are working from an early stage of fetal development [1]. Indeed, neonates have functional ascending (excitatory) pain pathways by 24 weeks' gestation, but descending (inhibitory) pathways appear immature until approximately 48 weeks' gestation [2, 3]. Here, the importance of reducing neonatal pain is discussed. Neonates have also many other immature pain responses, which expose them to a greater intensity of pain for a prolonged period of time [4]. Repeated invasive procedures occur routinely in neonates who require intensive care [5]. Therefore, ill full-term newborns and preterm infants are prone to significant painful stimuli at a time when the developing nervous system is notoriously sensitive to changes in sensory experience [6]. There is increasing clinical evidence that painful stimulations elicit specific behaviors, activate the somatosensory cortex, and stimulate neuroendocrine and physiological stress responses, thus leading to short- and long-term clinical consequences in newborns [7–10]. In preterm newborns, repeated and prolonged pain exposures alter their subsequent pain modulation, pain reactivity, neurodevelopment, and behavior [11, 12]. Full-term born children, who needed neonatal intensive care, showed enhanced perceptual sensitization to prolonged painful stimulation and hypoalgesia to brief heat pain stimuli at the age of 9–14 years [13]. On healthy full-term infants, only few early studies suggest an alteration in pain threshold because of pain experience [14–16]. Intercurrent illness, duration of care, and gestational age are some confounding factors that may alter the real role of painful stimuli or tissue damage in clinical cohorts [17]. Moreover, all comorbidities, different scales for pain assessment, and wide pharmacological and nonpharmacological approaches decrease the specificity of behavioral and physiological response to pain [18]. Therefore, identifying underlying mechanisms of pain-related consequences, along with reliable biomarkers to combine with pain scales, is of paramount importance for future researches.

A recent study in preterm babies showed a significant correlation between biomarkers of oxidative stress (OS) and

Table 1: Descriptive analysis of the population, reported by groups.

	10% oral glucose ($n = 69$)	Sensorial saturation ($n = 14$)	p
GA (weeks) mean (SD)	38 (1)	39 (1)	ns
BW (g) mean (SD)	3209 (477)	3365 (328)	ns
Gender absolute frequencies (%)			
M	41 (59.4)	9 (64.3)	ns
F	28 (40.6)	5 (35.7)	
AOPP presample (umol/l) mean (SD)	59.73 (49.50)	43.71 (37.21)	ns
TH presample (UCARR/l) mean (SD)	250.89 (107.48)	277.87 (78.09)	ns
ABC score at the beginning of heel prick median (IQ)	0 (0)	0 (0)	ns

pain score after tape removal during discontinuation of an indwelling central arterial or venous catheter [19]. Previously, Bellieni et al. demonstrated the same correlation also during a heel prick (HP) procedure [20]. Little is known about free radicals (FR) generation after painful procedures in newborns. Conversely, a lot of evidence reports the FR toxicity in the neonatal period [21]. The current study tested the hypothesis that OS could be elicited also in healthy full-term infants after their first HP. The correlation between OS, pain score, and grade was also tested. Our secondary aim was to analyze the variations in pain-related OS level between two different nonpharmacological approaches and its relation to newborns' sex.

2. Materials and Methods

A prospective research study was performed at the University Hospital of Siena between January 2014 and June 2014. All healthy term newborns with normal adaptation to extrauterine life, who underwent a routine HP to perform blood sampling for metabolic screening at 48 hours of life, were consecutively enrolled in the study. Exclusion criteria were congenital anomalies, chromosomopathies, newborns from mothers with drug abuse or any systemic analgesic treatment, night time samplings, newborns with any previous HP, need of multiple HP, and nonsufficient blood collection for a proper analysis. 83 newborn were included in the study (see Table 1 for details). Study protocol was approved by the local ethic board. An informed consent was collected from the parents before the enrollment.

After informed consent was obtained, newborns were consecutively enrolled and by lottery included into two different groups. The first group received 1 mL of oral 10% glucose solution (10% OG) before and during HP, a midwife instilled the solution in the newborn's mouth, and the sucking was induced with a finger enveloped by a glove. The second group received the sensorial saturation (SS) [22], in which mothers simultaneously attract their infant's attention by massaging the cheek, speaking to the baby gently, and administering 1 mL of 10% OG, the latter similarly to the previous group. The same assistant, who gave standardized information, trained each mother to the technique at least 4 hours before the sampling. In all cases in which mothers refused to perform the procedure at the time of the sampling (e.g., fear for the HP, stress feeling, impossibility to cope with sampling at the right time, or insecurity) 10% OG solution was administered and the infants were switched to the first group. The final population consisted of sixty-nine babies receiving 1 mL of 10% OG solution and fourteen babies receiving SS for analgesia.

Table 2: ABC scale.

Parameters	Score	
Acuteness of the first cry	Absent	0
	Present	2
Burst rhythmicity	Absent	0
	Present	2
Constancy of the crying intensity	Absent	0
	Intermediate	1
	Constant	2

2.1. Test Procedure. The sampling procedure was divided into 3 phases: (1) after heel cleansing and the prick, 100 μL of blood for the early OS measurements in a microtube (early-sample) was collected; (2) 12 drops of blood for the routine metabolic screening were taken; (3) 100 μL of blood for the late OS measurements in a microtube (late-sample) was collected. The average time that elapsed from the beginning to the end of the HP procedure was 1 minute and 30 seconds.

Immediately after the HP (during phase 1), pain was scored using the ABC pain scale by an independent assistant who was blinded to the study. This assistant received training for ABC pain scale use before the beginning of the study. To minimize bias due to interscore variability, a single assistant was used. The ABC scale is a validated and an easy to use pain scale to establish the grade of acute pain in both term and preterm babies [23]; it estimates the pitch of the first cry, the crying rhythmicity, and the constancy of the crying during the first 30 seconds after a definite painful stimuli; it ranges from no pain (ABC score = 0) to maximum pain (ABC score = 6) (see Table 2). This scale does not detect the effect of the squeezing.

Early- and late-samples were stored and analyzed to determine protein and lipid OS-induced injury by measuring blood levels of advanced oxidation protein products (AOPP) and total hydroperoxides (TH). These biomarkers provide information about the level of OS in newborns [21, 24]. In particular, TH are the intermediate oxidative products of

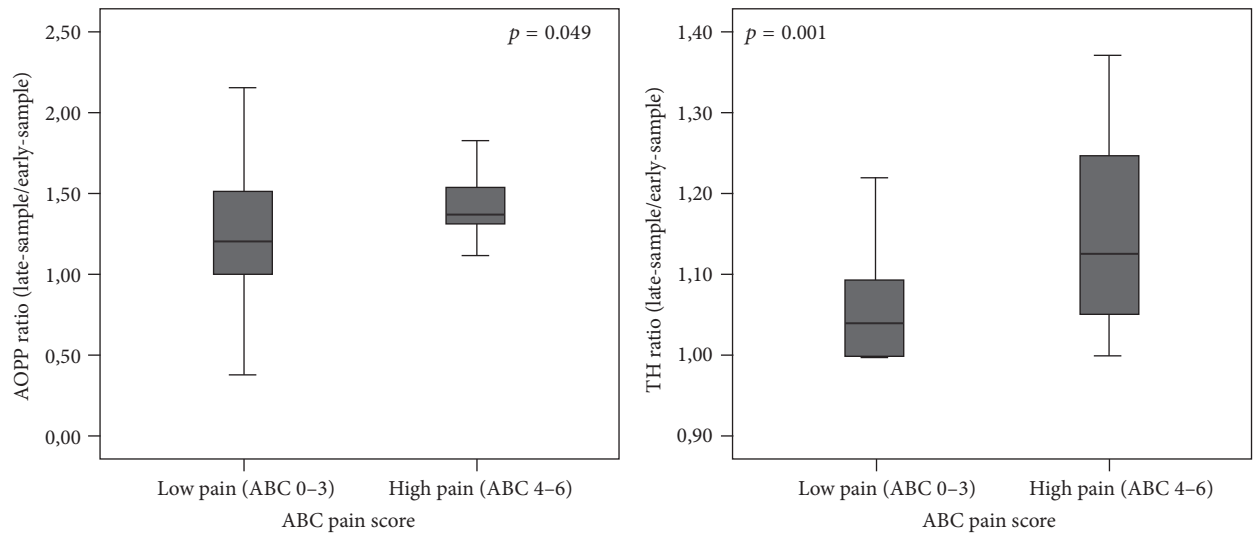

Figure 1: Mean of AOPP and TH ratio (late-sample/early-sample) depending on ABC pain scores.

lipids, protein, and amino acids, and therefore they represent a measure of overall OS. TH concentrations were evaluated using a d-ROMs Kit (Diacron srl, Italy) [24]. AOPP reflect oxidized plasma proteins and their concentrations were detected using spectrophotometry on a microplate reader. The changes (ratio) between the two time points for AOPP and TH were calculated to evaluate the increase in OS biomarkers before and after the HP.

2.2. Statistical Analysis. Analyses were performed using the SPSS v20 for Windows statistical package (SPSS Inc., Chicago, IL, USA). The paired t-test was used to verify the difference in the ABC pain score and in OS biomarkers between the two time points, while the unpaired t-test or the Mann–Whitney test was used, as appropriate, to evaluate pain in relation to gender, analgesia, and OS biomarkers. The Pearson test was used to verify the existence of a linear correlation between the pain ABC score and OS biomarkers levels.

3. Results

The descriptive analysis of the population is reported in Table 1.

Before the procedure, all the newborns were quiet and relaxed (mean ABC score before HP: 0.00 ± 0.00) and a significant increase in the ABC pain score was observed after HP (mean ABC score: 2.66 ± 1.81; $p = 0.000$). According to the type of analgesia, a significant decrease of the ABC pain score was found in infants treated with SS compared to that in those with 10% OG (respectively: 0.42±0.85 versus 3.11±1.61; $p < 0.0001$).

3.1. Pain-Related Oxidative Stress Results. After the HP AOPP blood levels significantly increased (AOPP, early-sample: 34.44 ± 16.70 versus AOPP late-sample: 42.91 ± 27.71; $p = 0.007$), while TH blood levels increased but not significantly (TH early-sample: 244.99 ± 85.16 versus TH late-sample: 257.07 ± 85.89; $p = 0.058$). Evaluating OS according to pain score, significantly higher levels of both AOPP and TH ratio were observed in high levels of pain (4–6) compared to low levels of pain (0–3) (resp., AOPP ratio 1.49 ± 0.35 versus 1.20 ± 0.45 $p = 0.049$; TH ratio 1.14 ± 0.11 versus 1.05 ± 0.06, $p = 0.001$, Figure 1). A significant linear correlation was found between pain scores and AOPP ratio and TH ratio (resp., $r = 0.453$, $p = 0.002$; $r = 0.423$, $p = 0.002$, Figure 2).

Significantly decreased AOPP ratio levels were also observed in newborns treated with SS compared to those without (resp., 1.38 ± 0.87 versus 1.76 ± 1.13, $p = 0.021$, Figure 3). No differences in TH ratio levels were found according to the type of analgesia (TH ratio in SS: 1.45 ± 1.93 versus TH ratio in 10% OG: 1.69 ± 2.30, $p = 0.694$).

3.2. Sex-Related Results. No difference in the ABC pain score was observed between males and females (resp., 2.60 ± 1.89 versus 2.75 ± 1.71; $p = 0.701$), independently of the type of analgesia. No sex differences were found in AOPP levels measured at the beginning and at the end of HP procedures. Males showed significant higher lipid peroxidation than females at the end of the HP (TH late-sample, resp., 277.50 ± 85.66 versus 222.76 ± 73.71, $p = 0.005$), which was also confirmed for higher degrees of pain (TH ratio, M: 1.18 ± 0.10 versus F: 1.07 ± 0.08, $p = 0.032$, Figure 4). The linear correlation existing between ABC pain score and OS markers reported by newborn's sex persisted only for males (AOPP ratio: $r = 0.583$, $p = 0.003$; TH ratio: $r = 0.506$, $p = 0.003$). AOPP ratio was significantly lower in males than in females after SS administration (0.96 ± 0.66 versus 1.87 ± 0.56, $p = 0.047$, Figure 5).

4. Discussion

The results of the present study were in agreement with the current literature that reports an association between a routine painful procedure and the subsequent FR production

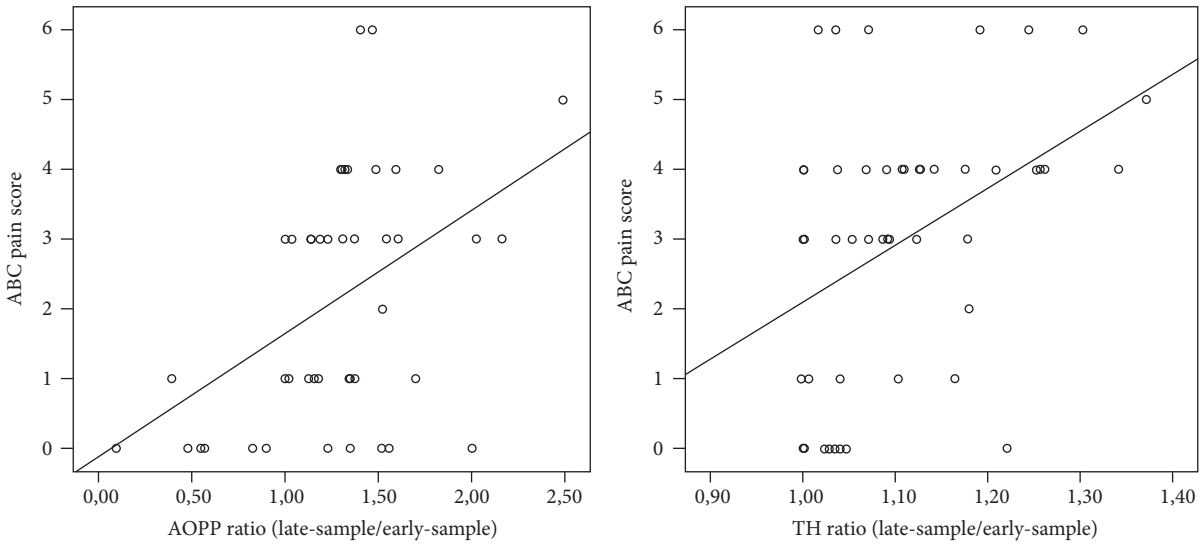

Figure 2: Linear correlation between OS biomarkers (AOPP and TH ratio, late-sample/early-sample) and ABC pain scores.

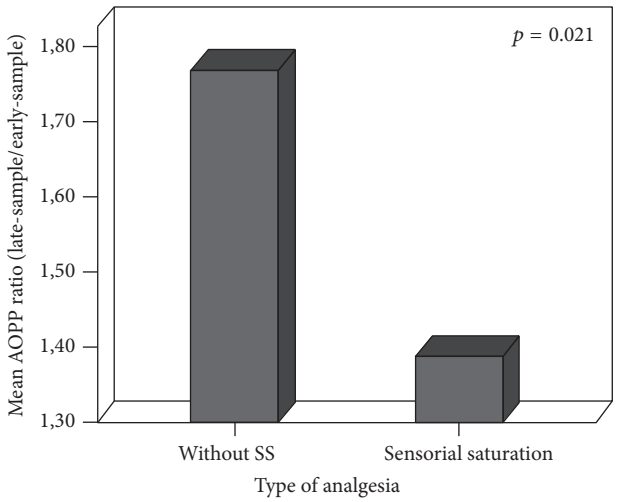

Figure 3: Mean of AOPP ratio (late-sample/early-sample) depending on type of analgesia.

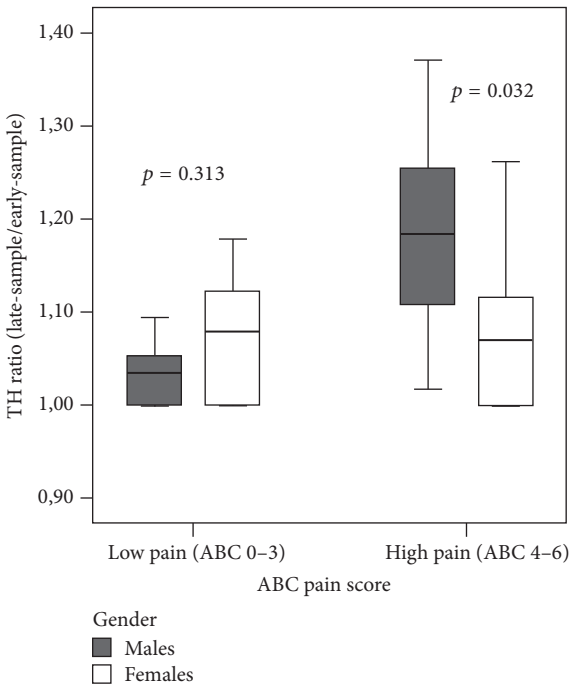

Figure 4: Mean values of TH ratio according to the level of pain, reported by gender.

in infants [19, 20]. This work also showed a linear correlation between OS biomarker levels and pain score. Slater et al. have found the same evidences using a different pain procedure (tape removal during discontinuation of an indwelling central catheter), biomarker (malondialdehyde), and pain scale (Premature Infant Pain Profile scale) [19], thus giving strength to the present finding. For the first time in literature, this pain-related stress response was evaluated in healthy full-term infants with no other pain experiences or confounding factors related to critically ill conditions or prematurity.

We believe that this physiologic reaction may play a crucial role in pain-related stress consequences. Many conditions of increased FR release occur in ill term babies who require intensive care and some long-term pain-related consequences were found also in these cohorts [13]. In healthy term infants, the hyperoxic challenge between intra- and extrauterine environment represents the main FR source and OS can consequently occur [25]. No data is available about term newborn's susceptibility to isolated increase in OS level after 48 hrs of life and literature lacks of follow-up studies in this kind of population. However, their less impaired antioxidant defenses and the rarity of the insults may generate an easier and faster neutralization of FR, thus preventing long-term consequences. Anyhow, FR production occurs soon after a painful stimulus also in term babies; thus OS could be finally

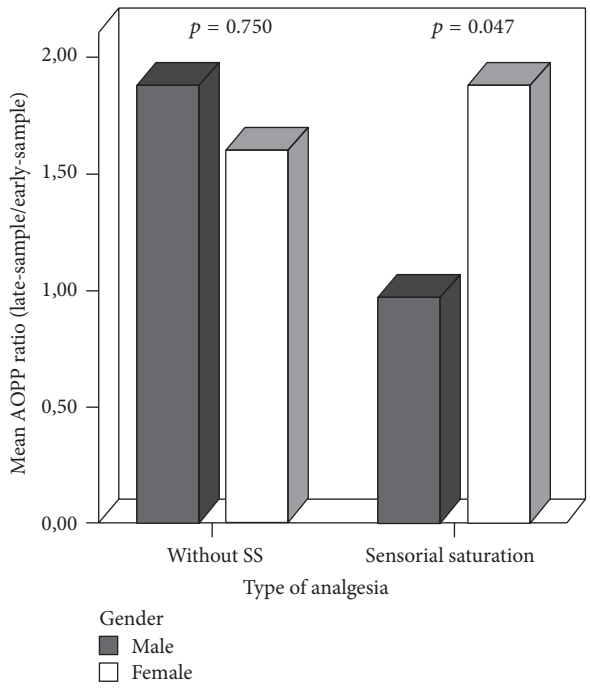

Figure 5: Gender differences in mean of AOPP ratio (late-sample/early-sample) depending on type of analgesia.

proposed as an independent physiological response to pain-related stress or tissue damage. We believe that glutamatergic stimuli can play a pivotal role in this correlation, but further studies are needed to deeply understand the underlying mechanisms. The significant increase only in AOPP blood concentrations following pain stimuli may reflect the role of proteins as the first line defense against oxidative injury, as previously suggested by our research group [26]. The present study also supports our previous findings showing a clear association between high pain degree and higher OS levels in newborns [20]. The latter association was found for both of the tested biomarkers; thus lipid and protein peroxidation could be involved during a stronger pain experience, but there are no data in literature supporting this theory.

In this study, a routine HP procedure causes pain-related stress in newborns, in both males and females. However, according to pain score, the entity of OS was sex-related, being higher in males than females. Males showed higher lipid peroxidation than females not only at the end of the prick, but also when the grade of pain was high. It was previously demonstrated that estrogen activates the membrane associated estrogen G protein-coupled receptor (GPR30), which influence the signaling cascades of opioid receptors. Therefore, sex differences in pain-related OS biomarkers level may be due to the organizational effects of steroids during a critical period of development [27]. The protective estrogen's effect against OS is also shown by several in vitro and in vivo experiments. Giordano et al. demonstrated in a recent animal study that estrogen modulates the cerebral expression of some antioxidant enzymes (paraoxonase 1 and paraoxonase 2, PON1 and PON2), thus increasing the resistance of female neurons to OS damage [28]. Though estrogen's neuroprotective effects are well known [29, 30], the absence of the estradiol's protective effect on cells from PON2 knockout ($PON2^{-/-}$) mice suggests that a major mechanism of estrogen-related neuroprotection may be represented by the induction of a specific antioxidant enzyme [28]. Gender disparities in OS levels and their relationship with the hormonal status were also underlined in another study from Minghetti et al. in which males showed higher lipid peroxidation and lower antioxidant capacity than females, contributing to a better understanding of the male disadvantage in OS injury [31]. The exact mechanisms through which these sex-related differences act are still largely unknown, but differential genotypes [32] and the disparity in the operation of the gonadal hormones- and opioid-modulating-pain circuits may be an explanation [33]. The downstream mechanisms involve cytosolic calcium increase [34], protein kinase A, protein kinase B, protein kinase C, phospholipase C, inositol triphosphate activation, neuronal membrane depolarization, and ROS accumulation [35].

In a previous work, we stated that SS is a noninvasive, easily performed, and reproducible technique for a complete analgesia during HP in neonates [36]. The present study showed that SS significantly reduces pain and its subsequent pain-related OS in full-term newborns, with significant lower AOPP levels in males than females. The evidence that a proper analgesic procedure could protect patients from pain-related OS is one of the main findings of our paper as this result may represent one of the most important future perspectives in this field. Unfortunately, the small sample size of SS avoids any kind of conclusion in this sense and further studies are required to confirm these data.

The present study has some limitations. First of all, the SS group number was small. Nevertheless, the disparity in numbers between groups did not affect the statistical power of the study. Second, the examiner was not blinded to the patient group, but since a complete blinded examination was impossible due to the visual difference between the two procedures, we involved a collaborator who was present during the procedure but not aware of the aims of the study, in order to limit the bias. Third, the choice of 10% oral glucose followed our previous study design in premature babies [20], in order to use a well-known tool that is also validated by the Italian Society of Neonatology guidelines [37]. There are some evidences that sweet solutions less than 18% are ineffective to reduce infant pain optimally [38]. Otherwise, studies conducted by Bellieni et al. reported the use of 10% OG as an effective analgesic treatment when associated with nonnutritive sucking [39]. We believe that research studies should be performed as homogeneously as possible; of course, with more concentrated glucose solutions for analgesia the average level of pain might be different but less physiologic and most babies would display a wide variety of responses.

5. Conclusions

For the first time in literature, the current study demonstrates that a relationship between degree of pain and OS exists also

in healthy full-term newborns at their first pain exposure. This evidence proposes a new insight into pain-related responses and contributes to suggesting a potential novel mechanism of damage after repeated and undermanaged painful experiences. The severity of OS injury is sex-related, higher in males than in females, thus indicating an innate susceptibility of males to OS. SS seems to be a promising procedure to reduce not only pain but also pain-related OS in newborns, giving chance to focusing on future researches in this field. Nevertheless more studies and a bigger sample size are needed to confirm our data.

Abbreviations

10% OG: 10% oral glucose solution
AOPP: Advanced oxidation protein products
FR: Free radicals
GPR30: G protein-coupled receptor
HP: Heel prick
OS: Oxidative stress
PON: Paraoxonase
$PON2^{-/-}$: Paraoxonase 2 knockout mice
SS: Sensorial saturation
TH: Total hydroperoxide.

Competing Interests

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

Acknowledgments

The authors thank EURAIBI foundation for its partial grant. Grants from the Tuscany Region and the Ministry of Health, General Directorate of Scientific and Technological Research for the 2011–2014 Project "Early Identification of Newborn at High Risk of Brain Injury through a Validated Biomarkers Profile and Specific Neuroimaging Patterns: A Follow-Up Study of Clinical and Neurodevelopmental Outcome in a Large Cohort Study" (RF-2009-1499651), are acknowledged.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 4569108, 10 pages
http://dx.doi.org/10.1155/2016/4569108

Research Article

Cord Blood Adiponectin and Visfatin Concentrations in relation to Oxidative Stress Markers in Neonates Exposed and Nonexposed *In Utero* to Tobacco Smoke

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Received 24 March 2016; Revised 6 June 2016; Accepted 16 June 2016

Academic Editor: Maria L. Tataranno

Aims. Maternal smoking is considered as a source of oxidative stress, which has been implicated to disrupted adipokines expression in adipose tissue. We examined the relationship between selected adipokines and markers of oxidative stress/antioxidant defence in the umbilical cord of neonates exposed and nonexposed *in utero* to tobacco smoke. *Methods.* Subjects including 85 healthy neonates (born to 41 smokers and 44 nonsmokers) were tested for adiponectin, visfatin, oxidized low density lipoprotein (ox-LDL), total oxidant capacity (TOC), and total antioxidant capacity (TAC). *Results.* Cord serum visfatin, ox-LDL, and TOC were significantly higher ($p < 0.001$) but adiponectin and TAC were lower ($p < 0.001$ and $p < 0.05$, resp.) in smoking group than in tobacco abstinents. In whole group of children (adjusted for smoking status, gender, and birth weight) adiponectin showed negative and visfatin positive correlations with ox-LDL. In the model estimated separately for smokers ox-LDL explained 36% of adiponectin and 35.5% of visfatin variance, while in the model of nonsmokers it explained 36.8% and 69.4%, respectively. *Conclusion.* Maternal smoking enhances oxidative status and depletes antioxidant potential in newborns. Lower level of adiponectin and higher visfatin concentration seem to be related with a less beneficial oxidative stress profile and higher level of lipid peroxidation in neonates exposed and nonexposed *in utero* to tobacco smoke.

1. Introduction

Oxidative stress (OS) has been associated with numerous adverse pregnancy outcomes and fetal disturbances [1, 2]. Due to overproduction of reactive oxygen (ROS) and reactive nitrogen species (RNS) as well as inadequate induction of antioxidant protection newborns are particularly susceptible to oxidative injury [3, 4]. The oxidized LDL, which is considered as a strong indicator of oxidative stress, can stimulate placental endothelial dysfunction and lead to intrauterine growth retardation (IUGR) and low birth weight [5]. Furthermore, it has been shown that oxidative stress in adipose tissue may also impair neonatal condition in consequence of disrupted adipokines expression [6]. Adipokines are bioactive molecules expressed and secreted mainly from adipose tissue playing critical roles in energy homeostasis and are regarded to be key regulators of insulin sensitivity [7, 8]. Moreover, adipokines are constitutively expressed by the fetoplacental unit and are present in cord blood suggesting an involvement of these molecules in fetal development [9]. Adiponectin is a 244-amino-acid protein with a molecular weight of 28 kDa which modulates many metabolic processes, especially the metabolism of carbohydrates and fatty acids, indirectly affecting insulin resistance [10, 11]. Described potentially anti-inflammatory and antioxidative properties of adiponectin make it extremely interesting as an active

factor in maintaining the balance between ROS generation and antioxidant defence production [12]. *In vitro* experiment showed that adiponectin protects against endothelial dysfunction and cellular disruption induced by oxidized low density lipoprotein (ox-LDL) [13]. In an *in vivo* study significantly lower levels of lipid peroxidation products in adiponectin-overexpressing transgenic mice compared with wild-type mice were documented [14]. Also in a few human studies this association was studied, but the results are ambiguous [6, 15–17]. Visfatin originally identified as pre-B-cell colony-enhancing factor (PBEF) is a 491-amino-acid protein with molecular weight 52 kDa which is thought to have insulin-mimetic metabolic effects [18]. Additionally visfatin activates cytokines release and phospholipid metabolism; therefore, increasingly, it is considered as a proinflammatory adipokine [19, 20]. Contrary to adiponectin, high level of visfatin and increased level of lipid peroxidation products were shown in obese subjects and in patients with unstable carotid and coronary atherosclerosis [21].

Maternal smoking has been considered as an additional source of oxidant stress in pregnant women and in newborns exposed *in utero*, leading to perinatal and postnatal health consequences [22]. Cigarette smoke rich in free radicals and oxidizing species depletes plasma antioxidant defence and causes maternal chronic inflammation, decreased trophoblast invasion, and impaired placental metabolism and function [23]. Nicotine through higher secretion of catecholamines increased lipolysis and levels of plasma-free fatty acids resulting in higher concentrations of oxidatively modified lipid products in mothers as well as fetoplacental units [23, 24]. Oxidative stress and inflammatory process appear to be the main factors of endothelial dysfunction in smoking pregnant women which may result in the progression of insulin resistance [25]. Due to disorders of insulin sensitivity, changes in concentrations of adiponectin and visfatin in neonates of smoking mothers might be a physiological response to vascular endothelial damage in placental vasculature [6, 26]. Although smoking has been associated with increased oxidative stress in a number of studies, the finding that oxidative markers are independently associated with adipokines status in newborn of smoking pregnant women is relatively new [6, 23, 27–29]. Thus, in the present study, we sought to assess the association between selected adipokines (adiponectin and visfatin) and oxidative stress markers (ox-LDL, TOC, total oxidant status, and OSI, oxidative stress index) in cord blood of neonates exposed and nonexposed *in utero* to tobacco smoke. Relationships between total antioxidant status (TAC) and adiponectin as well as visfatin were also studied.

2. Material and Methods

The study was performed in accordance with Helsinki Declaration for Human Research and the study protocol was approved by the Ethics Committee of the Institute of Mother and Child in Warsaw, Poland. All mothers of participating infants were informed of the study objectives and written consent was obtained for analysis of cord blood samples and linking results to the data collected from questionnaires.

2.1. Subjects. The study was conducted in the Institute of Mother and Child in Warsaw between January 2012 and March 2014. Cord blood samples were obtained from 86 healthy women at delivery following a pregnancy of 37–42 weeks. The study included a consecutive series of 41 active smokers who smoked minimum 5 cigarettes per day throughout their pregnancy and smoked minimum 2 years before conception and a series of 45 nonsmokers of similar age and age of gestation, who had never smoked and were not exposed to environmental tobacco smoke during their pregnancy (smoking spouse or coworkers). History of smoking was obtained by direct questioning of the pregnant women and the classification was confirmed by measurement of serum cotinine concentration in mothers and their children. A cut-off value of $\geq 13.7\ \mu g/L$ was used to separate smokers from nonsmokers according to Jarvis et al. [30] who selected the optimal value which, in relation to self-reported smoking, misclassified the fewest subjects. This observation was confirmed in two studies in big population of pregnant women (3550 and 1134 participants) [31, 32]. In our study the high correlation ($r = 0.9$, $p = 0.000$) between maternal and fetal cotinine level was observed; therefore we decided to use the same cut-off value.

Gestational age at birth was estimated by the last menstrual period and confirmed by ultrasound measurements. Inclusion criteria were uncomplicated singleton pregnancies, birth weight appropriate for the gestational age, Apgar score of fifth minute more than 9 points, gestational age between 37 and 42 weeks, and spontaneous labor. The exclusion criteria for the study were maternal diseases (preeclampsia, hypertension, diabetes mellitus, active hepatitis, renal and cardiovascular diseases, and inflammatory conditions), multiple pregnancy, birth defects detected during pregnancy, assisted reproduction, delivery complications, and prolonged labor. All of subjects remained on a mixed diet and lived in an urban area. None of the mothers reported drinking alcohol and using drugs or illicit substances.

Prepregnancy body mass index (BMI) was calculated using height and prepregnancy weight (BMI = kg/m^2). Newborn infants were evaluated in the first 24 hours of life. Neonatal length and weight were determined using a measuring board to the nearest 0.1 cm and a calibrated scale to the nearest 10 g.

2.2. Blood Sampling and Biochemical Analysis. Mixed venous and arterial umbilical cord blood samples (5 mL) were collected at the time of delivery from the umbilical vein before placental separation. In order to obtain serum, the blood was centrifuged at 2500 ×g, at 4°C for 10 minutes, and was stored in small portions for subsequent biochemical analysis.

Total oxidant capacity and total antioxidant capacity values were measured by colourimetric assay (Labor Diagnostika Nord GmbH&Co.KG, Nordhorn, Germany). The method is based on the enzymatic reaction of peroxides and peroxidases. Oxygen produced by this reaction oxidizes the chromogenic substrate tetramethylbenzidine (TMB), which changes its colour from colourless to blue. By addition of sulfuric acid the reaction cascade is stopped and the colour of mixture changes to yellow and can be detected

at 450 nm. Serum peroxide levels were calculated as the difference of the absorbance readings relating to the hydrogen peroxide standard curve. Antioxidants inhibit this reaction and can be detected analogously on the basis of the indirect proportionality of this inhibition reaction. The limit of detection was 0.06 mmol/L for TOC and 0.08 mmol/L for TAC, respectively. The intra- and interassay coefficients of variation were less than 4.9% and 7.33% for TOC and 2.5% and 3.33% for TAC, respectively. Oxidative stress index (OSI) was defined as the percentage ratio of TOC levels to TAC levels.

ox-LDL levels were determined by enzyme-linked immunosorbent assay (ELISA) (Immundiagnostik AG, Bensheim, Germany). The intra- and interassay coefficients of variability were found less than 5.7% and 9.0%. The limit of detection was 4.13 ng/mL.

Total adiponectin and visfatin concentrations were determined by immunoassay (ELISA) (ALPCO Diagnostics, Salem, USA; Ray Biotech Inc., Norcross, USA, resp.). The intra- and interassay coefficients of variability were found less than 5.0% and 5.3% for adiponectin and 10% and 15.0% for visfatin. The limit of detection was 0.019 ng/mL for adiponectin and 0.778 ng/mL for visfatin, respectively.

Cotinine levels were evaluated by immunoenzymatic method using a commercially available kit (Calbiotech Inc., Spring Valley, CA, USA). The detection limit was 1.0 ng/mL.

2.3. Statistical Analysis. All of the statistical analyses were performed using SPSS statistical software version 17.1 (SPSS Inc., Chicago, IL, USA). The normality of data was tested using Kolmogorov-Smirnov test. The results were presented as means with standard deviation (SD) for normally distributed data or median with interquartile range (25th–75th percentiles) for nonnormally distributed variables (gestational age, Apgar score, number of cigarettes/day, and time of smoking before conception). In the smoking and nonsmoking groups, the baseline characteristics were compared using the Student t-test or Mann-Whitney U test depending on the assumptions. The Chi-squared test was used for comparing nominal variables. Correlations between the plasma adipokines (adiponectin and visfatin) and other normally distributed variables were assessed by Pearson's coefficient of correlation. Stepwise linear regression analysis was performed to study the relationship between adipokines level and oxidative stress markers. Independent variables were presented in the order of importance. Results were presented as the value of B unstandardized regression coefficient with 95% confidence interval and change in R-squared coefficient after each variable was entered. Models were estimated separately for smokers and nonsmokers as well as for total group. Three oxidative stress markers (ox-LDL, TOC, and TAC) were main independent variables. Regression models were adjusted for child gender and birth weight (all models: total, smokers, and nonsmokers group), for smoking status (total group), and for number of cigarettes (smokers). A p value <0.05 was considered statistically significant.

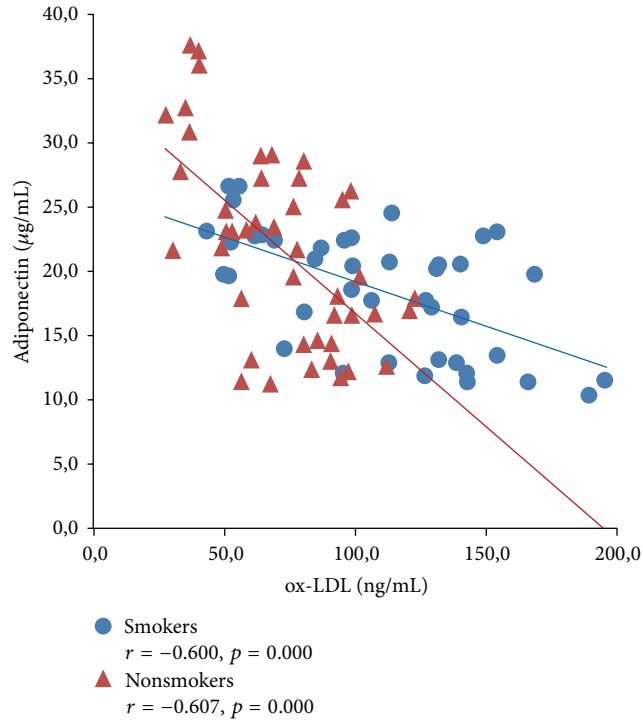

Figure 1: Correlations between cord serum adiponectin and ox-LDL levels in newborns of smokers and nonsmokers.

3. Results

The clinical characteristics of the study sample and biochemical findings of the subjects are given in Table 1. Nonsignificant differences were noted between the two groups with respect to clinical characteristics except for cigarette smoking habits. Birth weights and body length of the smokers' newborns were found to be lower than those of nonsmokers; however, in the case of length, there were no statistical differences. According to biochemical markers, the newborns of smoking mothers had significantly higher concentrations of serum visfatin, ox-LDL, TOC, and OSI ($p < 0.001$) but lower adiponectin ($p < 0.05$) and TAC ($p < 0.001$) levels compared with newborns of nonsmoking women.

In whole group we observed significant inverse correlation between studied adipokines ($p < 0.001$). Cord serum adiponectin was negatively associated with ox-LDL, TOC, and OSI levels and smoking status and positively correlated with TAC concentration and birth weight and length. On the contrary, cord serum visfatin was related positively with markers of oxidative stress (ox-LDL, TOC, and OSI) and negatively with TAC, smoking status, and anthropometric parameters (Table 2).

Figures 1–4 expressed correlation between studied adipokine and oxidative stress markers separately for smokers and tobacco abstinent group. There was significant inverse relationship between adiponectin and ox-LDL as well as OSI level in both studied groups (Figures 1 and 2). Cord serum visfatin correlated positively with ox-LDL concentration in newborn of smoking and nonsmoking

Table 1: Clinical characteristics and biochemical measurements of the study subjects ($N = 85$).

	Nonsmokers $N = 44$	Smokers $N = 41$	p value
Newborn			
[c]Male/female (%)	56.8/43.2	53.7/46.3	0.470
[b]Gestational age (week)	39 (39-40)	39 (38.5–40)	0.086
[b]Apgar score (5th min)	10 (10-10)	10 (10-10)	0.146
[a]Birth weight (g)	3511.4 ± 426.4	3123.7 ± 431.3	0.000
[a]Birth length (cm)	55.6 ± 2.7	54.4 ± 2.8	0.058
[a]Adiponectin (μg/mL)	21.8 ± 7.4	18.6 ± 4.8	0.019
[a]Visfatin (ng/mL)	9.0 ± 2.3	14.4 ± 3.5	0.000
[a]ox-LDL (ng/mL)	71.7 ± 25.4	108.9 ± 41.1	0.000
[a]TOC (mmol/L)	0.195 ± 0.102	0.352 ± 0.164	0.000
[a]TAC (mmol/L)	1.694 ± 0.382	1.189 ± 0.277	0.000
[a]OSI	0.128 ± 0.080	0.323 ± 0.195	0.000
[a]Cotinine (μg/L)	0	81.5 ± 29.3	—
Mother			
[a]Age (years)	28.9 ± 4.7	28.2 ± 4.4	0.465
[a]Maternal weight (kg)	64.8 ± 5.5	63.8 ± 5.2	0.398
[a]Maternal height (cm)	164.6 ± 4.5	165.1 ± 4.9	0.651
[a]Pregravid BMI (kg/m^2)	23.9 ± 1.4	23.4 ± 1.43	0.095
[b]Number of cigarettes/day	0	10 [5–10]	—
[a]Cotinine (μg/L)	0	89.2 ± 30.4	—

[a]Values are means ± standard deviation (SD), [b]values are median and interquartile range (25th–75th percentiles), and [c]values are percentage. ox-LDL: oxidized low density lipoprotein; TOC: total oxidant capacity; TAC: total antioxidant capacity; OSI: oxidative stress index; BMI: body mass index.

mothers, while association with OSI level was found only in tobacco abstinent group (Figures 3 and 4).

In multiple regression analysis performed in whole group (adjusted for smoking status, child gender, and birth weight) with ox-LDL, TOC, and TAC as independent variables, those associated with cord serum adiponectin were ox-LDL and birth weight; those associated with cord serum visfatin were ox-LDL and smoking status. In the model estimated separately for smokers (adjusted for child gender, birth weight, and number of cigarettes/day) the highest impact of the serum ox-LDL was indicated for adiponectin as well as visfatin levels. In these subjects ox-LDL explained 36% of adiponectin and 35.5% of visfatin variance. In the model of nonsmokers (adjusted for child gender and birth weight) ox-LDL, birth weight and TOC were of significant importance for adiponectin and ox-LDL for visfatin concentration. In those subjects ox-LDL explained 36.8% and 69.4% of adiponectin and visfatin variance, respectively (Table 3).

4. Discussion

The results of this study demonstrate a significant relationship between cord serum concentrations of adipokines and oxidative stress markers and confirmed the negative effect of tobacco smoking on these parameters. Pregnancy is a physiological state accompanied by high metabolic demand, changed glucose and lipid profile, and increased susceptibility to oxidative stress [23, 33]. In normal pregnancies, increased production of reactive oxygen species depletes antioxidant status in mothers, leading to oxidative stress in fetus, which may contribute to birth outcomes such as preterm birth, low birth weight, and developmental problems [1, 6, 34]. In women smoking during pregnancy, the risk of oxidative damage not only depends on the amount of physiologically enhanced ROS but can be affected also by intoxication with tobacco smoke radicals. Pregnant women who smoked had higher total oxidant status and concentration of lipid peroxidation products compared with tobacco abstinent [22, 23, 27, 28]. Exposure to cigarette smoke may also reduce the level of particular antioxidants and in consequence decreases total antioxidant capacity in the course of gestation [27, 35, 36].

There have not been many studies conducted concerning effect of maternal smoking on oxidant-antioxidant status in cord blood of newborns. No differences on MDA (malondialdehyde, breakdown product of lipid peroxidation) and antioxidant parameters as well as reduced level of total antioxidant capacity and increased levels of lipid peroxide products in cord blood of smokers were found [22, 28, 29, 35–37]. Because of the rapid metabolism and wide variation of MDA, we suggested that plasma ox-LDL level or TOC level could be a better marker of ROS activity in the fetus. In the presented study, conducted in group with confirmation of smoking status by cotinine levels, ox-LDL concentrations were higher in newborns of smoking mothers as compared with nonsmoking group. Increased susceptibility of LDL to oxidation in smoking group may be due to several reasons. One of them can be the raised

Table 2: The correlations between adipokines (adiponectin, visfatin) and clinical/biochemical parameters in studied subjects ($N = 85$).

	Adiponectin		Visfatin	
	r	p value	r	p value
Gender	0.096	0.384	0.028	0.799
Gestational age	0.203	0.062	−0.147	0.180
Birth weight	0.444	0.000	−0.377	0.000
Birth length	0.322	0.003	−0.223	0.040
Apgar	−0.039	0.720	−0.061	0.577
Adiponectin	—	—	−0.445	0.000
Visfatin	−0.445	0.000	—	—
ox-LDL	−0.582	0.000	0.758	0.000
TOC	−0.439	0.000	0.558	0.000
TAC	0.457	0.000	−0.586	0.000
OSI	−0.403	0.000	0.570	0.000
Newborn cotinine	−0.287	0.008	0.658	0.000
Maternal age	0.154	0.160	−0.050	0.651
Maternal weight	0.036	0.745	0.033	0.765
Maternal height	0.000	0.999	0.109	0.323
Maternal BMI	0.056	0.608	−0.058	0.600
Smoking status (no = 0; yes = 1)	−0.251	0.021	0.683	0.000

ox-LDL: oxidized low density lipoprotein; TOC: total oxidant capacity; TAC: total antioxidant capacity; OSI: oxidative stress index; BMI: body mass index.

Table 3: Multivariable regressions of adiponectin and visfatin with markers of oxidative status (ox-LDL, TOC, and TAC) as independent variables.

Dependent and independent variables	B	95% CI	p value	ΔR^2
Dependent variable: adiponectin, $N = 85$*				
ox-LDL	−0.081	−0.112/−0.049	0.000	0.339
Birth weight	0.003	0.001/0.006	0.009	0.053
Dependent variable: visfatin, $N = 85$*				
ox-LDL	0.057	0.043/0.0711	0.000	0.574
Smoking status	−3.243	−4.314/−2.171	0.000	0.130
Dependent variable: adiponectin, $N = 41$ (smokers)**				
ox-LDL	−0.069	−0.099/−0.039	0.000	0.360
Dependent variable: visfatin, $N = 41$ (smokers)**				
ox-LDL	0.050	0.028/0.072	0.000	0.355
Dependent variable: adiponectin, $N = 44$ (nonsmokers)***				
ox-LDL	−0.110	−0.190/−0.031	0.008	0.368
Birth weight	0.006	0.002/0.010	0.008	0.083
TOC	−20.528	−39.604/−1.451	0.036	0.058
Dependent variable: visfatin, $N = 44$ (nonsmokers)***				
ox-LDL	0.074	0.059/0.089	0.000	0.694

*Adjusted for infant gender and birth weight and mother smoking status.
**Adjusted for infant gender and birth weight and number of cigarettes.
***Adjusted for infant gender and birth weight.

amount of oxidizable substrate. Smokers have higher level of total cholesterol, LDL-cholesterol, and triglycerides [38, 39]. In addition, the pregnancy intensifies these changes [1]. As mentioned in Introduction, nicotine through higher secretion of catecholamines leads to intensification of lipolysis and increased free fatty acids, particularly PUFA (polyunsaturated fatty acids), a substrate for lipid oxidation [23, 24, 40]. Both phases of cigarette smoke (CS) contain high concentrations of oxidants and free radicals (estimated at 1×10^{15} radicals per puff) like superoxide anion ($O_2^{\bullet -}$), reactive hydroxyl radical ($HO^{\bullet}$), H_2O_2, and nitric oxide ($NO^{\bullet}$) which may directly influence the oxidation of serum LDL. A fast reaction between nitric oxide and superoxide anion produced the relatively long-lived potent prooxidant peroxynitrite anion ($ONOO^-$), which is highly toxic and initiates lipid peroxidation [41]. Yamaguchi et al. [42] demonstrated *in vivo* and *in vitro* studies that oxidative modification of LDL induced by cigarette smoke can be mediated via

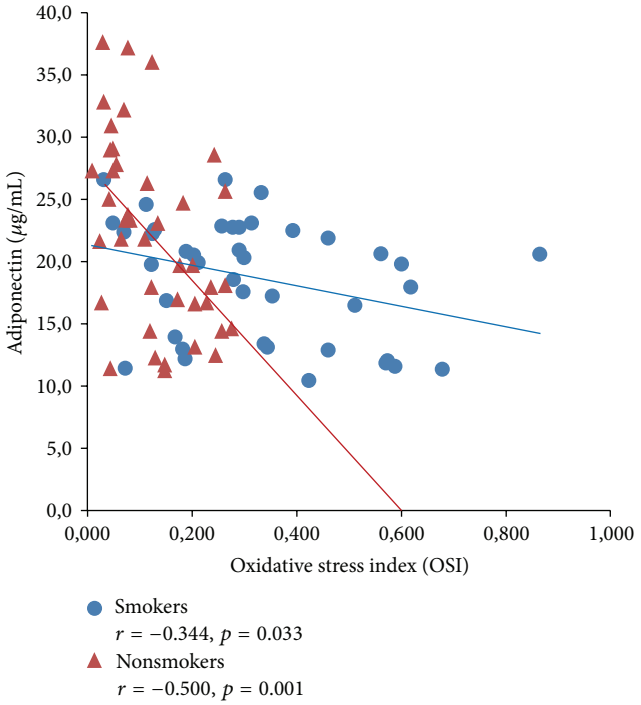

Figure 2: Correlations between cord serum adiponectin and OSI levels in newborns of smokers and nonsmokers.

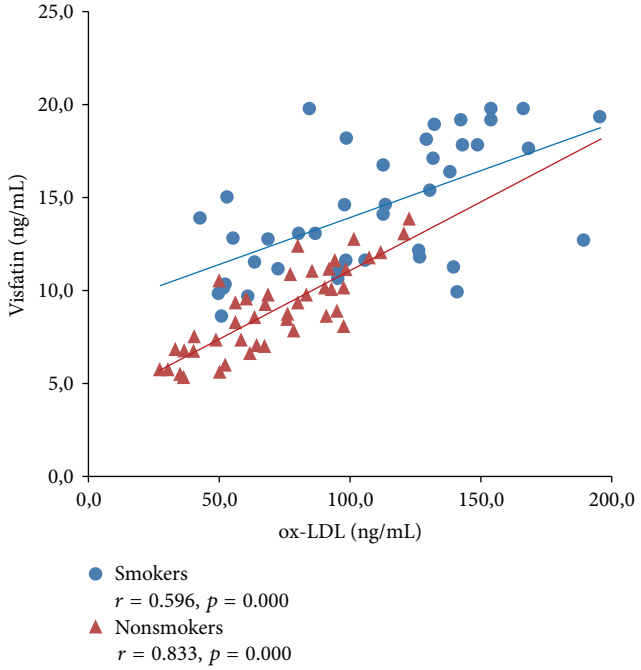

Figure 3: Correlations between cord serum visfatin and ox-LDL levels in newborns of smokers and nonsmokers.

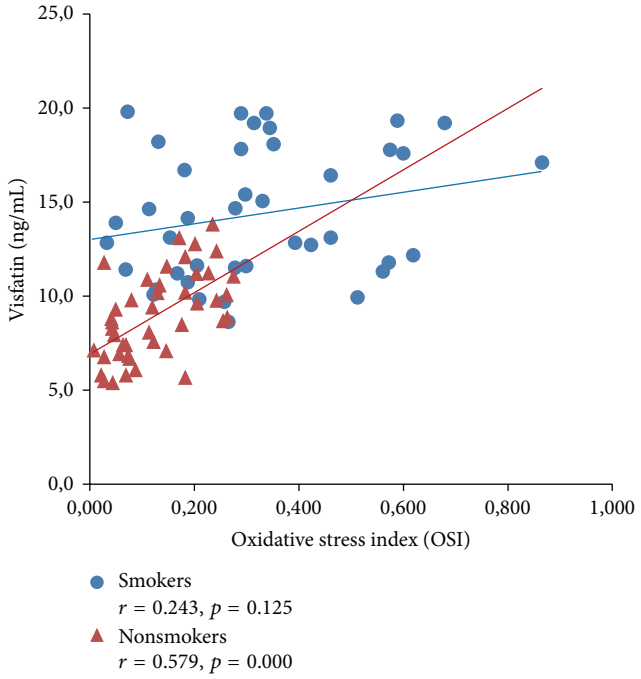

Figure 4: Correlations between cord serum visfatin and OSI levels in newborns of smokers and nonsmokers.

peroxynitrite (measured by formation of 3-nitrotyrosine in plasma LDL). Some authors postulated mechanisms by which LDL becomes oxidized by CS with actions of several enzymes within the artery wall [42–45]. Increased expression of lipoxygenase which can be considered as a potential protein to oxidize LDL was observed in smokers [43]. Cigarette smoke and thiol-reactive substances found in CS induce $O_2^{\bullet-}$ and H_2O_2 production by activating NADPH oxidase in endothelial cells [44]. Smokers have also higher level of myeloperoxidase (MPO) which can be regarded as a "radical starter," as it produces hypochlorous acid and in consequence might oxidize lipoproteins [45, 46]. Another important factor affecting the amount of ox-LDL may be insufficient concentrations of antioxidants. Cigarette smoking reduces the level of certain nonenzymatic antioxidants (glutathione, vitamins E and C, and β-carotene) as well as activity of free radical scavenging enzyme (glutathione peroxidase and superoxide dismutase) [22, 23, 27, 37]. Particularly vitamin E shows high effectiveness in preventing oxidation of LDL by cigarette smoke which was confirmed by other investigators [42, 45]. In our previous study we have shown the negative effect of maternal smoking on concentration of vitamin E, vitamin A, and β-carotene in umbilical cord blood [27].

In the present study we also evaluated the global oxidant/antioxidant status in newborns of smoking and nonsmoking mothers. For total oxidant capacity determination we used method described by Tatzber et al. [46] based on oxidation of TMB by horseradish peroxidase (HRP)/H_2O_2. This type of reaction is the basic mechanism for detecting both peroxide levels and peroxidase activity in blood serum. In the case of measurement of total peroxides HRP was added, while excess hydrogen peroxide was added for determination of enzyme activity [46]. Similar to Aycicek and Ipek [28] our findings showed significantly higher TOC and OSI levels in neonates exposed *in utero* to tobacco smoke compared with tobacco abstinent offspring. Because the above is accompanied with decreased levels of total

antioxidant capacity, we speculate the severity of oxidative stress in this group.

It has been recently shown that level of oxidation products could be associated with some adipokine concentrations. These molecules participating in the regulation of glucose and lipids metabolism play a major role to promote optimal fetal development [7–9]. Increased serum adiponectin was assumed to be compensatory for early vascular endothelial damage and may have anti-inflammatory and antioxidative properties [6, 17]. On the contrary, visfatin can serve as a marker of insulin resistance with proinflammatory and prooxidant condition [8, 19, 47, 48]. This is particularly important due to the fact that smoking even in small amounts may cause endothelial dysfunction and transient uteroplacental vasoconstriction [6, 49]. Abnormal development of the placental vasculature leads to placental insufficiency which could compromise nutrient and gas transfer to the fetus and cause fetal growth restriction and low birth [49, 50]. There are only a few studies which explored the effect of maternal tobacco smoking on the concentrations of adiponectin and visfatin in cord blood [26, 51–53]. Similar to Pardo et al. [51] and our previous results [53], in the presented study adiponectin concentrations were significantly lower in umbilical cord blood of the smokers than in tobacco abstinent ones. On the contrary, Fang et al. [52] show no differences in adiponectin levels in cord blood between smokers and nonsmokers, but the study group of those authors had only 18 smokers based on self-reports and smoking means 5 cigarettes per day. In parallel to other investigators, we observed the positive correlation between adiponectin and birth weight [51]. López-Bermejo et al. [26] found increased cord serum visfatin in smaller babies of smoking mothers who smoked ten and more cigarettes per day. These authors showed that in the whole group cord serum visfatin was inversely associated with birth weight. Our results confirmed this observation.

To our knowledge, this is the first study to explore the effect of maternal smoking status on relations between oxidative stress markers and adiponectin as well as visfatin levels in newborns. Contrary to the study of Makedou et al. [6], we found negative correlations between adiponectin and ox-LDL in whole group of children (adjusted for smoking status, gender, and birth weight) as well as separately in smoking and nonsmoking group. Thus, Makadou et al. [6] conducted their research in a group of pregnant women, while fetal adipose tissue, rather than maternal production, may be a major source of adiponectin production in newborns [53]. Similar to our results, evidences from both humans and animals studies support the inverse correlations between oxidative stress and adiponectin level [15–17, 54]. Moreover, we observed negative effect of TOC and OSI on adiponectin level of our patients. These results, in conjunction with the positive relation with TAC, may confirm antioxidative role of this protein. In accordance with the findings of other authors, in our study visfatin correlated positively with ox-LDL, TOC, and OSI and negatively with TAC, which can suggest its prooxidative capacity [55, 56]. The lower level of visfatin coexisting with higher levels of TOC, OSI, and lipid hydroperoxide in women with premenstrual syndrome was observed [47]. The current study reported that in both the smoking and nonsmoking groups ox-LDL was a main predictor of adiponectin and visfatin concentrations in cord blood.

Recent study indicated that ox-LDL promotes the expression of visfatin through its activation of the endoplasmic reticulum (ER) stress in endothelial cells and macrophages [55]. Relationship between adiponectin and visfatin concentrations and ox-LDL production dependent on inflammation status was also observed [17, 21, 57]. As we described above, placenta is a site of active oxygen metabolism continuously generating oxidative stress. The placenta is also particularly susceptible to ER stress leading to poor placental development. Moreover, the activation of ER stress has been shown to induce inflammatory pathways [2, 24]. The adverse effect of nicotine in the placenta mediated through increased ER stress, inflammation, and oxidative stress was documented [24]. Therefore it is possible that tobacco smoking during pregnancy may affect adipokine levels by increased production of ox-LDL. Due to the transition from hypoxic intrauterine environment to extrauterine life, oxidative stress appears after birth in all newborns, regardless of the exposure to the tobacco smoke *in utero* [3]. Thus, as shown by us antioxidative property of adiponectin and prooxidant tendency of visfatin in cord blood cells of nonsmokers seem to be important for maintaining the oxidative-antioxidative balance in newborns.

The present study has some limitations. First, our sample is relatively of small size. However, both studied groups were similar for maternal BMI, age of gestation, type of delivery, and fetal gender, which are recognized as important factors for adipokine and oxidative stress marker levels [18, 20, 58–60]. Second, we cannot achieve the percentage of smokers representative of a normal pregnant population with the enrolment method we used. However, we were able to compare groups that did not differ in terms of their size and basic characteristics (listed above). The applied research scheme is not typical for a case-control study with retrospective assumptions [61]. This is a prospective comparison of cohorts exposed and not exposed to the effect of one factor, in this case tobacco smoking. Third, we did not measure any inflammatory markers in cord blood, but our study group consists of newborns of healthy pregnant women without inflammation process confirmed by negative c-reactive protein. Fourth, levels of cord serum lipids were not determined, but it is known for long time that maternal smoking during pregnancy markedly affects lipid metabolism in the fetus [39].

In conclusion, our data imply that maternal smoking enhances oxidative status and depletes antioxidant potential in newborns. Lower level of adiponectin and higher level of visfatin seem to be related with a less beneficial oxidative stress profile and higher level of lipid peroxidation in neonates exposed and nonexposed *in utero* to tobacco smoke.

Competing Interests

The authors declare that they have no competing interests.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 8651820, 7 pages
http://dx.doi.org/10.1155/2016/8651820

Review Article

"Cumulative Stress": The Effects of Maternal and Neonatal Oxidative Stress and Oxidative Stress-Inducible Genes on Programming of Atopy

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Received 25 January 2016; Revised 27 May 2016; Accepted 22 June 2016

Academic Editor: Daniela Giustarini

Although extensive epidemiological and laboratory studies have been performed to identify the environmental and immunological causes of atopy, genetic predisposition seems to be the biggest risk factor for allergic diseases. The onset of atopic diseases may be the result of heritable changes of gene expression, without any alteration in DNA sequences occurring in response to early environmental stimuli. Findings suggest that the establishment of a peculiar epigenetic pattern may also be generated by oxidative stress (OS) and perpetuated by the activation of OS-related genes. Analyzing the role of maternal and neonatal oxidative stress and oxidative stress-inducible genes, the purpose of this review was to summarize what is known about the relationship between maternal and neonatal OS-related genes and the development of atopic diseases.

1. Introduction

Allergic diseases including atopic dermatitis, allergic rhinitis, and asthma are some of the most common chronic diseases in the world [1]. Although extensive epidemiological and laboratory studies have been performed to identify the environmental and immunological causes of atopy, genetic predisposition seems to be the biggest risk factor for allergic diseases [2, 3]. It is known that several foetal adaptive responses to environmental factors are mediated by epigenetic changes which, impacting early-life morbidity, may exercise effects on the immune system, lung development, airway remodelling, allergen predisposition, and atopic and nonatopic inflammation, through numerous pathways [2, 4]. In particular, the onset of atopic diseases may be the result of heritable changes of gene expression, without any alteration in DNA sequences, occurring in response to early (prenatal) or later (perinatal) environmental stimuli [4]. Findings suggest that the establishment of a peculiar epigenetic pattern may also be generated by oxidative stress (OS) and perpetuated by the activation of OS-related genes [5]. Reactive oxygen species (ROS), known to be important cell-signalling molecules [6], could, in fact, set up a positive-feedback loop that induces and perpetuates atopic injury. OS, also influencing T-cell signal transduction and gene expression [7], modulates T-cell polarization toward a T helper- (Th-) 2 cellular subset [8] which might be, in turn, a further source of ROS.

OS is a specific setting also occurring in normal events such as pregnancy and birth.

Pregnancy is a physiological period associated with enhanced OS related to high metabolic turnover and elevated tissue oxygen requirements [9]. During pregnancy, increased oxygen demand augments the rate of production of ROS, and women, even during normal pregnancies, experience elevated serum OS levels [9]. Increased OS levels and reduced antioxidative capacities may contribute to the pathogenesis of perinatal [10, 11] and postnatal disorders [12, 13], such as atopic diseases [14, 15], as newborns are more prone to OS than individuals later in life [16]. Moreover, it has also been reported that OS-related maternal genetics, independently of

transmission of specific alleles, may influence a child's atopic risk beginning in the uterus [17, 18].

Also during pregnancy, newborns are also continually exposed to elevated levels of ROS. At birth, newborns transit from a hypoxic intrauterine to a normoxic extrauterine environment. This increased OS further favours neonatal morbidity, also including atopy [14].

Analyzing the role of maternal and neonatal oxidative stress and oxidative stress-inducible genes, the purpose of this review was to summarize what is known about the relationship between maternal and neonatal OS-related genes and the development of atopic diseases.

2. Cumulative Effects of Maternal and Neonatal Oxidative Stress on the Immune System

It is well known that OS occurs early in pregnancy and continues in the postnatal period [12]. In particular, pregnancy is associated with enhanced OS related to high metabolic turnover and elevated tissue oxygen requirements [34]. On the other hand, newborns, exhibiting an accelerated production of free radicals and limited antioxidant protection, are also constitutively vulnerable to OS. Therefore, during pregnancy and intrauterine life, many factors such as hypoxia, inflammation, and infections can easily induce overproduction of free radicals (FRs) [11], exceeding the capacity of defensive mechanisms to neutralize them. The release of FRs leads to the oxidation of lipids, proteins, and polysaccharides and to DNA modifications [7–9, 19] which, in turn, increase the susceptibility of rapidly growing tissues to damage [35], as well as modulation of the immune system [10, 36].

With regard to the immune system, different immunological responses to ROS production have been reported, depending on environmental oxidative status. While normal ROS amounts have been shown to be important for T-cell function and for adequate, beneficial antimicrobial protection [37], high ROS concentrations can negatively modulate immune system responses leading to inhibited T-cell proliferation [37] and to hyporesponsivity to exogenous and/or endogenous activating stimuli [9]. In particular, OS plays a critical role as a secondary messenger in the initiation and amplification of signalling, miming antigenic effects. The antigen receptors are themselves OS-generating enzymes, contributing further to enhancing the cellular "oxidative burst" against exogenous pathogens as well as neighbouring cells [10], causing autoinflammatory and/or allergic diseases [17, 38].

Moreover, it has been suggested that OS, leading to secretion of a variety of proinflammatory cytokines and chemokines [38], elicits a polarized immune response which is closely associated with a breakdown in immune tolerance [39]. In particular, when immunoglobulin- (Ig-) E binds to specific membrane receptors, peripheral blood is activated to produce more superoxide and hydrogen peroxide (H_2O_2), contributing to elevated environmental OS and sterile inflammation [40] in upper and lower airways [41–43], and in the skin [44]. Furthermore, immune cells, because of higher production of ROS, are themselves particularly sensitive to OS, creating a vicious circle for the production of proinflammatory mediators and supporting a prooxidant status [9]. The activation of both the redox-sensitive transcription factor nuclear factor-kappa B (NF-κB) and activator protein- (AP-) 1 and the release of proinflammatory proteins involved in immune response (e.g., interleukin- (IL-) 1, IL-6, tumour necrosis factor- (TNF-) α, and interferon- (INF-) α, as well as H_2O_2) are critical events in immunity, promoting stimulus-specific genes expression [17, 38]. These findings confirm the evidence that foetal immune response is prenatally influenced [45] and that the activation of maternal and neonatal OS-inducible genes may influence a child's atopic risk, early in the uterus [46, 47] (Tables 1, 2, and 3).

3. Epigenetic Effects on Atopic Predisposition

Epigenetics refers to information that is heritable through cell division. Epigenetic mechanisms include DNA methylation, chromatin remodelling and noncoding RNA, histone variations, and posttranslational histone modifications [48]. Epigenetic alterations can occur prenatally, perinatally, and later in life during developmental stages, with unique susceptibility to the effects of environmental exposures [48]. Uterine life is the most critical time in developmental programming; when negative environmental exposures occur, the foetal structure and its functions are irreversibly modified and subjects can be predisposed to several diseases, including allergy [49]. T-cellular differentiation into Th1, Th2, Th17, and Treg is influenced by changes in DNA/histone methylation and/or histone acetylation in naive T-cells and in cytokine promoter regions. Thus, the well-known correlation between epigenetic modifications and Th lineage has led to hypothesize that triggers inhibit Th1 and T regulatory cell differentiation, promote Th2-response, and could favour the risk of atopic predisposition [50]. Although the mechanism of this process is not fully understood, environmental changes, such as microbial burden [51], dietary changes [52, 53], and environmental pollutants [54], appear essential to initiate the cascade of epigenetic modifications that stabilize Th2 gene expression [55]. It is also likely that effects of environmental triggers are also mediated by oxidative stress which, by NF-κB-induced expression of proinflammatory cytokines and methylation-mediated changing, can induce histone modifications and chromatin remodelling of proinflammatory genes, exercising further implications on foetal immune programming, atopic predisposition, and increased IgE production following allergen sensitization [49, 56].

4. Cumulative Effects of Oxidative Stress-Inducible Genes on the Immune System

Genetic linkage and transmission alleles analyses have highlighted the important role of oxidative stress-inducible genes on the neonatal immune system response [26, 57]. In particular, the concurrent presence of higher ROS levels and antigenic exposure has been reported to alter the methylation of T helper genes [58]. All these changes impair the differentiation

Table 1: Oxidative stress-inducible genes and allergic asthma.

Gene	Clinical relevance	References
Glutathione S-transferases M1 (GSTM1) and P1 (GSTP1)	GSTs conjugate endogenous byproducts of OS with glutathione, enabling rapid elimination and thus defending tissues against oxidant damage; common polymorphisms exist in genes coding for various GSTs including glutathione S-transferases M1 (GSTM1) and P1 (GSTP1)	[19]
Antioxidant defence enzymes (ADE) Glutamate cysteine ligase (GCLM) Glutathione peroxidase (GPX1) Myeloperoxidase (MPO) NADPH oxidase (CYBA, p22phox subunit) NAD(P)H: quinone oxidoreductase type 1 (NQO1) Microsomal epoxide hydrolase (EPHX1) Glutamate cysteine ligase (GCLM)	They are associated with allergic and nonallergic asthma, inducing increased oxidative stress status	[11, 20, 21]
Tumor necrosis factor G-308A	It may have a protective role in asthma pathogenesis, depending on airway oxidative stress levels	[22]
Methylenetetrahydrofolate reductase (MTHFR) ORM1-like 3 (ORMDL3) Gasdermin A and B (GSDM)	In addition to foetal smoke exposure, it seems to be associated with lower airway responsiveness, lung function, and increased risk of transient wheezing, a phenotype of childhood asthma	[23] [24] [25]
Antioxidant enzyme paraoxonase (PON1)	It is inversely correlated to plasma total oxidant status and to severity of asthma	[26]
Nuclear factor (NF), erythroid-derived 2-related factor 2 (NRF2)	It has been found to be a critical regulator in protecting cells and tissues under highly oxidative microenvironments, including airways that interface with the external environment and are exposed to pollutants and other oxidant stressors	[27]
Toll-like receptor 4 (Tlr4)	It is associated with O_3-induced lung inflammation and increased airway hyperpermeability	[28]
Heme oxygenase-1 (HMOX-1)	In addition to ozone exposure, it is responsible for the onset of allergic asthma	[29]
Transforming growth factor- (TGF-) beta1 C-509T polymorphism	This genotype is associated with an increased risk of asthma in addition to maternal smoking exposure in the uterus or to traffic-related emissions	[30]
Arginases (*ARG1* and *ARG2*)	It may play an important role in asthma pathogenesis through effects on nitrosative stress	[31]

Table 2: Oxidative stress-inducible genes and allergic rhinitis.

Gene	Clinical relevance	References
Glutathione S-transferases- (GSTs-) 1 polymorphism	It may exert protective effects in allergic rhinitis, decreasing oxidative stress status	[19]
Tumour necrosis factor (TNF) rs1800629 Toll-like receptor 4 (Tlr4) rs1927911	They are associated with a higher risk of allergic rhinitis	[22, 28]

of T helper cells, increasing the risk of allergic sensitization [58]. More recently, changes in the expression of small noncoding regulator microRNAs have also been suggested as being critical for mediation of imbalanced responses to allergens [59]. However, to date, it is still unclear what genes and pathways are active during pregnancy and/or at birth and which systems are down- and/or upregulated in response to perinatal OS.

There is increasing evidence that ROS, also at physiologic concentrations, might, acting as cell-signalling mediators and promoting a shift toward a Th2-skewed immune response [17, 38], play additional roles in the onset of allergic disorders [17, 38].

The lung, due to its anatomy, provides an extensive surface area available to interact with all sources of reactive O_2 species, and a large variety of lung diseases, including allergic asthma, may be induced by ROS [26, 43]. In particular, pulmonary epithelial cells of alveolar structure appear to be the principle target for oxidant injury which, inhibiting cellular cycle progression, promotes a delayed reepithelialization process and irreversible cellular damage [60]. Moreover, airway inflammatory cells, such as macrophages [61], eosinophils,

Table 3: Oxidative stress-inducible genes and atopic dermatitis.

Gene	Clinical relevance	References
Glutathione S-transferases- (GSTs-) 1 polymorphism	It is associated with atopic dermatitis susceptibility in a Korean population	[19]
MicroRNA-223 or hypomethylation of the thymic stromal lymphopoietin (TSLP) gene 59-CpG island (CGI)	It predisposes the host to development of atopic dermatitis when combined with exposure to oxidative stress	[32]
Tumour necrosis factor (TNF) promoter region (TNF-a-308G/A) and linked	It is linked to oxidative stress-mediated atopic dermatitis	[22]
Nitric oxide polymorphism (T276 (276C/T, nNOS) + C186 (-186A/C, nNOS) + X (CCTTT), nNOS + G954 (-954G/C, iNOS) +220 (TAAA), niNOS + G894 (894C/G, eNOS) + a (VNTR), eNOs)	It is related to clinical and functional manifestations of bronchial asthma and atopic dermatitis	[33]

and peripheral blood monocytes [40], are themselves a likely source of ROS production [62]. Confirming these findings, studies have shown higher H_2O_2, nitric oxide, and superoxide levels in exhaled gases from asthmatic patients than from control subjects [63–66]. A prooxidant status also induces a wide range of biological and molecular damage in the lung. Increased release of isoprostanes and ethane, both in epithelial and in endothelial cell membranes, as well as diminished activity of proteins, such as α1-protease inhibitor, ascorbate, α-tocopherol, and superoxide dismutase (SOD), has been reported [67].

Acting on other targets, such as airway smooth muscle, inducing acetylcholine-mediated contraction [68], mucin secretion [69], and nitric oxide- (NO-) mediated neurogenic inflammation [69], ROS can also impair broncho- and vasoregulation [70, 71].

Finally, large-scale genome-wide association studies (GWAS) have demonstrated that genetic susceptibility to allergic asthma is also determined by complex interactions between genes involved in OS, such as glutamate cysteine ligase (GCLM), glutathione peroxidase (GPX1), catalase (CAT), myeloperoxidase (MPO), NADPH oxidase (CYBA, p22phox subunit), NAD(P)H, quinone oxidoreductase type 1 (NQO1), and microsomal epoxide hydrolase (EPHX1) [26] (Table 1).

As the primary cell of interface between internal and external environments, nasal mucosal epithelial cells are known to initiate the release of a cascade of proinflammatory mediators through redox pathways [20]. Moreover, these cells also exhibit the capacity to upregulate an effective antioxidant defence [20]. However, natural allergen exposure agents show the ability to interfere with oxidant/antioxidant balance, enhancing OS and upper airway inflammation [72].

Although it has been hypothesized that the role of OS in allergic rhinitis is similar to that of asthma, the exact underlying mechanism is still not understood. However, it has been reported that OS, playing a critical role in allergic asthma, can also contribute to the onset of allergic rhinitis and to enhancing the asthma-rhinitis link, as expression of united airways disease [73].

It has been widely assessed that the loss of antioxidant activities characterizes patients affected by allergic rhinitis. Studies reported that decreased activities of both antioxidant enzyme paraoxonase (PON1) [74] and reduced glutathione [20] are inversely correlated to plasma total oxidant status and to severity of disease [20]. Consequently, increased nasal fraction of exhaled NO (FENO), 8-isoprostane, leukotriene- (LT-) B4, and PGE2 levels was detected in patients with allergic rhinitis [75]. An impaired function and distribution of superoxide anion, NADPH oxidase (NOX)1, and NOX4 in allergic nasal rhinitis has also been noted, as further confirmation of the possible influence of OS on the development of allergic rhinitis [76] (Table 2).

The ability to interfere with the immune system allows ROS to induce and perpetuate skin injury, also in atopic dermatitis. In particular, authors reported that ROS, acting mainly on keratinocytes and partially on lymphocytes [77], induce oxidative protein damage in the stratum corneum, leading to the disruption of barrier functions and the exacerbation of atopic dermatitis [78]. Therefore, in response to a variety of oxidant reactants, the skin upregulates transactivating AP-1 components such as Fos and Jun, whereas it downregulates anti-inflammatory components [79]. Precisely, it has been suggested that upregulation of AP-1 may be associated with a defect in ceramide generation which could result in enhanced protein kinase-C activation, leading to excessive release of proinflammatory cytokines by keratinocytes [79]. Generally, peroxisome proliferator-activated receptors (PPARs), a member of the nuclear factor family, also influence the biological activity of keratinocytes. To be precise, PPAR isoform-α (PPAR-α) counteracts the inflammatory response by inhibition of the expression of proinflammatory genes, as well as cytokines and metalloproteases. PPAR-α activation also induces antioxidant enzymes (catalase, SOD) which would reduce oxidative damage and inflammatory response [21].

The oxidant/antioxidant balance is also altered in atopic dermatitis. ROS reduce the physiological antioxidant levels of a number of compounds, such as α-tocopherol (VE), ubiquinol-10 (CoQH2-10), ascorbic acid (VC), and glutathione (GSH), in the epidermis and dermis and thus impair the cellular redox system [80]. Evidence of enhanced protein and lipid-oxidative damage was also found in atopic dermatitis patients, as demonstrated by the increase of carbonyl moieties both in lesional and in nonlesional skin, along with higher activity of SOD, an effective scavenger of ROS [81]. Recent experimental studies support a role for

oxidative/antioxidative imbalance also in the shift toward a Th2-skewed immune response, probably NO-mediated [38]. Accordingly, the administration of antioxidants to human T-cells culture downregulated Th2 polarization, with a decrease in the expression of IL-4 and IL-5, and simultaneous skewing toward a Th1- phenotype [38]. Finally, data suggest epigenetic changes linked to the development of atopic dermatitis through OS-mediated immune dysregulation [82] (Table 3).

5. Conclusions

To date, the exact underlying mechanisms of atopic disease are still not understood. Recently, more attention has been given to the critical role of OS-inducible genes in the pathogenesis of atopic diseases. However, in spite of much evidence linking atopic predisposition, inflammatory status, and maternal and neonatal OS, much more remains to be investigated. Moreover, a genomic approach would clarify the role of oxidant/antioxidant pathways, in order to better understand the pathogenesis of atopic diseases and identify innovative therapeutic strategies.

Competing Interests

The authors have declared no conflict of interests.

Authors' Contributions

All authors had equally contributed to the manuscript.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 9247493, 13 pages
http://dx.doi.org/10.1155/2016/9247493

Research Article

Erythropoietin Restores Long-Term Neurocognitive Function Involving Mechanisms of Neuronal Plasticity in a Model of Hyperoxia-Induced Preterm Brain Injury

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Received 22 March 2016; Revised 31 May 2016; Accepted 13 June 2016

Academic Editor: Serafina Perrone

Cerebral white and grey matter injury is the leading cause of an adverse neurodevelopmental outcome in prematurely born infants. High oxygen concentrations have been shown to contribute to the pathogenesis of neonatal brain damage. Here, we focused on motor-cognitive outcome up to the adolescent and adult age in an experimental model of preterm brain injury. In search of the putative mechanisms of action we evaluated oligodendrocyte degeneration, myelination, and modulation of synaptic plasticity-related molecules. A single dose of erythropoietin (20,000 IU/kg) at the onset of hyperoxia (24 hours, 80% oxygen) in 6-day-old Wistar rats improved long-lasting neurocognitive development up to the adolescent and adult stage. Analysis of white matter structures revealed a reduction of acute oligodendrocyte degeneration. However, erythropoietin did not influence hypomyelination occurring a few days after injury or long-term microstructural white matter abnormalities detected in adult animals. Erythropoietin administration reverted hyperoxia-induced reduction of neuronal plasticity-related mRNA expression up to four months after injury. Thus, our findings highlight the importance of erythropoietin as a neuroregenerative treatment option in neonatal brain injury, leading to improved memory function in adolescent and adult rats which may be linked to increased neuronal network connectivity.

1. Introduction

Over the last 20 years considerable progress in the care of high-risk prematurely born infants has led to increased survival, but also to a change in the pattern of pathology associated with neurological impairments [1]. Cystic focal lesions leading to cerebral palsy are now less common [2, 3], but the predominant neuropathological hallmark is a more subtle and diffuse type of damage involving impaired development of grey and white matter [4]. Recent findings from clinical MRI studies at term equivalent age led to the assumption that adverse neurodevelopmental outcome is

primarily attributed to disturbed glial maturation and neural connectivity rather than to cell death alone [5]. As a result, survivors of preterm birth suffer from altered function ranging from severe motor impairment to cognitive problems, attention deficit disorders, behavioural alterations, and psychiatric disease [6, 7]. The latter have brought the search for neuroprotective and/or regenerative therapies into the focus of preclinical experiments to prepare for clinical trials. Since preterm brain injury involves a complex pathophysiology with acute and chronic phases, preclinical testing of potential therapeutic compounds targeting multiple mechanisms of neural cell injury and maturation in adequate experimental models is highly warranted. Epidemiological studies revealed that exposure to high oxygen concentrations at atmospheric pressure is a contributor to a poor outcome in survivors of preterm birth [8, 9], leading to caution with the use of supplementary oxygen during the perinatal period [10]. Therefore a preterm rodent model of oxygen-induced brain damage has been developed mimicking the clinical situation [11, 12]. Hyperoxia-triggered subtle neurodegeneration in rodents is associated with inflammation, oxidative stress response, growth factor deficiency, and transient hypomyelination with long-lasting microstructural changes in the white matter [13–17]. Recent studies in rodents further revealed hyperactivity and coordination deficits at adolescent age [18] and cognitive impairment persisting into adulthood [19], which parallel the clinical situation in preterm infants.

Erythropoietin (Epo) is an endogenous 30.4 kDa protein which is in clinical use for years to prevent anaemia of prematurity [20]. Retrospective evaluation of several clinical trials primarily addressing stimulation of erythropoiesis suggested Epo as a potential therapeutic agent for neonatal brain injury [21]. Epo is produced in the developing brain by multiple cell types (neurons, oligodendrocytes, microglia, and astrocytes) and may act as a growth factor, providing endogenous neuroprotection upon an injurious stimulus [22, 23]. In the past the effects of Epo treatment have been studied in rodents and nonhuman primates in hypoxic-ischemic injury representing a model of term asphyxia and stroke and recently also in combination with therapeutic hypothermia [24–26]. Clinical trials are underway evaluating its safety and neuroprotective properties for term asphyxia, neonatal stroke, and congenital cyanotic heart disease and also include the preterm population [27]. However, very few experimental studies in the past focused on preterm models such as intrauterine hypoxia-ischemia [28, 29] and hypoxia-ischemia at postnatal day 3 (P3) [30]. Hardly any experimental model addressed subtle diffuse brain injury types such as perinatal inflammation or hyperoxia [31]. However, in the context of the use of Epo in oxygen-induced cerebral injury in rodents, modulation of inflammatory cascades, growth factor signalling, and autophagy activity have been shown besides its antiapoptotic and antioxidative capacities [16, 32–34]. Still, current knowledge of its impact on myelination, neuronal networks, and long-term functional outcome is limited.

Our goal in the present study was to test the hypothesis that single intraperitoneal injection of high-dose Epo in 6-day-old rats attenuates the long-term consequences of experimental hyperoxia-induced brain injury. Therefore we investigated white matter injury and elucidated mechanisms involved in synaptogenesis and formation of neuronal networks up to adulthood. In addition, long-term neurobehavioural, motor, and cognitive testing was applied at adolescent and adult age.

2. Materials and Methods

2.1. Animals and Experimental Procedures. All animal experiments were approved and performed in accordance with the guidelines of the University Hospital Essen, Germany, and with permission of the local animal welfare committee.

6-day-old Wistar rat pups were placed together with their lactating dams in an oxygen chamber (OxyCycler, BioSpherix, Lacona, NY, USA) for 24 hours containing 80% oxygen (80%). Hyperoxic dams underwent treatment only once and control animals were kept under normoxic conditions at 21% oxygen (21%). Based on our previous work [33] animals received a single intraperitoneal (i.p.) dose of 20,000 IU/kg body weight of recombinant erythropoietin (Epo, NeoRecormon®, Boehringer-La Roche, Grenzach, Germany) or equal amounts of normal saline (10 mL/kg body weight) at the onset of hyperoxia, resulting in four study groups: normoxia + normal saline (21%), normoxia + Epo (21% + Epo), hyperoxia + normal saline (80%), and hyperoxia + Epo (80% + Epo). A total of 139 sex-matched rat pups were enrolled in the study and randomly assigned to the treatment groups with at least two litters per experiment and per analysis protocol to ensure heterogeneity. Bodyweight was recorded regularly and there were no significant changes in weight gain between experimental groups. For different analysis protocols, pups were sacrificed at postnatal day 7 (P7) (57 pups, 5 litters), P11 (36 pups, 3 litters), and P125 (44 pups, 4 litters) under deep anaesthesia. In accordance with our previous observations [11, 13, 19] acute and subacute white matter impairment with cellular oligodendrocyte degeneration and myelin basic protein (MBP) expression were determined at P7 and P11, respectively. Functional deficits and behavioural abnormalities were analysed at adolescent (P30) and adult (P90) developmental stage and the same animals were partially used for postmortem diffusion tensor imaging (DTI) performed at P125 to evaluate long-term microstructural white matter changes and mRNA analysis of neuroplasticity-associated genes. mRNA analysis was moreover conducted at P7 and p11 (latter animals also used for MBP protein analysis). For protein and mRNA analysis, rats were transcardially perfused with phosphate buffered saline (PBS) and brain hemispheres were snap-frozen in liquid nitrogen. For histological and DTI studies, pups were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA, Sigma-Aldrich, Munich, Germany). Brains were postfixed in 4% PFA overnight at 4°C and embedded in paraffin or sent for diffusion tensor imaging.

2.2. Behavioural Studies. Behavioural testing was initiated in adolescent animals from P30 to P38 and repeated from P90 to P98 in adulthood. From P20, animals were familiarised with the investigator through every-other-day-handling during the active phase under an inverse 12-hour light-dark cycle. Testing started with one day of open field followed by four

days of novel object recognition and completed with four days of Barnes maze. To avoid intramaze cues due to odour, mazes were carefully cleaned after each animal. Data was recorded using an automatic tracking system (Video-Mot2, TSE Systems, Bad Homburg, Germany) and exported for statistical analysis.

For the open field test [35], animals were placed into the centre of a dimly lit open field arena (50 × 50 × 40 cm for adolescent or 75 × 75 × 40 cm for adult animals) placed upon an infrared-box (850 nm, TSE Systems). Movements were recorded by the tracking system for 10 minutes. General motor activity, that is, travelled distance and velocity, was analysed. The novel object recognition test was performed according to Chambon et al. with minor adaptations [36]. Animals were placed into the centre of a Y-maze (arm length: 60 cm; width: 26 cm; wall height: 56 cm) under red light. From inside the maze no external cues were visible. The first day, during habituation, animals were allowed to explore the empty arena. The next two days, three identical objects (white pyramids) were placed at the end of the maze arms for familiarisation. For the testing phase on day four, one of the familiar objects was replaced by a novel object consistent in height and biologically inert material but differently shaped (black cylinder). In each phase animals were exposed to the arena for five minutes and the time spent at each object was detected by the software. According to Chambon et al. analysis was performed for the first two minutes of testing time [36]. Spatiotemporal memory was assessed by the Barnes maze [37] as described previously [19]. Briefly, the animals were placed into the centre of the maze (1.22 m width, 0.8 m height, 20 holes at the border, TSE Systems) under red light followed by bright light to allow the animal to recognise extra-maze cues. The animals were allowed to explore the maze and find the escape box within 120 seconds; afterwards the animals were left in the escape box for 1 minute. Animals who did not find the escape box were gently placed into it for 1 minute. To avoid intramaze cues due to odour the escape box was rotated clockwise for every other animal, with the same escape location for each animal as on the three training days. The latency to find the trained escape box was assessed on the fourth day of each experiment when all holes were closed [38].

2.3. Immunohistochemistry and Confocal Microscopy. After deparaffinisation, 10 μm coronal sections (−3.72 ± 0.7 mm from bregma) were rehydrated. Antigen-retrieval was performed in a preheated 10 mM sodium-citrate buffer (pH 6.0) for 30 minutes. After blocking with 1% bovine serum albumin and 0.3% cold fish skin gelatin in 0.1% Tween-20 Tris-buffered saline (Sigma-Aldrich), all slides were incubated with primary antibodies overnight at 4°C followed by appropriate secondary antibody incubation for 1 hour at room temperature. Degeneration of oligodendrocytes was evaluated at P7 via colabelling with Olig2 (1 : 100, polyclonal rabbit anti-Olig2, Millipore, Darmstadt, Germany), followed by appropriate secondary antibody staining (1 : 300, anti-rabbit Alexa Fluor 594, Invitrogen, Karlsruhe, Germany) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL, in situ cell death detection kit, FITC, Sigma-Aldrich), performed according to the manufacturer's instructions. Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (1 μg/mL, Invitrogen, Karlsruhe, Germany). For exemplary image acquisition three laser lines (laser diode, 405 nm; Ar laser, 514 nm; G-HeNe laser, 543 nm) and three different filters (450/50-405 LP, 515/20-540 LP, and 585/65-640 LP) were used. Confocal z-stacks of 10 μm thickness (z-plane distance 1 μm) were converted into 2-dimensional images using maximum intensity projections. Analysis was performed by an observer blinded to treatment. Degenerating oligodendrocytes were analysed in 2 sections per animal by counting triple-positive ($Olig2^+/TUNEL^+/DAPI^+$) cells, respectively, at 20x magnification under an inverted confocal fluorescence microscope system (A1 Eclipse Ti, Nikon, Düsseldorf, Germany). Oligodendrocyte degeneration was assessed in four different regions of interest: corpus callosum, deep cortical white matter, cortex, and thalamus. Data are expressed as the average of Olig2/TUNEL positive cells per mm^2.

2.4. Immunoblotting. Western blotting was performed with hemisphere protein lysates (40 μg per sample) as described previously [13]. Membranes were incubated overnight at 4°C with a primary monoclonal mouse anti-MBP antibody (1 : 10,000, Abcam, Cambridge, UK) detecting two classical isoforms at 18.5 kDa and 21.5 kDa and with polyclonal rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (37 kDa; 1 : 1000, Santa Cruz, Heidelberg, Germany). Membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary anti-mouse (1 : 5000, Dako, Hamburg, Germany) or anti-rabbit (1 : 2000, Dako) antibody. All antibodies were diluted in 5% nonfat dry milk in Tris-buffered saline and 0.05% Tween-20 (Sigma-Aldrich). Antibody binding was detected by using enhanced chemiluminescence (GE Healthcare Life Sciences, Munich, Germany). For visualisation and densitometric analysis ChemiDoc XRS+ imaging system and Image-Lab software (Bio-Rad, Munich, Germany) were used. Since several isoforms of MBP exist, the 21.5 kDa isoform known to be increased during early active myelination [39] was quantified. Data were expressed as density ratio of analysed protein/reference protein GAPDH with control group set to 1.

2.5. Diffusion Tensor Imaging. At P125, *ex vivo* brains from behaviour tested animals were subjected to diffusion tensor imaging. All experiments were performed on an actively shielded 9.4 T/31 cm magnet (Varian/Magnex Scientific, Oxford, UK) equipped with 12 cm gradient coils (400 mT/m, 120 μs) with a transceiver 25 mm birdcage volume RF coil. First- and second-order shims were adjusted manually, with a water bandwidth ranging between 20 and 30 Hz. Diffusion gradients were applied along 6 spatial directions in a spin echo sequence [40]. The intensity (G_d), duration (δ), and separation time of the pulsed diffusion gradients were set to 22 G/cm, 3 ms, and 20 ms, respectively (b-value of 1185 s/mm^2). A field of view of 27 × 27 mm^2 was sampled on a 128 × 128 Cartesian grid and 12 slices of 0.8 mm thickness were acquired in the axial plane with 20 averages. The echo time was set to 35 ms and the repetition time between consecutive measurements was 2000 ms. Using homemade Matlab

(Mathworks, Natick, MA, USA) software, the radial diffusivity ($D_{\perp}$), the axial diffusivity ($D_{//}$), the mean diffusivity (MD), and the fractional anisotropy (FA) were derived from the tensor. The program allows manual delineation of region of interest (ROI) on the direction encoded colour maps. On two different structures of the brain (corpus callosum (CC) and external capsule (EC)), ROIs were carefully manually delimited at 6 different image-planes of the brain from the genu to the splenium of the corpus callosum.

2.6. RNA Extraction and Semiquantitative Real-Time PCR. Total RNA was isolated from snap-frozen tissue by acidic phenol/chloroform extraction (peqGOLD RNAPure™; PEQLAB Biotechnologie, Erlangen, Germany) and 2 μg of RNA was reverse transcribed. Gene expression analysis was performed as previously described using the PCR ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA [13, 41]). The PCR products of *synaptophysin*, *neuregulin-1*, *neuropilin-1,* and *β-actin* (as internal standard) were quantified in real time, by fluorogenic reporter oligonucleotide probes and primers (Metabion, Munich, Germany) with the following sequences and corresponding GenBank accession numbers: *synaptophysin* (*Syp*, NM_012664) sense 5′-TTCAGGCTGCACCAAGTGTA-3′, antisense 5′-TTCAGCCGACGAGGAGTAGT-3′, probe 5′-AGGGGGCACTACCAAGATCT-3′; *neuregulin-1* (*Nrg1*, NM_001271118) sense 5′-GGGACCAGCCATCTCATAAA-3′, antisense 5′-ATCTTGACGGGTTTGACAGG-3′, probe 5′-ACTTTCTGTGTGAATGGGGG-3′; *neuropilin-1* (*Nrp1*, NM_145098) sense 5′-TGAGCCCTGTGGTCTATTCC-3′, antisense 5′-CCTCTGGCTTCTGGTAGTGC-3′, probe 5′-TGTGGGTACACTGAGGGTCA-3′; *β-actin* (*Actb*, NM_031144) sense 5′-GTACAACCTCCTTGCAGCTCCT-3′, antisense 5′-TTGTCGACGACGACGGC-3′, probe 5′-CGCCACCAGTTCGCCATGGAT-3′. Real-time PCR and detection were performed in triplicate, measurements repeated 3 times for each sample. Target gene expression was quantified according to the $2^{-\Delta\Delta CT}$ method [42].

2.7. Statistical Analysis. Data are presented as mean + standard deviation and differences between groups were determined by one-way analysis of variance (one-way ANOVA) followed by Bonferroni post hoc test for multiple comparison with Prism 6 (GraphPad Software, La Jolla, CA, USA). For MRI results, nonparametric Mann-Whitney U test was used. p values less than 0.05 were considered as statistically significant.

3. Results

3.1. Erythropoietin Attenuates Hyperoxia-Induced Long-Term Cognitive Deficits. Since neonatal exposure to hyperoxia is associated with motor-cognitive impairment in rodents [18, 19], we assessed the potential protective effect of Epo treatment on motor activity and cognitive function in adolescent (P30) and adult (P90) rats following neonatal hyperoxia (P6, 24 hours of 80% oxygen). General motor activity analysed in the open field test was affected by neither hyperoxia nor Epo treatment (Figure 1(a)). For evaluation of memory deficits we performed the novel object recognition and the Barnes maze test. For novel object recognition, the time animals spent at the familiar and novel objects was recorded. In accordance with physiological exploration behaviour [36], control animals spent significantly more time with the novel object, whereas animals in the hyperoxia group did not show preference to any object. However, upon Epo treatment we detected normalised object recognition (Figure 1(b)), suggesting that hyperoxia-induced long-term cognitive impairment can be attenuated by Epo. The exploration preference was more prominent in adolescent animals with overall reduced exploratory activity in adulthood. In the Barnes maze we found a significant increase in the latency to find the trained escape hole in adolescent as well as adult animals after neonatal hyperoxia which was absent in the Epo treated animals (Figure 1(c)). Thus, behavioural testing showed long-term cognitive impairment after neonatal hyperoxia, whereas single Epo treatment improved hyperoxia-induced memory deficits.

3.2. Erythropoietin Improves Oligodendrocyte Survival after Hyperoxia but Does Not Influence Hypomyelination and Long-Term Structural White Matter Injury. White matter changes have been associated with cognitive deficits of preterm born infants later in life [43] and experimental models of hyperoxia revealed striking effects on myelination and microstructural white matter changes [13, 19, 44, 45]. Since Epo application has been shown to ameliorate white matter injury [46–48], we tested whether Epo-induced restoration of cognitive function might be associated with preservation of white matter structures. To investigate the effect of hyperoxia and Epo on oligodendrocyte survival we performed immunohistochemical staining of the pan-oligodendrocyte marker Olig2 and TUNEL. We detected a significant increase in oligodendrocyte cell death at P7 after 24 hours of hyperoxia, which was significantly diminished in Epo treated animals (Figure 2(a)). To elucidate whether the preserved oligodendrocyte survival following Epo treatment was associated with protection of subacute and long-term hyperoxia-induced white matter injury we performed MBP protein expression analysis at P11 and postmortem diffusion tensor imaging (DTI) of *ex vivo* brains at P125. A significant decrease of MBP protein expression was detected in hyperoxia-exposed animals as compared to normoxic controls, which was not influenced by Epo treatment (Figure 2(b)).

In accordance with MBP protein levels, fractional anisotropy (FA) as a sign of altered white matter microstructure and myelination deficit measured by DTI (Figures 3(a)–3(c)) was significantly reduced in the corpus callosum of hyperoxic animals and remained unchanged after Epo application (Figure 3(b)). The analysis of FA in the external capsule showed similar results (Figure 3(c)), although not reaching statistical significance. Of note, a single Epo injection ameliorates the neonatal hyperoxia-induced oligodendrocyte degeneration without long-lasting influence on myelination and preservation of white matter structures.

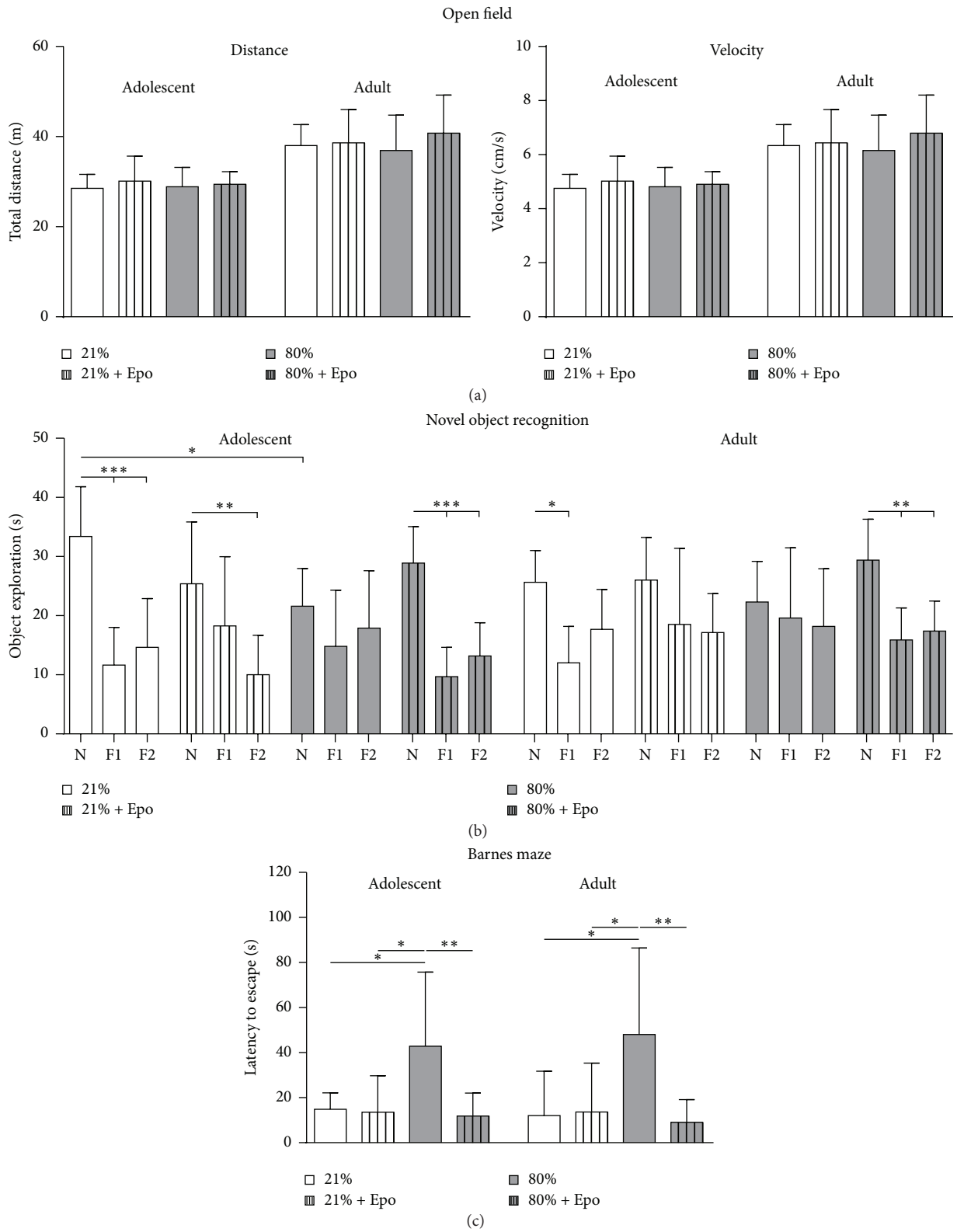

Figure 1: Erythropoietin improved cognitive function following neonatal hyperoxia. Motor-cognitive development was assessed by open field, novel object recognition, and Barnes maze starting at P30 (adolescent) and P90 (adult) after exposure to neonatal normoxia (21% oxygen (21%)) or hyperoxia (24 h, 80% oxygen (80%)) at P6 combined with i.p. administration of normal saline or 20,000 IU/kg Epo. (a) To test general motor activity animals were placed into the open field maze for 10 minutes. Movement of animals was tracked automatically by the software through three-point detection and the centre of the animals was analysed. Motor activity was expressed by the mean velocity and the total distance. (b) Cognitive function was assessed in the novel object recognition task presented as the exploration time at the novel object (N) versus familiar objects (F1 and F2). (c) Memory function was determined in the Barnes maze test expressed as the latency to find the trained escape hole after a 3-day training period. $n = 8$–10 rats/group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

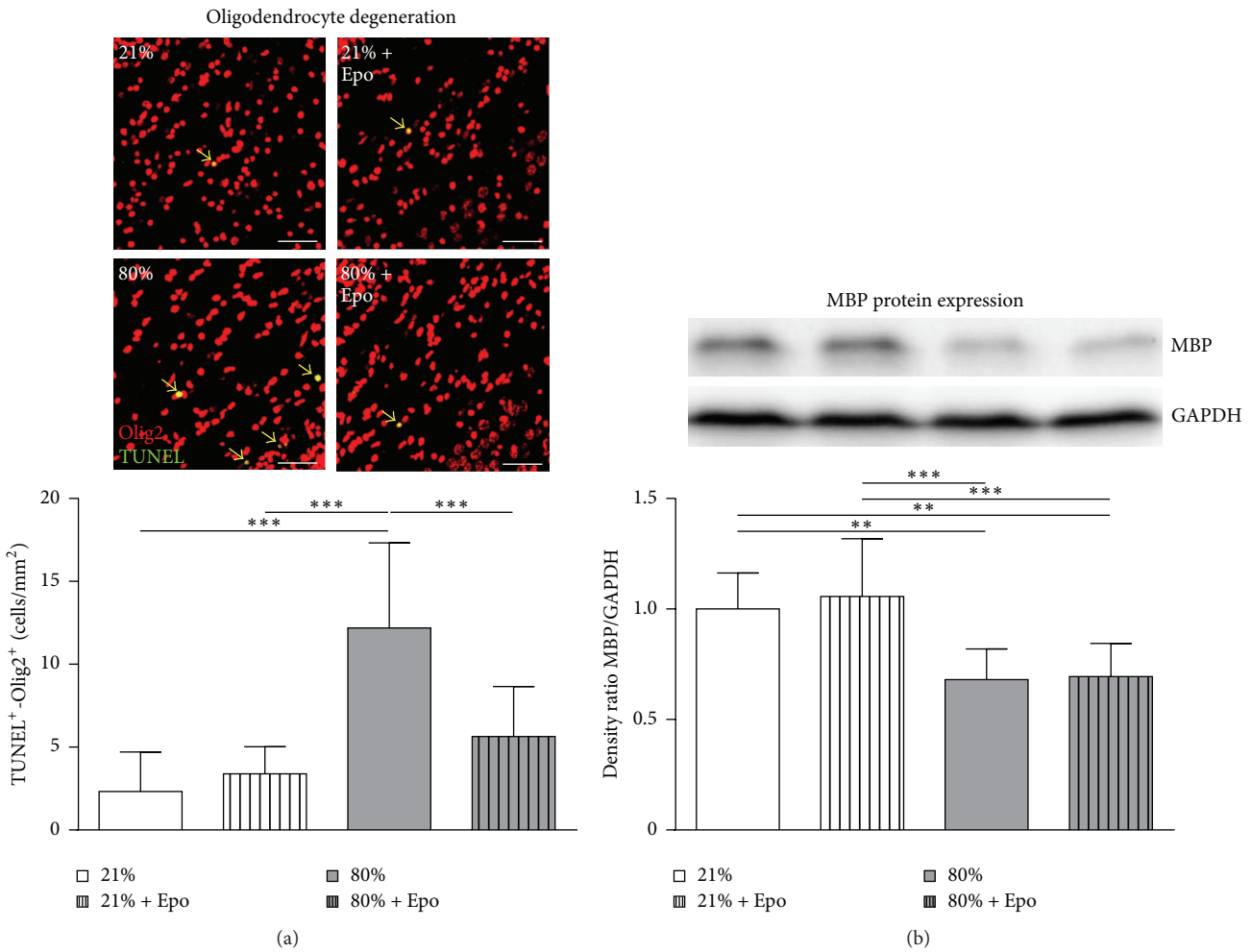

FIGURE 2: Erythropoietin ameliorates oligodendrocyte degeneration but not hyperoxia-mediated hypomyelination. (a) Oligodendrocyte degeneration was determined in brain sections from P7 rats that were exposed to either normoxia (21% oxygen (21%)) or hyperoxia (24 h, 80% oxygen (80%)) at P6 and treated with normal saline or 20,000 IU/kg Epo. Oligodendrocyte degeneration was determined by immunohistochemical TUNEL (green)/Olig2 (red) and DAPI (not depicted) costaining (positive counted cells appear yellow and are marked by arrows). Scale bar = 50 μm, n = 8–10 rats/group. (b) Myelin basic protein (MBP) expression was analysed 4 days after hyperoxia in protein lysates of complete hemispheres (excluding cerebellum). n = 8–10 rats/group. $^{**}p < 0.01$, $^{***}p < 0.001$.

3.3. Hyperoxia-Induced Downregulation of Neuronal Plasticity-Associated Genes Is Ameliorated by Epo Treatment. Since Epo-induced improvement of cognitive function after neonatal hyperoxia was not directly associated with preservation of white matter structures, we investigated whether amelioration of memory deficits might be linked to changes in neuronal connectivity. We assessed mRNA expression of the synaptic plasticity-related markers *synaptophysin* (*Syp*), *neuregulin-1* (*Nrg1*), and *neuropilin-1* (*Nrp1*) 24 hours (P7) and 4 days (P11) after hyperoxia exposure and in adult animals (P125) to evaluate potential persisting effects. Interestingly, we detected significant acute (Figure 4(a)) and subacute (Figure 4(b)) downregulation of all investigated markers in hyperoxic tissue which were still prominent in adulthood (Figure 4(c)). Treatment with Epo led to significant upregulation at all investigated time points (Figures 4(a)–4(c)). Thus, hyperoxia-triggered changes in neuronal plasticity gene expression were significantly ameliorated by single Epo treatment.

4. Discussion

The present study demonstrates long-term cognitive improvement following Epo treatment in a neonatal rodent model of oxygen-induced brain injury. A single Epo injection at the beginning of hyperoxia (24 hours of 80% oxygen) at P6 induces oligodendrocyte preservation, however without obvious protection against hypomyelination or long-lasting microstructural white matter changes. Nevertheless, we provide clear evidence that Epo improves cognitive function which is associated with long-lasting restoration of hyperoxia-induced changes of neuronal plasticity processes.

The neuroprotective effect of Epo has been widely described in experimental neonatal rodent models like hypoxia-ischemia, excitotoxicity, and stroke [27, 49]. Single-dose Epo application at the onset of 24 hours of hyperoxia was likewise repeatedly identified as neuroprotective [33, 50]. In previous studies we demonstrated that Epo mediates its protection through reduction of apoptosis, inflammation,

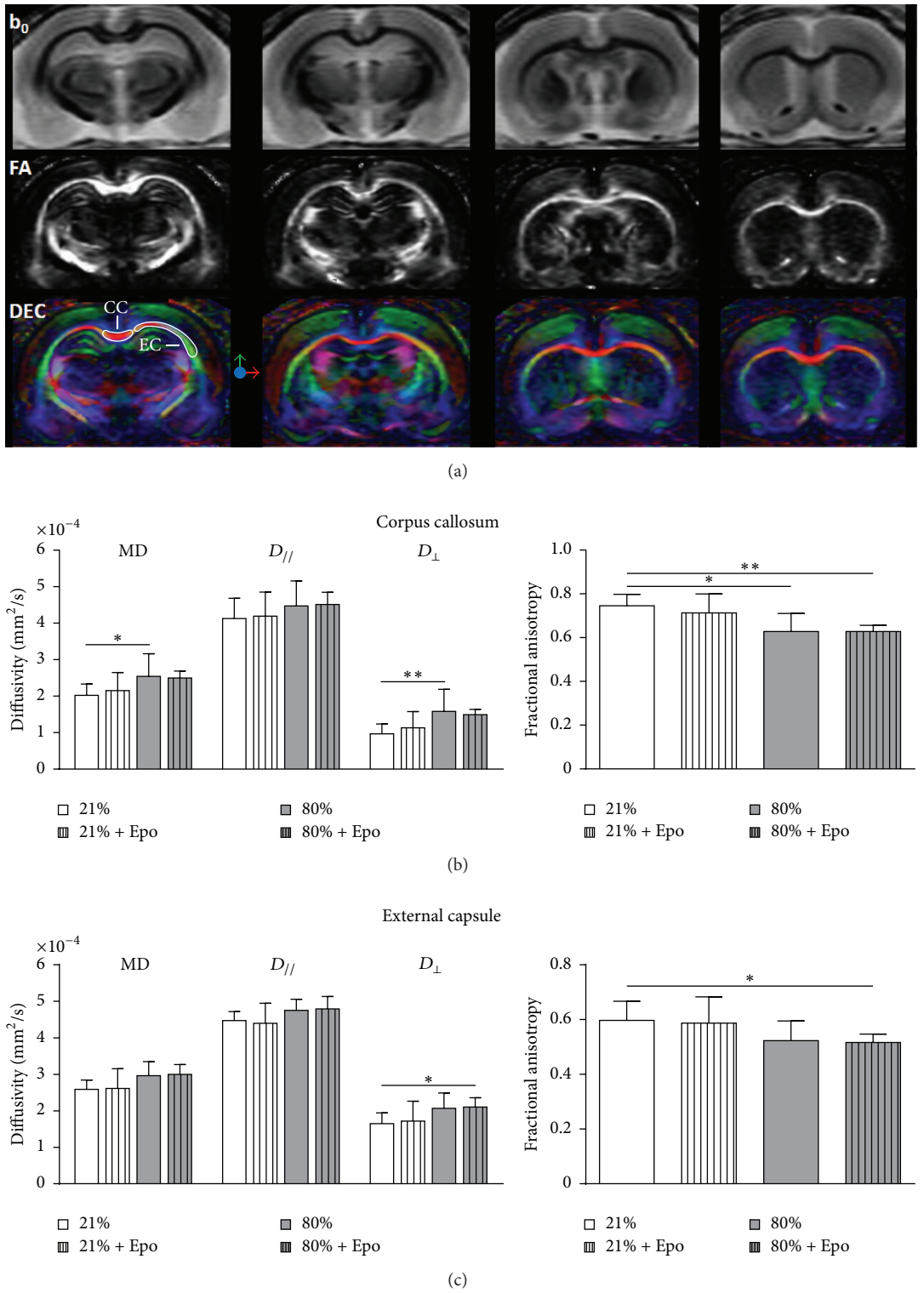

Figure 3: Long-term white matter microstructural development is not improved by a single injection of erythropoietin. (a) Representative T_2W images (b_0), fractional anisotropy (FA) maps, and direction encoded colour maps (DEC) of a P125 control rat (21%) derived from diffusion tensor imaging showing the different levels used for quantitative analysis. Corpus callosum (CC) and external capsule (EC) are displayed on the DEC. Diffusivity values of radial diffusivity ($D_\perp$), axial diffusivity ($D_{//}$), mean diffusivity (MD), and fractional anisotropy in corpus callosum (b) and external capsule (c) determined by diffusion tensor imaging out of rats exposed to normoxia (21% oxygen (21%)) or hyperoxia (24 h, 80% oxygen (80%)) at P6 and treated with normal saline or 20,000 IU/kg Epo. $n = 6$ rats/group. $^*p < 0.05$, $^{**}p < 0.01$.

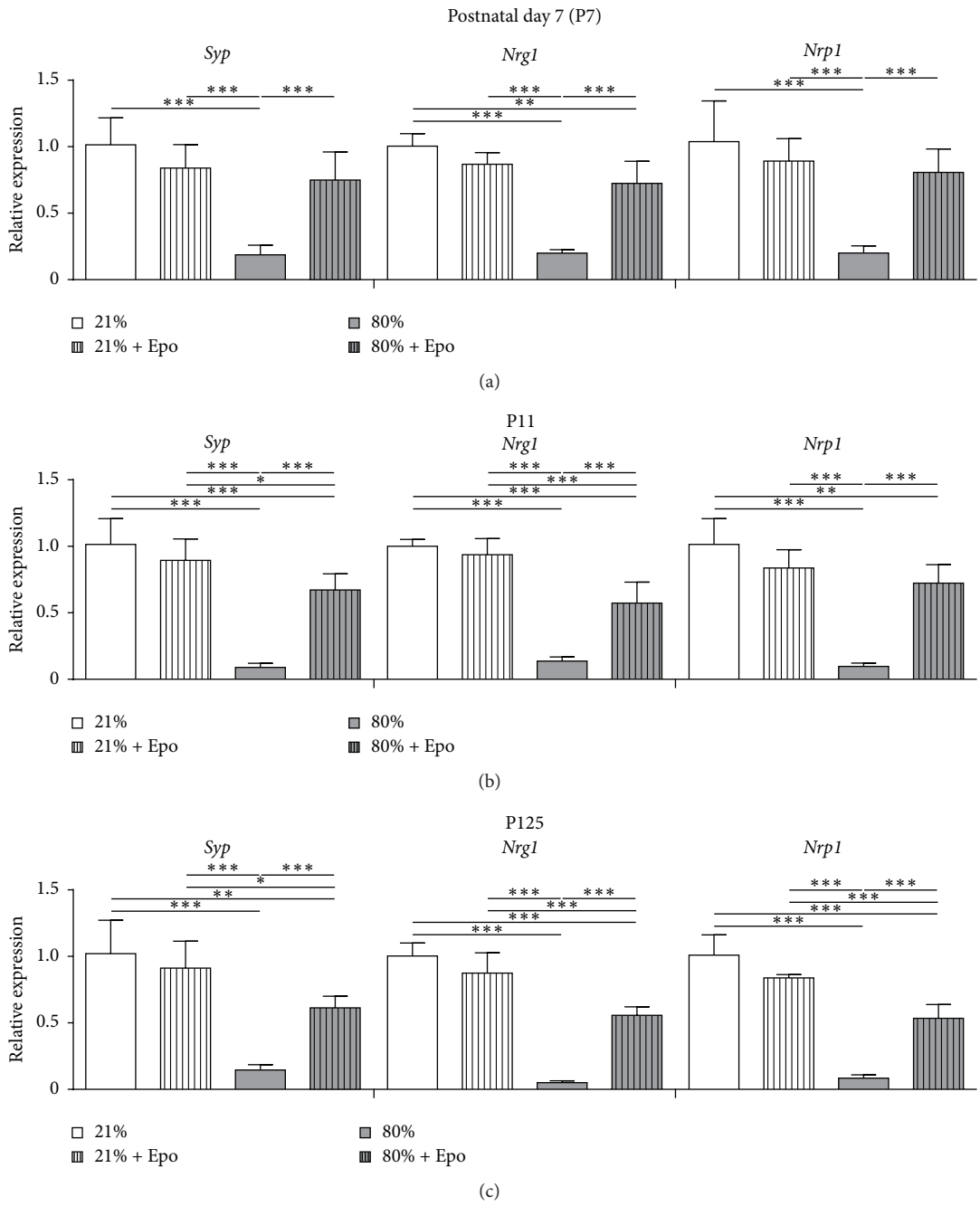

Figure 4: Erythropoietin restores plasticity-related genes following neonatal hyperoxia. *Synaptophysin* (*Syp*), *neuregulin-1* (*Nrg1*), and *neuropilin-1* (*Nrp1*) mRNA expression in hemispheres of (a) P7, (b) P11, and (c) P125 rats exposed to either normoxia (21% oxygen (21%)) or hyperoxia (24 h, 80% oxygen (80%)) at P6 and treated with normal saline or 20,000 IU/kg Epo i.p. $n = 4$–8 rats/group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

oxidative stress responses, and modification of autophagy-associated processes [16, 32–34]. Here we show that single-dose administration of Epo significantly ameliorates oxygen-induced memory impairment in adolescence, persisting into adulthood. Whereas Barnes maze revealed similar results for both developmental stages, the exploration preference obtained by novel object recognition was more prominent in adolescent animals with generally reduced exploratory activity at the novel object in adulthood. The latter results are in accordance with Stansfield and Kirstein describing adolescent animals spending more time with a novel object relative to adults [51]. Epo's cognitive improvement properties have also been found in other neonatal injury models, that is, neonatal stroke/hypoxia-ischemia, which revealed a long-term improved neurological outcome after Epo treatment [52, 53]. It has been shown that the neuroprotective dose in animal

models is much higher than the one used for support of erythropoiesis in the newborn [54]. This is further supported by reports in other experimental neonatal brain injury models, that is, hypoxia-ischemia. Here it was shown that that plasma concentrations shown to be neuroprotective in seven-day-old rats receiving 5000 IU/kg equal those obtained by 1000 IU/kg Epo per dose in term infants. These studies suggest that higher doses need to be administered in rats compared to humans [55, 56]. The Epo dose in the present work was chosen according to our own previous studies and based on the literature with numerous experimental evidences of neuroprotection in six-day-old rats [16, 32–34, 50].

In the clinical setting, several safety studies revealed a good tolerance for high Epo doses administered to preterm infants [48, 54, 55, 57–59]. Follow-up of patients included in these studies as well as first clinical trials designed to investigate neurological outcome after Epo treatment indicated that cognitive performance is improved up to the age of four years. However, these trials differed in design and dose of Epo used: Mcadams et al. did a retrospective analysis of the neurodevelopmental outcome of 60 patients included in a pharmacokinetic trial evaluating the effects of high-dose Epo over a short period of time. Ohls and coworkers aimed at investigating the difference between chronic application of low-dose Epo and Darbepoetin on the outcome in preschool age [60–62]. Since further investigation of the adolescent and adult developmental outcome will take decades in the clinic, our data provide first evidence for a long-lasting neuroprotective effect of Epo on the cognitive outcome in a preterm model of brain injury.

Although there is an increased understanding regarding the neuroprotective mode of action of Epo in experimental models of neonatal brain injury, the underlying mechanisms are not completely understood and may differ between models. One aim of this study was to investigate the possible impact of Epo on white matter preservation. In general, the phase of greatest vulnerability of the developing brain coincides with the peak of the brain growth spurt, in humans starting at mid-pregnancy and extending into the third year of life. Rodents show a delay in brain maturation and myelination, starting in the first postnatal days of life and therefore corresponding to immature born infants (23–36 weeks of gestation) [63, 64]. In preterms, the leading cause of motor and cognitive disturbances in later life consists of cerebral grey and white matter injuries [65]. Particularly, microstructural abnormalities in particular white matter regions are related to long-lasting impairment [66]. Previous experimental studies revealed that neonatal hyperoxia-triggered neural cell degeneration leads to a transient hypomyelination with disrupted axon-oligodendrocyte integrity and long-lasting microstructural changes in white matter up to the fully adult developmental stage [11, 13, 14, 19, 44, 45, 67, 68]. The present study shows that a single Epo application significantly decreases oligodendrocyte cell death in the developing brain following neonatal hyperoxia. Interestingly, there is no protection from hypomyelination. The absence of a protective effect differs from findings in other injury models of the mature and immature brain, where systemic Epo application induced an increased proliferation and differentiation of oligodendrocyte progenitor cells as well as preservation of the white matter [47, 69–72]. Even chronic Epo administration over a period of three weeks in healthy young mice was shown to increase the number and differentiation of neural cells without increased proliferation or decreased cell degeneration [73]. The majority of studies did not investigate changes up to the adult developmental stage, leaving long-term myelination partially undefined. Our data reveal hyperoxia-induced changes in white matter microstructures on diffusion tensor imaging, that is, reduced fractional anisotropy of adult rat brains, which are likewise not restored by Epo treatment. These findings differ from clinical trials, where Epo-mediated protection improved white matter integrity assessed by MRI in preterm infants [48, 59]. However, imaging was performed at term equivalent age and therefore at a different developmental stage in comparison to our study. Keeping the complex phenotype and the multiple origins of pathology of preterm infants in mind, limitations of our single-hit experimental model have to be taken into account. In contrast to the present work, other studies applied different Epo treatment regimes, using chronic applications of lower dosage instead of single high-dose treatment. Our data suggest that single Epo administration leads to protection of degenerating oligodendrocytes after neonatal hyperoxia. However, in addition to oligodendrocyte cell death hyperoxia also alters differentiation and maturation [13, 19, 44, 45, 67], which have not been investigated on a cellular level in the current study. Thus, it cannot be excluded that the lack of myelin-preservation might have been caused by insufficient promotion of differentiation by single-dose usage of Epo, possibly resulting in a reduced number of myelin-producing, mature oligodendrocytes. Therefore, a multiple-dose treatment regime should be investigated in further studies to elucidate additional cellular effects regarding white matter development.

The detrimental effect of neonatal hyperoxia on neuronal survival and differentiation has been described previously [74–76]. It has been further shown that Epo protects neurons from degeneration in different disease models of the mature and immature brain [53, 77, 78]. The impact of hyperoxia with and without application of Epo on neuronal plasticity, however, has not been addressed so far. Synaptic plasticity is the ability of synapses to modify transmission in strength or efficacy. Changes in synaptic plasticity have been reported to play a critical role in memory formation. In addition to the key effector mechanisms of Epo associated with neuroprotection, high expression of the Epo receptor on cortical and hippocampal neurons has been related to higher synaptic plasticity and cognitive performance [79]. Moreover, administration of Epo decreases the excitatory neurotransmitter release probability, enhances synaptic plasticity in mice hippocampal slices, and improves hippocampus dependent memory by modulating plasticity, synaptic connectivity, and activity of memory-related neuronal networks [80, 81]. To investigate the effect of hyperoxia and Epo application on neuronal plasticity we assessed mRNA expression of the associated markers *synaptophysin* (*Syp*), *neuregulin-1* (*Nrg1*), and *neuropilin-1* (*Nrp1*). *Synaptophysin* is an integral synaptic vesicle protein considered to be a representative for synaptic

density and synaptogenesis [82]. *Neuregulin-1*, a member of the epidermal growth factor family, shows high affinity to ErbB4 receptor, whereas Nrg1-ErbB4 signalling has been described to play a critical role in neurotransmission, synaptic plasticity, and synchronisation of neuronal network activity [83–85]. *Neuropilin-1*, a transmembrane protein receptor, is supposed to be important for axon patterning of motor and sensory nerves after binding to *semaphorin-3A* [86]. Our data indicate that the protective effects of single-dose Epo administration are linked to changes in neuronal plasticity. These findings are in accordance with increasing evidence suggesting that age-related decreases of Epo expression in the hippocampal region may contribute to Alzheimer (AD), a disease well known to be associated with neurodegeneration and apoptosis which impair synaptic function [87–90]. Moreover, in adult injury models of AD, administration of Epo has been shown to improve synaptic plasticity and memory deficits which were associated with increased cerebral synaptophysin expression [91, 92]. After rodent spinal cord injury, injection of nonreplicating herpes simplex virus based vector coding for Epo also improved the functional outcome and increased synaptic proteins synaptophysin and postsynaptic density protein-95 [93]. Therefore, Epo-mediated regulation of synaptic plasticity genes may be one effector mechanism to improve the cognitive outcome after neonatal brain injury. Further studies will be required to unravel the detailed molecular mechanisms underlying the observed regulation of Epo-mediated alterations in plasticity-related gene expression.

5. Conclusion

In summary, our study reveals that administration of Epo in oxygen-induced brain injury leads to an improved cognitive outcome. Analysis of neuronal plasticity markers demonstrates a hyperoxia-triggered persistent downregulation, which can be reversed by Epo treatment. Therefore, changes in plasticity processes might contribute to an ameliorated neurocognitive outcome.

These findings are highly relevant from a clinical perspective since oxygen administration in neonates is sometimes inevitable and premature infants are already exposed to oxygen concentrations after birth fourfold higher compared to intrauterine conditions. Therefore, any therapy needs to be applied immediately at the onset of hyperoxia. To support the very vulnerable phase of brain maturation and enhance the developmental and regenerative potential of the preterm brain, it is crucial to investigate safe treatment options, like erythropoietin, to better understand the underlying molecular and cellular mechanisms and facilitate transition into the clinical setting. In conclusion, our results underline once more the importance of Epo as a candidate for neuroprotective therapy in neonates.

Competing Interests

The authors declare that they have no conflict of interests with any financial organisation regarding the commercial identities mentioned in the paper.

Acknowledgments

The authors gratefully thank Christian Koester and Mandana Rizazad for their excellent technical assistance and David Hill for proofreading the paper as a native speaker. This work was supported by the "IFORES-Program, Interne Forschungsförderung" of the University Hospital Essen (Germany), the Karl-Heinz Frenzen- and C. D.-Stiftung, and the Centre d'Imagerie Biomédicale (CIBM) of the Université de Genève (UNIGE), Université de Lausanne (UNIL), Hôpitaux Universitaires de Genève (HUG), Centre Hospitalier Universitaire Vaudois (CHUV), Ecole Polytechnique Fédérale de Lausanne (EPFL), and the Leenards and Jeantet Foundations.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 2768365, 9 pages
http://dx.doi.org/10.1155/2016/2768365

Review Article

Oxidative Stress Related Diseases in Newborns

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Received 25 March 2016; Revised 27 April 2016; Accepted 18 May 2016

Academic Editor: Serafina Perrone

We review oxidative stress-related newborn disease and the mechanism of oxidative damage. In addition, we outline diagnostic and therapeutic strategies and future directions. Many reports have defined oxidative stress as an imbalance between an enhanced reactive oxygen/nitrogen species and the lack of protective ability of antioxidants. From that point of view, free radical-induced damage caused by oxidative stress seems to be a probable contributing factor to the pathogenesis of many newborn diseases, such as respiratory distress syndrome, bronchopulmonary dysplasia, periventricular leukomalacia, necrotizing enterocolitis, patent ductus arteriosus, and retinopathy of prematurity. We share the hope that the new understanding of the concept of oxidative stress and its relation to newborn diseases that has been made possible by new diagnostic techniques will throw light on the treatment of those diseases.

1. Introduction

(1) Oxidative Stress. There is a crucial balance between free radical production and antioxidant defense mechanisms. While human bodies are producing energy, molecules with one or more unpaired electrons in their outer shell, called free radicals, occur in the respiratory chain, phagocytosis, prostaglandin synthesis, and the cytochrome P450 system [1–3]. Free radicals are formed from molecules via the breaking of a chemical bond such that each fragment keeps one electron, via cleavage of radicals to give other radicals and via redox reactions [3, 4]. Current known free radicals are hydroxyl (OH•), superoxide (O_2^-•), nitric oxide (NO•), nitrogen dioxide (NO_2•), peroxyl (ROO•), and lipid peroxyl (LOO•). In addition, hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), and lipid peroxide are not free radicals but are called oxidants, because they can easily lead to free radical reactions in organisms [5]. Reactive oxygen species (ROS) include both free radicals and nonfree radical oxygenated molecules. Reactive nitrogen, iron, copper, and sulfur species are also encountered. Oxidative stress and imbalance of the redox reaction can be originated by those radical species. ROS/reactive nitrogen species- (RNS-) induced damage in oxidative stress is considered a contributing factor to the pathogenesis and pathophysiology of many health problems, either as a source or as an outcome [1, 6, 7]. Overexpression of oncogenes and generation of mutagen compounds or inflammation leads to some diseases such as cancer, and neurodegeneration may be affected by the involvement of ROS/RNS species [1, 5, 8, 9].

ROS and RNS are generated from either endogenous or exogenous sources. Endogenous free radicals are produced from immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, cancer, and aging. Exogenous ROS/RNS is caused by air and water pollution; cigarette smoke; alcohol; heavy or transition metals; certain drugs including cyclosporine, tacrolimus, gentamycin, and bleomycin; industrial solvents such as asbestos; cooking (smoked meat, used oil, and fat); and radiation. After penetration into the body by different routes, these exogenous compounds are decomposed or metabolized into free radicals [6, 10–15].

However, free radicals are not always harmful; at low or moderate concentrations, ROS and RNS are necessary for the maturation process of cellular structures and play an important role in the host defense system. Indeed, phagocytes (neutrophils, macrophages, and monocytes) release free radicals to destroy invading pathogenic microbes as part of the body's defense mechanism against disease [8, 16]. The importance

of ROS production by the immune system is clearly shown by patients with granulomatous disease. These patients have defective membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase production that makes them unable to produce the superoxide anion radical ($O_2^{-}\bullet$), thereby resulting in multiple and persistent infections [8, 13]. Macrophages are activated when there is infection or inflammation, with toll-like receptors releasing NO or oxygen free radicals that may damage the tissue. Free radicals also induce proinflammatory cytokines [17]. Antioxidants are inhibitors of oxidation, either produced endogenously or received from exogenous sources. The role of antioxidants is to neutralize an excess of free radicals, to contribute to disease prevention, and to protect the cells against the toxic effects of oxidants, such as deoxyribonucleic acid (DNA) mutations and malignant transformations [1, 6]. It was reported that the use of antioxidants greatly enhances immune cell function, helping to control many bacterial and viral infections, reverse the imbalance between oxidants and antioxidants at the site of oxidant injury, and prevent progressive tissue damage [18]. Antioxidants are suggested for treatment of HIV, hepatitis C, Japanese encephalitis, and tuberculosis with anti-inflammatory features [18–21]. Currently known endogenous antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, peroxiredoxin, thioredoxin reductase, glutathione, flavonoids, bilirubin, uric acid, melatonin, thiols, reduced coenzyme Q, alpha-lipoic acid, endogenous organic selenium, and the metal-binding proteins transferrin, ferritin, lactoferrin, ceruloplasmin, and albumin. Exogenous antioxidants include vitamin C, vitamin E, carotenoids, stilbene antioxidants, phenolic acids, flavonoids, oil lecithins, acetylcysteine, exogenous selenium, zinc, magnesium, and copper [1, 2]. All of those molecules seem to be probable targets in the management of oxidative stress-induced diseases.

Oxidative stress, which occurs when there are more toxic free radicals produced than can be neutralized by antioxidant mechanisms, is an increasingly important topic among biological researchers. Under normal conditions, it is a continuing process of our bodies that begins before birth [22, 23]. ROS and RNS play dual roles as both toxic and beneficial compounds. The delicate balance between their two opposite effects is clearly an important aspect of life. At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. At high concentrations, they generate oxidative stress, a deleterious process that can damage cell structures such as DNA, lipids, and proteins [6]. Oxidative stress initiates structure modifications and function modulation in nucleic acids, lipids, and proteins. Of these, lipids are the most susceptible to oxidation. Oxidative degradation of lipids yields malondialdehyde, 4-hydroxynonenal, and isoprostanes, from unsaturated fatty acids. Protein damage may occur with thiol oxidation, carbonylation, side-chain oxidation, fragmentation, unfolding, and misfolding, resulting in loss of backbone and the side chain of proteins. ROS damage nucleic acids, and 8-hydroxydeoxyguanosine is an index of DNA damage [24]. Oxidative injury occurs when excessive production of ROS/RNS emerges and cannot be counteracted by the antioxidants. The imbalance between the oxidative and antioxidative systems may trigger some factors that cause oxidative damage in the cell. This leads to disease such as bacterial, viral, and parasitic infections, autoimmune disorders, malignancies, atherogenic activity, diabetes, kidney diseases, skin diseases, and neurodegeneration [1, 22, 25]. Moreover, bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), necrotizing enterocolitis (NEC), patent ductus arteriosus (PDA), periventricular leukomalacia (PVL), respiratory distress syndrome (RDS), intrauterine growth retardation (IUGR), and congenital malformation have also been reported to be oxidative stress-related neonatal diseases [20, 21, 23, 26–28].

2. Oxidative Stress-Related Disease in Preterm and Newborn Infants

In 1988, Saugstad hypothesized that BPD, NEC, intracranial hemorrhage, PDA, and other possible diseases are not distinct, but all belong to one entity, "the oxygen radical disease of neonatology," that has different symptoms according to which organs are mostly affected (Figure 1) [29]. Premature infants are especially susceptible to oxidative stress, and newborns are also susceptible for reasons including the following.

(1) Hypoxic-Hyperoxic Challenge. In the uterus, infants have a hypoxic environment, with 20–25 mmHg oxygen tension (PO_2). However, they are born into an extrauterine normoxic environment of approximately 100 mmHg PO_2 [30]. Some newborns require resuscitation with supplemental oxygen in the delivery room after being exposed to the hyperoxic environment. Rizzo et al. reported that increased oxygen tension induces elevated production of ROS in animal studies [31].

(2) Infections. Infants, especially preterm infants, are more susceptible to infections because infants are relatively immunodeficient [32].

(3) Antioxidant Defense Deficiency. Preterm infants and newborns have reduced antioxidant defense processes, including decreased levels of vitamin E, β-carotene, melatonin, ceruloplasmin, transferrin, and erythrocyte SOD [30]. Some antioxidants such as ascorbate and bilirubin are present in high concentration in newborns but only for a short time after birth [33].

(4) High Levels of Free Iron. Newborns have higher levels of free iron than older children, which cause an increased Fenton reaction, leading to the production of the highly toxic hydroxyl radical [17].

2.1. Periventricular Leukomalacia. In 2014, 1 of every 10 babies was born premature in the United States [34]. Every year, more than 20 million infants are born weighing less than 2.5 kg. Very-low-birth-weight infants, those infants born weighing less than 1.5 kg, are susceptible to adverse outcomes [35]. The incidence of PVL based on ultrasonographic findings ranges from 5% to 15% in very-low-birth-weight infants [36]. Neuropathologic evidence of PVL is found in 25% to 75% of very-low-birth-weight infants who die. PVL refers to

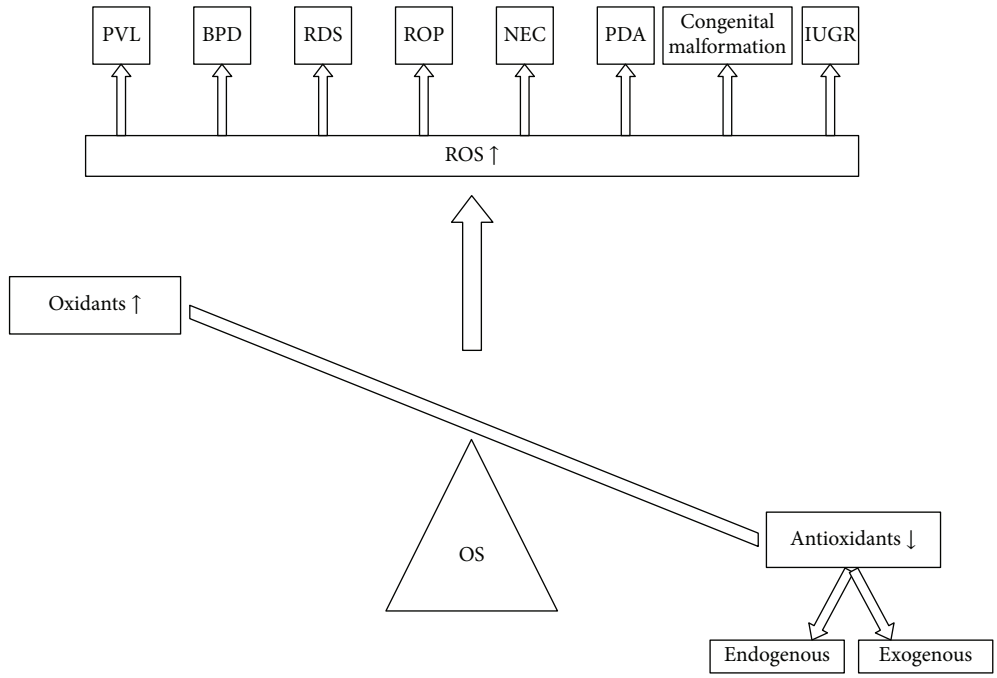

Figure 1: The imbalance between prooxidants and antioxidants in "oxygen radical disease of neonatology." BPD, bronchopulmonary dysplasia; ROP, retinopathy of prematurity; NEC, necrotizing enterocolitis; PVL, periventricular leukomalacia; PDA, patent ductus arteriosus; RDS, respiratory distress syndrome; IUGR, intrauterine growth retardation; OS, oxidative stress; ROS, reactive oxygen species.

injury to cerebral white matter that occurs in a characteristic distribution and consists of periventricular focal necrosis, with subsequent cystic formation, and more diffuse cerebral white matter injury [37].

Prevention of PVL will require new insights into its pathogenesis. The pathogenesis of this disease is related to the following factors:

(i) Incomplete development of the vascular supply in the cerebral white matter.

(ii) A maturation-dependent impairment in the regulation of cerebral blood flow underlying a propensity to ischemic injury to cerebral white matter.

(iii) Oligodendroglial precursor cells being the major cellular target of maturation-dependent vulnerability in PVL.

(iv) Maternal/fetal inflammation or infection causing oxidative stress.

(v) Elevation in extracellular glutamate causing toxicity to oligodendroglial precursors.

It has been shown that attacks by radicals, deficiency of antioxidant defenses, and active acquisition of iron derived from hemorrhage contribute to the pathological processes of disease during oligodendroglial differentiation. In consequence, deadly ROS and apoptotic oligodendroglial death may be the underlying reasons for PVL [38, 39].

2.2. Respiratory Distress Syndrome. More than half of extremely-low-birthweight (<1 kg) newborns will have some type of respiratory distress, and in that population RDS is the most common diagnosis (50.8%). The main factor for RDS is prematurity [40]. The pathophysiological factors include the following:

(i) Insufficient/dysfunctional surfactant resulting in collapsed alveoli, atelectasis, ventilation-perfusion mismatching, and subsequent hypoxemia and respiratory acidosis [40].

(ii) Sudden increase in oxygen supply after birth leading to an overproduction of ROS and depletion of antioxidants.

(iii) Hyperoxygenation destroying the vascular and alveoli endothelial cells.

(iv) Oxidant stress promoting expression of cytokines and the inflammatory process (interleukin-6, interleukin-8, and tumor necrosis factor-α) [41, 42].

2.3. Bronchopulmonary Dysplasia. Damage starts with the first postnatal breaths in lungs of premature infants. Infants weighing less than 1500 g at birth have BPD ranges between 15% and 50%, and ranges decrease by gestational age [43, 44]. The pathogenesis of BPD is complex. Some defined factors include the following:

(i) Reduced alveolar volume.

(ii) Deficiency of surfactant.

(iii) Immature extracellular matrix.

(iv) Inflammation unrelated to infection [43, 45].

(v) Oxygen free radicals produced after exposure to oxygen.

(vi) Oxidative stress activating inflammatory cells and increasing proinflammatory cytokines.

(vii) Oxidative stress causing injury to the respiratory tract epithelium and inactivating surfactant [44].

(viii) High tidal volume positive-pressure ventilation producing inflammation.

(ix) PDA with increased pulmonary blood flow triggering the inflammatory cascade and stimulating neutrophil margination and activation in the lung [43, 45].

2.4. Retinopathy of Prematurity. ROP, a proliferative retinopathy affecting premature infants, continues to be a leading cause of lifelong visual impairment among children in the developed countries. ROP causes visual loss in 1300 children and severe visual impairment in 500 children each year in the United States alone. The overall incidence of ROP is 0.17%, but it is nearly 16% for premature infants [46]. Basic research into the pathogenesis of ROP contributes to further understanding of retinal development, angiogenesis, and intraocular neovascularization [28, 46]. Currently known ROP pathogenetic factors after birth by hyperoxia include the following:

(i) Inhibition of retinal vascularization.

(ii) Loss of the nutrients and growth factors at the maternal-fetal interface.

(iii) Stopped blood vessel growth, with subsequent hypoxia because of retinal maturation and increasing metabolic demand.

(iv) The hypoxic retina stimulating expression of the oxygen-regulated factors that stimulate retinal neovascularization by using erythropoietin and vascular endothelial growth factor [47].

(v) Oxygen fluctuations inducing cells to produce NADPH oxidase, which causes increased ROS as well as apoptosis of endothelial cells, which contribute to avascular retina [38].

2.5. Necrotizing Enterocolitis. The incidence of NEC is approximately 1 per 1000 live births. For infants under 1500 g, the incidence increases to between 2.3% and 12%. Both the incidence and case fatality rate of NEC are inversely correlated with birth weight; about 30% of babies <1500 g with NEC will not survive [48, 49]. Pathogenetic factors of NEC include the following:

(i) Bacterial lipopolysaccharides increasing inducible nitroxide synthase activation in the enterocytes of neonates, triggering ROS production [41].

(ii) Multifactorial etiologies, including inflammation, ischemia, and cytokines (tumor necrosis factor-α, interleukin-6) producing a high level of free radicals [50, 51].

(iii) Local ischemia of the intestinal tissue and reperfusion triggering production of reactive species through some enzymes such as xanthine oxidase [50].

(iv) ROS and free radicals contributing to the disruption of the immature gut barrier [48, 51].

(v) Platelet-derived growth factor [52].

2.6. Patent Ductus Arteriosus. PDA, which has an incidence of 1 per 2000 in term neonates, is the persistence of the fetal communication between the descending aorta and left pulmonary artery. This congenital defect allows blood to bypass the fetal lungs and be directed into the descending aorta to supply structures below this region. It usually closes soon after birth under the physiologic effects of elevated oxygen level. Defects can range in size from so small as to be undetectable to large enough to cause volume loading of the left ventricle and pulmonary hypertension. PDA is seen more frequently (20% to 60%) in preterm infants [53], particularly those born at <30 weeks' gestation [54].

In 1971 Fay showed that there were oxygen sensors in the ductus arteriosus [55]. Further studies showed that the ductus arteriosus is affected by changes in PO_2, with changes in the redox state producing ROS [56, 57]. Intrauterine hypoxia maintains the patency of the ductus arteriosus. However, at the time of birth, ROS increases when PO_2 changes from fetal to neonatal levels [58]. In addition to this, hemodynamically significant PDA may cause hypoperfusion of organs, which can cause diseases such as NEC, BPD, and acute renal insufficiency [59, 60]. Hypoperfusion, ischemia, and chronic hypoxia lead to the production of oxygen radicals [61].

2.7. Congenital Malformation. Congenital anomalies are important causes of childhood death, chronic illness, and disability all over the world and they may have a significant impact on individuals, families, healthcare systems, and societies. It is estimated that 276,000 newborns die in the first month of life every year from congenital anomalies. The most common severe congenital anomalies are heart defects, neural tube defects, and trisomy 21. Although congenital anomalies may be genetic, infectious, nutritional, or environmental in origin, most often it is difficult to identify the exact causes [62]. It was stated in recent reports that there may be an association between oxidative stress and congenital malformations [63–65]. Oxidative stress has been defined as harmful radicals attacking biological molecules such as DNA, lipids, and proteins [66]. However, this relationship between oxidative stress and congenital malformation and the exact nature of the damage are not clear and need further investigation.

2.8. Intrauterine Growth Restriction. IUGR is a complication of pregnancy, often described when the fetus is estimated to be small for gestational age [67]. The reported incidence of IUGR ranges between 3% and 7%. IUGR is most probably a consequence of placental ischemia/hypoxia [68]. Some mechanisms involving IUGR and oxidative stress are as follows:

(i) High metabolic demand and elevated requirements for tissue oxygen in pregnancy [69].

(ii) Increased rate of production of ROS, oxidative stress, and lipid peroxidation compared with nonpregnant women.

(iii) Uncontrolled production of lipid peroxides resulting in additional oxidative stress [70].

(iv) Placental ischemia/hypoxia resulting in the release of products into the maternal circulation, which triggers preeclampsia as well as IUGR [71].

(v) Development during the late second or third trimester when the mother's antioxidant capacity to cope is limited.

(vi) Increased antioxidants such as vitamin E, ceruloplasmin, and erythrocyte thiols and increased iron-binding capacity; otherwise, serum iron concentrations progressively decrease.

(vii) Insufficient increase in antioxidants trying to counter the increase in oxidative stress and lipid peroxidation.

In conclusion, damage to cell integrity, cell membrane function, organelle membranes, and protein synthesis is a major cause of maternal and fetal morbidity [70].

3. Diagnosis of Oxidative Stress-Related Neonatal Disease

Currently it is known that oxidative stress is important in the pathogenesis of various kinds of neonatal disease, and there is a need for more information about and investigations of the manifestations and diagnosis of oxidative stress in neonates.

Giuffrè et al. [72] stated that glutathione, lipid hydroperoxides, and heat shock protein chaperonin 60 in the newborn's serum might have functional and diagnostic significance for oxidative stress. There are some studies of the diagnosis of oxidative stress-related neonatal disease in both humans and animals. Serum selenium is a constituent of the oxidant enzyme glutathione peroxidase and is vital for antioxidant defense [73]. El-Mazary et al. [74] showed that neonates with hypoxic-ischemic encephalopathy had lower serum selenium levels than normal healthy neonates. Mukhopadhyay et al. [75] reported that levels of antioxidants such as vitamin C and glutathione are reduced, and levels of serum malondialdehyde and protein carbonyl are different, in children with congenital malformation and healthy children. Therefore, these markers may be a target for studies that focus on the diagnosis of oxidative stress-related diseases [75]. Kumar et al. [76] claimed that increased levels of plasma and cerebrospinal fluid malondialdehyde are related to perinatal asphyxia.

To evaluate oxidative stress markers in neonates with IUGR, antioxidant enzyme (SOD, catalase, and glutathione peroxidase) activities and levels of antioxidants were measured. It was found that there were significantly lower levels of enzyme activities in the IUGR group [77] than in a control group. Cancelier et al. [78] demonstrated that thiobarbituric acid-reactive substances, which are an oxidative stress marker, were significantly higher in cord blood of infants with neonatal sepsis, and thiobarbituric acid-reactive substance levels were independently related to the development of neonatal sepsis. Lista et al. [79] noted that lung inflammatory response in preterm infants with RDS may be assessed by measuring proinflammatory cytokines in tracheobronchial aspirate fluid; if there is inflammation, lungs are more susceptible to oxidant stress. In 2015, Tataranno et al. [80] reported that discovery and validation of specific plasma oxidative stress markers of neonatal brain injuries give an idea of neonatal neuroprotection. According to the authors, prostanoids and nonprotein bound iron could be used as specific plasma oxidative biomarkers reflecting oxidative stress injury to neuronal cells. Eventually Marseglia et al. [81] concluded that visfatin could be a new marker of oxidative stress in preterm newborns. Visfatin is an adipocytokine involved in oxidative stress and an important mediator of inflammation that induces dose-dependent production of both proinflammatory and anti-inflammatory cytokines.

4. Therapeutic Approach to Oxidative Stress-Related Neonatal Disease

Neonates, particularly those born prematurely, have an incomplete detox response to free radicals. To passivate oxidative stress-related damage in newborns, many therapeutic strategies to promote antioxidant status in newborns have been proposed. Supplementation with enzymatic and/or nonenzymatic antioxidants has been experimented with, but the results were mixed [50]. It was reported that an antioxidant supply can prevent oxidant stress-related disease, support the immune systems of neonates, reduce stillbirths, and enhance neonatal vitality [41].

Antioxidant defense mechanisms include endogenous antioxidant enzymes such as SOD, catalase, and glutathione peroxidase and nonenzymatic compounds such as glutathione, proteins (ferritin, transferrin, ceruloplasmin, and even albumin), and uric acid, coenzyme Q, and lipoic acid, which are all low-molecular-weight scavengers. Vitamins C and E, carotenoids, and phenolics (flavonoids-flavonols) have been identified as the major exogenous antioxidants [1]. Many potential therapeutic antioxidants have already been investigated, particularly in diseases of newborns [38]. It was shown in some reports that oxidative stress-mediated intestinal injury was reduced by the addition of SOD, glutathione peroxidase, and N-acetylcysteine, which reduces concentrations of intestinal tissue tumor necrosis factor-α via its anti-inflammatory and antioxidant properties [82, 83]. In various studies, some therapies including hyperbaric oxygen, medical ozone, and enteral glutamine alone or in combination with arginine have shown favorable effects on NEC by modulating antioxidative defense mechanisms [84–86]. Surech et al. [87] concluded that intratracheal administration of recombinant human copper zinc SOD caused an improvement in the antioxidant activity of some enzymes in premature infants. Melatonin and its metabolites are strong antioxidants and they have important functions to prevent mutilation of crucial molecules by free radicals. It was demonstrated that melatonin reduces all aspects of the ensuing damage in the ischemia and subsequent reperfusion model of the heart, kidney, liver, intestine, and brain in cases of excessive ROS

[50]. In 2004, Gitto et al. [42] showed that melatonin treatment can reduce the severity of RDS in preterm newborns by reducing inflammation. Additionally, it has been shown that melatonin can be used in the treatment of hypoxic-ischemic encephalopathy in newborns [88]. Melatonin for PVL has been studied in animal models, and agomelatine and melatonin seem to be likely neuroprotective agents for the prevention of PVL [38].

Resveratrol (a phytoalexin synthesized by some plants) and epicatechin (a green tea extract) are considered new treatment modalities for ROP. Resveratrol as a nitric oxide mechanism modulator and caffeic acid were investigated in the pathogenesis of retinal neovascularization and some effects beneficial for the prevention of ROP were found [89, 90]. Vitamin E, D-penicillamine, intratracheal recombinant human SOD, and allopurinol are used for the treatment and prevention of ROP [91]. Paraoxonase-3 has been identified as an antioxidant that is systemically upregulated in late gestation of human fetuses and some animals such as rats and sheep. According to the findings of the study designed by Belteki et al. [92], paraoxonase-3 may be a therapeutic candidate for preterm infants. For neonatal brain injury, there are possible agents such as vitamins C and E, inhibitors of nitric oxide synthase, allopurinol, erythropoietin, albumin, docosahexaenoic acid, deferoxamine, prostaglandin inhibitors, magnesium sulfate, N-acetylcysteine, melatonin, lutein, and omega-3 polyunsaturated fatty acid [93]. Endotracheal administration of recombinant human SOD, melatonin, and surfactant replacement can reduce the lung injury in preterm newborns receiving mechanical ventilation for RDS [94]. Exogenous antioxidants such as vitamins A and E and recombinant human SOD are considered able to prevent BPD [38].

Saugstad [17] stated that oxidative stress may be triggered or already occurs in prenatal life or before initiation of therapy. Currently there is no accurate single or combination antioxidant or anti-inflammatory agent that has been found and routinely used [17].

5. Future Directions

5.1. New Human Oxidative Stress Source: Wireless Local Area Networks. New studies have started to focus on the relationship between oxidative stress and wireless local area networks (WLANs). Nowadays, WLANs are everywhere, such as workplaces, homes, and public places. Scientists are investigating possible biological effects of exposure to WLAN signals because of the increased usage [95]. There are a few human studies in addition to animal studies. In animal investigations, it has been shown that the oxidative stress of organs such as the brain, liver, testes, ovary, kidney, and eye increases with exposure to WLANs, especially in pregnant or newborn animals [96, 97]. Human studies have generally focused on fertility [98].

5.2. Genetic Mutations. Gene mutations related to oxidative stress have been discovered. Tuxworth et al. [99] showed that the lack of CLN3 function leads to a failure to control the response to oxidative stress and this causes juvenile neuronal ceroid lipofuscinosis (also known as Batten disease), a disease characterized by neuronal degeneration. In one study, preterm infants born by cesarean delivery were compared with preterm infants born by vaginal delivery in terms of H_2O_2-induced oxidative DNA damage and repair capacity (residual DNA damage) in peripheral blood mononucleated cells [100]. The authors reported that preterm infants born by cesarean delivery repair oxidative DNA damage more slowly than preterm infants born by vaginal delivery. It is currently unknown how gene expression is affected; however, there are some hypotheses. Schlinzig et al. [101] observed significantly higher global DNA methylation in white blood cells of newborns delivered by cesarean section, and physiological hypoxemia during vaginal delivery was conducted to increase antioxidant defenses, whereas in the normoxemic planned cesarean section there might be a slower cell cycle, possibly favored by prenatal administration of corticosteroids to the mother, or there could be a different regulation of DNA repair enzymes [100]. Decordier et al. [102] found that H_2O_2 repair capacity and chromosome/genome mutations in newborns are different from those in adults. They found no genotype with a significant effect on DNA repair capacity for the introduction of chromosome/genome mutations by oxidative stress. However, maternal antioxidant supplementation during pregnancy is important for protecting newborns against oxidative DNA damage [102].

6. Conclusion

Neonatal tissues are especially sensitive to oxidative damage because of the rapidly growing nature of their tissues, which makes them vulnerable to the harmful effects of free radicals. However, there are still gaps in our knowledge of the potential role of oxidative injury in the pathogenesis of neonatal diseases. Moreover, there are few publications on validated roles of oxidative stress biomarkers and antioxidants and their protective roles in this field. New studies should more extensively investigate the diagnostic and therapeutic value of various oxidative stress biomarkers and antioxidants to reduce oxidative tissue injury to developing newborns.

Competing Interests

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

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5763743, 11 pages
http://dx.doi.org/10.1155/2016/5763743

Research Article

Immediate Remote Ischemic Postconditioning Reduces Brain Nitrotyrosine Formation in a Piglet Asphyxia Model

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Received 22 January 2016; Revised 21 March 2016; Accepted 27 March 2016

Academic Editor: Serafina Perrone

Remote ischemic postconditioning (RIPostC) is a promising therapeutic intervention that could be administered as an alternative to cooling in cases of perinatal hypoxia-ischemia (HI). In the current study we hypothesized that RIPostC in the piglet model of birth asphyxia confers protection by reducing nitrosative stress and subsequent nitrotyrosine formation, as well as having an effect on glial immunoreactivity. Postnatal day 1 (P1) piglets underwent HI brain injury and were randomised to HI (control) or HI + RIPostC. Immunohistochemistry assessment 48 hours after HI revealed a significant decrease in brain nitrotyrosine deposits in the RIPostC-treated group ($p = 0.02$). This was accompanied by a significant increase in eNOS expression ($p < 0.0001$) and decrease in iNOS ($p = 0.010$), with no alteration in nNOS activity. Interestingly, RIPostC treatment was associated with a significant increase in GFAP ($p = 0.002$) and IBA1 ($p = 0.006$), markers of astroglial and microglial activity, respectively. The current study demonstrates a beneficial effect of RIPostC therapy in the preclinical piglet model of neonatal asphyxia, which appears to be mediated by modulation of nitrosative stress, despite glial activation.

1. Introduction

Hypoxic-ischemic encephalopathy (HIE) occurs after intrapartum asphyxia [1] and is responsible for 23% of neonatal deaths worldwide [2]. Hypothermia therapy has been established as standard clinical care for infants diagnosed with moderate to severe neonatal encephalopathy (NE) in the developed world. Cooling started within six hours of birth ameliorates secondary energy failure and cell death, significantly lowering the risk of death and severe disability in treated infants [3, 4]. In the UK, 45% of infants have adverse outcome after HIE despite cooling, with 25% dying and 20% developing cognitive cerebral palsy and other life-long debilitating conditions [5]. Adjunct therapies for hypothermia are needed to enhance overall protection and improve outcome. In low and mid resource settings where cooling is not routine, alternative therapies may be important.

The developing neonatal brain is particularly vulnerable to oxidative stress because free-radical scavenging systems have not yet matured, resulting in insufficient synthesis of antioxidant enzymes/scavengers following injury [6]. The free-radical nitric oxide (NO) is an ubiquitous neurotransmitter and an important signalling molecule with multiple functions within the CNS. In the brain, NO is synthetized from arginine, nicotinamide adenine dinucleotide phosphate (NADPH), and oxygen by three distinct isoforms of NO synthase (NOS). Neuronal NOS (nNOS) is a calcium-dependent enzyme that is upregulated during ischemia. Resulting NO production reacts with ROS to produce several radicals detrimental to neuronal survival [7–12]. Inducible NOS (iNOS) is a calcium-independent enzyme associated with inflammatory markers and can be produced by macrophages and various other cells under pathological conditions. Like nNOS, iNOS has also shown a link with neuronal loss [6, 8, 9, 11, 13]. Calcium-dependent endothelial NOS (eNOS) enzyme, unlike nNOS and iNOS isoenzymes, is thought to be protective under ischemic conditions, potentially as a result of its

vasodilating effect and subsequent improvement in cerebral perfusion [9, 11, 14–17]. Oxidative stress results in excessive production of NO within different brain regions. NO then combines with superoxide radicals to produce peroxynitrite [18, 19]. Nitrotyrosine is a product of tyrosine nitration from peroxynitrite, and its formation in proteins is an indicator of cell damage. This has been shown both in animal models as well as in clinical cases of brain perinatal asphyxia [11, 20–23]. In addition to directly causing apoptotic cell death in the brain, oxidative stress products induce a profound inflammatory response characterised by neuroglial activation [24]. Reactive oxygen species and important transcription factors such as the inflammatory and antiapoptotic NF-κB [11] stimulate astrocytes and microglia, which respond by secreting a number of proinflammatory cytokines such as interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) [19, 25].

RIPostC is the application of "brief intermittent cycles of ischemia alternating with reperfusion" [26] in the limbs after an ischemic insult. RIPostC has been shown to reduce cerebral ischemia-induced infarct size and neuronal apoptosis in rats [27] and could be similarly effective in neonates. Whilst the underlying neuroprotective mechanism is yet to be understood, RIPostC may interfere with apoptotic pathways by reducing oxidative stress to indirectly inhibit NF-κB activity [27]. In addition, animal models of myocardial injury have shown attenuation of nitrosative stress following postconditioning [28]. We have previously shown in a neonatal piglet model that RIPostC treatment confers white matter protection [29]. In this study we aimed to investigate whether RIPostC protection was associated with changes in oxidative stress, which are also linked to glial activation/inflammation.

2. Materials and Methods

2.1. Animal Experiments and Surgical Preparation. All animal experiments were approved by the Ethics Committee of the University College London and carried out by licensed personnel in concordance with the UK Home Office Guidelines [Animals (Scientific Procedures) Act, 1986] and in compliance with the ARRIVE guidelines. Large white newborn female piglets at postnatal day 1 (P1) were sedated, anesthetized, and surgically prepared as described previously [30–33]. In brief, animals were sedated with intramuscular midazolam (0.2 mg/kg) and anaesthetised with isoflurane (4% v/v) to facilitate tracheostomy and intubation and maintained with 3% during surgery, 2% v/v otherwise. Mechanical ventilation was titrated to ensure partial arterial pressure of oxygen (PaO_2) at 8–13 kPa and carbon dioxide ($PaCO_2$) at 4.5–6.5 kPa. After the airway was secured, both common carotid arteries were isolated at the level of the fourth cervical vertebra and encircled by remotely controlled vascular occluders (OC2A, In Vivo Metric). An umbilical arterial catheter was inserted to enable continuous monitoring of heart rate (HR), mean arterial blood pressure measurement, and arterial blood extraction to measure PaO_2, $PaCO_2$, pH, electrolytes, glucose (3–10 mmol/L), and lactate (I-Stat, Abbott Laboratories, Maidenhead, UK). Mean arterial blood pressure was maintained at approximately 40 mmHg using saline boluses and infusions of inotropes (dopamine and dobutamine 5–20 μg/kg/min each). Hyperglycemia (>10 mmol/L) was treated by substituting 10% with 5% dextrose; Hyperglycemia (>20 mmol/L) was treated by substituting 5% dextrose for saline. Hyperkalemia ($K^+ > 7$ mmol/L) was treated with 4 mcg/kg salbutamol (10 mcg/mL) through the umbilical venous catheter over 10 minutes. Metabolic acidosis (base excess < -10) was corrected with sodium bicarbonate (8.4% wt/vol). All animals received continuous physiological monitoring (SA Instruments, Stony Brook, NY) and extensive life support throughout experimentation. Arterial lines were maintained by infusing 0.9% saline solution (1 mL/h); heparin sodium (1 IU/mL) was added to prevent blockage.

After surgery, and before securing the piglets in a prone position in an acrylic pod with their heads immobilized, the purpose built postconditioning device was placed over both inguinal canals and strapped securely diagonally across the inguinal region with an inflatable bladder placed underneath for further fixation. To assess limb blood perfusion during and after RIPostC, a separate pulse oximeter was secured to the right hind limb, while a laser Doppler assessed perfusion on the left hind limb. An additional pulse oximeter was attached to the right fore limb to measure systemic oxygen saturation [29].

2.2. Cerebral Hypoxia-Ischemia. A 70 × 50 mm, elliptical transmit/receive surface coil tuned for ^{31}P signal acquisition was secured to the head and the animal placed into the bore of a 9.4 Tesla Agilent spectrometer. Whilst in the MRS system, transient HI was induced by remote occlusion of common carotid arteries using inflatable vascular occluders and reducing fractional inspired oxygen (FiO_2) to 6% v/v. During HI, the β-nucleotide trisphosphate (NTP) was continuously monitored using in-house Matlab (Mathworks) software. When β-NTP fell to 40% baseline value, FiO_2 was titrated to maintain 30–40% β-NTP for 12.5 minutes. At the end of insult, the occluders were deflated and FiO_2 normalized. This leads to loss of neurons and TUNEL and caspase 3 positive cell death as well as microglial activation [31–33].

2.3. Experimental Groups. Following resuscitation, piglets were randomised into two groups: HI ($n = 8$) and HI + RIPostC ($n = 8$). Resuscitation after the end of transient HI was taken at time point zero. Piglets were maintained at their target rectal temperature of 38.5 ± 0.5°C using a warmed water mattress. RIPostC was administered with a purpose built device fitted after surgery around the piglet hind limbs and was induced by inflating the device to occlude the femoral artery and then deflating it induced reperfusion. Immediately after resuscitation, piglets underwent 4 cycles of 10 minutes ischemia followed by 10 minutes reperfusion in both hind limbs [29]. In the RIPostC study, cerebral HI resulted in similar insult severity between both groups; however, white matter lactate/N acetyl aspartate was significantly reduced in RIPostC-treated animals 48 hours after HI, and NTP/epp was substantially higher in the treated group [29].

2.4. Brain Histology. Forty-eight hours after HI, piglets were euthanized with pentobarbital. The brains were fixed via cardiac perfusion with phosphate-buffered saline (PBS) followed by cold 4% paraformaldehyde/PBS, dissected out and postfixed at 4°C in 2% paraformaldehyde for 9 days. Coronal slices (5 mm thickness) of the whole right hemisphere were embedded in paraffin wax and sectioned to 8 μm thickness. For each animal, 2 sections (bregma 00 and −2.0) were stained issuing immunohistochemistry and 12 different brain regions were blindly examined. To assess nitrosative stress, adjacent brain sections were stained for nitrotyrosine, inducible NO synthase (iNOS), neuronal NO synthase (nNOS), and endothelial NO synthase (eNOS), respectively. To assess glial activation, sections were also stained for glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule 1 (IBA1).

The brain sections were treated as previously described [32]. In brief, sections were rehydrated followed by heat treatment for antigen retrieval. Sections were then blocked with 5% goat serum (Sigma-Aldrich, USA) for 30 minutes and incubated overnight with nitrotyrosine (1 : 4000, BD Biosciences, UK), iNOS (1 : 200, Novus Biologicals, UK), nNOS (1 : 200, Bioss, UK), eNOS (1 : 1000, BD Biosciences, UK), IBA1 (1 : 2000 Wako, Japan), GFAP (1 : 1000 EMD Millipore, USA), or GFAP (1 : 5000, eBiosciences, USA). Sections were incubated with biotinylated secondary antibody (1 : 250). The staining was visualized using ABC (Vector Laboratories, UK) and DAB (Thermo Scientific, USA). The sections were counterstained with haematoxylin before being dehydrated in graded alcohol and xylene and mounted with Depex (Leica Biosystems, USA). Naïve controls were stained for the aforementioned markers to demonstrate baseline levels.

For fluorescence double labelling, sections were stained as described above. Nitrotyrosine colabel with eNOS and GFAP sections were incubated with both primary antibodies overnight and then incubated with a biotin-conjugated donkey anti-rabbit Ig and an AlexaFluor488-conjugated goat anti-mouse Ig antibody, followed by tertiary AlexaFluor488-conjugated donkey anti-goat Ig antibody, and Texas Red-Avidin (Vector Laboratories, USA, 1 : 1000) was added. For nitrotyrosine with iNOS, nNOS, and IBA1, sections were incubated with primary, secondary, and tertiary antibodies for nitrotyrosine only, blocked with goat and donkey serum (Sigma-Aldrich, USA) and then incubated with antibodies for the second marker. In both protocols, sections were counterstained with DAPI for nuclear visualisation following incubation with the tertiary antibody and then stored in the dark at 4°C.

2.5. Data Analysis. For each animal and section, 12 brain regions were assessed blindly (Figure 2). For nitrotyrosine, nNOS, iNOS, and eNOS, 3 fields per region were scored using a light microscope at ×20 magnification. The semiquantitative scoring system used is described in Table 1 (adapted from [34, 35]). For GFAP and IBA1 assessment was carried out using quantitative thresholding image analysis (adapted from Rahim et al., 2012 [36]). 10 nonoverlapping images (per region) were captured using a live video camera (Nikon, DS-Fi1) mounted onto a Nikon Eclipse E600 microscope at ×40 magnification. Immunoreactivity was determined using Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) with the threshold setting kept constant for all subsequent images for each respective antibody.

Table 1: Brain semiquantitative score system.

Score	Staining	% positive cells
0	None	0
1	Weak	≤25
2	Moderate	≥25–≤75
3	Severe	≥75

2.6. Statistical Analysis

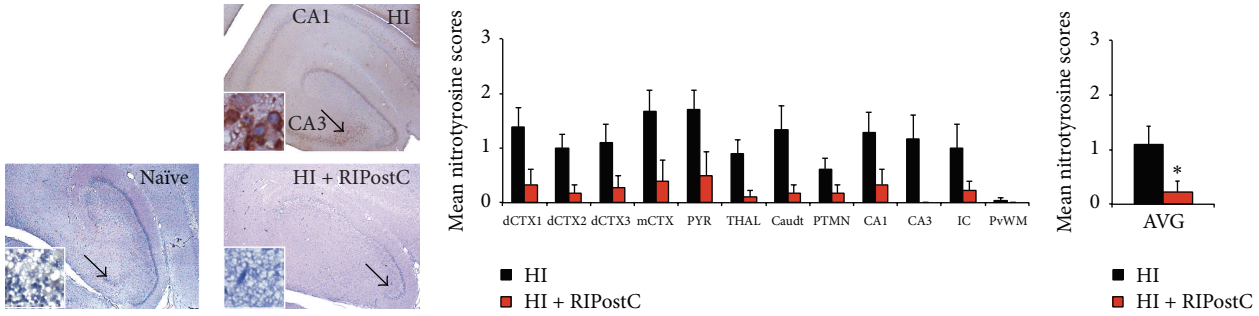
2.6.1. Comparison of Treatment Groups. For each study and for each measurement type, three separate field (nitrotyrosine, iNOS, eNOS, and NOS) and ten separate field (GFAP and IBA1) results were averaged for each subject for each region. Analysis of variance (ANOVA) was performed on the mean data (overall brain effect) with terms for Treatment, Region, and the interaction between Treatment and Region fitted in the model. The significance (p value) for the Treatment effect was assessed to see whether there was evidence of a difference between the overall treatment group means.

2.6.2. Correlation between Measurements. For both treatment groups, the correlation between the different measurements was assessed. A matrix plot which shows a grid of XY scatter plots for each possible pairing of two of the measurements was produced with each of the points on the plot being the average values of X and Y for a particular subject and region. The points in the plot have been identified by the treatment group for the subject and linear regression lines have been plotted so as to assess the strength of a linear relationship between the two measurements. Correlation coefficients R have been tabulated for each pairing of variables for each study.

Results are presented as mean (±SD), and statistical significance was assumed for $p < 0.05$.

3. Results

3.1. Formation of Nitrotyrosine Deposits. Nitrotyrosine is a reaction product of peroxynitrite, a powerful oxidising and nitrating agent that damages lipids, proteins, and DNA [19, 37]. In human neonates, nitrotyrosine deposits are present after perinatal asphyxia [23] and positive staining of the neuronal cytoplasm is a marker of cell damage, oxidative stress, and inflammation that correlates with severity of brain damage and poor outcomes [38]. However, little to no nitrotyrosine deposits are found in healthy organs [39] or in an infant human case control of spinal muscular atrophy without HIE [23]. In the current study, 48 hours after HI insult, nitrotyrosine staining was seen in all brain regions in both HI (controls) and HI + RIPostC groups. Overall semiquantitative scoring assessment showed that levels of nitrotyrosine deposits 48 hours after HI was significantly



(a) (b) (c)

Figure 1: RIPostC decreases nitrotyrosine deposits in the brain 48 hours after HI. (a) Representative nitrotyrosine staining from the hippocampus at 48 hours after HI in HI alone (upper panel) and HI + RIPostC (lower panel). Naïve brain has also been stained to demonstrate the lack of nitrotyrosine deposits in health tissue (left panel). (b) Mean nitrotyrosine scores across multiple brain regions in HI alone and in RIPostC-treated piglets. (c) Overall brain nitrotyrosine scores 48 hours after HI. Data are expressed as mean ± SEM in ANOVA, $n = 8$ per group, and $^{*}p < 0.05$. dCTX1–3 = dorsal cortex 1–3; mCTX = midtemporal cortex; PYR = pyriform cortex; THAL = thalamus; Caudt = caudate; PTMN = putamen; IC = internal capsule; PvWM = periventricular white matter.

reduced across the entire brain hemisphere in RIPostC-treated animals compared to controls (from 1.1 ± 0.3 to 0.2 ± 0.2, $p = 0.02$) (Figure 1(c)).

3.2. Production of Oxidative Stress Biomarkers (NO Synthases). The presence of nitrotyrosine is regarded as a marker of peroxynitrite and suggestive of a role of NO toxicity (nitrosative stress) in HI brain injury. To determine the potential source of nitrotyrosine, NOS –nNOS, iNOS, and eNOS were subsequently assessed using the same semiquantitative scoring system performed in the nitrotyrosine assessment.

3.2.1. Neuronal Nitric Oxide Synthase Expression (nNOS). Assessment of nNOS expression showed no significant difference in nNOS expression in the HI + RIPostC group versus HI alone (Figure 2(c)).

3.2.2. Inducible Nitric Oxide Synthase Expression (iNOS). We observed significantly less iNOS expression across the brains of the HI + RIPostC group than in the HI control group (from 0.8 ± 0.3 to 0.4 ± 0.2, $p = 0.010$) (Figure 2(f)).

3.2.3. Endothelial Nitric Oxide Synthase Expression (eNOS). There was a significant overall brain increase in eNOS expression in the HI + RIPostC group compared to the HI control group (from 0.4 ± 0.2 to 1.2 ± 0.3, $p < 0.0001$) (Figure 2(i)).

3.3. Glial Activation. To determine whether neuroprotection was associated with glial activation, sections were stained for markers of astrogliosis (GFAP) and microglial activation (IBA1). Analysis of these stainings consisted of quantitative threshold analysis of 10 nonoverlapping images per assessed brain region and immunoreactivity determined using Image-Pro Plus software.

3.3.1. Microglia. The overall level of microglial activation was significantly higher in RIPostC-treated animals in comparison to controls (from 2.8 ± 0.8 to 4.9 ± 1.7, $p = 0.006$) (Figure 3(c)).

Table 2: Correlation matrix between nitrotyrosine deposits and markers of NOS and glia activity.

RIPostC	eNOS	GFAP	IBA1	iNOS	Nitrotyrosine	nNOS
eNOS		0.22	0.06	−0.29	−0.30	−0.16
GFAP			0.05	−0.20	−0.18	−0.07
IBA1				0.01	−0.12	−0.05
iNOS					0.46	0.36
Nitrotyrosine						0.31

3.3.2. Astrocytes. Assessment of astrocyte activation (GFAP) showed overall significant increase in the RIPostC group versus control (from 9.0 ± 2.6 to 13.3 ± 3.4, $p = 0.002$) (Figure 3(f)).

3.4. Correlation of Nitrotyrosine, Oxidative Stress Biomarkers, and Glial Activation. Our results showed that nitrotyrosine deposits, expression of NO synthases microglia, and astroglial activation were affected by RIPostC treatment. Therefore, we assessed potential histological correlations between nitrotyrosine deposits and each of the NOS and glial markers used. Overall, nitrotyrosine deposits correlated positively with nNOS ($r = 0.31$) and iNOS ($r = 0.46$) expression and correlated negatively with eNOS expression ($r = -0.30$). Although moderate R^2 values indicate that these correlations are not very strong, the consistent effect suggests a reasonable link. There was no correlation between nitrotyrosine and IBA-1 ($r = -0.12$) and GFAP ($r = -0.18$) markers (Table 2).

3.5. Colocalization of Nitrotyrosine and NOS in Different Cell Types. Colabeling of nitrotyrosine with different cell types as well as NOS showed that nitrotyrosine deposits were present mostly in neuronal cells of both treatment groups but to a greater extent in HI animals (Figures 4(a)-4(b)). Nitrotyrosine deposits were also identified in some endothelial cells of HI control animals (Figure 4(g)) as well as nNOS (Figures 4(i)-4(j)) and iNOS positive cells in both groups (Figures 4(k)-4(l)). Some nitrotyrosine deposits were also present

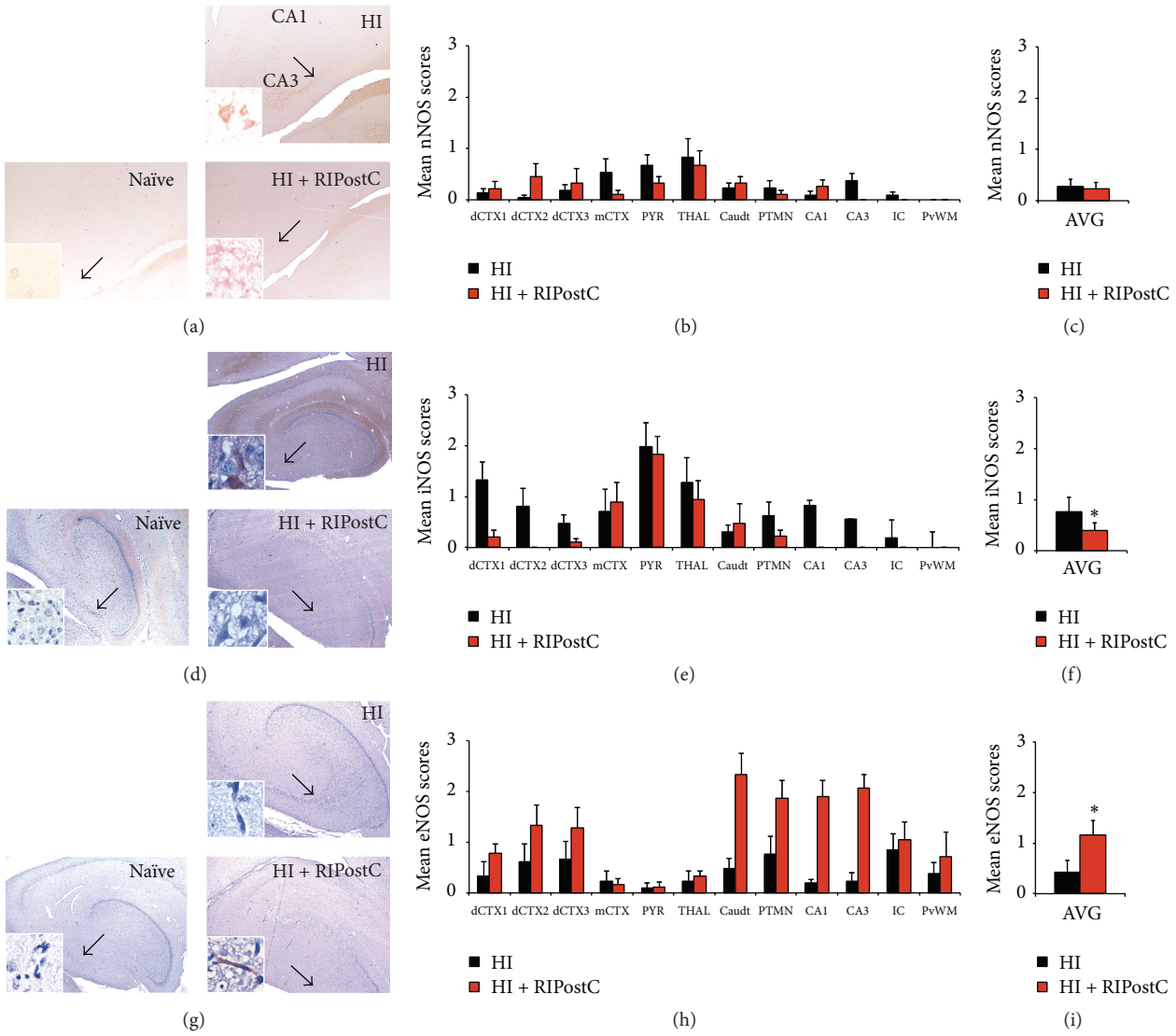


Figure 2: Effect of RIPostC treatment on nitric oxide synthases. (a–c) nNOS levels remain unaffected by RIPostC treatment 48 hours after HI. (a) Representative nNOS staining from the hippocampus at 48 hours after HI in HI alone (upper panel) and HI + RIPostC (lower panel). (b) Mean nNOS scores across multiple brain regions in HI alone and in RIPostC-treated piglets. (c) Overall brain nNOS scores 48 hours after HI. (d–f) RIPostC treatment significantly reduced overall iNOS levels in the brain 48 hours after HI. (d) Representative micrographs of iNOS staining from the hippocampus at 48 hours after HI in HI alone (upper panel) and HI + RIPostC (lower panel). (e) Mean iNOS scores across multiple brain regions in HI alone and in RIPostC-treated piglets. (f) Overall brain iNOS scores 48 hours after HI showing significant reduction in iNOS semiquantitative scoring for the RIPostC-treated group ($p = 0.010$). (g–i) RIPostC treatment was associated with a significant increase in endothelium-derived NO 48 hours after HI injury. (g) Representative eNOS immunohistochemistry from the hippocampus at 48 hours after HI in HI alone (upper panel) and HI + RIPostC (lower panel). (h) Mean eNOS scores across multiple brain regions in HI alone and in RIPostC-treated piglets. (i) Overall significant increase in brain eNOS semiquantitative scores in RIPostC-treated piglets 48 hours after HI. (a, d, g) For comparison, naïve tissue is shown in the left column. Data are expressed as mean ± SEM in ANOVA, $n = 8$ per group, and $^*p < 0.05$. dCTX1–3 = dorsal cortex 1–3; mCTX = midtemporal cortex; PYR = pyriform cortex; THAL = thalamus; Caudt = caudate; PTMN = putamen; IC = internal capsule; PvWM = periventricular white matter.

in microglia IBA1-stained cells of RIPostC-treated animals (Figures 4(c)-4(d)) but not in astroglia (Figures 4(e)-4(f)). iNOS marker was identified in neurons (Figures 4(m)-4(n)) and astrocytes (Figures 4(q)-4(r)) of both HI and RIPostC-treated animals, as well as in microglia of RIPostC group (Figure 4(p)). However, no iNOS deposits were observed in endothelial cells (Figures 4(s)-4(t)).

4. Discussion

Our results demonstrate that RIPostC treatment has a significant association with decrease in nitrotyrosine deposits in the brain, as well as iNOS. Conversely, eNOS was significantly upregulated, with also a substantial increase in IBA1 and GFAP glial markers, suggesting that RIPostC may

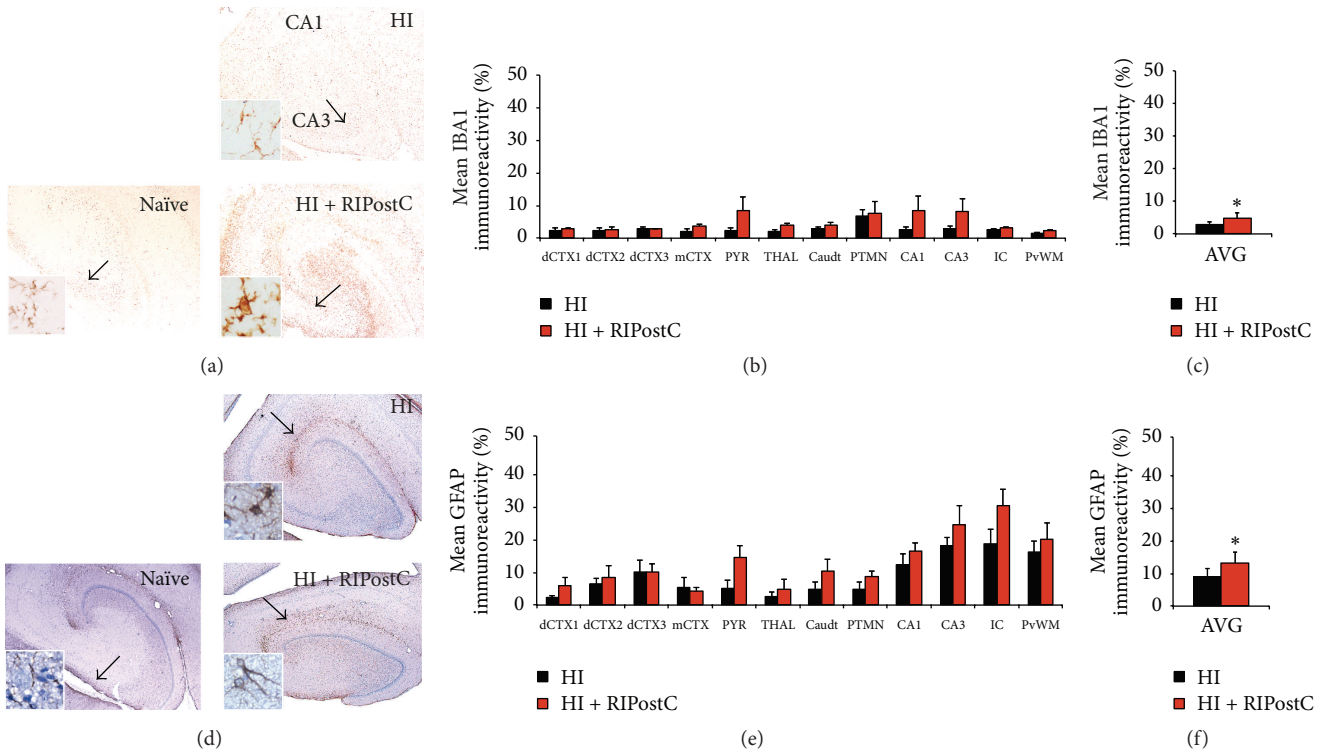


Figure 3: RIPostC-treated animals have increased glial activation. (a–c) IBA1 quantitative thresholding analysis revealed significant increase in microglial IBA1+ staining in the HI + RIPostC group 48 hours after HI. (a) Representative IBA1 staining from the hippocampus at 48 hours after HI in HI alone (upper panel) and HI + RIPostC (lower panel). (b) Mean IBA1 quantitative thresholding analysis across multiple brain regions in HI alone and in RIPostC-treated piglets. (c) Overall IBA1 immunoreactivity was significantly increased in the RIPostC-treated group when compared to HI alone piglets ($p = 0.006$). (d–f) RIPostC treatment significantly increased GFAP+ astrocytes 48 hours after HI. (d) Representative micrographs of GFAP immunohistochemistry from the hippocampus at 48 hours after HI in HI alone (upper panel) and HI + RIPostC (lower panel). (e) Mean GFAP quantitative thresholding analysis across multiple brain regions in HI alone and in RIPostC-treated piglets. (f) RIPostC treatment resulted in an overall significant increase in GFAP+ astrocyte immunoreactivity 48 hours after HI ($p = 0.002$). (a, d) Naïve comparative controls are shown on the left panel. Data are expressed as mean ± SEM in ANOVA, $n = 8$ per group, and $^*p < 0.05$. dCTX1–3 = dorsal cortex 1–3; mCTX = midtemporal cortex; PYR = pyriform cortex; THAL = thalamus; Caudt = caudate; PTMN = putamen; IC = internal capsule; PvWM = periventricular white matter.

work through reduction in oxidative/nitrosative stress despite increased glial activation.

4.1. Remote Ischemic Postconditioning Reduces Nitrotyrosine Deposits. Several neurological conditions are associated with formation of nitrotyrosine deposits, which in turn is associated with brain tissue damage. Groenendaal et al. described the presence of nitrotyrosine deposits, particularly in the thalamus and inferior olives of 22 full-term infants [23], as well as in the spinal cord of 5 out of 18 full-term neonates [40] who died following HIE. Nitrotyrosine forms from peroxynitrite, a powerful nitrating and oxidising agent that is the reaction product of NO and superoxide anions. Pathological conditions, such as HI, excessively activate neuronal and inducible NOS, leading to elevated NO and subsequent increased peroxynitrite production [19, 41]. This is known to cause oxidative damage to cellular constituents and trigger cell death [42]. Our results demonstrate that RIPostC administered immediately after the end of transient global HI and consisting of 4 cycles of 10-minute hind limb ischemia followed by 4 cycles of 10-minute reperfusion significantly reduced the presence of cytoplasmic nitrotyrosine deposits in the brain 48 hours after HI. The reduction in nitrotyrosine deposits seen with RIPostC treatment indicates a reduction in oxidative/nitrosative stress. This is well known to cause oxidative damage to cellular constituents and trigger cell death [42]. This suggests that one of the protective effects of RIPostC is most likely due to a reduction in upstream production of NO.

4.2. Remote Ischemic Postconditioning Reduces iNOS Expression. At the onset of ischemia, nNOS is responsible for the overproduction of NO and consequent cytotoxicity described above [8]. iNOS, which is not normally present in healthy tissue, is activated by a number of inflammatory and immunologic signals [43–45] as well as cerebral ischemia. In contrast to nNOS, iNOS activity peaks in the later stages of ischemic damage [46], suggesting that the NO it generates contributes to delayed cell damage. In neonatal encephalopathy it is the delayed cell death as a result of secondary energy failure that is correlated with the severity of adverse outcomes [47, 48], and iNOS inhibitors have shown protective effects even

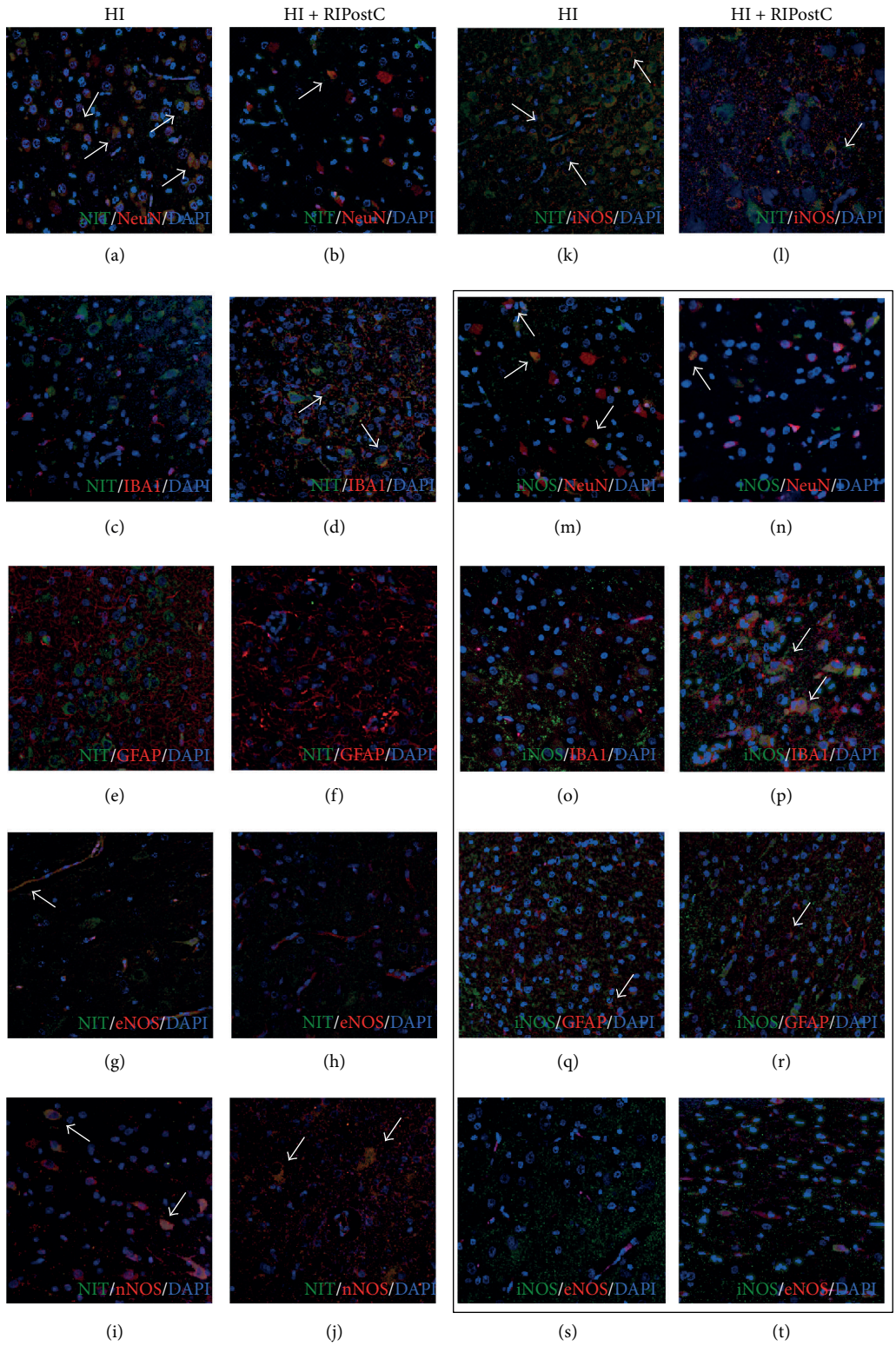


Figure 4: Colocalization of nitrotyrosine deposits and oxidative stress/neural markers. Nitrotyrosine deposits were mostly visible in neurons of HI animals (white arrows, (a)), with some colocalization also present in RIPostC-treated piglets (b). This colocalization was also observed to a much smaller extent in microglia of RIPostC animals (c-d), endothelial cells in the HI group (g-h), and toxic nNOS (i-j) and iNOS (k-l) for both treatments but not in astroglial (e-f). iNOS double labelling (black box) showed its expression in neurons (m-n) and astrocytes (q-r) of both HI and RIPostC animals but not in endothelial cells (s-t). iNOS was also expressed by microglia in the RIPostC group (o-p).

when administered 24 hours after MCA [49]. Whilst in the current study, nNOS levels were unchanged in the RIPostC group compared with untreated animals; levels of iNOS were significantly reduced. In rats, selective inhibition of iNOS activity with aminoguanidine attenuates postischemic iNOS activity and reduces infarct volume following middle cerebral artery occlusion (MCA) [46]. Similarly, iNOS-knockout mice do not exhibit ischemia-induced iNOS expression and have smaller tissue infarction than wild-type mice after MCA occlusion [50]. Additionally, a study by Wei and colleagues has shown that ischemic postconditioning attenuated iNOS and nitrotyrosine production following focal MCA occlusion [51]. Patients with acute myocardial infarct who underwent ischemic postconditioning demonstrated reduction in iNOS activity in white blood cells as well as decreased plasma nitrotyrosine. The same group showed similar effect in the rat model of myocardial ischemia/reperfusion injury [28]. This suggests that RIPostC may confer neuroprotection in our model by reducing iNOS expression and subsequent production of superoxide to generate peroxynitrite resulting in nitrotyrosine formation.

4.3. Remote Ischemic Postconditioning Increases eNOS Expression. In contrast to iNOS and nNOS, eNOS-derived NO has a functionally protective role. In the brain, eNOS is expressed in cerebral endothelial cells [52] and acts as a potent vasodilator; the NO it generates is critical for regulating vascular tone. This facilitates cerebrovascular perfusion and protects against ischemic brain damage by improving blood flow to ischemic tissue [41]. Accordingly, eNOS-knockout mice have reduced cerebral blood flow and enlarged cerebral infarcts after stroke [53]. Conversely, upregulation of eNOS reduces infarct volume after focal [54] and global [55] cerebral ischemia in rats. In the current study, RIPostC-treated animals demonstrated significant upregulation of eNOS expression 48 hours after HI. This is in agreement with the effect of RIPostC on eNOS expression already reported by Peng et al., 2012, where the neuroprotective effect of RIPostC resulted in significant upregulation of eNOS via the PI3K/Akt pathway. This protective effect was reversed following the administration of NG-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor [17, 55]. Therefore, an increase in eNOS-derived NO probably mediates some of the neuroprotective effect of RIPostC demonstrated in our piglet model of global HI.

4.4. Remote Ischemic Postconditioning Activates Microglia. In the current study, levels of IBA1 were significantly increased in RIPostC-treated animals compared to untreated controls. Depending on the extent of injury microglia are classified as either classical (M1) or alternative (M2) and exert neurotoxic or neuroprotective functions, respectively. In ischemic conditions, M1 microglia produce proinflammatory cytokines, reactive oxygen species, and neurotoxic factors and destructively phagocytose tissue whereas M2 produce anti-inflammatory cytokines and neurotrophic factors and phagocytose dying neurons [56, 57]. Accordingly, selective ablation of microglia in mice increases infarct size and apoptosis after stroke [58], but use of microglial inhibitors provides significant neuroprotection against global ischemia [59]. This highlights the complexity of the role of microglia-mediated inflammation in neurological damage or disease. In the current study we observed a significant increase in IBA1 immunoreactivity. This is not in concordance with our previous results, where IBA1 was significantly reduced in the corpus callosum of RIPostC-treated piglets [29]. This difference in results may be a consequence of different analyses, with threshold imaging proving to be a more thorough assessment encompassing a greater area of the different assessed brain regions. The current results raise questions on whether RIPostC treatment is associated with increase in M2 microglial activation or whether there is a neuroprotective effect provided by RIPostC despite microglia activation.

4.5. Remote Ischemic Postconditioning Upregulates Astroglial Immunoreactivity. GFAP expression was significantly increased in the RIPostC-treated group. Similarly to NO, there is evidence of a dual role of astrocyte activation following HI. Many studies have shown that attenuation of astrogliosis often correlates with reduced infarct size—nonspecific inhibition of cell proliferation [60] or inhibition of astrocyte component synthesis [61] reduce infarct size accompanied by an attenuated astroglial response. Activated astrocytes secrete a number of proinflammatory cytokines and chemokines, responsible for inducing apoptotic cell death, increasing production of toxic NO, and attracting inflammatory cells to the injured site [62]. However, astroglia also support neurons through providing an antioxidant effect, reducing excitotoxicity and downstream oxidative stress by taking up excess glutamate, and producing neurotrophic factors [63] to reduce injury and promote recovery. Administration of astrocyte-derived factors reduces cerebral oedema and lesion size in rats with induced focal ischemia [64]. GFAP-knockout mice exhibit larger lesions and a greater reduction in cerebral blood flow following focal cerebral ischemia [65]. This suggests that the increase in astrocyte activation seen in the current study with RIPostC treatment could be a contributor to its neuroprotective effect by assisting with vasogenesis. Interestingly, one of the mechanisms in which RIPostC is thought to be involved is the humoral pathway, where circulation of blood-borne protective factors is released by the ischemic limb as well as efferent nerve activation [29]. Therefore, it could be possible that astrocytes may assist with increased blood flow and activation of prosurvival factors and repair mechanisms. The current findings on both microglia and astroglial activation following RIPostC treatment are intriguing and represent a very interesting avenue for further investigation.

5. Conclusion

In the piglet model of perinatal asphyxia, RIPostC treatment provided significant white matter protection as observed through TUNEL assay [29]. In the current study, this RIPostC-induced neuroprotection appears to be associated with observed reduced nitrotyrosine deposits across the brain 48 hours after HI. This reduction may be a result of an overall

increase in eNOS, as well as reduction of iNOS expression across all assessed brain regions. This RIPostC-mediated reduction in nitrosative stress has also been observed in other in vivo and clinical studies [28, 51, 55]. Interestingly, we have also shown significant increase in astroglial and microglia activation not previously observed. This raises question on whether increased glial activation is somehow protective or whether RIPostC has a beneficial effect reducing nitrosative stress irrespective of glial activation. Furthermore, nitrotyrosine deposits were mostly present in neurons but were also found to a smaller extent in glial cells. Our findings demonstrate the need for better understanding the potential dual role of glial activation following HI. Overall, this study suggests that the neuroprotective effects previously reported are mediated, at least in part, by alteration of nitrosative stress despite glial activation. The effective but safe and noninvasive nature of RIPostC makes it an attractive potential treatment for NE.

Competing Interests

The authors declare no competing interests regarding the research, authorship, and/or publication of this paper.

Acknowledgments

The authors thank Debbie Kraus for the statistical analysis. This study was funded by the MRC (Grant no. MR/J00457X/1). This research received a proportion of funding from the UK Department of Health's National Institute for Health Research Biomedical Research Centres funding scheme.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5057610, 12 pages
http://dx.doi.org/10.1155/2016/5057610

Research Article

Oxidative Stress in the Developing Rat Brain due to Production of Reactive Oxygen and Nitrogen Species

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Received 15 January 2016; Accepted 16 March 2016

Academic Editor: Maria L. Tataranno

Oxidative stress after birth led us to localize reactive oxygen and nitrogen species (RONS) production in the developing rat brain. Brains were assessed a day prenatally and on postnatal days 1, 2, 4, 8, 14, 30, and 60. Oxidation of dihydroethidium detected superoxide; 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate revealed hydrogen peroxide; immunohistochemical proof of nitrotyrosine and carboxyethyllysine detected peroxynitrite formation and lipid peroxidation, respectively. Blue autofluorescence detected protein oxidation. The foetuses showed moderate RONS production, which changed cyclically during further development. The periods and sites of peak production of individual RONS differed, suggesting independent generation. On day 1, neuronal/glial RONS production decreased indicating that increased oxygen concentration after birth did not cause oxidative stress. Dramatic changes in the amount and the sites of RONS production occurred on day 4. Nitrotyrosine detection reached its maximum. Day 14 represented other vast alterations in RONS generation. Superoxide production in arachnoidal membrane reached its peak. From this day on, the internal elastic laminae of blood vessels revealed the blue autofluorescence. The adult animals produced moderate levels of superoxide; all other markers reached their minimum. There was a strong correlation between detection of nitrotyrosine and carboxyethyllysine probably caused by lipid peroxidation initiated with RONS.

1. Introduction

Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, and hydroxyl radicals together with reactive nitrogen species (RNS) derived from nitric oxide constitute common intermediates of cellular metabolism and they are collectively referred to as RONS. Their overproduction is the cause of the oxidative stress; however, at very low concentrations, they can function as signaling molecules [1, 2]. Oxidative stress participates in many pathologies and is also considered a major factor in the ageing process [3]. Brain reveals high specific oxygen consumption due to abundant mitochondria and highly active respiratory enzymes [4]; therefore, the effect of oxidative stress on brain ageing is widely recognized [5]. Nitric oxide (NO) is enzymatically synthesized by three isoforms of nitric oxide synthase, that is, neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). It was shown that transcripts for all isoforms are present in the brain during embryonal and postnatal development [6].

Brain oxygen concentration highly increases after birth compared to the foetus, thus creating a condition of oxidative stress. The expression of both nNOS and iNOS increases after birth reaching a peak on the fifth postnatal day [7]. Excessive NO production injures the adjacent tissues by formation of peroxynitrite which originates from the rapid interaction with superoxide. These reactions result in the formation of nitrotyrosine moieties in affected proteins. Nitrotyrosine was detected in the cortex of aging rats [8] and also during the early postnatal period [7]. Besides that, the early postnatal period is characterized by apoptotic elimination of both neurons and glia which proliferated in

overabundance [9]. Such an elimination and further proliferation of glial and even nerve cells manifest some phasing during the first two postnatal months [10]. Apoptotic cells are phagocyted by microglia with concomitant production of RONS. Superoxide was detected by staining the living brain slices with nitroblue tetrazolium, and microglial respiratory burst was detected in vivo using a fluorescent probe [11]. Peroxynitrite can also initiate membrane lipid peroxidation [12, 13] and catalyze glycoxidation and lipoxidation reactions [14]. Thus, the products of glycoxidation reactions such as carboxyethyllysine may be formed in the sites of NO-induced lipid peroxidation [15].

The increased production of free radicals in the early postnatal period suggests that oxidative stress is generally occurring after normal birth. An important study in this regard showed ROS-mediated oxidative damage to DNA in rat liver, kidney, and skin during the first few hours after normal birth. The lesions were considered substantial, having been similar to or exceeding the levels in 24-month-old rats [16]. This concept was further supported by the finding of pronounced neonatal decreases in the hepatic glutathione/glutathione disulfide (GSH/GSSG) ratio in rats [17, 18]. Also, the product of membrane lipid peroxidation, malondialdehyde, exhibited a transient rise after birth in rat liver and kidney [19]. In the guinea pig brain, the increase in lipid peroxidation was observed during prenatal period [20]. Studies in the rat brain recognized proteins modified by lipid peroxide decomposition product, 4-hydroxynonenal (HNE), by immunocytochemistry and electron microscopy. A moderate increase in labeling was observed in the corpus callosum in the 7-day-old and 8-day-old rats, on day 10 it was maximum, sharp decrease was observed on day 14, and no staining was observed in adult rats. Electron microscopy showed dense HNE staining on the basal laminae of blood vessels and on the plasma membranes of unmyelinated axons [21]. In the human brain, HNE protein adducts increased in parietal white matter from gestational week 40 to postnatal year 1.5 encompassing the peak period of myelin sheath synthesis at this site. It was concluded that human brain development involves basal levels of oxidative stress [22]. In the recent study [23], ROS production was found to increase in developing mouse heart from postnatal day 1 to day 7, when it was maximal, and correlated with DNA oxidative damage.

In a preceding work we analyzed the quantitative aspects of free radicals production in the developing rat brain [24]. We have documented the usefulness of redox-sensitive fluorescent probes in connection with fluorescence microscopy for the detection of RONS in vivo in further studies [25, 26]. In the present study, we have focused our aim at the localization of the sites of RONS production. We used dihydroethidium (DHE) for the detection of superoxide and 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) as a probe for hydrogen peroxide. Antibodies against protein nitrotyrosine detected the results of nitric oxide overproduction, antibodies against carboxyethyllysine localized the sites of nonenzymatic glycation and lipid peroxidation, and blue autofluorescence detected oxidized proteins. This integrative approach has enabled the detailed localization of the sites of RONS production in the developing brain.

2. Materials and Methods

2.1. Animals. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication number 85-23, revised 1996). Experiments were approved by the Animal Protection Expert Commission of the Faculty.

Twelve pregnant female Wistar rats were used in the experiments. They had free access to water and standard laboratory diet. The offspring (a total of 65 neonates) of both sexes were investigated 1 day before birth (group F, $n = 12$, mean weight: 4.32 ± 0.30 g) and then on days 1 (group D1, $n = 10$, mean weight: 6.29 ± 0.41 g), 2 (group D2, $n = 10$, mean weight: 7.91 ± 0.23 g), 4 (group D4, $n = 10$, mean weight: 10.15 ± 0.99 g), 8 (group D8, $n = 8$, mean weight: 16.64 ± 2.00 g), 14 (group D14, $n = 7$, mean weight: 25.68 ± 1.79 g), 30 (group D30, $n = 4$, mean weight: 110.00 ± 2.5 g), and 60 (group D60, $n = 4$, mean weight: 275.15 ± 7.07 g) of postnatal life. The preliminary studies did not reveal any gender difference in structures of interest; therefore, distribution of males and females in groups was random.

On the sampling day, rats were weighed and sacrificed under general anaesthesia (Thiopental, 40 mg kg^{-1} b.w., i.p., VUAB Pharma a.s., Roztoky, Czech Republic) by decapitation (groups F, D1, D2, D4, and D8) or by cutting the spinal cord (groups D14, D30, and D60). In F group, mothers were anaesthetized as well. Isolated brains were cut transversally approximately at the bregma level, snap-frozen in the liquid nitrogen, and stored at −80°C.

2.2. Detection of ROS Production by Fluorescence Microscopy. Brains were cut at −20°C using the cryostat Leica CM 1950 (Leica Microsystems, Germany) at 5 μm. The nonfixed sections were used for the experiments. Slides were assessed by the Olympus BX 53 microscope in the bright field and by epifluorescence (filter U-FUW: excitation 340–390 nm, barrier 420 nm; filter U-FBW: excitation 460–495 nm, barrier 510 nm; filter B/G: excitation 481–520 nm, barrier 568–643 nm), respectively. Microphotographs were taken by the ProgRes C5 digital camera using the NIS Elements AR software (Laboratory Imaging, Czech Republic).

For the detection of hydrogen peroxide, we used 6-carb oxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes, USA) as specified in our previous work [24]. In brief, the stock solution of H2DCFDA was made by dissolving 5 mg of the dye in 1 mL of ethanol; for staining, it was diluted to 10 μg/mL in phosphate-buffered saline, pH 7.4 (PBS). Slides with frozen brain sections were washed in PBS and incubated with H2DCFDA for 10 min.

A similar procedure was used for the detection of superoxide using dihydroethidium (DHE; Molecular Probes, USA). The stock solution of DHE was made by dissolving 5 mg of the dye in 1 mL of water; for staining, it was diluted to 5 μg/mL in PBS.

All sections (except for autofluorescence) were counterstained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; 0.5 μg/mL, 2 min, Sigma-Aldrich Chemie, Germany).

For autofluorescence measurements the thin sections were mounted after PBS washing only.

Finally, sections were mounted into Fluoroshield mounting medium (Sigma-Aldrich Chemie, Germany) under a coverslip and sealed with a lacquer.

Microphotographs were taken by a camera in the bright field under UV to reveal autofluorescence and nuclei stained by DAPI and under appropriate filter to reveal oxidized H2DCFDA or DHE fluorescence. The figures showing fluorescent nuclei were electronically merged with figures showing the specific fluorescence. To elucidate specific structures, some figures taken in the bright field were merged with the figures showing the respective fluorescence.

2.3. Immunohistochemistry of Nitrotyrosine and Carboxyethyllysine. Formalin-fixed and paraffin-embedded brain sections cut at 4 μm were used for immunohistochemistry. Three deparaffined and rehydrated tissue sections from each group were treated with 3% H_2O_2 in methanol for 30 minutes to block endogenous peroxidases. The washed sections were incubated with the primary mouse monoclonal antibodies NO-60-E3 (against protein-bound 3-nitrotyrosine) diluted 1 : 100 [27, 28] or CEL-9-H11 (against N^{ε}-(carboxyethyl)lysine) diluted 1 : 200 for 90 min [29]. PBS with 10 mg/mL bovine serum albumin was used for dilution of all antibodies as well as for washings between the incubation steps. Washed sections were incubated with the secondary antibody against mouse IgG labeled with horseradish peroxidase (P260, DakoCytomation, Denmark) diluted 1 : 100. The bond was visualized using diaminobenzidine (Sigma FAST™ DAB Peroxidase Substrate Tablets, Sigma-Aldrich Chemie, Germany); the reaction was enhanced by 1% $CuSO_4$. The sections were counterstained with hematoxylin and mounted into the Solacryl BMX permanent mounting medium. Negative controls were provided by omission of the primary antibody.

3. Results

3.1. Detection of Superoxide Production with DHE. Oxidation products of DHE were used for the detection of superoxide generation giving orange fluorescing fluorophores. These products can also bind to DNA and as we have counterstained nuclei with the blue fluorescing DAPI, we can encounter in the nuclei three situations: (1) the fluorescence intensity of DHE oxidation products is higher than fluorescence of DAPI and the nucleus will appear as red; (2) the fluorescence intensities of both DHE oxidation products and DAPI are about the same and the resulting light composed of orange and blue will be seen as white; (3) no oxidation of DHE in the vicinity of a nucleus results in the blue fluorescence.

This situation is illustrated in Figure 1 for the foetal brain (panels (a)–(c)). Panel (a) shows lateral ventricle between fimbria of the hippocampus and amygdala. There are whole regions containing blue, red, and white fluorescing nuclei of neurons and glia, respectively. As it is apparent from the detailed view of a blood vessel in panel (b), the cytoplasm of the endothelial and smooth muscle cells is positive as well, not only the respective nuclei. Panel (c) shows the positive region of panel (a) in greater detail. Indicated by arrows are the neuronal dendrites containing high amount of DHE oxidation products. The situation has changed quickly on D1 (panel (d)). The positivity of cytoplasm of neurons and glial cells was reduced; still positive was the cytoplasm of endothelial cells.

On D2 (Figure 2(a)), the positivity of blood vessels changed in comparison with the foetal brain (compared to Figure 1(b)). The cytoplasm of the endothelial cells was less positive and fluorescence intensity of the neuronal cytoplasm and glial cytoplasm was increased. Panel (b) illustrates both high positivity of the endothelial cells in a blood vessel localized within pia mater and cortical cellular positivity limited to the external granular layer on D4. Panel (c) shows increased positivity observed in the nuclei localized both in the cortex and in the endothelial cells on D8. The red fluorescence was also apparent in the cytoplasm of the respective cells. D14 (panel (d)) was characterized by a total lack of fluorescence in the blood vessel cell cytoplasm and nuclei. The cytoplasm of neurons and glia showed some red fluorescence; however, the highest intensity of bright orange fluorescence was found in the arachnoidal membrane cells. On D30 (panel (e)), the positivity in the blood vessel cells was partially restored and the fluorescence in the neuronal cytoplasm and glial cytoplasm was increased in comparison with D14. All the cortex nuclei demonstrated white to red fluorescence; the cytoplasm was moderately and uniformly active on D60 (panel (f)).

3.2. Detection of Hydrogen Peroxide Production with H2DCFDA. Oxidized H2DCFDA shows green fluorescence which appears as yellow-green at high intensity. The situation in the foetal brain is illustrated in Figure 3(a). We can see uniformly low intensity fluorescence, which is not different in the blood vessel wall cells. On D1, fluorescence in neuronal cytoplasm and glial cytoplasm was without change; however, cells of developing blood vessels appeared intensively bright (panel (b)). A moderate fluorescence could have been observed both in the blood vessel wall cells and in the neuronal cytoplasm and glial cytoplasm on D2 (panel (c)). A distinguished increase in H2DCFDA fluorescence occurred in the blood vessel wall cells on D4 (panel (d)). Intensive fluorescence originated from the internal elastic membrane. Fluorescence intensity in the neuronal cytoplasm and glial cytoplasm was approximately on the same level as on D2. A cessation of fluorescence intensity in the cortical cells was observed on D8; low intensity fluorescence was retained in pia mater and in the blood vessel wall (panel (e)). Fluorescence intensity in the blood vessel wall on D14 was about the same magnitude as on D8; however, new fluorescence appeared in the cytoplasm of neurons and glia (panel (f)). On D30, the intensity of fluorescence of internal elastic membrane was higher than that on D14; fluorescence

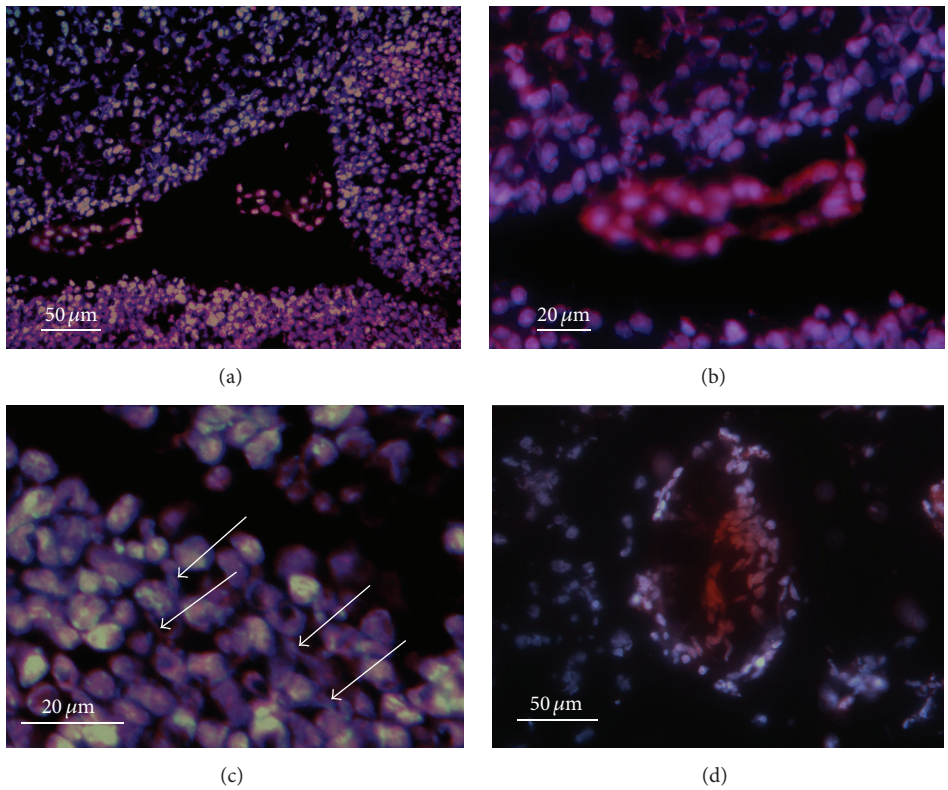

Figure 1: DHE oxidation in the perinatal brain (colocalization with DAPI). (a) Overview of a lateral ventricle between fimbria of the hippocampus and amygdala of the foetal brain. (b) A detailed view of a blood vessel. (c) A detail of the active region; arrows indicate the positive dendrites. (d) The situation on D1.

in the cytoplasm of neurons and glia was practically the same (panel (g)). Faint but still apparent fluorescence can be observed in the brain on D60 (panel (h)).

3.3. Localization of the Sites of Protein Nitration with Antibodies against Protein Nitrotyrosine. Production of reactive nitrogen species results in the formation of protein-bound nitrotyrosine (NT) and nitrated proteins can be detected by specific antibodies. The summary of immunohistochemical detection of nitrated proteins in the brain is given in Figure 4. A lot of positive sites can be found in the foetal brain: cytoplasm of most neurons including their dendrites as well as glia, some nuclei, and cytoplasm of endothelial cells (panel (a)). The situation was practically without change on D1 (panel (b)). There was a marked positivity in superficial glial limiting membrane on D2; cytoplasm of neurons, glia, and blood vessel wall cells was without change (panel (c)). On D4, there was the highest extent of positivity found in the whole time range of the study. Cortical neurons and glia in molecular, external granular, and external pyramidal layers were intensively stained. Blood vessel wall was not changed in comparison with D2; however, border arachnoidal cells were highly positive (panel (d)). D8 was characterized by cessation of positivity in neuronal cytoplasm and glial cytoplasm; endothelial cells and border arachnoidal cells maintained high staining intensity (panel (e)). The decrease in the intensity of staining of neurons/glia continued on D14; for the first time, a decrease in the staining intensity in the cytoplasm of endothelial cells was observed. Border arachnoidal cells still stained intensively (panel (f)). The intensity of staining further decreased on D30 and the decrease concerned also the border arachnoidal cells, though their intensity of staining was still highest in comparison with other sites (panel (g)). The situation on D60 was without change in relation to D30 (panel (h)).

3.4. Localization of the Sites of Protein Glycation and Lipid Peroxidation with Antibodies against Carboxyethyllysine. Carboxyethyllysine (CEL) is a product that originates from both protein glycation and lipid peroxidation. As such it represents an important marker of free radical activity. The summary of its immunohistochemical detection in brain is presented in Figure 5. Several positive sites can be found in the foetal brain, especially the cytoplasm of endothelial cells and neurons/glia, the fibrous component of pia mater, and nuclei of neurons/glia in external granular layer (panel (a)). Little less intensive staining was observed on D1 (panel (b)). D2 differs from D1 by increased positivity in border arachnoidal cells; markedly positive was superficial glial limiting membrane (panel (c)). On D4, new cortical positivity appeared in cytoplasm and nuclei of neurons/glia localized in molecular, external granular, and external pyramidal layers. Other sites were without change (panel (d)). More intensive staining of the border arachnoidal cells was the major change

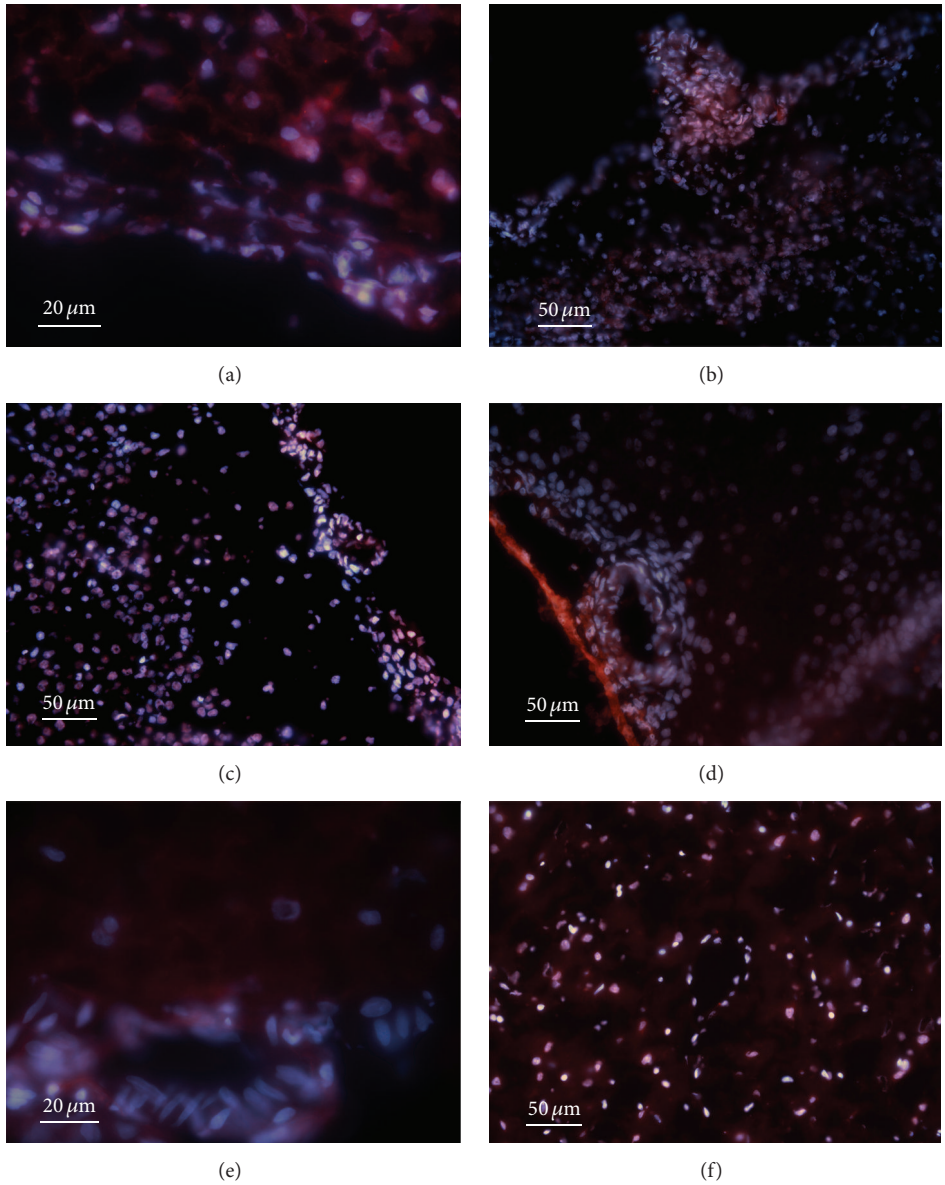

Figure 2: DHE oxidation during brain development (colocalization with DAPI). (a) D2, (b) D4, (c) D8, (d) D14, (e) D30, and (f) D60.

on D8 in comparison to D4 (panel (e)). D14 was characterized by a general decrease in the intensity of staining (panel (f)). This decrease continued on D30; positive staining was found in pia mater; border arachnoidal cells and blood vessels showed decreased staining (panel (g)). This pattern continued to D60 (panel (h)).

3.5. Localization of Blue Autofluorescence. Blue autofluorescence did not appear until D4. Its following time course is illustrated in Figure 6. Isolated areas of pia mater showed low intensity of blue autofluorescence on D4 (panel (a)) and on D8 (panel (b)). No fluorescence of blood vessels was encountered on these days. Starting from D14, the vessel wall was the site of intensive blue fluorescence (panel (c)). The highest intensity of blue fluorescence was observed in the vessel wall on D30 (panel (d)). It was so bright that the sensitivity of the camera had to be reduced which erased the low level fluorescence outside the vessel wall. On D60, the blue autofluorescence in the vessel wall decreased and higher sensitivity of the camera revealed low level autofluorescence in other parts of the brain (panel (e)).

4. Discussion

The reaction of dihydroethidium is considered to be relatively specific for superoxide, with minimum reaction with hydrogen peroxide [30]. DHE undergoes a two-electron oxidation to form a DNA-binding fluorophore. In the case of mitochondrial generation of superoxide it binds mitochondrial DNA; at higher concentrations it can bind nuclear

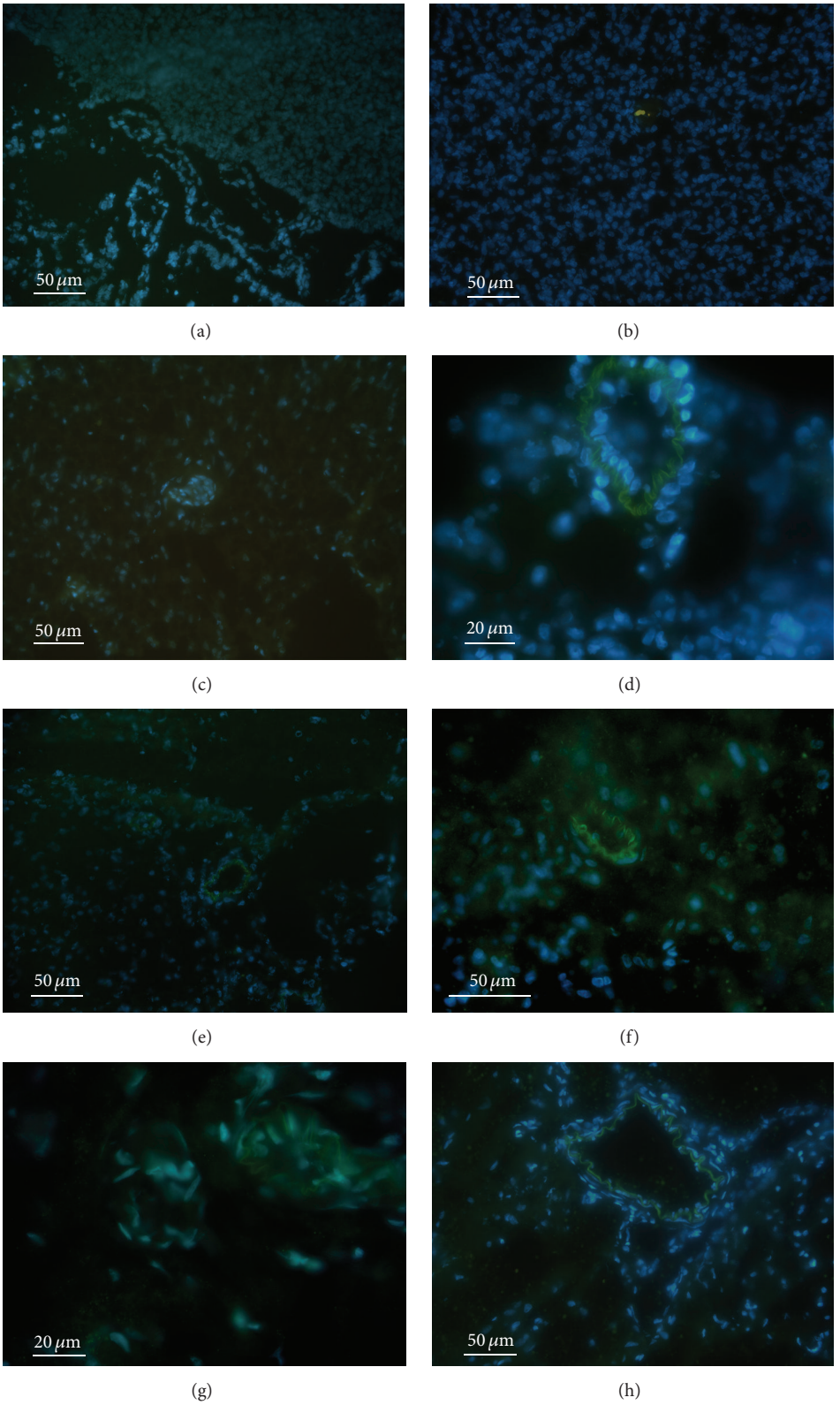

Figure 3: Oxidation of H2DCFDA during brain development (colocalization with DAPI). (a) foetal brain, (b) D1, (c) D2, (d) D4, (e) D8, (f) D14, (g) D30, and (h) D60.

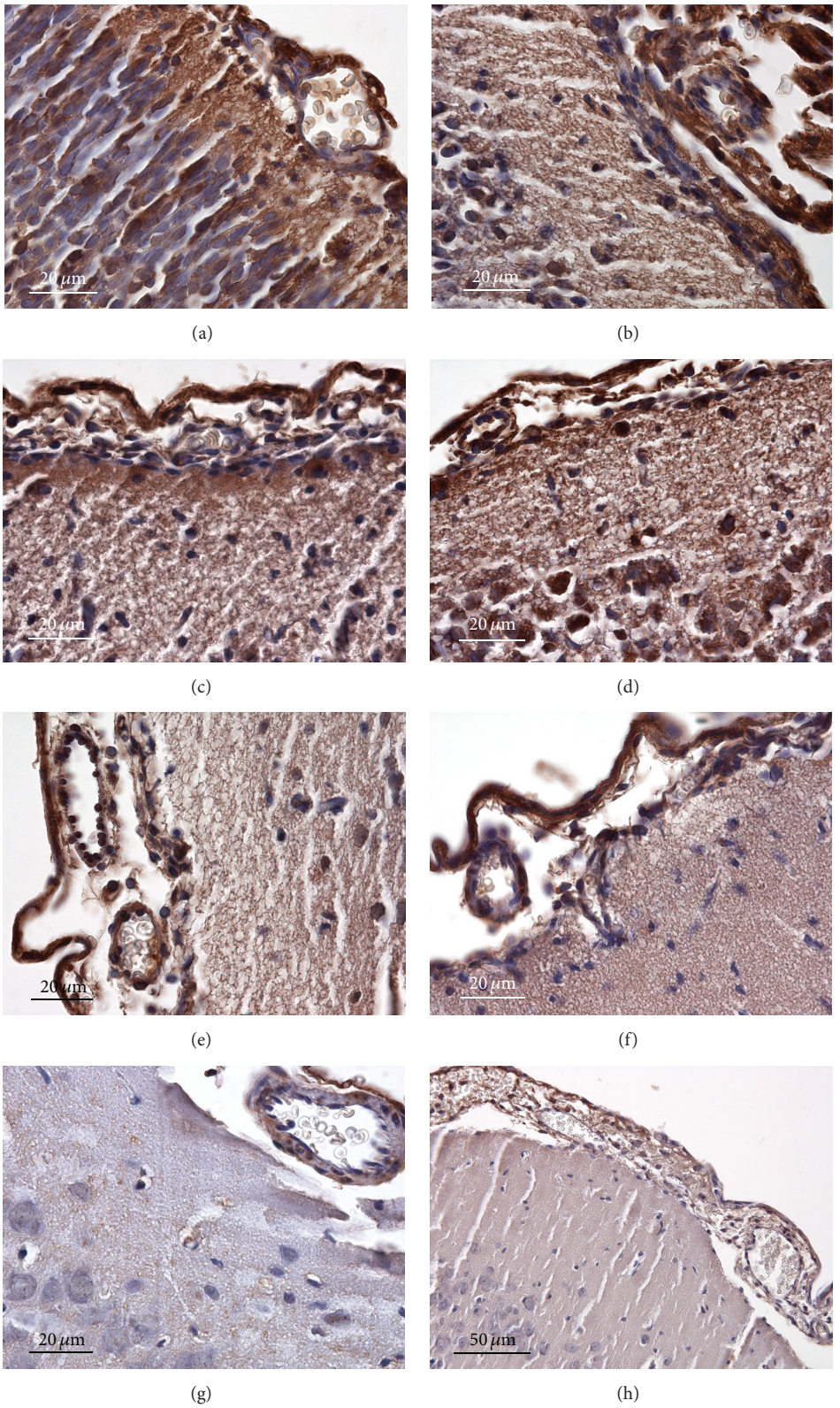

Figure 4: The summary of immunohistochemical detection of nitrated proteins during brain development. (a) foetal brain, (b) D1, (c) D2, (d) D4, (e) D8, (f) D14, (g) D30, and (h) D60.

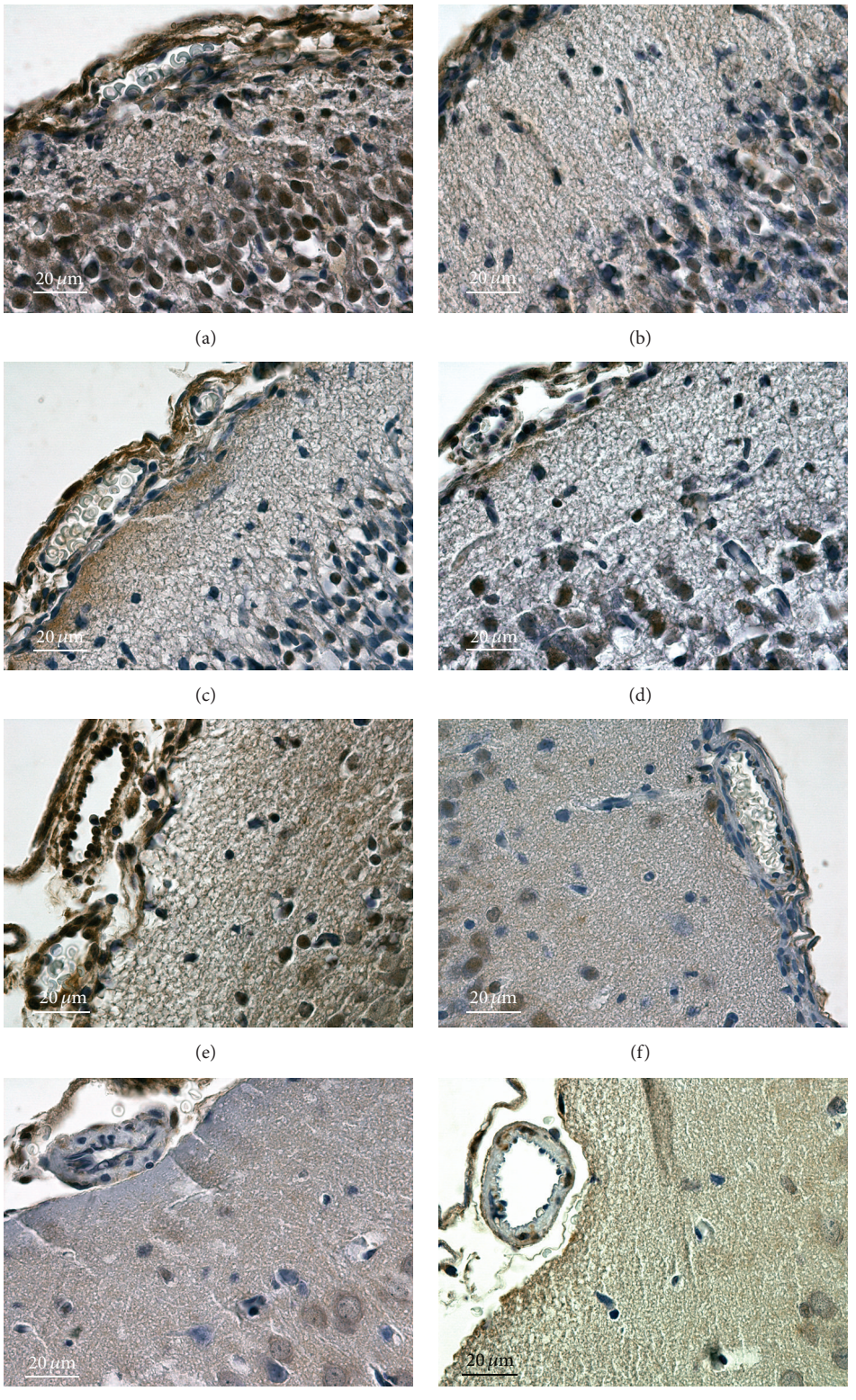

Figure 5: Immunohistochemical detection of carboxyethyllysine during brain development. (a) foetal brain, (b) D1, (c) D2, (d) D4, (e) D8, (f) D14, (g) D30, and (h) D60.

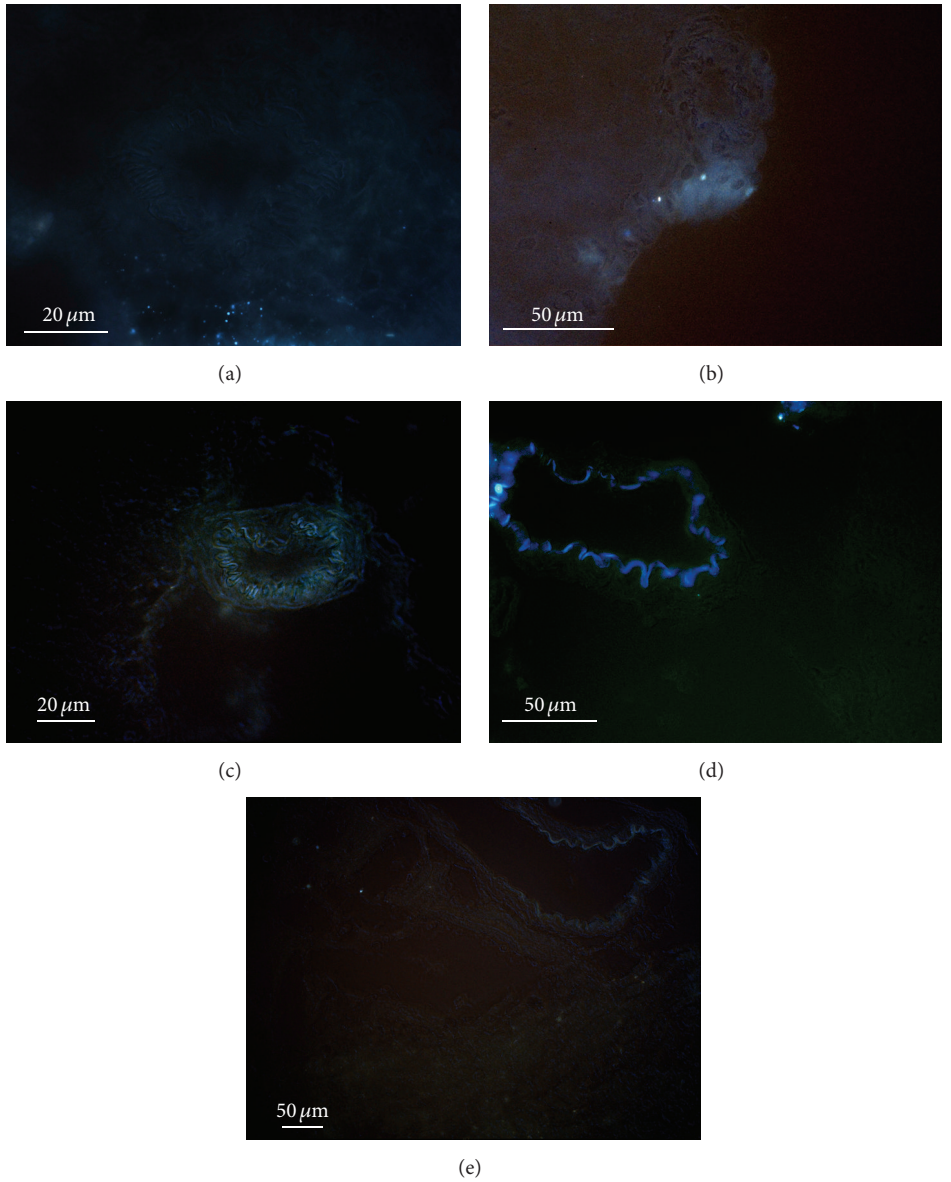

Figure 6: Occurrence of blue autofluorescence during brain development. (a) D4, (b) D8, (c) D14, (d) D30, and (e) D60.

DNA [31]. DHE oxidation yields two fluorescent products, 2-hydroxyethidium (EOH), which is more specific for superoxide, and the less specific product, ethidium. Both EOH and ethidium are fluorescent in the absence of DNA; however, in the presence of DNA, their fluorescence is highly increased [32]. We have documented fluorescence due to DHE oxidation by superoxide in the nuclear membrane devoid of DNA which contributed to staining of the nuclei [25].

The oxidation of 2′,7′-dichlorofluorescein (DCFH) to fluorescent dichlorofluorescein (DCF) was originally used for the detection of hydrogen peroxide [33]. For the intracellular detection, use is made of the diacetate form of DCFH (DCFH-DA). DCFH-DA is taken up by the cells and then deacetylated by intracellular esterases and the resulting DCFH becomes trapped inside the cell being ready for the oxidation. However, intracellularly formed DCFH can escape to extracellular space, where it is accessible by extracellular oxidants [34]. Besides hydrogen peroxide, DCFH can be oxidized by peroxidases, even in the absence of hydrogen peroxide [35]. In addition to peroxidase-dependent oxidation of DCFH, other substances can produce DCF in the absence of H_2O_2. These substances comprise lipid peroxides [36] and reactive nitrogen species such as peroxynitrite [37]. We have used DCFH for the detection of hydrogen peroxide production in rat heart in our previous studies [25, 26]. In the present study, we employed carboxy-derivative H2DCFDA, which proved to be a more sensitive stain for intracellular hydrogen peroxide production.

Exposure of proteins to free radical-producing systems induces appearance of blue autofluorescence [38, 39]. In a

previous study, we have observed blue fluorescence in isolated collagen I exposed to UV irradiation in vitro [40]. Using confocal microscopy, elastin autofluorescence was found in mesenteric arteries of 10-day-old rats. The intensity of fluorescence was much higher in 1-month-old and 6-month-old animals [41].

In the rat hearts, we have observed first signs of autofluorescence of the coronary vessels internal elastic lamina in 7-day-old heart, albeit very faint. On day 15, the autofluorescence was bright enough to be photographed, and the fluorescence intensity reached maximum on day 60. It was still highly pronounced in 7-month-old animals. From day 60 on, there was also observable autofluorescence originating from the cells. To the opposite of the fluorescence of the vessel wall, this fluorescence was extractable to chloroform and its intensity was increasing with age and was higher in 7-month-old animals. We have suggested that this cellular autofluorescence originated from the lipofuscin-like pigments, the end products of lipid peroxidation [26].

As the individual stains reveal specific parts of the general free radical mediated processes, the comparison of their time course can provide an insight into the mechanisms of the oxidative stress during brain development. The foetal brain just before birth contained several markers of active free radical generation. Superoxide production could have been found in many sites, while production of hydrogen peroxide was relatively lower. Production of RONS is revealed by increased staining for NT in the cytoplasm and nuclei of neurons/glia and endothelial cells. It corresponded to CEL staining which could have been the result of lipid peroxidation in the areas of RONS production. According to Bandeira et al. [10], more than 90% of the brain cells are neurons in this period.

Immediately after birth on D1, there was a decrease in neuron/glia superoxide production which was accompanied by low level of hydrogen peroxide. It thus appears that increased oxygen concentration after birth does not contribute to increased ROS generation in neurons/glia. The first three days represent dormant phase concerning cell proliferation [10]. On the other hand, the marked change was observed in the vessel wall which intensively produced hydrogen peroxide, and endothelial cells actively generated superoxide. This could have been related to the intensive metabolism of developing blood vessels. The amount of NT was without change in comparison to foetuses; CEL was decreased a little.

Dynamic changes in RONS production appeared on D2. Production of superoxide in neurons/glia increased, while H_2O_2 production stayed low. Endothelial cells produced less superoxide and blood vessels showed lowered production of H_2O_2, indicating that metabolic activity in blood vessels is not increasing linearly. The amount of NT in neurons/glia and endothelial cells was practically without change. Positivity in superficial glial limiting membrane appeared newly as well as intense positivity for CEL. So this is the possible site of lipid peroxidation initiated by RONS.

With regard to RONS production, D4 appears to be the critical point in development. Superoxide production by endothelial cells was high again, accompanied by intense production of hydrogen peroxide in the vessel wall. The most pronounced positivity was observed in the vessel internal elastic membrane. In our previous study on rat hearts we found that H_2O_2 in the internal elastic membrane is produced by lysyl oxidase [26]. The neuronal/glial superoxide production decreased a little; the most significant change, however, was in the site of production, as cortical positivity was limited to the external granular layer. The striking increase was observed in NT staining which was highest throughout the whole development. From this time point on, the intensity of NT staining was decreasing. Newly appearing CEL positivity in neuronal/glial cytoplasm could have been associated with increased RONS production, again. D4 was also the first time period when blue autofluorescence appeared. This might have been the result of lipid peroxidation. From D4 till D7, number of neurons duplicated and the brain volume increased due to neuronal growth as well [10].

Superoxide production was restarted in cortical neurons/glia on D8, accompanied by decreased production of H_2O_2. Increased NT staining was maintained in the cytoplasm of endothelial cells and border arachnoidal cells, which also stained for CEL and showed blue autofluorescence. These two parameters might indicate initiation of lipid peroxidation.

During the second and third weeks, glial cells proliferated fifty times more, while number of neurons decreased by 70% during the second week [10]. D14 represented another period when the character of RONS production has changed dramatically. There was no indication of superoxide production in the blood vessel cell cytoplasm and nuclei. On the other hand, highest level of superoxide, which was maximal throughout the development, was found in the arachnoidal membrane. In contrast to superoxide, H_2O_2 production in the cytoplasm of all cells was on the highest level in the development. Moderate H_2O_2 production was observed in the internal elastic lamina which for the first time in the development gave blue autofluorescence. Both NT and CEL were generally decreased.

New increase in superoxide production in neurons/glia and blood vessel cells was found on D30, accompanied with increased H_2O_2 production in the internal elastic membrane. Also, the blue autofluorescence of the internal elastic membrane was the most intensive in the whole development. NT staining further decreased and the decrease concerned also the border arachnoidal cells; CEL continued to decrease.

From D25 till D60, the second wave of growth came [10]. A moderate superoxide production was found in all cortex cells on D60, the levels of all other markers reached their minimum, and the blue autofluorescence was less intensive than that on D30.

The generation of superoxide and hydrogen peroxide in the developing brain had a cyclical character. The periods of their production have differed as well as the sites of maximum production, indicating that they have been generated by independent processes. DCFH has been shown to be an indicator of peroxynitrite in vitro [42]; however, maximal oxidation of H2DCFDA in our experiments did not correlate with NT staining; thus, it appears that H2DCFDA oxidation is influenced by peroxynitrite only a little in the developing

brain. On the other hand, there was a strong correlation between staining for NT and that for CEL, which suggests that CEL could have been produced in lipid peroxidation initiated with RNS. This view is also supported by the appearance of cellular blue autofluorescence. Generation of blue autofluorescence in the internal elastic membrane of the blood vessels started on D14, reached a peak on D30, and decreased on D60. This behaviour differs from that in the heart, where the maximum was found on D60 [26]. It indicates that blood vessels develop in the heart and brain in a specific way, although both processes involve hydrogen peroxide.

A comparison of these events to events of the brain growth [10] shows a time correlation, especially with the end of the postnatal neurogenesis by D7-8 and starting gliogenesis by the second week. During the third week, late neurogenesis takes place. It is important to note that the output of pathologic or toxicologic studies in young animals might depend on the timing, as the periods of intrinsic oxidative stress would modify the response to other treatments.

This study documents that oxidative stress is an inherent component of normal postnatal brain development and it has a characteristic time course, closely related to growth and apoptotic changes. The output of additional external stress factors might be dependent on their timing, that is, whether they appear during a period of high or low free radical production. The oxidative damage to proteins with a long half-life early in the development, such as elastin and collagen which was detected by blue fluorescence, suggests that these effects might be revealed later in life and even influence the animal longevity.

Abbreviations

RONS:	Reactive oxygen and nitrogen species
ROS:	Reactive oxygen species
RNS:	Reactive nitrogen species
NO:	Nitric oxide
nNOS:	Neuronal nitric oxide synthase
eNOS:	Endothelial nitric oxide synthase
iNOS:	Inducible nitric oxide synthase
GSH:	Glutathione
GSSG:	Glutathione disulfide
HNE:	4-Hydroxynonenal
DHE:	Dihydroethidium
H2DCFDA:	6-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate
PBS:	Phosphate-buffered saline, pH 7.4
DAPI:	4′,6-Diamidino-2-phenylindole dihydrochloride
NT:	Nitrotyrosine
CEL:	Carboxyethyllysine
EOH:	2-Hydroxyethidium
DCFH:	2′,7′-Dichlorofluorescein
DCF:	Dichlorofluorescein
DCFH-DA:	Dichlorofluorescein diacetate.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

The study was supported by the Grant Agency of the Czech Republic (Grant no. P303/11/0298).

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 1971452, 10 pages
http://dx.doi.org/10.1155/2016/1971452

Review Article

Oxidative Stress after Surgery on the Immature Heart

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Received 17 January 2016; Revised 11 March 2016; Accepted 15 March 2016

Academic Editor: Serafina Perrone

Paediatric heart surgery is associated with increased inflammation and the production of reactive oxygen species. Use of the extracorporeal cardiopulmonary bypass during correction of congenital heart defects generates reactive oxygen species by various mechanisms: haemolysis, neutrophil activation, ischaemia reperfusion injury, reoxygenation injury, or depletion of the endogenous antioxidants. The immature myocardium is more vulnerable to reactive oxygen species because of developmental differences compared to the adult heart but also because of associated congenital heart diseases that can deplete its antioxidant reserve. Oxidative stress can be manipulated by various interventions: exogenous antioxidants, use of steroids, cardioplegia, blood prime strategies, or miniaturisation of the cardiopulmonary bypass circuit. However, it is unclear if modulation of the redox pathways can alter clinical outcomes. Further studies powered to look at clinical outcomes are needed to define the role of oxidative stress in paediatric patients.

1. Introduction

The stress response to surgery compromises a series of humoral, metabolic, or cellular reactions [1]. Cardiac surgery with use of cardiopulmonary bypass (CPB) is a major activator of the systemic inflammatory response (SIRS) [2, 3]. In some instances, SIRS, rather than being a homeostatic mechanism, can be overactivated and result in multiorgan failure and increased mortality after surgery [2]. Inflammation, resulting in neutrophil activation, plays a central role in the production of reactive oxygen species (ROS) [4, 5]. However, other pathways such as haemolysis, ischaemic reperfusion injury, or reoxygenation of the hypoxic myocardium can also generate free radicals. When there is an imbalance between the production of ROS and the antioxidant capacity of the body, *oxidative stress* occurs resulting in cellular injury. Very few studies looked at the impact of oxidative stress on the immature heart, and little is known about the differences between the developing heart and the mature heart during oxidative stress. Although the immature heart has a greater antioxidant capacity than the adult heart, certain congenital conditions that we address surgically make it more susceptible to free radical oxidation [6]. Theoretically, modulation of these oxidative pathways could be of great importance to clinical practice. However, it is unclear how oxidative stress correlates with clinical outcomes after paediatric heart surgery. The current review focuses on the mechanisms of free radical production during paediatric heart surgery, the particularities that make the immature heart more prone to oxidative damage but also on the possible interventions that could mitigate ROS production.

2. Free Radicals, Reactive Oxygen Species, Mitochondrial Oxidative Stress, and Antioxidant Enzymes

Free radicals are chemical species with a single unpaired electron making them highly unstable and reactive. The role of oxygen derived free radicals was studied in the context of cell injury secondary to ischaemic reperfusion injury, inflammation, phagocytosis, chemical or radiation injury, oxygen toxicity, and cell aging [7]. Reactive oxygen species refers to free radicals and other oxidants without an unpaired electron such as the highly reactive hydrogen peroxide (H_2O_2).

The main types of reactive oxygen species are the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), the hydroxyl

radicals ($^{\bullet}OH$), peroxynitrite ($ONOO^{-}$), and the hypochlorite radical (OCl^{-}) [2, 5–8].

Reactive oxygen species are produced under physiological conditions during the mitochondrial respiration process by the reduction-oxidation reactions (redox). A toxic reactant escaping this reaction is the $O_2^{\bullet-}$ radical that can convert, spontaneously or under the action of the superoxide dismutase, to H_2O_2. The so-called *Fenton reaction* can further generate from H_2O_2 the highly reactive hydroxyl radical ($^{\bullet}OH$).

Mitochondria are the energy factory of the body but also a major source of reactive oxygen. The mitochondrial transport chain can leak electrons to molecular oxygen resulting in superoxide radical production. When the mitochondrial antioxidant capacity is overwhelmed, *mitochondrial oxidative stress* can cause mitochondrial DNA damage, impaired mitochondrial respiration, and lipid or protein peroxidation. Such processes are responsible for DNA mutations, cellular aging, and death. An important mechanism is the peroxidation of *cardiolipin*, a mitochondrial lipid protein that results in cytochrome C release and subsequent caspase activation and apoptosis [9–13].

A recently discovered antioxidant system is the mitochondrial *thioredoxin* and *peroxiredoxin* family of proteins [14]. This scavenger complex is an important regulator of the redox state but also a regulator of cell apoptosis via the apoptosis stress kinase 1 (ASK 1). The mitochondrial thioredoxin-2 can preserve cardiac function by suppressing ROS within the mitochondria according to a study [15].

Within the activated phagocyte, a similar chain of reactions occurs. Of great importance is the production of the superoxide anion ($O_2^{\bullet-}$) by the reaction of oxygen with nicotinamide adenine dinucleotide phosphate (NADPH). This reaction is catalysed by the NADPH-oxidase, a membrane specific enzyme. Another key enzyme is the *myeloperoxidase* that catalyses the reaction of the halide ions (Cl^{-}) with H_2O_2, thus generating the OCl^{-}, the "bleach" of the phagocyte [5].

Cell injury occurs by three main mechanisms: lipid peroxidation, protein oxidative damage, and DNA damage (Figure 1) [7, 8]. Nonspecific markers of lipid peroxidation such as the thiobarbituric acid-reactive substances (TBARS) had been used in previous studies to measure outcomes after paediatric heart surgery [16]. Antioxidant enzymes such as the *superoxide dismutase*, the *glutathione* synthetic enzymes, and the *catalases* can protect cells against the oxidative stress. As we will see later, both endogenous and exogenous antioxidants such as vitamins C, E, and A or beta-carotene can also enhance the antioxidant capacity [7].

Apart from their important role in microbial killing, ROS are also involved in cell signaling [7, 17, 18]. The NF-κB family of transcription factors are involved in inflammation, immunity, cellular growth, or apoptosis. There is a complex cross talk between ROS and NF-κB: transcription of the NF-κB gene that regulates the production of ROS in the cell but also NF-κB activity can be influenced by ROS. Furthermore, NF-κB can mediate the expression of antioxidant proteins such as superoxide dismutases, ferritin heavy chain, thioredoxins, and glutathione peroxidase [18].

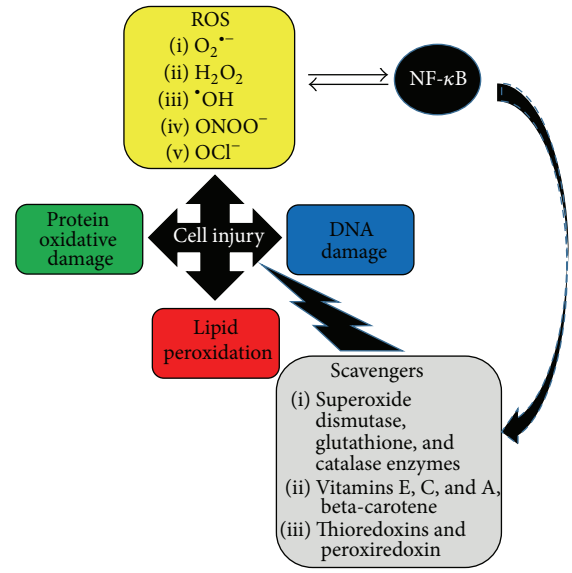

Figure 1: The various reactive oxygen species (ROS) injure cells by three main mechanisms: lipid peroxidation, protein oxidative damage, and DNA damage. Cellular injury is limited by the action of the main antioxidant enzymes (scavengers). There is a complex cross talk between ROS and NF-κB transcription factors that regulate the production of antioxidants.

3. Paediatric Extracorporeal Circuits and Oxidative Stress

Use of the extracorporeal circuits induces oxidative stress in various ways, ultimately resulting in organ system dysfunction (Figure 1).

3.1. Extracorporeal Circuits, Inflammation, Oxidative Stress, and Markers of Oxidation. Several factors cause a more profound systemic inflammatory response in neonates and infants compared to older children or adults: (1) the surface and the volume of the CPB circuit relative to the blood volume and patient size, (2) more frequent use of hypothermic circulatory arrest, and (3) more pronounced haemodilution [6]. It is well known that CPB induces systemic inflammation by mechanisms such as contact activation with the non-self-circuit surfaces, translocation of intestinal endotoxins, general surgical trauma, blood loss, or hypothermia [2, 19]. This activates the complement system, the production of cytokines, neutrophil adhesion and aggregation, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and xanthine oxidase systems, ultimately resulting in ROS production [2, 4, 5, 8, 20]. Certainly, the recruitment and activation of neutrophils play a pivotal role in the production of ROS. Activation of neutrophils leads to an increase of the plasma neutrophil elastase and the increase of *myeloperoxidase* enzyme linked to oxidative stress [21]. Measurement of the myeloperoxidase can be used as a marker of cardiovascular disease [22].

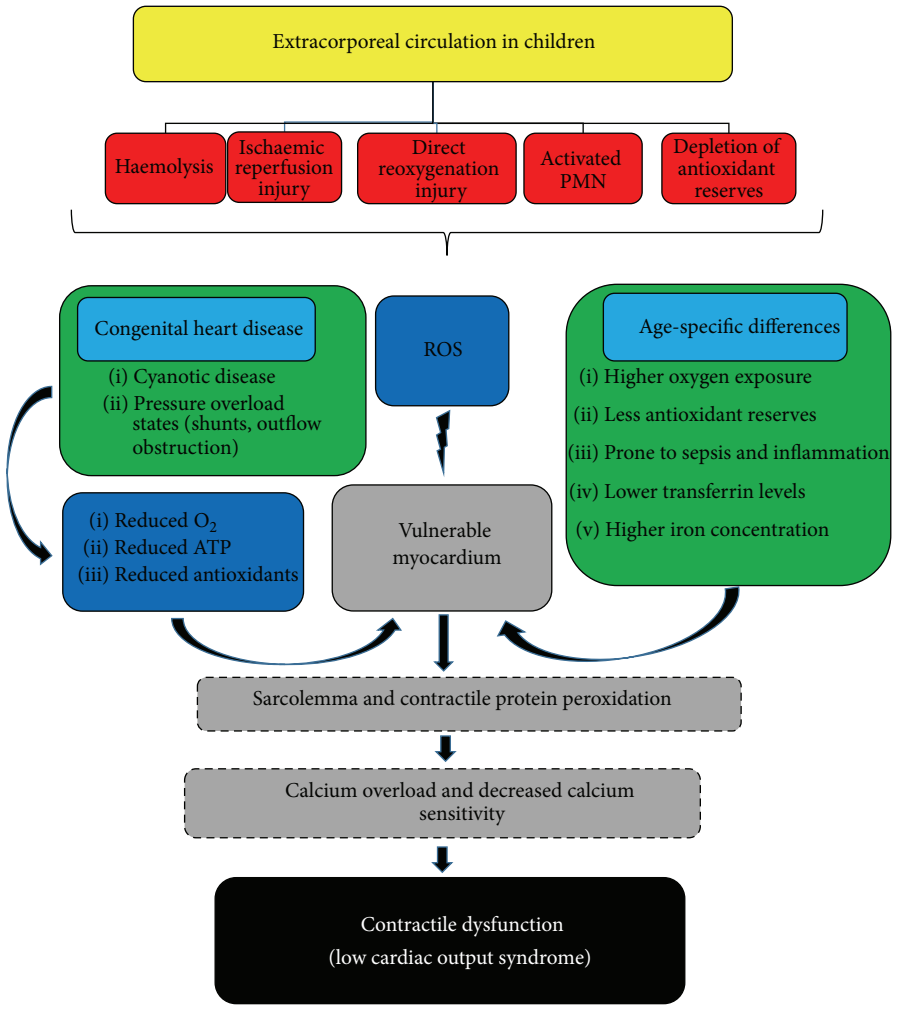

Figure 2: The various mechanisms of production of ROS with use of extracorporeal circuit. The immature myocardium is vulnerable to ROS injury because of age specific differences compared to the adult heart but also because of coexistent congenital heart disease. The end-result is contractile dysfunction.

Calza et al. [23] evaluated the glutathione redox cycle in the context of paediatric CPB. They found increased free radical production before and during CPB by measuring an increase of the total and oxidised glutathione (reduction of the glutathione/glutathione oxidase ratio).

Gil-Gómez et al. [24] found a direct correlation between the time of extracorporeal circulation, the duration of post-operative mechanical ventilation, and the amplitude of the oxidative stress response. The authors measured glutathione levels but also the lipid peroxidation product, malondialdehyde. Other products of lipid peroxidation such as isoflurane or 8-isoprostane were found to be increased during paediatric CPB [23, 25, 26].

Extracorporeal membrane oxygenation (ECMO) is commonly used in paediatric heart surgery. Similar to the CPB circuit, contact activation initiates an inflammatory cascade and oxidative stress. Extracorporeal membrane oxygenation is a major activator of SIRS because the whole blood of the patient is in contact for days or weeks with the ECMO circuit [8]. Hirthler et al. [27] in the nonsurvivors after paediatric ECMO found elevated levels of the lipid peroxidation markers: thiobarbiturate acid-reactive substances (TBARS) and malondialdehyde.

3.2. Ischaemic Reperfusion Injury and Oxidative Stress in the Immature Myocardium. Ischaemic reperfusion injury (IRI) following cardioplegic arrest results in increased ROS production. The main sources of ROS during IRI are (1) uncoupling of the mitochondrial electron transport, (2) circulating polymorphonuclear leukocytes generating superoxide anions from NADPH (reaction catalysed by the NAPDH oxidase), and (3) coronary endothelial cells that generate the superoxide anion from hypoxanthine, reaction catalysed by the *xanthine oxidase* enzyme [28]. Ischaemic reperfusion injury results in cardiac dysfunction, a major cause of mortality and morbidity in paediatric cardiac surgery [16, 29, 30]. Reactive oxygen species can induce contractile dysfunction (low cardiac output syndrome) by peroxidation of the sarcolemma and of the contractile proteins. This results in calcium overload and calcium desensitization (Figure 2) [28].

Previous studies suggested that the immature heart is more tolerant to ischaemia than the adult heart. Possible mechanisms implicated are as follows: (1) the sarcolemma of the cell is more resistant to calcium, (2) the immature myocardium relies on fatty acid for energy production and hence less potential for anaerobic glycolysis, and (3) the larger amount of amino acids provides more substrates of anaerobic metabolism [6, 16, 23]. Despite such developmental differences, the myocardium in cyanotic heart disease and heart failure might be more susceptible to ischaemia and subsequently oxidative stress [16, 23, 30]. The pressure overload of the myocardium in left to right shunts or outflow obstructions, or the reduced oxygen availability in cyanotic heart disease, further reduces the ATP stores and antioxidant enzymes, thus making the myocardium more susceptible to oxidative injury [23, 31, 32]. Cabigas et al. [33] compared oxidative stress in the newborn and adult heart in a model of ischaemic reperfusion injury. They found age and chamber specific differences related to oxidative stress. Interestingly, the newborn right ventricle myocytes showed significantly higher production of H_2O_2 compared to the adult heart, the superoxide dismutase activity increased only in the right ventricle heart, and the catalase activity and levels were all reduced in the newborn heart.

Reactive oxygen species play a fundamental role in reperfusion injury and their effect had been extensively studied in this context [8]. During IRI, oxidants are produced by various mechanisms such as increased production of xanthine oxidase production by the endothelial cells, decreased function of the glutathione peroxidase enzyme, or leakage of electrons from the mitochondria (resulting in superoxide anions) [8]. The activated neutrophil with subsequent fabrication of ROS seems to play a role in more prolonged periods of ischaemia that are associated with tissue necrosis [8, 34]. Oliveira et al. [35] in an immunohistological study of infants that underwent repair of cardiac malformations found lipid peroxidation to be the mechanism responsible for myocardial injury. The authors tested immunoreactivity to 4-hydroxynonenal, a lipid peroxidation product, and nitrotyrosine, a tyrosine nitration product, mediated by the peroxynitrite radical.

Manso et al. [16] questioned the role of oxidative stress in myocardial dysfunction or low cardiac output after heart surgery. The authors found no correlation between TBARS and carbonyl moieties and the development of low cardiac output syndrome in a retrospective study of 55 children. Certainly, further studies powered to look at clinical outcomes and oxidative stress in paediatric surgery are required.

3.3. Hypoxemic/Reoxygenation Induced Oxidative Stress in Cyanotic Heart Disease. Buckberg et al. [29, 36] demonstrated in animal studies that the reintroduction of oxygen in the initial stages of CPB or mechanical ventilation can induce *per se* myocardial injury in the hypoxaemic myocardium. In cyanotic heart disease, the reduction of the antioxidant reserve increases the vulnerability of the myocardium. The authors demonstrated that reoxygenation reduced the myocardial reserve capacity (measured by incubating myocardial tissue in the oxidant t-butyl hydroperoxide) and increased the products of lipid peroxidation (conjugated dienes) measured in coronary sinus and the myocardial tissue.

Caputo et al. randomized cyanotic patients to receive either normoxic or hyperoxic cardiopulmonary bypass. The normoxic arm of patients had less oxidative stress (significantly lower 8-isoprostane levels) compared to the hyperoxic group [26]. Later studies by the same group demonstrated less oxidative stress with controlled reoxygenation in the single ventricle compared to double ventricle patients [25].

3.4. Extracorporeal Circuits and the Antioxidant Reserve. Oxidative stress during CPB is an imbalance between the production of free radicals and the antioxidant capacity of the body. As discussed earlier, congenital cardiac conditions are associated with a decrease of the myocardial antioxidant capacity. However, CPB can also deplete the plasma antioxidant capacity. Cavarocchi et al. demonstrated depletion of vitamin E after bypass [37, 38]. Pyles et al. [39] investigated in vitro the plasma ability to prevent lipid peroxidation (malondialdehyde production) in beef brain homogenate media. They found the plasma antioxidant capacity significantly reduced after congenital heart surgery with CPB.

3.5. Haemolysis, Blood Transfusion, and Iron Overload Promote Oxidative Stress . The contact with the nonphysiological surfaces of the bypass circuit and the associated mechanical shear stress associated with CPB leads not only to inflammation but also to haemolysis [40–42]. The resulting free haemoglobin can react with H_2O_2 and generate redox active low molecular mass iron that can lead to lipid peroxidation and the highly reactive hydroxyl radical ($^{\bullet}OH$) [42]. Iron overload in paediatric cardiac surgery can result also from blood cardioplegia, ischaemia reperfusion injury, blood transfusion, or the use blood prime [40].

Blood transfusion is a common practice in heart surgery that promotes oxidative stress by both the decreased antioxidant properties of stored blood and increased erythrocyte fragility resulting in haemolysis and ROS generation [4]. Low molecular mass iron is present in small amounts within cells for synthesis of fetoproteins or DNA. Under normal conditions, iron is regulated by ligands such as transferrin that inhibits transfer of electron from iron to molecular oxygen [42, 43]. When the iron binding capacity of transferrin is exceeded, free iron can be detected in the plasma. Notably, Mumby et al. [40] found a higher plasma iron overload in neonates undergoing CPB compared to older children. A possible explanation was the lower transferrin concentration within this group. Another study of patients undergoing tetralogy of Fallot repair found acute right ventricular failure due to restrictive physiology to be associated with severe iron loading of transferrin and increased oxidative stress markers compared to the nonrestrictive physiology cohort [44]. Two paediatric studies [42, 45] found associations between haemolysis and renal dysfunction that was believed to be mediated by lipid peroxidation.

Christen et al. [19] found the peak of oxidative stress markers (malondialdehyde and carbonyls) to occur before the rise of the inflammatory cytokines (interleukin-6 and

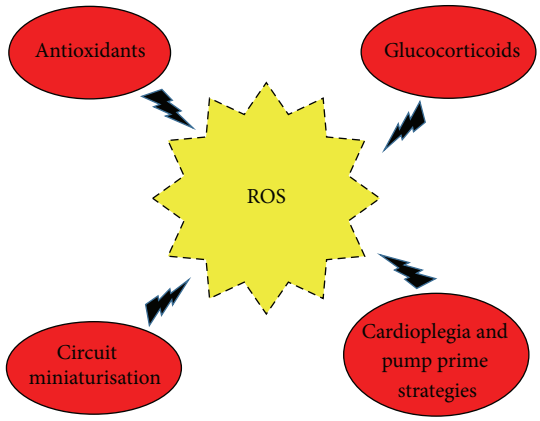

Figure 3: Interventions to reduce oxidative stress in paediatric heart surgery.

interleukin-8) in paediatric patients undergoing heart surgery with CPB. This further suggests that mechanisms such as haemolysis could be responsible for the propagation of oxidative stress.

4. Modulation of Oxidative Stress in Paediatric Heart Surgery

Figure 3 summarises the various interventions on oxidative stress after heart surgery.

4.1. Antioxidants. Children, particularly newborns, are more prone to oxidative stress because of several factors: (1) surfactant deficiency that exposes them to higher oxygen concentration, (2) less efficient antioxidant reserves, (3) being more prone to sepsis and inflammation, and (4) higher levels of free iron than older children [14]. Buckberg [29] studies defined the concept of antioxidant reserve capacity on the immature heart by incubating cardiac muscle with oxidants and measuring the products of lipid peroxidation. The dose response was similar to the Starling volume load curves and suggested the relation between antioxidant depletion and oxidative stress. Cardiopulmonary bypass had been shown to reduce plasma antioxidant capacity [19, 39]. Christen et al. [19] found a decrease in ascorbate (vitamin C) levels coupled with an increase in dehydroascorbate (oxidised vitamin C) and malondialdehyde levels in paediatric patients undergoing heart surgery. Therefore, increasing the antioxidant reserve capacity by administration of exogenous agents seems beneficial.

As seen previously, iron can play an important role in free radical formation; hence, chelators such deferoxamine could prove to be advantageous. Morita et al. [32] found administration deferoxamine to increase the heart antioxidant reserve in piglets undergoing CPB.

Data from critically ill patients or adult patients undergoing heart surgery suggests that supplementation of antioxidant micronutrients such as vitamin C, selenium, zinc, or vitamin E could increase the antioxidant defence [2, 4, 8, 46]. Amino acids are well known to have antioxidants properties [4]. For example, L-arginine reduced markers of oxidative stress in adult cardiac surgery when added to the cardioplegia solution [47]. The nonessential amino acid glutamine is another important endogenous antioxidant that could be supplemented [8]. N-acetylcysteine, antioxidant, and mucolytic agent used in respiratory medicine reduced the incidence of postoperative atrial fibrillation in a randomized controlled trial [48]; however, a later meta-analysis of randomized trials showed no benefit in reducing renal dysfunction, haemodialysis, or death [49]. To our knowledge, there are no studies investigating the effect of the above agents on oxidative stress in children undergoing heart surgery.

England et al. [50] found mannitol to reduce lipid peroxidation in patients undergoing adult heart surgery; however, its use was not evaluated in paediatric heart surgery. Allopurinol, an inhibitor of the purine metabolism, acts on the xanthine oxidase pathway generation [50] of superoxide. Some studies suggested reduced oxidative stress in paediatric heart surgery [50, 51] while others suggested it might not be beneficial [52]. One study on neonates with respiratory failure requiring ECMO demonstrated reduced purine degradation and uric acid production, hence with possible reduction in free radicals during reperfusion and reoxygenation injury [53].

The induction and maintenance anaesthetic agent, propofol, exerts antioxidant effects on the adult heart [4]. Xia et al. [54] in a study on 20 children undergoing CPB demonstrated reduced expression of NF-κB in the propofol group. As seen earlier, oxidative stress is associated with the activation of the NF-κB transcriptional factor. The same research group compared the antioxidant effects of the sedative midazolam to those of propofol in children undergoing congenital heart surgery. The propofol group had significantly less oxidative stress (lower superoxide dismutase and malondialdehyde levels) compared to the midazolam group [55].

Salvia miltiorrhiza, a herb extract with potent antioxidant effects, prevented the increase of lipid peroxidation products (malondialdehyde) in children undergoing CPB according to a study [56].

Melatonin is an endogenous indolamine, known to be a potent anti-inflammatory molecule but also a direct antioxidant [46, 57, 58]. In neonatal sepsis [46] but also after neonatal gastrointestinal surgery [57], melatonin reduced markers of oxidative stress. In several studies, using human cardiomyocytes cultures or isolated perfused rat heart, melatonin effectively reduced oxidative damage [59]. Further clinical studies in paediatric heart surgery are required to validate the above findings.

Aprotinin is used in congenital heart surgery as haemostatic agent but is also known to exert anti-inflammatory effects. Several paediatric studies suggested reduced oxidative stress with its use [60, 61].

As seen earlier, the mitochondrion is the main contributor to ROS production. Recently, agents that target the mitochondrial redox systems are being developed. MitoQ is a ubiquinone derivate conjugated to triphenylphosphonium that accumulates within the mitochondria because of an electrochemical gradient [4]. Ubiquinone is known for inhibiting the production of lipid peroxyl in the cell [13].

MitoQ reduced IRI in murine models of heart infarction [62] or heart transplant [12]. As opposed to the MitoQ lipophilic antioxidant that accumulates within the mitochondria in a potential-dependent manner, a novel class of small peptides (Szeto-Schiller) that selectively permeate the mitochondrial membrane had also been developed [10]. Szeto-Schiller peptides reduced IRI in several in vivo and ex vivo experimental models [10].

4.2. Glucocorticoids and CPB-Induced Oxidative Stress. Glucocorticoids are widely used in paediatric heart surgery to blunt the systemic inflammatory response to surgery but also to treat presumed postoperative adrenal insufficiency. Checchia et al. [63] in an international survey on steroid use in paediatric heart surgery found that 35 out of 36 centres (97%) give steroids but there is very wide variability in dose, timing, regimens, and type of steroid given. A later UK survey by Allen et al. [64] demonstrated that 80% of the centres give steroids. According to a more recent US large database retrospective study in 2010, steroids were used in 54% of the paediatric cases. Despite wide use of glucocorticoids and proven anti-inflammatory effects in heart surgery with CPB [65–67], the majority of the studies have failed to show a survival benefit with steroids use [68–73]. Furthermore, large retrospective studies reported higher infection rates with steroids use [70, 71]. A recent large retrospective randomized trial in adult heart surgery found no significant effect of methylprednisolone on mortality or morbidity after cardiac surgery [74]. Similarly, a large, multicentre randomized trial in the paediatric population is warranted before firm recommendations can be made.

Steroids exert an anti-inflammatory effect by activating the cytoplasmic glucocorticoid receptor (GR) in the target tissues. The glucocorticoid-glucocorticoid receptor complex dissociates from its chaperone (the heat shock protein family hsp90) and translocates to the nucleus where it associates with the *glucocorticoid response elements* of various genes to induce expression of anti-inflammatory genes [75–77]. Steroids can also inhibit the activation of the NF-κB redox sensitive transcription factors that play a central role in oxidative stress [76, 77].

Very few studies looked at the effect of steroids on inflammation and oxidative stress and cardiac function [76, 78]. Valen et al. [76] found increased activity of the tissue catalase and glutathione peroxidase in the isolated rat heart pretreated with methylprednisolone. Withington et al. [78] randomized 54 infants undergoing heart surgery with CPB to three different regimens of methylprednisolone (preoperative, at induction, and in the prime fluid). The prime administration group had less oxidative stress (higher glutathione to oxidised glutathione ratio).

Despite some studies [76] suggesting an increase of the antioxidant capacity with steroid treatment in the myocardium or other tissues [79], effects of glucocorticoids on oxidative stress remain controversial. For example, studies on hippocampal cell cultures demonstrated that steroids could promote oxidative stress induced death [80, 81]. Recently, several studies demonstrated a proinflammatory action of steroids depending on time and context of administration thus creating more controversy [82, 83].

4.3. Miniaturisation of the Extracorporeal Circuit. Mini-CPB bypass was developed in adult heart surgery in an attempt to reduce contact activation, air fluid interface, and cell damage by cardiotomy suction and haemodilution. Minimising such disadvantages of conventional CPB tempers the proinflammatory response to surgery but also the associated oxidative stress [84]. Adult studies demonstrated less oxidative stress with the use of mini-CPB [85]. The need to maintain an adequate haematocrit but also to overcome the patient's size and circuit discrepancy led to development of paediatric minicardiopulmonary. As seen earlier, the possibility of the bloodless prime eliminates the deleterious effects of blood transfusion. This is achieved in several ways: creation of biocompatible-coated circuits and oxygenators, vacuum assisted drainage systems, reducing the length of the circuit, or eliminating certain circuit components [84]. Miyaji et al. [86] successfully used mini-CPB in neonates weighing more than 4 kg. A more recent study by the same group demonstrated reduced inflammatory response with the use of mini-CPB in patients undergoing the Fontan procedure [87]. To our knowledge, there are no studies investigating differences between oxidative stress with conventional CPB and mini-CPB in paediatric heart surgery.

4.4. Cardioplegia, Pump Prime, and Oxidative Stress. Reactive oxygen species production can be controlled by enrichment of cardioplegia solution with antioxidants according to both experimental [88–90] and adult studies [91–95]. Early experimental studies demonstrated the superiority of blood cardioplegia over crystalloid cardioplegia in reducing oxidative damage [96]. This could be explained by the natural antioxidants blood constituents: haemoglobin, superoxide dismutase, catalase, and glutathione [90]. Despite these potential benefits of the blood cardioplegia, other studies suggested increased oxidative stress by either iron overload [40] or increase of hypoxanthine levels [97]. Finally, adult studies demonstrated less oxidative stress with intermittent antegrade warm blood cardioplegia compared to cold blood cardioplegia [98]. Calza et al. [23] found controlled antegrade low oxygen warm reperfusion to reduce oxidative stress and promote myocardial recovery.

Pump prime solutions used in neonatal heart surgery can also influence the antioxidant plasma capacity. This can prove to be significant because of the relation of the blood circulating volume and the volume of the extracorporeal circuit [99]. Previous animal studies demonstrated that supplementation of the prime solution with catalase reduces myocardial oxidative damage in the piglet heart [100]. Molicki et al. [99] investigated the antioxidant capacity of two different prime solutions for cardiopulmonary bypass in neonates: albumin or fresh frozen plasma based. Both prime solutions had no total radical antioxidant parameter value (ability of the investigated prime solution to inhibit peroxidation of a target lipid). However, the ferric-reducing ability (the capacity of the prime to reduce ferric ion to the ferrous form) was reduced in the prime solution compared to the undiluted, standard albumin and fresh frozen plasma. In their study, they also demonstrated that ultrafiltration led to loss of antioxidants and was ineffective in clearing lipid peroxidation products.

In addition, adding mannitol to the prime had no antioxidant effect. The above study highlights not only the dilutional effect that results during prime preparation but also the depletion of antioxidants during ultrafiltration.

5. Conclusion

Cardiac surgery with the use of CPB is associated with altered redox states in children. Reactive oxygen species cause injury not only by both direct oxidation and peroxidation of cell membranes but also by cellular signalling. Contact with the paediatric bypass circuit surfaces activates the inflammatory cascade in which the neutrophil plays a central role in manufacturing of ROS. However, early inflammation-independent mechanisms such as haemolysis can also contribute to oxidative stress. The generation of ROS during ischaemic reperfusion injury can result in contractile dysfunction but nonreperfusion injury by reoxygenation of the chronically hypoxic heart can also occur. The lack of an antioxidant reserve or the coexistence of congenital heart defects associated with hypoxia or pressure load increases the vulnerability of the immature heart to oxidative damage. A multitude of interventions aimed at reducing ROS production or at increasing the antioxidant reserve were reviewed: exogenous antioxidants, steroids, miniaturisation of the CPB circuit, prime fluid, or cardioplegia strategies. The effect of such strategies on clinical outcomes, however, remains controversial. Further clinical studies looking at the effect of oxidative stress modulation on clinical outcomes after paediatric heart surgery are needed.

Competing Interests

The authors declare that they have no competing interests.

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