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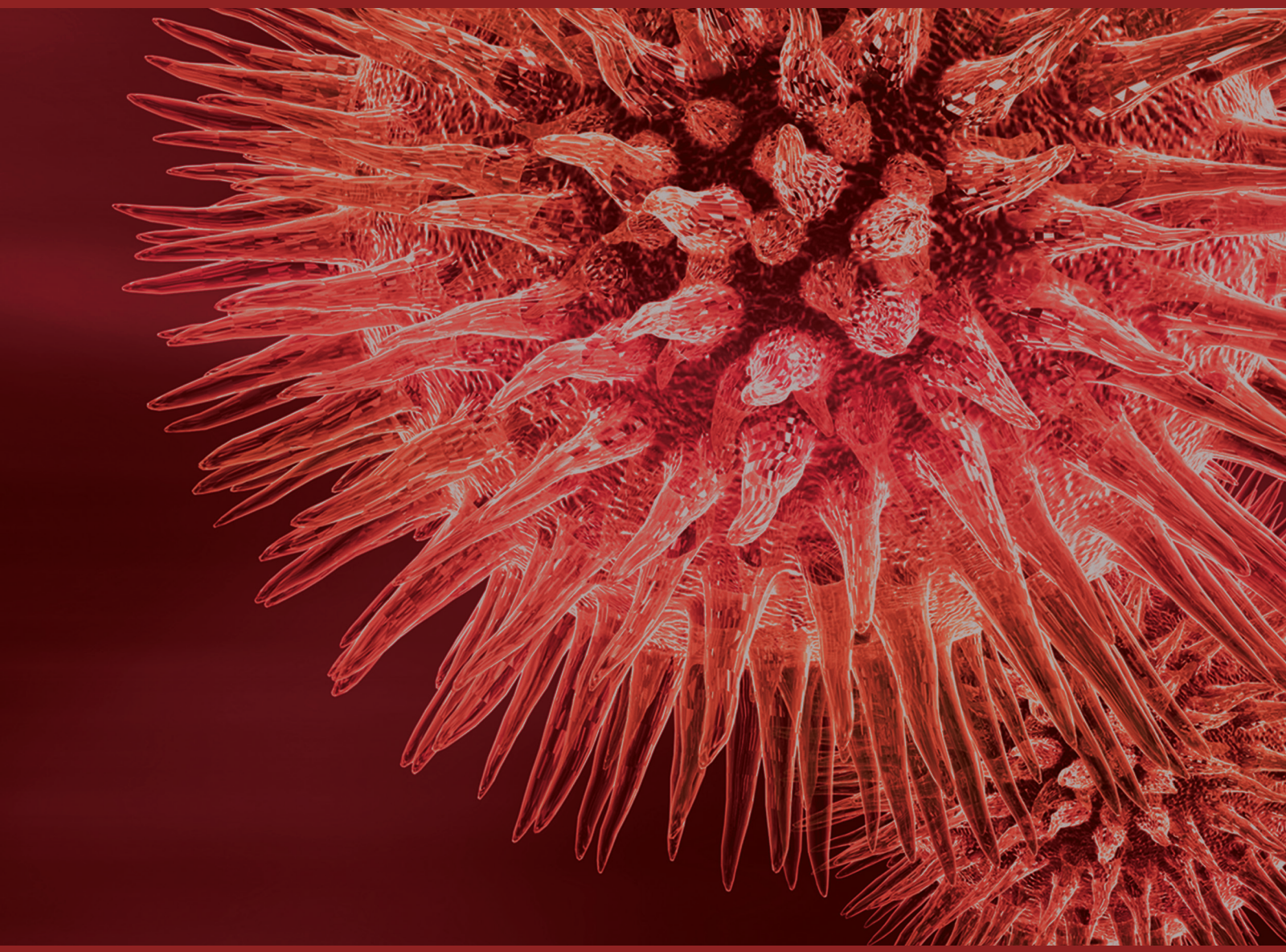
Pharmacology: The Pharmacodynamics of Nutrients and Nutrient Interactions in Biological Functions

Guest Editors: Christopher M. Butt, Gunter Eckert, M. Hasan Mohajeri, and James R. Pauly



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Editorial

Pharmacology: The Pharmacodynamics of Nutrients and Nutrient Interactions in Biological Functions

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Epidemiological studies and randomized controlled trials (RCTs) have shown that nutrition and nutritional habits may play a critical role in the optimal functioning of biological systems from conception to old age [1]. Epidemiological studies, due to their methodology, can only provide correlations between consumption of nutrient(s) and biological outcomes, whereas RCTs normally study just one dose of a certain nutrient. Both study types are therefore ill-suited to study the mechanisms by which nutrients exert their benefits. Moreover, the nutrients' functions may depend on each other. For example, B-vitamins' functions are known to be interdependent [2]. While the exact mechanisms are unclear, the course and severity of conditions such as obesity, cellular aging, cancer, and neurological disorders can be affected by nutritional approaches [3]. Thus, food and nutrition play an intimate and inextricable role in human health. Despite growing interest in adequate nutrition, the effects of nutrient interaction, the possible varying effects on different organs, and the dependency of such effects on age or health status are complicated topics that deserve careful examination.

The pharmacodynamics of nutrients, or the so-called nutridynamics, is the term used to describe how a food component is affected by other food ingredients and what a particular ingredient does in the body [4]. The intention is to systematically study the mechanism of action, that is, how an effect is produced. Obviously, this is a challenging task due to presence of manifold targets and the involvement of multiple biological systems for each nutrient. As an example, depending on the available amount of a given micronutrient, some biological functions may be put on hold, so that the limiting micronutrient can be used for biological functions that are indispensable to life [5].

This special issue presents several research articles studying a few selected nutrients and their function in human biology by *in vitro*, *in vivo,* and human experiments. E. Adamska et al. aimed to determine the metabolic response after intake of standardized meals with various fat and carbohydrate contents and to determine the differences among normal-weight and overweight/obese men. Glucose, insulin, triglyceride, and free fatty acid levels were measured at fasting state and 30, 60, 120, 180, and 240 minutes after meal intake. The results showed that the postprandial response depended on both the meal macronutrient content and the body mass index (BMI). In another study, R. A. O. Cruz and colleagues compared the antioxidant activity of sorghum kafirin and sorghum flour and their influence on lipids and antioxidant capacity in rats. Sorghum is a grain that has a high content of fiber, protein, mineral, and polyphenols. α-kafirin is the main storage protein in sorghum [6]. Rats supplemented with sorghum kafirin extract exhibited improved lipid metabolism and increased serum antioxidant potential, especially when cholesterol was added to the feed. These data may suggest that sorghum kafirin extract may be beneficial against atherosclerotic conditions. The effects of nutritional intervention on markers of oxidative stress were also studied in large domestic animals. C. Tan et al.,

supplemented oregano essential oil (OEO) to the diets of 60 white sows during gestation and lactation. Oxidative stress status, colostrum and milk composition, lactation feed intake of sows, and piglet growth performance were determined. The OEO supplementation led to a dramatic reduction of serum levels of several reactive oxygen species at all ages studied and improved piglet growth, which was attributed to the reduction in oxidative stress.

In two *in vitro* studies, G. Ravacci et al. and G. La Fata et al. set out to examine the roles of docosahexaenoic acid (DHA) and vitamin E in cellular models related to breast cancer and aging, respectively. The hypothesis that lipid metabolism may stimulate malignancy in breast cancer was tested by overexpressing the HER2 proto-oncogene (human epidermal growth factor receptor 2) in a normal breast cell line. Overexpressing and normal cells were then treated with trastuzumab (interfering monoclonal antibody) and DHA. The data provided good evidence that the oncogenic transformation of breast cells by *HER2* overexpression may require a reprogramming of lipid metabolism that is independent of the mTORC1 pathway and peroxisome proliferator-activated receptor gamma (PPARγ) activity. Moreover, DHA inhibited the reprogramming of cancerous cells. Last, but not least, it was shown by using an unbiased automated quantification method in two different human primary cell types that acute or chronic vitamin E treatments may slow down cellular senescence. Mechanistically, this antiaging vitamin E effect could be due to downregulation of the expression of the cyclin-dependent kinase inhibitor P21. Further studies are warranted to clarify whether this effect is dependent on antioxidative properties of vitamin E or whether this possible antiaging affect belongs to new emerging functions of vitamin E which go beyond the antioxidative function.

In summary, the interaction of food ingredients may affect their biological functions as well as the extent of their benefits on growth, repair, and maintenance of biological systems. These areas of research need to be intensified in order to develop meaningful recommendations of dietary guidelines to the general public and/or to individuals with specific needs.

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Gunter P. Eckert
James R. Pauly
Christopher M. Butt

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Research Article

Docosahexaenoic Acid Modulates a HER2-Associated Lipogenic Phenotype, Induces Apoptosis, and Increases Trastuzumab Action in HER2-Overexpressing Breast Carcinoma Cells

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In breast cancer, lipid metabolic alterations have been recognized as potential oncogenic stimuli that may promote malignancy. To investigate whether the oncogenic nature of lipogenesis closely depends on the overexpression of HER2 protooncogene, the normal breast cell line, HB4a, was transfected with HER2 cDNA to obtain HER2-overexpressing HB4aC5.2 cells. Both cell lines were treated with trastuzumab and docosahexaenoic acid. HER2 overexpression was accompanied by an increase in the expression of lipogenic genes involved in uptake (CD36), transport (FABP4), and storage (DGAT) of exogenous fatty acids (FA), as well as increased activation of "de novo" FA synthesis (FASN). We further investigate whether this lipogenesis reprogramming might be regulated by mTOR/PPARγ pathway. Inhibition of the mTORC1 pathway markers, p70S6 K1, SREBP1, and LIPIN1, as well as an increase in DEPTOR expression (the main inhibitor of the mTOR) was detected in HB4aC5.2. Based on these results, a PPARγ selective antagonist, GW9662, was used to treat both cells lines, and the lipogenic genes remained overexpressed in the HB4aC5.2 but not HB4a cells. DHA treatment inhibited all lipogenic genes (except for FABP4) in both cell lines yet only induced death in the HB4aC5.2 cells, mainly when associated with trastuzumab. Neither trastuzumab nor GW9662 alone was able to induce cell death. In conclusion, oncogenic transformation of breast cells by HER2 overexpression may require a reprogramming of lipogenic genetic that is independent of mTORC1 pathway and PPARγ activity. This reprogramming was inhibited by DHA.

1. Introduction

Cell lipogenic metabolism has traditionally been considered a minor anabolic energy-storage pathway, yet its role in various cancers is increasingly being recognized [1–5]. Endogenous fatty acid (FA) biogenesis may constitute an oncogenic stimulus that drives normal epithelial cells towards malignancy [1–5]. Moreover, emerging evidence indicates that the oncogenic nature of human lipogenesis depends on the activity and/or expression of key protooncogenes, such as human epidermal growth factor receptor 2 (*HER2*) [1, 2, 5]. Amplification and overexpression of *HER2* are detected in approximately 20–30% of breast carcinomas and are associated with a poor prognosis [6–10]. Hyperactivation of HER2 promotes aberrant cell proliferation and tumorigenesis, thereby making HER2

an important therapeutic target against breast cancer [6–10].

Currently, the primary treatment for HER2-overexpressing tumors is trastuzumab (Herceptin) [11–14]. Trastuzumab is a monoclonal antibody that is designed to target the extracellular domain of HER2 and block its function. However, response rates for trastuzumab monotherapy have been reported to range from 12% to 34% with a median duration of 9 months [9, 10]. Thus, it appears that the mechanism of action of HER2 is not yet fully understood.

We previously showed that HER2 hyperactivation and signaling in breast cancer cells depend strongly on the location of the receptor within membrane lipid rafts [15]. In breast cancer cells, HER2 overexpression may be accompanied by an increase in cell membrane lipid raft microdomains, thereby establishing a vicious cycle of aberrant cell signaling [1, 15]. Recent experimental evidence revealed that the dimerization of HER2 (as a homo- or heterodimer with members of its own family) is associated with lipid rafts [1, 16]. In addition, HER2-mediated proliferation and survival signals depend on the colocalization of HER2 with other membrane proteins (e.g., integrins and extranuclear factor of the estrogen receptor [ER]) in lipid rafts [17, 18]. Accordingly, it is possible that an increase in the number of lipid rafts in HER2-overexpressing cells can enhance the activation of these oncogenic receptors [15].

To ensure lipid raft synthesis, HER2 promotes the activation of fatty acid synthase (FASN). Its final product, palmitate, is frequently used to synthesize membrane microdomains [1, 15, 19]. In a previous study, when this pathway was inhibited by omega-3 docosahexaenoic fatty acid (DHA), lipid rafts were disrupted and cell apoptosis was induced [15]. Thus, HER2 overexpression in breast cancer cells is associated with constitutive upregulation of the endogenous FASN-catalyzed biogenesis of palmitate. The upregulation of palmitate biogenesis represents a "lipogenic benefit" for the proliferation and survival of breast cancer cells by providing lipid raft components for the proper localization and activation of HER2 in the cell membrane [1, 2, 15, 19]. However, accumulation of palmitate in nonadipose tissue promptly stimulates lipolysis and apoptosis and can act as an inhibitory feedback signal for endogenous FA synthesis [1, 2, 20–22].

On the other hand, these events seem to be avoided in HER2-overexpressing breast carcinoma cells, through the conversion and storage of FAs as triglycerides by peroxisome proliferator-activated receptor gamma (PPARγ) [1, 2]. Rather than preventing lipotoxicity, the transcriptional activation of PPARγ increase the expression of genes related to uptake and transport of exogenous FA, contributing to the establishment of lipogenic phenotype in HER2-overexpressing cells [1, 2]. Therefore, in these cells, upregulation of FASN appears to be a downstream manifestation of an early and common deregulation of upstream regulatory circuits that affect the lipogenic genetic program [2]. It is believed that the regulation of lipogenesis occurs through mTOR protein [1, 2]. The HER2/mTOR pathway results in SREBP1 activation which can increase the transcription of PPARγ endogenous ligands and regulates the expression of *FASN* [1, 2]. However, the details of this process remain unclear, since activation of components of the mTOR pathway, as mTORC1, may limit the survival signs by reducing Akt activity [1, 2]. Accordingly, it remains to be determined whether HER2 overexpression-mediated oncogenic transformation requires the activation of a genetic switch of lipogenic cell metabolism to maintain aberrant signaling that affects cell survival and proliferation.

From a molecular perspective, we hypothesized that the HER2 overexpression-mediated oncogenic transformation of breast cells involves a distinct lipogenic program that, in addition to FA synthesis, requires the coordinated expression of genes involved in the following: (a) the conversion and storage of excess FAs (e.g., palmitate) to triglycerides, thereby avoiding lipotoxicity; and (b) the uptake and transport of other exogenous FAs, which are necessary to maintain a constant supply of lipids/lipid precursors, membrane lipid raft production, and lipid-based posttranslational protein modifications in these highly proliferative cells. From a clinical perspective, the dependence of cancer cells on lipogenesis for survival and proliferation may represent the "Achilles' heel" of HER2-driven oncogenesis. Thus, lipogenic enzyme inhibitors, modulators of PPARγ transcriptional activity, and, perhaps, dietary omega-3 polyunsaturated FAs (e.g., DHA) may provide novel therapeutic strategies for the clinical management of HER2-positive breast carcinomas and may increase the efficacy of standard therapies [2, 20, 21].

DHA is a potent PPARγ regulator that has been shown to suppress adiposity in rodents and block adipogenesis in many adipocyte cell lines [23, 24]. As a modulator of cell membrane lipid composition, DHA can disrupt lipid rafts, thereby impairing HER2-regulated pathways and inducing cell apoptosis [15]. Therefore, our second hypothesis was that DHA could effectively modulate the lipogenic genetic switch associated with HER2 overexpression. In addition, we investigated whether DHA increases the trastuzumab action in HER2-overexpressing breast carcinoma cells.

2. Methods and Materials

2.1. Cell Culture. Parental, nontransformed HB4a cells and HER2-overexpressing HB4a variant cells, HB4aC5.2, were cultured in RPMI-1640 with 10% fetal bovine serum (FBS) (GIBCO, Invitrogen, Brazil) plus ampicillin, hydrocortisone, and insulin (Sigma-Aldrich, Brazil) at 37°C in a 10% CO_2 humidified incubator [25, 26]. HB4a cells were derived from normal breast luminal cells. HB4aC5.2 cells were generated by cotransfecting HB4a cells with pJ5E.c-erbB-2, a plasmid containing the full-length normal human *HER2* cDNA, derived from the established breast cancer line BT474, under the control of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) promoter and SV40 polyadenylation signals [25, 26]. Five copies of pJ5E.c-erbB-2 were detected in the genome of the C5.2 clone, resulting in the expression of ~10^6 HER2 receptors per HB4aC5.2 cell [25, 26]. Cells were tested periodically for mycoplasma (data not shown) and were authenticated by real-time reverse transcription polymerase chain reaction (RT-PCR) to evaluate *HER2* overexpression [15, 25, 26]. The HB4a and HB4aC5.2 cell lines were a generous gift from Michael J. O'Hare (Ludwig

Table 1: Primers used for RT-PCR.

GENE	Forward (5′-3′)	Reverse (5′-3′)
HER2/neu	GGGCTGGCCCGATGTATTTGAT	ATAGAGGTTGTCGAAGGCTGGGC
FAT/CD36	TGCAAAACGGCTGCAGGTCA	TGGTTTGTGCTTGAGCCAGGTTTAT
FABP4	GGAGTGGGCTTTGCCACCAGG	CGCCTTTCATGACGCATTCCACC
DGAT	TCGCCTGCAGGATTCTTTAT	GCATCACCACACACCAGTTC
DEPTOR	GCGGAGCTGCCCCGAACAAA	GTGCAGCCTGAGCCGTAGCTG
SREBP1	ACAGTGACTTCCCTCGCCTAT	GCATGGACGGCTACATCTTCAA
FASN	CCGAGACACTCGTGGGCTA	CTTCAGCAGGACATTGATGCC
UBC	ACCCAAGAAAAGCACAAGG	AGCCCAGTGTTACCACCAAG
HMBS	CAAAGATGAGAGTGATTCGC	CACACTGTCCGTCTGTATGC
β-actin	GGGACGACATGGAGAAAATC	GGGTGTTGAAGGTCTCAAAC

Institute for Cancer Research and University College London Breast Cancer Laboratory, Department of Surgery, London, UK).

2.2. Cell Treatments. Briefly, cells were seeded in flasks with medium containing 10% FBS and were allowed to adhere. After 24 h, the culture medium was replaced with fresh medium containing 10% FBS plus treatment agent. Cells were incubated for 72 h without changing the medium. Then, the cells were harvested with trypsin-EDTA (Sigma-Aldrich), and the viable cells were counted by Trypan Blue exclusion (Sigma-Aldrich) and a hemocytometer. Only samples with more than 95% viable cells were used.

2.2.1. Treatment with the PPARγ Inhibitor GW9662. The HB4a and HB4aC5.2 cell lines were treated with GW9662 diluted in dimethyl sulfoxide (DMSO) to 1 μL/mL, on the basis of previous experimental studies using breast cancer cells [27]. The final concentration of DMSO did not exceed 0.1% in any case and was not cytotoxic in any of the cell lines tested at this concentration. GW9662 was kindly provided by Professor William Festuccia (University of Sao Paulo, Brazil).

2.2.2. Treatment with Trastuzumab. The HB4a and HB4aC5.2 cell lines were treated with trastuzumab diluted in phosphate-buffered saline (PBS) to a concentration of 15 μg/mL, on the basis of previous experimental and clinical studies [11–14]. Trastuzumab (Herceptin/Genentech, EUA) was kindly provided by Professor José Ernesto Belizário (University of Sao Paulo, Brazil).

2.2.3. Treatment with DHA. DHA (C22:6n-3, Sigma-Aldrich) was dissolved in ethanol prior to emulsification in medium containing serum proteins. The final concentration of ethanol in the culture medium did not exceed 0.05%. This concentration was previously shown to be nontoxic to cells [28, 29]. The HB4a and HB4aC5.2 cell lines were treated with 100 μM DHA for 72 h, based on previous testing of both cell lines with varying doses of DHA (25, 50, 75, and 100 μM DHA for 24, 48, and 72 h in standard medium). Standard medium plus ethanol was used as a control.

2.3. Proliferation Experiments. To compare proliferation rates between the HB4a and HB4aC5.2 cell lines, 10^3 cells were plated in triplicate and were allowed to attach to 96-well plates overnight in culture medium. The following day, the cells were washed with PBS and received fresh culture medium. After 5 d, the cells were harvested and combined with 0.5 mg/mL MTT. Four hours later, solubilization buffer was added and the cells were incubated for an additional 15 h. Spectrophotometry of the cells was then performed at 595 nm with a 655 nm reference filter. Calibration curves were established with a known number of cells, allowing the absorbance values to be converted into cell numbers.

To compare proliferation rates between treatments, cells were washed with PBS and received culture medium containing trastuzumab or GW9662 for 72 h. At several time points during this 72 h incubation (e.g., baseline and 12, 24, 48, and 72 h after treatment), a portion of the cells were harvested and combined with 0.5 mg/mL MTT to assess proliferation. Detection of proliferation was performed as described above.

2.4. RT-PCR. Total RNA was extracted from cells with the RNeasy Mini Kit (Qiagen, Brazil), in accordance with the manufacturer's instructions. RNA concentration and purity were determined with a spectrophotometer (NanoDrop ND-1000 UV-Vis Spectrophotometer, NanoDrop Technologies) by calculating the ratio of optical density at wavelengths of 260 nm and 280 nm. The cDNA was synthesized by reverse transcription from 2 μg of RNA with the Superscript III Reverse Transcriptase Kit (Invitrogen, Brazil), according to the manufacturer's instructions.

Each PCR sample included 2.5 μL of cDNA, 5 μL of SYBR Green I (Molecular Probes), 1.1 μL of $MgCl_2$, 0.9 μL of H_2O DEPC, and 0.5 μL of primers specific for the gene of interest (Table 1). PCR samples were amplified using a Rotor Gene 3000 System. After each run, the melting curve was analyzed to assess the reaction specificity.

2.5. Western Blotting. The HB4a and HB4aC5.2 cell lines (10^5 cells/25 cm^2 flask) were treated with 5 mL of medium containing 10% FBS, with or without DHA or trastuzumab. After 72 h, the cells were pelleted and proteins were extracted in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium

dodecyl sulfate (SDS), and protease inhibitors. Protein concentrations were determined with the Bradford assay. Cell lysates (40 μg) were boiled for 5 min in Laemmli buffer before being loaded on 10% acrylamide gels for SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membranes. The FASN protein was separated on a 6% acrylamide gel. Membranes were blocked for 1 h in Tris-buffered saline containing 0.05% Tween-20 and 5% skim milk before being incubated with primary antibodies (Cell Signaling Technology, USA, 1 : 1000). After 16 h at 4°C, membranes were incubated with anti-rabbit IgG antibodies (1 : 5000). Bound antibodies were visualized by enhanced chemiluminescence reagent (GE). Membranes were subjected to autoradiography, and quantitative densitometric analysis was performed with the Scion Image software package.

2.6. Flow Cytometry Analysis of Cell Death. The HB4a and HB4aC5.2 cell lines (10^5 cells/25 cm^2 flask) were treated with 5 mL of medium containing 10% FBS, with DHA, trastuzumab, GW9662, or DHA plus trastuzumab, for 72 h. Treated cells were fixed in 70% ethanol at −20°C and stained for 30 min at room temperature with 20 μg/mL propidium iodide (PI) (Sigma-Aldrich), Triton-X (0.1% v/v), and 200 μg/mL DNase-free RNase diluted in PBS. Cells from each sample were analyzed for DNA content with a Becton Dickinson FACS Caliber instrument. Percentages of cells in the sub-G1, G0/G1, and S/G2/M phases of the cell cycle were determined with the Cell Quest software package. Cell death was measured according to the percentage of cells in the sub-G1 region of the fluorescence scale that contained hypodiploid DNA.

2.7. Statistical Analysis. Data are presented as mean ± standard error of the mean (SEM) of three independent experiments performed for each variable. For both cell lines, relative gene expression was expressed as the ratio between target gene expression and the mean expression of the constituent genes (i.e., *UBC*, *HMBS*, and *β-actin*, selected because they did not present significant variations in expression between the untreated and treated cell lines). For comparisons between cell lines, gene expression values obtained by real-time PCR were normalized to the results obtained from the HB4a cell line. For comparisons between treatments, gene expression values obtained by real-time PCR were normalized to results obtained from cell lines treated with ethanol, DMSO, or PBS (controls [CNTs]). Data were obtained from experiments performed in quadruplicate (or duplicate, for real-time PCR assays). The statistical significance of differences was assessed by one-way analysis of variance (ANOVA), followed by the Bonferroni post-test. The significance level was set at $P \leq 0.05$.

3. Results

3.1. In HB4aC5.2 Cells, HER2 Overexpression Is Associated with Activation of a Lipogenic Genetic Switch. To test the hypothesis that HER2 overexpression requires activation of a lipogenic genetic program for oncogenic transformation, the immortalized human mammary luminal epithelial cell line, HB4a, was transfected with *HER2* cDNA to generate the HB4aC5.2 cell line. Oncogenic transformation was assessed by RT-PCR. The HB4aC5.2 cell line expressed *HER2* mRNA at levels equivalent to the tumor-derived cell line, SKBR3, but should be identical to the HB4a cell line in all other aspects (including *ER* mRNA levels) (Figure 1(a)). The ER-negative SKBR3 cell line is characterized by *HER2* amplification.

Real-time PCR analysis detected increased expression of lipogenic genes related to FA uptake (fatty acid translocase gene/cluster of differentiation 36, *FAT/CD36*), FA transport (fatty acid binding protein 4, *FABP4*), and lipid storage (diacylglycerol acyltransferase, *DGAT*) in HB4aC5.2 cells compared to HB4a cells (Figure 1(b)). Interestingly, *FASN* expression was not altered by HER2 overexpression (Figure 1(b)). However, according to Western blot analysis, HB4aC5.2 cells exhibited increased activation of the FASN protein compared to HB4a cells (Figure 1(c)).

3.2. DEPTOR, but Not mTOR/PPARγ, May Be Associated with Activation of a Lipogenic Genetic Program in HB4aC5.2 Cells. Activation of mTOR pathway components, mainly complex 1 (mTORC1) and the p70S6K1 (p70 ribosomal S6 kinase 1) protein, and expression of *SREBP1* (sterol regulatory element-binding protein 1) and *LIPIN1* may contribute to lipogenesis by promoting the production of endogenous ligands for PPARγ [2, 30, 31]. Unexpectedly, both RT-PCR (Figure 2(a)) and Western blotting (Figure 2(b)) assays showed that all of these mTORC1 pathway markers were decreased in HB4aC5.2 cells, but not in HB4a cells (Figure 2).

When HB4a cells were treated with a PPARγ selective antagonist (GW9662) and RT-PCR assays were performed, all of the PPARγ-target regulatory genes via mTORC1 were decreased (with the exception of *FAT/CD36*, which showed increased expression) (Figure 3). Intriguingly, when HB4aC5.2 cells were treated with GW9662, expression levels of *FAT/CD36* increased, but the expression levels of the other genes remained unchanged (Figure 3).

Consistent with these findings, an increase in expression of *DEPTOR* (the main inhibitor of the mTOR pathway) was detected in HB4aC5.2 cells, but not in HB4a cells (Figure 2(a)). When overexpressed, *DEPTOR* can inhibit the activation of both complexes of the mTOR pathway, but especially mTORC1 [32–35]. The observed downregulation of *SREBP1* (Figure 2(a)) and its regulatory protein p70S6K1 (Figure 2(b)) confirmed the inhibition of mTORC1 activation and supported the possibility that DEPTOR activity was increased in HB4aC5.2 cells.

Although an association between activation of the mTORC1 pathway and cell proliferation has been observed in several cancers, this relationship was not observed in the present study [31, 32, 36]. Instead, the hyperproliferative phenotype induced by HER2 in HB4aC5.2 cells appeared to be independent of this pathway. The increased rate of proliferation of HB4aC5.2 cells compared to HB4a cells was independent of additional mitogen stimulation (Figure 3(b)). Treatment with GW9662 did not affect the proliferation rate of either cell line (Figure 3(c)).

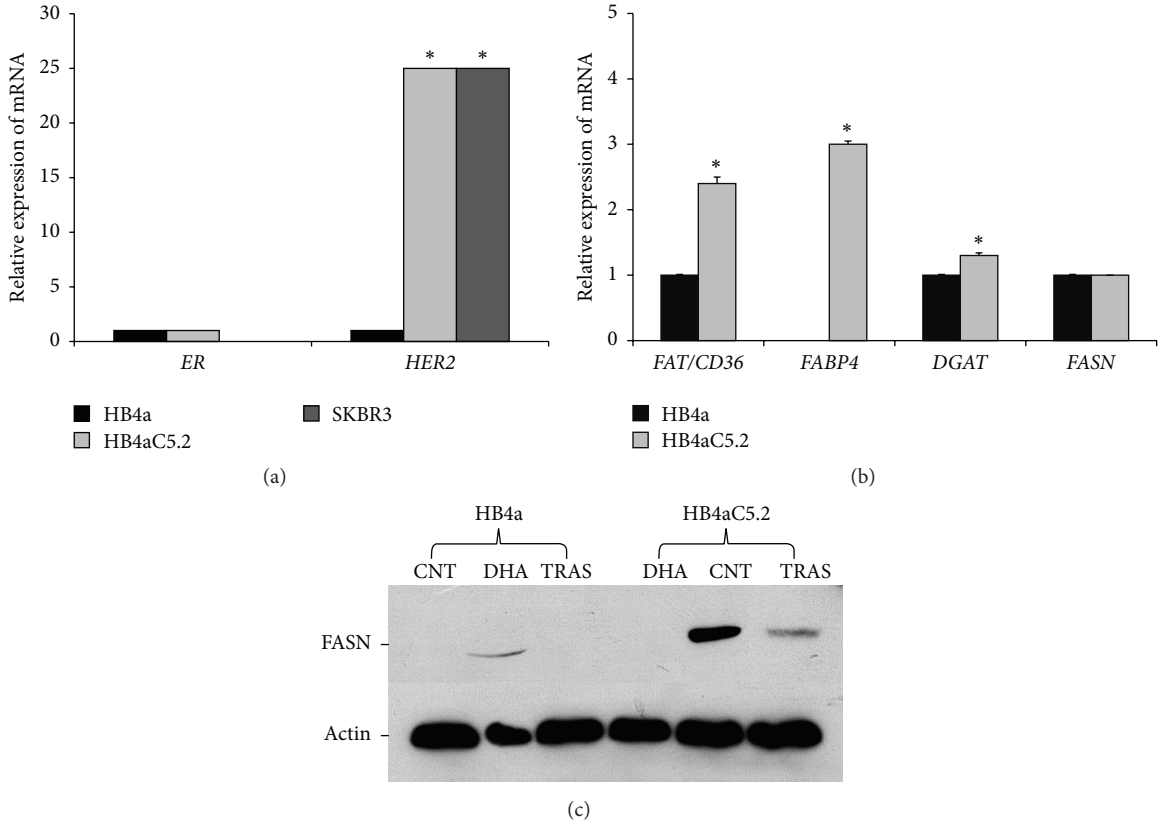


Figure 1: HER2 overexpression and activation of a lipogenic genetic program. (a-b) Relative expression levels of *HER2* and *ER* mRNAs in the HB4a, HB4aC5.2, and SKBR3 cell lines (a) and levels of *FAT/CD36*, *FABP4*, *DGAT*, and *FASN* mRNAs in the HB4a and HB4aC5.2 cell lines (b). The experiment was performed in quadruplicate. The PCR reaction was performed in duplicate. $^{*}P < 0.001$ versus HB4a. (c) Activation of FASN protein in HB4a and HB4aC5.2 cells. Immunoprecipitated proteins were subjected to Western blotting for FASN and β-actin, as controls. CNT: control cells; DHA: cells treated with 100 μM DHA for 72 h; TRAS: cells treated with 15 μg/mL trastuzumab for 72 h.

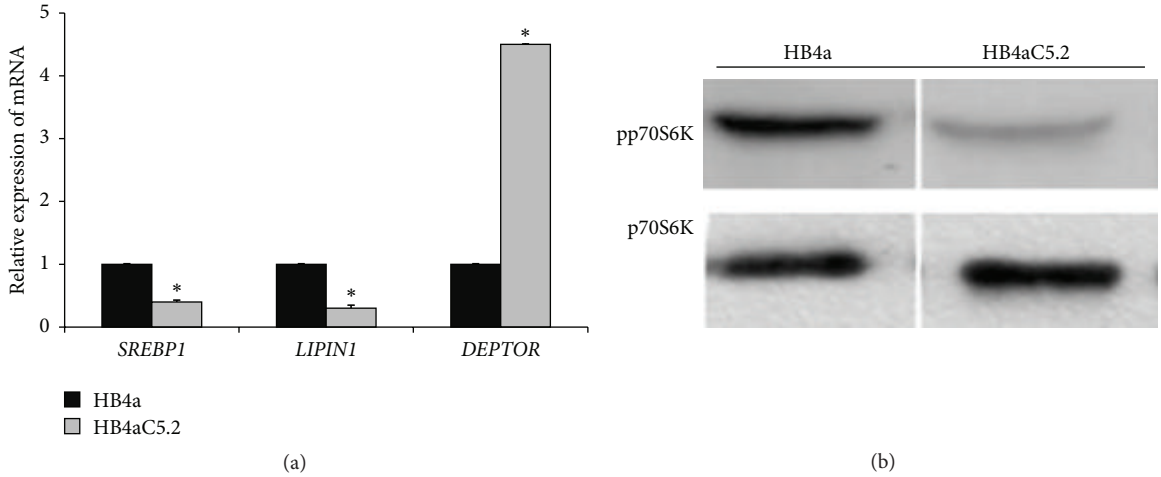


Figure 2: Relative expression of mTORC1 pathway markers. (a) Relative expression of *SREBP1*, *LIPIN1*, and *DEPTOR* in HB4a and HB4aC5.2 lines. Data were obtained from an experiment performed in quadruplicate. The real-time PCR reaction was performed in duplicate. $^{*}P < 0.001$ versus HB4a. (b) Detection of phosphorylated and nonphosphorylated forms of p70S6K (pp70S6K and p70S6K, resp.) by Western blot in HB4a and HB4aC5.2 cells.

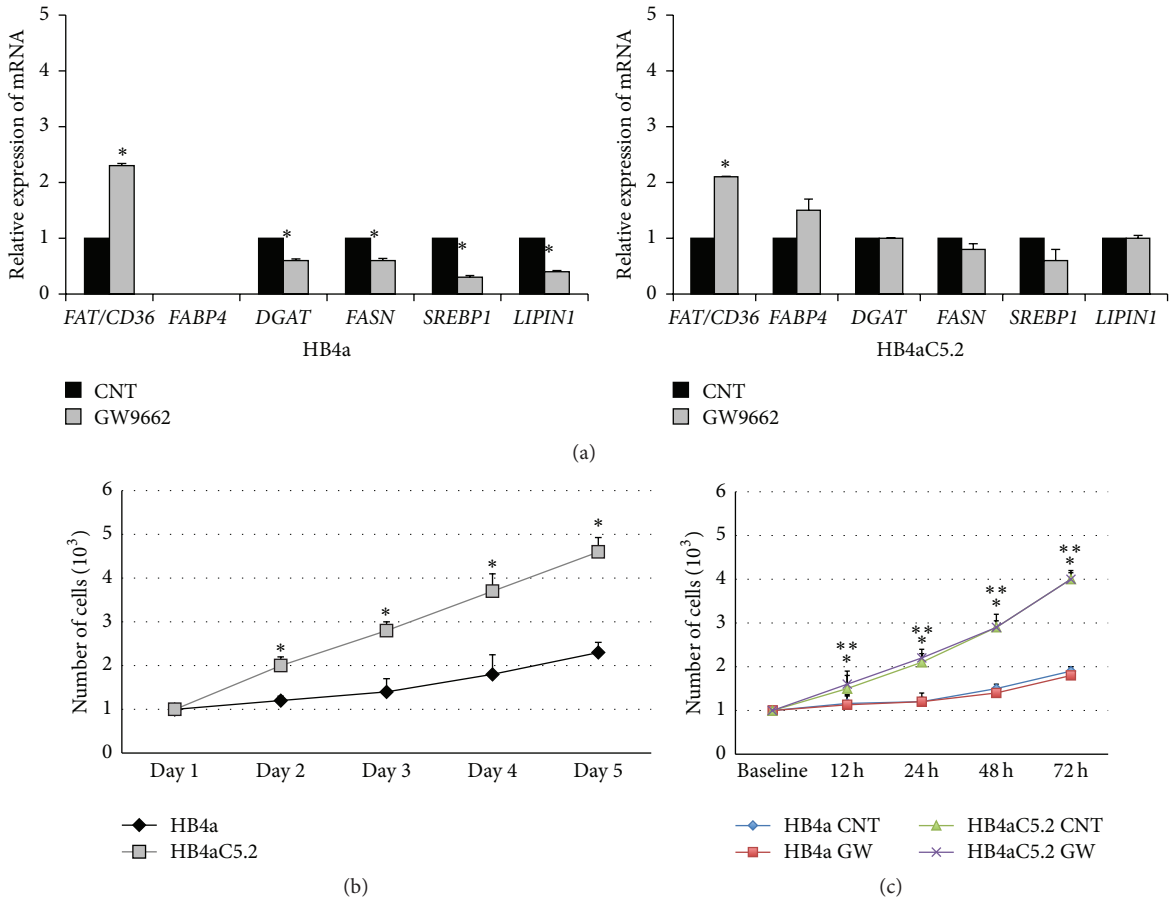


Figure 3: Effects of the PPARγ inhibitor GW9662 on HB4a and Hb4aC5.2 cells. (a) Relative expression of *FAT/CD36*, *FABP4*, *DGAT*, *SREBP1*, *LIPIN1*, and *FASN* in HB4a and HB4aC5.2 cells treated with GW9662 (1 μL/mL) for 72 h. DMSO was used as a control (CNT). Data were obtained from an experiment performed in quadruplicate. The real-time PCR reaction was performed in duplicate. $^{*}P < 0.001$ versus CNT. Proliferation rates for untreated (b) and treated (c) HB4a and HB4aC5.2 cells. Untreated cells were cultured in standard medium without additional stimulation for 5 d. Treated cells were incubated with medium containing GW9662 (GW) or DMSO as a control (CNT) for 0, 12, 24, 48, and 72 h. (b) $^{*}P < 0.001$ versus HB4a. $^{**}P < 0.001$ versus HB4a CNT. (c) $^{*}P < 0.001$ versus HB4aC5.2 CNT. $^{**}P < 0.001$ versus HB4aC5.2 GW.

3.3. In HB4aC5.2 Cells, Trastuzumab Treatment Was Accompanied by Increased FASN Gene Expression and Decreased FASN Protein Activation, While the Cell Proliferation Rate Remained Unchanged. Real-time PCR analyses of *FAT/CD36*, *FABP4*, *DGAT*, *FASN*, *SREBP1*, and *LIPIN1* revealed that trastuzumab treatment was associated with a decrease in *DGAT* mRNA and increases in *SREBP1* and *FASN* mRNA levels in HB4aC5.2 cells (Figure 4(b)). However, these results were not observed in HB4a cells (Figure 4(a)). Moreover, despite the significant increase in *FASN* transcription that was induced by trastuzumab treatment in HB4aC5.2 cells, activation of the FASN protein was inhibited in this cell line (Figure 1(c)).

In HB4aC5.2 cells, trastuzumab treatment did not influence cell proliferation, as detected by MTT assays (Figure 4(b)).

3.4. In HB4aC5.2 Cells, DHA Treatment Affected the Activation of a Lipogenic Genetic Program to Induce Cell Death and Improve Trastuzumab Action in Parallel with a Decrease in DEPTOR Transcription. Previous studies have shown that DHA exhibits a triacylglycerol-lowering effect *in vitro* and *in vivo* and reduces the expression levels of lipogenic genes [37, 38]. However, the mechanisms responsible for these effects remain unknown. Real-time PCR analyses of *FAT/CD36*, *FABP4*, *DGAT*, *FASN*, *SREBP1*, and *LIPIN1* were performed for HB4aC5.2 and HB4a cells, with or without DHA treatment. DHA reduced the expression levels of all of the genes assayed, except *FABP4*, and inhibited the activation of FASN in HB4aC5.2 cells (Figure 5). Although both cell lines achieved very similar expression levels for the genes (except *FABP4*), DHA treatment induced cell death only in HB4aC5.2 cells (Figure 6(a)). Neither trastuzumab nor GW9662 alone affected the rate of cell death for either cell line (Figure 6(a)); however, combined treatment with DHA and trastuzumab increased cell death in the HB4aC5.2 cells when compared with DHA and trastuzumab alone (Figure 6(a)).

Finally, the relative expression of *DEPTOR* in the HB4a and HB4aC5.2 cells treated with DHA, trastuzumab, DHA plus trastuzumab, GW9662, or culture medium (as a control)

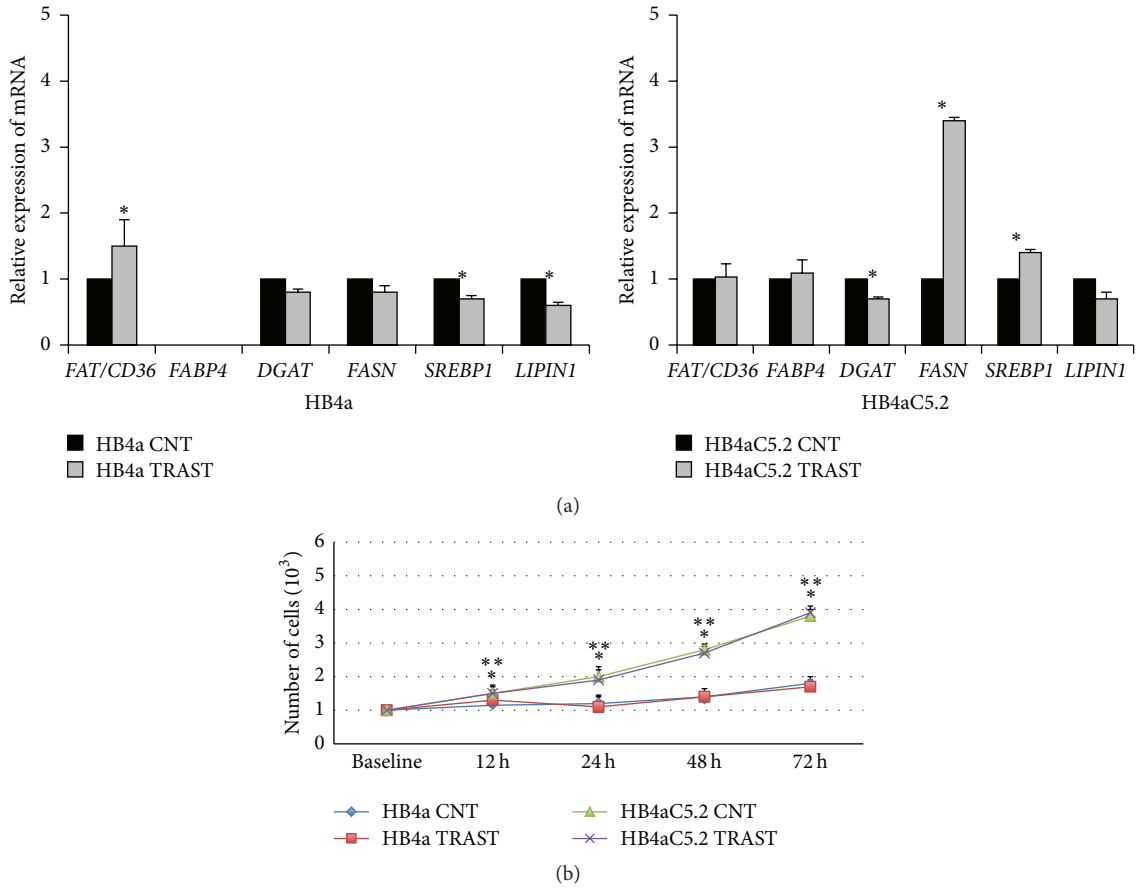


Figure 4: Effect of the HER2 inhibitor trastuzumab on HB4a and Hb4aC5.2 cells. (a) Relative expression of *FAT/CD36*, *FABP4*, *DGAT*, *FASN*, *SREBP1*, and *LIPIN1* in HB4a and HB4aC5.2 cells treated with trastuzumab (Herceptin, 15 μg/mL for 72 h) or PBS as a control (CNT). Data were obtained from an experiment performed in quadruplicate. The real-time PCR reaction was performed in duplicate. $^{*}P < 0.001$ versus CNT. (b) Proliferation rates of HB4a and HB4aC5.2 cells treated with trastuzumab (TRAST) or PBS as a control (CNT) for 0, 12, 24, 48, and 72 h. $^{*}P < 0.001$ versus HB4aC5.2 CNT. $^{**}P < 0.001$ versus HB4aC5.2 TRAST.

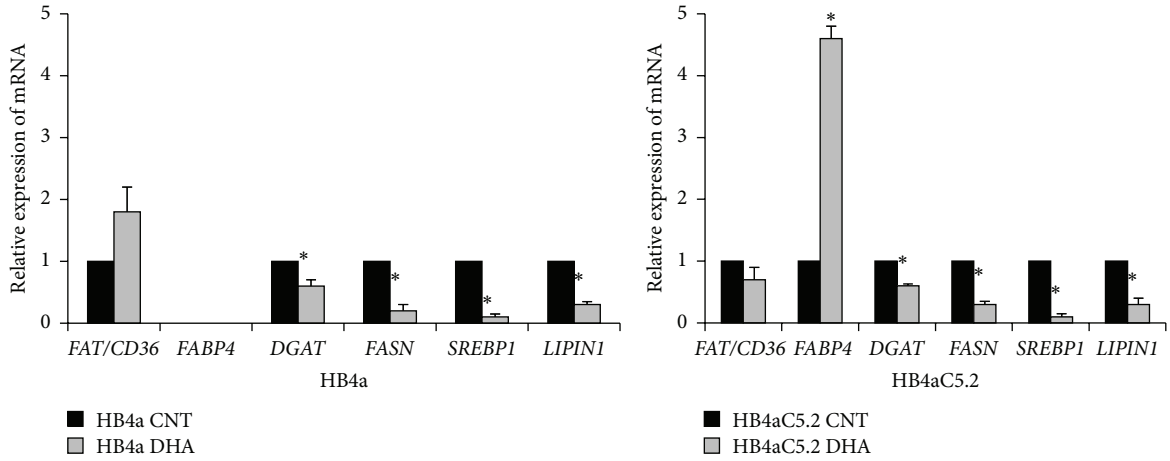
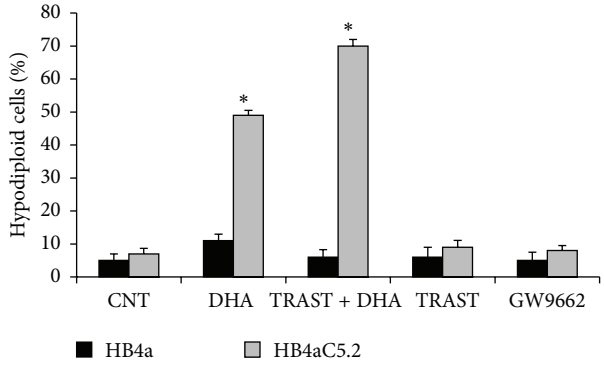
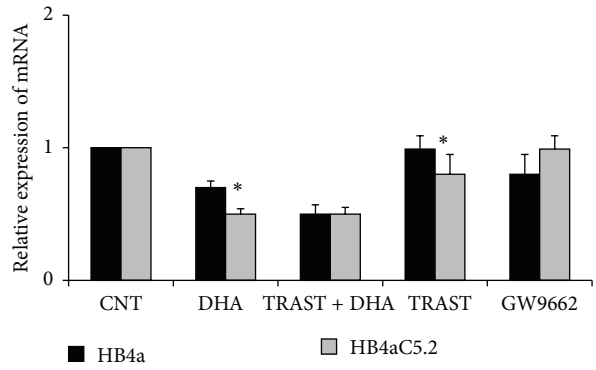


Figure 5: Effect of DHA on HB4a and Hb4aC5.2 cells. Relative expression of *FAT/CD36*, *FABP4*, *DGAT*, *FASN*, *SREBP1*, and *LIPIN1* in HB4a and HB4aC5.2 cells treated with DHA (100 μM for 72 h) or ethanol as a control (CNT). Data were obtained from an experiment performed in quadruplicate. The real-time PCR reaction was performed in duplicate. $^{*}P < 0.001$ versus CNT.



(a)



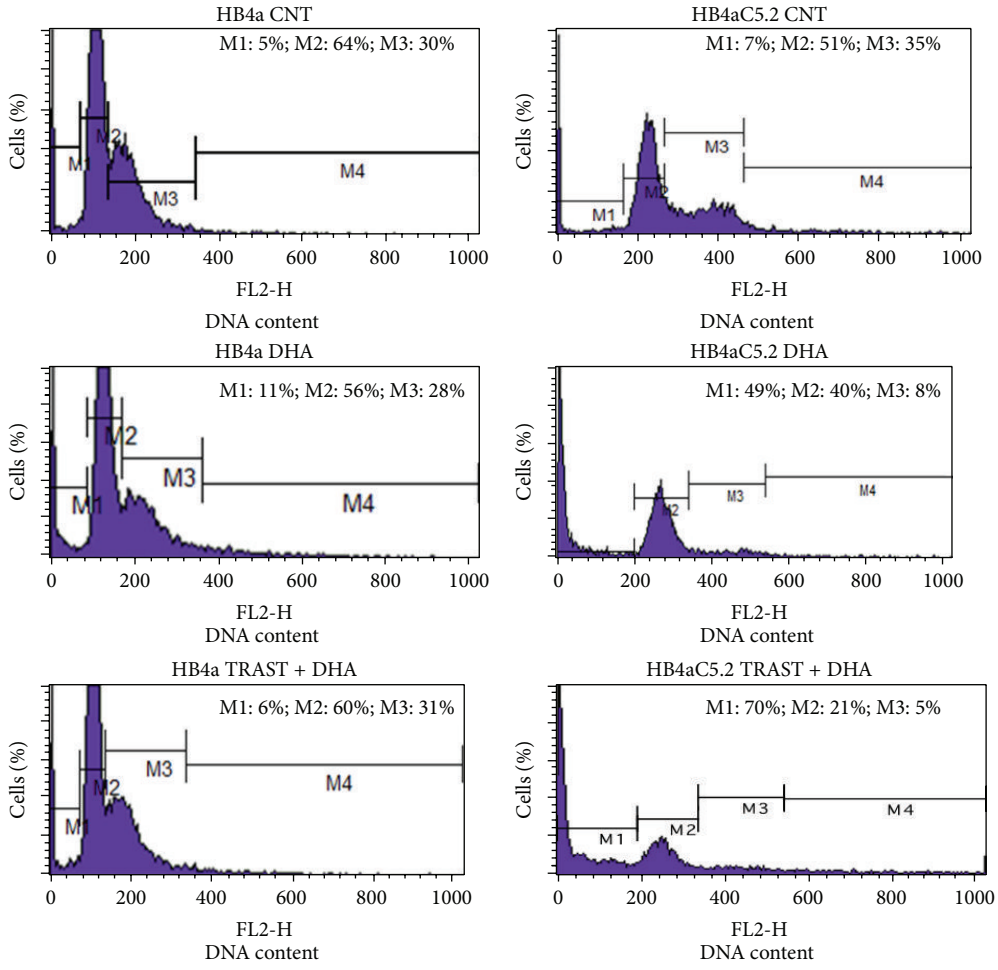
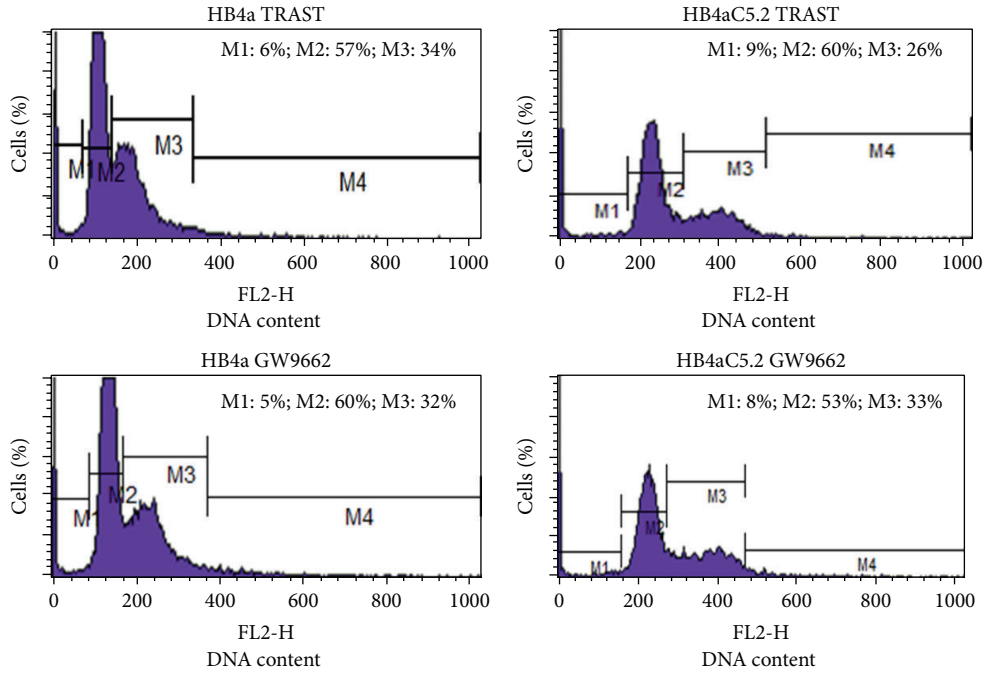


Figure 6: Continued.



(b)

Figure 6: Percentage of cell death induced by different treatments and relative expression of *DEPTOR*. (a) Percentage of cell death induced by 100 μM DHA, 15 μg/mL trastuzumab (TRAST), 15 μg/mL trastuzumab + 100 μM DHA, and 1 μL/mL GW9662 for 72 h in HB4a and HB4aC5.2 cells. Culture medium was used as a control (CNT). Subsequently, all cells were stained with PI and analyzed by flow cytometry. Cells exhibiting hypodiploid DNA content were considered to have undergone cell death. $^{*}P < 0.001$ versus HB4a. $^{*}P < 0.001$ DHA versus CNT, TRAST + DHA, TRAST, and GW9662. Data were obtained from an experiment performed in triplicate. (b) Relative expression of *DEPTOR* gene in HB4a and HB4aC5.2 cells treated with 100 μM DHA, 15 μg/mL trastuzumab (TRAST), 100 μM DHA plus 15 μg/mL trastuzumab (TRAST + DHA), 1 μL/mL GW9662, or culture medium as a control (CNT) for 72 h. Data were obtained from an experiment performed in quadruplicate. The real-time PCR reaction was performed in duplicate. $^{*}P < 0.001$ versus CNT. Results from PBS and DMSO treatments (vehicle dilution) did not differ from those obtained from treatment with standard culture medium. The histograms below the graphs represent the data obtained by flow cytometry. M1: percentage of hypodiploid cells (e.g., cell death); M2: percentage of cells in the G0/G1 phase of the cell cycle; M3: percentage of cells in the S/G2/M phase of the cell cycle; and M4: cell debris.

was detected by real-time PCR. In HB4aC5.2 cells, DHA-induced toxicity was accompanied by a decrease in *DEPTOR* transcription. A greater decrease in *DEPTOR* transcription was induced by DHA plus trastuzumab treatment compared to treatment with DHA alone (Figure 6(b)).

4. Discussion

We hypothesized that the HER2 overexpression-mediated oncogenic transformation of breast cells involves a distinct lipogenic program that, in addition to FA synthesis, requires the coordinated expression of genes involved in the following: (a) the conversion and storage of excess FAs to triglycerides, thereby avoiding lipotoxicity; and (b) the uptake and transport of other exogenous FAs, which are necessary to maintain a constant supply of lipids/lipid precursors, in these highly proliferative cells.

For a model, we chose a transformed, immortalized cell line with a strictly luminal phenotype that has been specifically engineered to overexpress HER2 (HB4aC5.2) but is identical to its parental strain (HB4a) in all other aspects. This permits a cleaner analysis of the specific effects of enhanced HER2 levels on luminal epithelial cell function and phenotype, unlike most tumor cell lines already established and described in *in vitro* studies, which show multiple genetic aberrations other than overexpression of HER2 receptors. By using HB4aC5.2 and HB4a cells, we were able to analyze the specific effects that enhancing HER2 levels had on the lipogenic phenotype [25, 26].

Elevated levels of HER2 expression have been observed in human breast cancers, with levels of *HER2* amplification ranging from 2-fold to greater than 20-fold [25, 26, 39, 40]. One consequence of HER2 overexpression in epithelial cells is hyperproliferation [25, 26], which requires an increase in FA synthesis in order to provide building materials for new membranes and lipid rafts [1–3, 15]. This requirement to lipogenesis for survival and proliferation may represent a target treatment in HER2-driven oncogenesis [1–3, 15]. Experimental and clinical studies have shown that the early stages of tumorigenesis in HER2-overexpressing breast cancer cells are associated with increased activation of the FASN-mediated synthesis of palmitic acid, which is often used to form lipid rafts [1–3, 5, 15, 19, 41, 42]. In the present study, HER2 overexpression was accompanied by an increase

in FASN protein activation, in parallel with the increased expression of *DGAT*, a gene that encodes an enzyme involved in the final step of triglyceride synthesis [43]. Thus, breast cancer cells that overexpress HER2 and have increased FASN activity may sustain their proliferation and avoid lipotoxicity by converting and storing excess palmitate as triglycerides.

The increase in *DGAT* expression was accompanied by an increase in expression levels of *FAT/CD36* and *FABP4*, which encode proteins involved in the uptake and transport of FAs. These processes facilitate the synthesis of cellular membranes during cell proliferation suggesting that HER2-overexpressing breast cancer cells alter their metabolism to improve triglyceride synthesis and lipid uptake/incorporation for cell proliferation [1–3]. These findings suggest that a lipogenic phenotype is required and possibly induced by HER2 overexpression and the observed increase in FASN activation comprises only one part of a much larger lipogenic program in such cells.

Although the exact mechanism linking HER2 signaling with lipogenesis remains unknown, accumulating evidence indicates that activation of PPARγ via the mTOR pathway may regulate this process [1, 2, 31, 33–35]. According to our results, the mTORC1 activity (component of the mTOR pathway) in HB4aC5.2 cells was low suggesting that PPARγ may be regulated through a pathway other than mTOR. Indeed, this lipogenic program required for oncogenic transformation in HB4aC5.2 cells was not found to be coordinated by PPARγ activity because the blockage of its activity did not alter the expression of the lipogenic genes assayed in these cells. This finding raises the possibility that HER2 overexpression may employ another mechanism to maintain or generate the lipogenic phenotype [33, 34]. Based on the present findings, *DEPTOR* may be a potential mediator.

DEPTOR is the main inhibitor of the mTOR pathway and may regulate the lipogenesis process [33, 34]. In adipocytes, *DEPTOR* expression promotes adipogenesis, whereas inhibition of *DEPTOR* blocks this process [33]. In animal models, *DEPTOR* overexpression is responsible for the accumulation of white adipose tissue, and in humans, it is associated with some degree of obesity [33].

Experimental evidence suggests that *DEPTOR* is a potent activator of Akt-mediated survival pathways [33, 34]. For example, when *DEPTOR* is overexpressed, mTORC1 activity is reduced and the PI3K/mTORC2/Akt pathway is activated via release of the inhibitory feedback that mTORC1 imposes on mTORC2 [32]. Interestingly, this indirect mode of Akt protein activation appears to be important for the viability of thyroid carcinoma and multiple myeloma cells [35]. *DEPTOR* overexpression and reduced mTORC1 complex activity have been detected in approximately 28% of patients with multiple myeloma, and these patients had lower survival rates [35]. Experimentally, *DEPTOR* overexpression has been shown to reduce protein synthesis and cell growth in multiple myeloma cells, while activating survival signals from PI3K/Akt proteins. *DEPTOR* downregulation has also been shown to promote cell death [35].

We previously reported that HER2 overexpression in HB4aC5.2 cells is accompanied by hyperactivation of Akt [15]. In the present study, a significant increase in *DEPTOR* expression and decreases in the expression levels of mTORC1 pathway members (i.e., p70S6K and *SREBP1*) were observed in HB4aC5.2 cells. It may be that the increase in *DEPTOR* expression provides an important oncogenic advantage for HB4aC5.2 cells. *DEPTOR* may help regulate lipogenesis to facilitate proliferation and may enhance Akt activation in favor of cell survival. Akt can promote cell survival by various mechanisms, including regulation of transcription factors other than PPARγ [44, 45]. As an Akt activator [35], *DEPTOR* may enhance the expression of lipogenic genes that are targeted not only by PPARγ but also by other transcription factors. This explanation would account for the observed increase in lipogenic gene expression in HER2-overexpressing cells while PPARγ activity was not detected.

Overall, the present results show that the oncogenic transformation of HB4aC5.2 cells by *HER2* overexpression appears to promote a lipogenic environment conducive to cell proliferation and cell survival. Furthermore, this environment may be potentially dependent on *DEPTOR* overexpression. Accordingly, inhibitors of lipogenic enzymes, modulators of *DEPTOR* gene expression, and FA supply could impair HER2-mediated oncogenesis.

Several reports have indicated that omega-3 polyunsaturated FAs, such as DHA, can act as efficient anti-HER2 therapeutics [2, 15, 46]. Previous studies have largely attributed the DHA sensitivity of HER2-positive cells to the ability of this FA to suppress HER2 expression or HER2-mediated pathways by different mechanisms (e.g., lipid raft disruption). However, it is possible that the supplementation of highly lipogenic HER2-positive cells with FAs other than palmitate could trigger the generation of reactive oxygen species and cell death [1, 2, 15, 46]. In the present study, DHA treatment of the HB4aC5.2 cells led to a decrease in *DGAT* expression and an induction of apoptosis. These findings indicate the diminished ability of these cells to mediate the nontoxic accumulation of lipids in their triglyceride form.

Interestingly, the increased expression levels of *FAT/CD36* and *FABP4* that were observed in HB4aC5.2 cells were maintained after DHA treatment of these cells. It is possible that these cells were able to capture and transport DHA for cell membrane formation. Such preferential use of DHA would inhibit cell survival and proliferation, due to alterations in the formation of cell membrane lipid rafts [47–49]. Previously, we reported that DHA treatment of HB4aC5.2 cells increased DHA and decreased palmitic acid percentages in cell membranes [15]. These changes were observed concomitantly with a decrease in the number of lipid rafts, which, in turn, may have impaired HER2-mediated signaling [15]. This possibility was supported by the simultaneous decrease in activation of the Akt and ERK1/2 proteins [15]. In the present study, DHA treatment led to decreased activity of the FASN protein and lower expression levels of *DGAT* and *DEPTOR*. Besides, the DHA treatment plus trastuzumab was able to increase cell death percentage, in HB4aC5.2 cells, when compared with DHA and trastuzumab alone.

Taken together, these data support the possibility that the mechanism responsible for DHA-related toxicity in HB4aC5.2 cells includes a disturbance of the lipogenic genetic

program. This disturbance may be mediated, at least in part, by *DEPTOR* and is distinct from trastuzumab, which seems to disturb FASN activity by different mechanisms. The cytotoxic pathways that are involved seem to be complementary, to improve cell death only in HER2-overexpressing HB4aC5.2 cells. Moreover, DHA appears to increase the sensitivity of cells to death by modulating a HER2-driven lipogenic genetic program. These findings support the use of DHA as a candidate therapeutic agent for minimizing HER2-mediated oncogenesis in breast cancer cells by disturbing a PPARγ-independent lipogenic phenotype associated with *HER2* overexpression.

One limitation of the present study design is its specificity. For example, the HB4aC5.2 cell line was designed to overexpress HER2. These cells exhibited noninvasive and proliferative characteristics and expressed luminal epithelial markers. Consequently, the present results should be analyzed with caution when extrapolated to more complex models or other types of cells representing different breast cancer tumor stages. Additionally, the results provided in this study are valid only for DHA, which may have distinct or different antitumoral effects than other omega-3 fatty acids, such as eicosapentaenoic acid (EPA), and omega-6 fatty acids, such as arachidonic acid (AA) [50]. Indeed, it is widely recognized that DHA reduce and AA increase the risk of breast cancer in experimental and clinical studies [50–53]. However, it is noteworthy that the opposite effects between these FA also seem to extend to the cellular lipogenesis [23, 24, 54, 55]. Some authors have shown that DHA suppress adiposity in rodents and block adipogenesis in many adipocyte cell lines [23, 24], while AA is associated with increase of adipogenesis [54, 55]. According to our results, the DHA antitumoral effect was accompanied by cellular lipogenesis decrease. In this scenario, an important question emerges: Is it possible that AA effect toward tumorigenesis [50–53] might be associated with its capacity to increase cellular lipogenesis [54, 55]? Moreover, are tumor lipogenic phenotype and FA (DHA and AA) effects associated? From a clinical perspective, considering that dietary fat is part of modifiable risk of breast cancer, further studies should be conducted to evaluate the role of different FA in lipogenesis and breast cancer progression.

Together, our data demonstrate that an oncogenic transformation of *HER2*-expressing breast cancer cells supercharges cell lipogenesis via coexpression of various genes involved in the synthesis, uptake, transport, and storage of FAs. DHA treatment disturbs this lipogenic state by inducing cell death and increasing the action of trastuzumab. Therefore, DHA may represent a useful tool for controlling the aberrant signaling triggered by HER2. Nutritional interventions may constitute a new approach for improving conventional therapies, without adversely affecting patient quality of life. In particular, DHA supplementation in combination with other drugs, such as inhibitors of HER2 (trastuzumab), should be explored as a treatment strategy for breast cancer.

Conflict of Interests

The authors have no potential conflict of interests to disclose.

Authors' Contribution

Graziela Rosa Ravacci was responsible for the conception, design, acquisition, and analysis of data, interpretation of the results, and drafting of the paper. Maria Mitzi Brentani contributed to the conception and design of the research, reviewing of the paper, and interpretation of the results. Tharcisio Citrângulo Tortelli was responsible for the acquisition and analysis of data and reviewing of the paper. Raquel Suzana M. M. Torrinhas, Jéssica Reis Santos, and Angela Flávia Logullo participated in the writing of the paper. Dan Linetzky Waitzberg contributed to the conception of the research and reviewing of the paper.

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Research Article

Vitamin E Supplementation Delays Cellular Senescence *In Vitro*

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Vitamin E is an important antioxidant that protects cells from oxidative stress-induced damage, which is an important contributor to the progression of ageing. Ageing can be studied *in vitro* using primary cells reaching a state of irreversible growth arrest called senescence after a limited number of cellular divisions. Generally, the most utilized biomarker of senescence is represented by the expression of the senescence associated β-galactosidase (SA-β-gal). We aimed here to study the possible effects of vitamin E supplementation in two different human primary cell types (HUVECs and fibroblasts) during the progression of cellular senescence. Utilizing an unbiased automated system, based on the detection of the SA-β-gal, we quantified cellular senescence *in vitro* and showed that vitamin E supplementation reduced the numbers of senescent cells during progression of ageing. Acute vitamin E supplementation did not affect cellular proliferation, whereas it was decreased after chronic treatment. Mechanistically, we show that vitamin E supplementation acts through downregulation of the expression of the cycline dependent kinase inhibitor P21. The data obtained from this study support the antiageing properties of vitamin E and identify possible mechanisms of action that warrant further investigation.

1. Introduction

Ageing is a process characterized by gradual functional decline [1, 2] and associated with an increased risk of developing life-threatening diseases [1] such as cancer, diabetes, cardiovascular diseases, and neurodegenerative disorders.

Human aging can be, to some extent, recapitulated *in vitro* using human primary cells which usually undergo a limited number of cellular divisions before reaching a state of irreversible growth arrest termed replicative senescence [3, 4], also known as the "Hayflick Limit" [5, 6]. Moreover, senescent cells become enlarged and lose cell-cell contacts [7, 8].

The changes associated with cellular senescence progression include morphological and biochemical modifications such as increased (and unique) expression of the senescence associated β-galactosidase enzyme (SA-β-gal) [9] as well as upregulation of the cyclin-dependent kinases (CDKs) inhibitors p16 and p21, which are both negative regulators of cell proliferation [1]. Together, these changes represent important biomarkers used for qualitative and quantitative determination of cellular senescence *in vitro* that is largely accepted to be related to ageing of the organisms *in vivo* [1].

An important contributor to the development of the senescence process is oxidative stress. Oxidative stress usually occurs when the production or the exposure to reactive oxygen species (ROS) overwhelms the antioxidant systems of the cells [10]. ROS are molecules that contain a reactive unpaired electron that can damage biomolecules vital for correct functioning and survival of complex systems [11, 12]. Vitamins E and C are important natural antioxidants capable of neutralizing the deleterious effects of ROS. For this reason and considering their safety, low cost, and the absence of effective alternative treatments, adequate vitamin E and C intake is promoted as a preventive (and potentially curative) treatment for specific pathologies that are typical of old age [12–14].

In this study we monitored the progression of the senescence process in human umbilical vein endothelial cells (HUVECs) and human fibroblasts. We show that the increasing expression of the SA-β-gal represents a sensitive and reliable marker to quantify senescent cells in both cellular models. Moreover, we show that addition of vitamin E to the cellular systems is sufficient to reduce the percentage of senescent cells *in vitro*. Lastly, we outline common mechanistic pathways by which vitamin E may exert its antisenescent effect.

2. Experimental Section

2.1. Cell Culture

2.1.1. HUVECs. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland). Cells were cultured at 37°C, in atmosphere of 5% CO_2 in Clonetics Endothelial Cell Growth Media (EBM) supplemented with the BulletKit containing bovine brain extract (BBE), epidermal growth factor (hEGF), hydrocortisone, gentamicin, amphotericin B, fetal bovine serum (FBS), and ascorbic acid according to the manufacturer's instructions (Lonza, Basel, Switzerland). Vitamin E (DL-α-Tocopherol acetate) (Sigma-Aldrich, Buchs, Switzerland) was added to the growth medium at the final concentration of 25 μM. Cells were passaged before they reached the confluence at a ratio of 1 : 3–1 : 6 according to their proliferating properties over time and at regular intervals. After trypsinization using 0.25% Trypsin/EDTA (Life Technologies Europe, Bleiswijk, Netherlands) cells were seeded at different densities. Cells between passages 3 and 12 were used for the experiments described here.

2.1.2. Fibroblasts. Human dermal fibroblasts were originally isolated from foreskin tissue of a healthy young male subject (DSM Nutritional Products, Kaiseraugst, Switzerland). Fibroblasts were cultured in a humidified incubator at 37°C, 5% CO_2 in DMEM medium supplemented with 4.5 g/L D-glucose, 4 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (Life Technologies Europe, Bleiswijk, Netherlands), and 10% heat-inactivated FBS (Sigma-Aldrich, Buchs, Switzerland). Subconfluent cells were trypsinized using 0.25% Trypsin/EDTA (Life Technologies Europe, Bleiswijk, Netherlands) and passaged at a ratio of 1 : 3–1 : 5 at regular intervals. Cells between passages 10 and 39 were used for experiments described in this paper.

2.1.3. Vitamin E Supplementation. Vitamin E (DL-α-Tocopherol acetate) (Sigma-Aldrich, Buchs, Switzerland) was originally dissolved in 100% EtOH as a stock solution of 50 mM, prediluted 1 : 10 in growth medium, and further diluted 1 : 200 in growth medium to result in a final concentration of 25 μM and a final EtOH concentration of 0.05%. The EtOH concentration was kept constant for all treatment conditions. The vitamin E stock solution in EtOH was stored at −20°C and prepared fresh every week.

2.2. Senescence Assessment

2.2.1. SA-β-Gal Staining. The senescence state was measured using the SA-β-gal kit (Cell Signaling, Beverly, USA) according to the manufacturer's instructions. Briefly, cells were seeded at comparable and nonconfluence density in Costar 48-well plates and cellular senescence was assessed 24 hours (hrs) later. Cells were washed with DPBS and fixed with 2% formaldehyde and 2% glutaraldehyde for 10 min at room temperature (RT). After washing, cells were incubated over night at 37°C in a CO_2-free incubator with freshly prepared SA-β-gal staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl-βD-galactopyranoside in dimethylformamide, 40 mM citric acid/sodium phosphate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 150 mM NaCl, 2 mM $MgCl_2$) at pH 6.0. Four wells per condition were not stained and used as background control (subtracted from the values of the stained wells). Cellular nuclei were stained with 5 μg/mL Hoechst 33342 solution (Life Technologies Europe, Bleiswijk, Netherlands) for 30 min at RT.

2.2.2. SA-β-Gal Automated Analysis. 50 adjacent images (fields) of each well were acquired with a 10x objective using an ArrayScan VTI high-content screening system (Thermo Fisher Scientific, Pittsburgh, USA), resulting in a field width of 660 microns. Channel one (Ch1) is the focus channel in which objects (Hoechst-stained nuclei) were identified. SA-β-gal staining was detected in Ch2 using the Brightfield module which offers transmitted (white) light illumination for the ArrayScan VTI and subsequently analyzed with the provided Spot Detector BioApplication. Masks were set for measurements within the stained cytoplasmic region by expanding a ring around the nucleus. Dark signal was detected as large spots (cellular debris, vacuoles, or elongated structures associated with refraction of the cell membrane were excluded from the analysis). Reference levels on the "Spot Total Area" feature were used to define a cell as positive (senescent). The percentage of SA-β-gal-positive cells was determined as the number of cells developing blue color in their cytoplasm relative to the total number of cells analyzed.

2.3. Proliferation.

The proliferation rate of the fibroblasts and endothelial cultures was measured using the *in situ* staining for the EdU and Ki67 proliferation markers according to the manufacturer's instructions (see the following). Briefly, cells were seeded at comparable and nonconfluence density in Costar 48-well plates and cellular proliferation was assessed 24 hrs later.

2.3.1. EdU (5-Ethynyl-2′-deoxyuridine) Detection. Cells were incubated with EdU for three hours and fixed in 4% formaldehyde for 15 min at RT. EdU staining was performed according to manufacturer's instructions (Click-iT Plus EdU Alexa Fluor 647 Imaging Kit, Life Technologies Europe, Bleiswijk, Netherlands).

2.3.2. Ki67 Detection. Following EdU staining, also the Ki67 was detected according to manufacturer's instructions (Ki-67 Monoclonal Antibody, Mouse (7B11), FITC Conjugate, Life Technologies Europe, Bleiswijk, Netherlands). Incubation with Ki-67 antibody was performed for 4 hrs at RT.

2.3.3. Proliferation Analysis. Images of three separate fluorescent channels (Hoechst, FITC, and Deep Red) were acquired on the ArrayScan VTI high-content screening system (Thermo Fisher Scientific, Pittsburgh, USA) using a 10x objective with suitable filter sets. Hoechst 33342 was used to stain and identify the nuclei. 50 fields per well were counted

using object selection parameters and a fluorescence intensity threshold to select only Ki67 and EdU positive cells. Image analysis was performed using the provided Cell Health Profiling BioApplication; the critical well-level output parameters reported the percentage of selected cells for each channel.

2.4. Real Time Polymerase Chain Reaction (PCR)

2.4.1. RNA Isolation. Cells were seeded in a 6-well plate and cultured as already described. Three days after seeding, cells were washed in DPBS and RNA was extracted using the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to manufacturer's instructions. Quantity and quality of the RNA were assessed using the Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland), respectively, according to manufacturer's instructions.

2.4.2. RT-PCR. Reverse transcription was performed using the Omniscript RT Kit with 2 μg of RNA according to the manufacturer's instructions (Qiagen, Hombrechtikon, Switzerland). Each PCR was performed in triplicate using 40 ng of cDNA and the 7900 HT real time PCR system (Applied Biosystems, Foster City, California, USA). All values were normalized to the expression of the 18S ribosomal RNA gene (internal standard). The sequences of the primers and probes used are the following:

Human P21 (NM_000389.4): Forward 5′-TGGAGACTCTCAGGGTCGAAA-3′; Reverse 5′-GGCGTTTGGAGTGGTAGAAATC-3′; Probe (FAM conjugated): 5′-CGGCGGCAGACCAGCATGAC-3′.

Human P16 (NM_00077.4): Forward 5′-CATAGATGCCGCGGAAGGT-3′; Reverse 5′-AAGTTTCCCGAGGTTTCTCAGA-3′; Probe (FAM conjugated): 5′-CCTCAGACATCCCCGATTGAAAGAACC-3′.

Human 18S (NR_003286.2): Forward 5′-CGGCTACCACATCCAAGG-3′; Reverse 5′-CGGGTCGGGAGTGGGT-3′; Probe (VIC conjugated): 5′-TTGCGCGCCTGCTGCCT-3′.

Fold changes were measured as follows: $2^{-\mathrm{ddCt}}$ (ddCt = dCt each value – dCt baseline (younger stage CTR); dCt = Ct value target gene – Ct value internal standard (18S). All fold change values were shown as relative to the younger stage and control (CTR) condition that was indicated as 100% of gene expression.

3. Results

3.1. SA-β-Gal: Marker of Cellular Senescence. To verify that the SA-β-gal quantification could be used as a reliable marker of cellular senescence, we tested our two *in vitro* models at different passages. Using an automated analysis system, we detected a significantly ($p < 0.001$) increased percentage (%) of SA-β-gal positive cells in HUVEC cultures at passage 10 (42%) when compared with younger ones at passage 3 (8%) (Figure 1(a)). Passage 10 HUVECs also showed larger nuclei (299 μm^2 versus 277 μm^2 for cultures at passage 3) ($p = 0.007$), abnormal morphology (bigger cell surface, flatter cells, and less cell-cell contacts) (Supplementary Figure 1(a)) (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/563247), and consistently longer doubling time (cultures at early passages = 1.8 to 2.1 days versus cultures at late passages = 2.7 to 3.9 days) as well as significant reduced percentage of EdU positive cells (passage 10 cultures: 15% versus passage 3 cultures: 31%) ($p < 0.001$) (Supplementary Figure 1(b)) and Ki67 positive cells (passage 10 cultures: 23% versus passage 3 cultures: 57%) both used as markers of cellular proliferation. EdU (5-ethynyl-2′-deoxyuridine) is a nucleoside analog of thymidine incorporated into DNA during active DNA synthesis, while Ki67 is a protein that is expressed in the nucleus during interphase and therefore strictly associated with cell proliferation [15, 16]. Similar results were also obtained in human fibroblasts where the significant higher percentage of SA-β-gal positive cells ($p < 0.001$) was detected in cultures at passage 31 (31%) when compared to younger ones at passage 15 (10%) (Supplementary Figure 2(a)). Moreover, in this cellular model, morphological (Supplementary Figure 2(a)) and proliferation differences were detected during the progression of senescence (passage 15 cultures: 57% and 49% Ki67 and EdU positive cells, resp.; passage 31 cultures: 26% and 21% Ki67 and EdU positive cells, resp.) (Supplementary Figure 2(c)).

3.2. Expression of the P21 and P16 Genes in HUVEC and Human Fibroblast Cultures at Early and Late Passage. The morphological and behavioral changes occurring during the progression of cellular senescence are inevitably associated with changes in the expression of specific genes. Several studies have shown an increased protein expression of the two CDKs inhibitors, p21 and p16, in senescent human fibroblasts [17–19] as well as in senescent HUVECs [4]. Therefore, changes in the expression of both of these proteins are considered markers of senescence.

To verify that these genes were also differently expressed in our cellular models, we measured their expression levels using real time polymerase chain reaction (RT-PCR). HUVEC cultures with a large number of senescent cells (refer to Figure 1) showed an increased expression of both the P16 and P21 genes when compared with cultures having lower percentage of SA-β-gal positive cells ($p < 0.001$ and $p = 0.035$, resp.) (Figure 1(b)). Similar results were also observed when comparing the P21 expression in fibroblasts, although the P16 expression was not increased (Supplementary Figure 3). Of note in HUVECs, the induction of P16 expression was much higher than P21 induction (Figure 1(b): comparing fold changes).

Therefore these experiments established that the measurement of SA-β-gal positive cells represents a reliable marker to quantify senescence in HUVECs and human fibroblasts.

3.3. Effect of Vitamin E Acute Supplementation on Senescence and Proliferation. We tested whether supplementation with

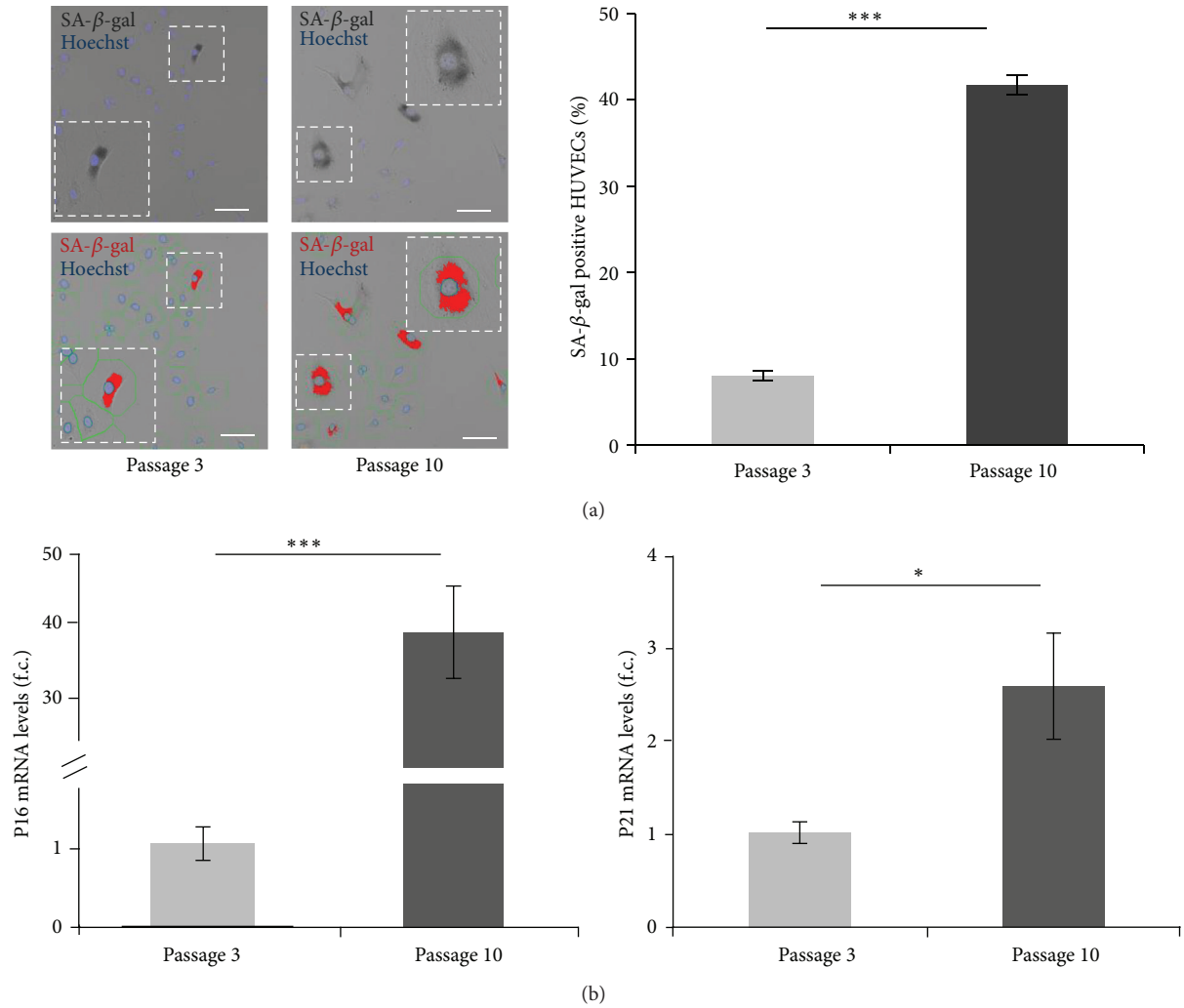

FIGURE 1: Comparison of HUVEC cultures at early and late passage. (a) Representative images of HUVEC cultures stained for the SA-β-gal (upper panels, dark grey cells) at passage 3 and passage 10. Lower panels show the digital images used to quantify the % SA-β-gal positive cells (red color; see materials and methods for details). Nuclei are stained with Hoechst (blue). Scale bars = 100 μm. Graph: percentage of SA-β-gal positive cells. $N = 8$ for both analyses. t test, $p < 0.001$ (***). Error bars indicate the standard error mean (s.e.m.). (b) Graphs show the expression levels of the P16 (left) and P21 (right) genes measured in HUVEC cultures. Values indicate the fold change (f.c.). All values were normalized to the expression levels of the housekeeping gene 18S. Error bars represent the s.e.m. For both analyses $n = 4$. P16 passage 3 = 1.1, passage 10 = 38.6. t test: $p < 0.001$ (***); P21 passage 3 = 1.0, passage 10 = 2.6. t test: $p = 0.035$ (*).

the antioxidant compound vitamin E could reduce the number of senescent cells that appeared *in vitro* over time (refer to Figure 1).

HUVEC cultures at passages 5 and 9 grown in optimal conditions (see materials and methods) were supplemented with vitamin E (25 μM) for 24 hrs and the percentage of SA-β-gal positive cells was measured (Figure 2). As expected, the number of SA-β-gal positive cells significantly ($p < 0.001$) increased at later passages (Figure 2(b): comparing percentage of SA-β-gal positive cells in control (CTR) of passage 5 cultures = 22% with CTR of passage 9 cultures = 42%). Importantly, 24 hrs vitamin E treatment was sufficient to significantly reduce ($p < 0.001$) the number of SA-β-gal positive cells at both stages (CTR = 22% versus vitamin E = 16% and CTR = 42% versus vitamin E = 36%, resp.) (Figure 2(b); see the caption of Figure 2 for details).

Senescent cells are characterized by irreversible proliferation arrest [1, 5, 20]; therefore we verified whether the reduced number of SA-β-gal positive cells following vitamin E treatment was also associated with an increased proliferating rate of the HUVECs cultures. 24 hrs after vitamin E treatment no difference, in the number of dividing cells (EdU positive cells), was observed neither in the early passages nor in the late passages (Figure 3). Similar results were observed when the percentage of Ki67 positive cells was analyzed (Supplementary Figure 4). As expected, a reduced number of cells positive for both of these proliferation markers were measured at later passages (Figure 3 and Supplementary Figure 4).

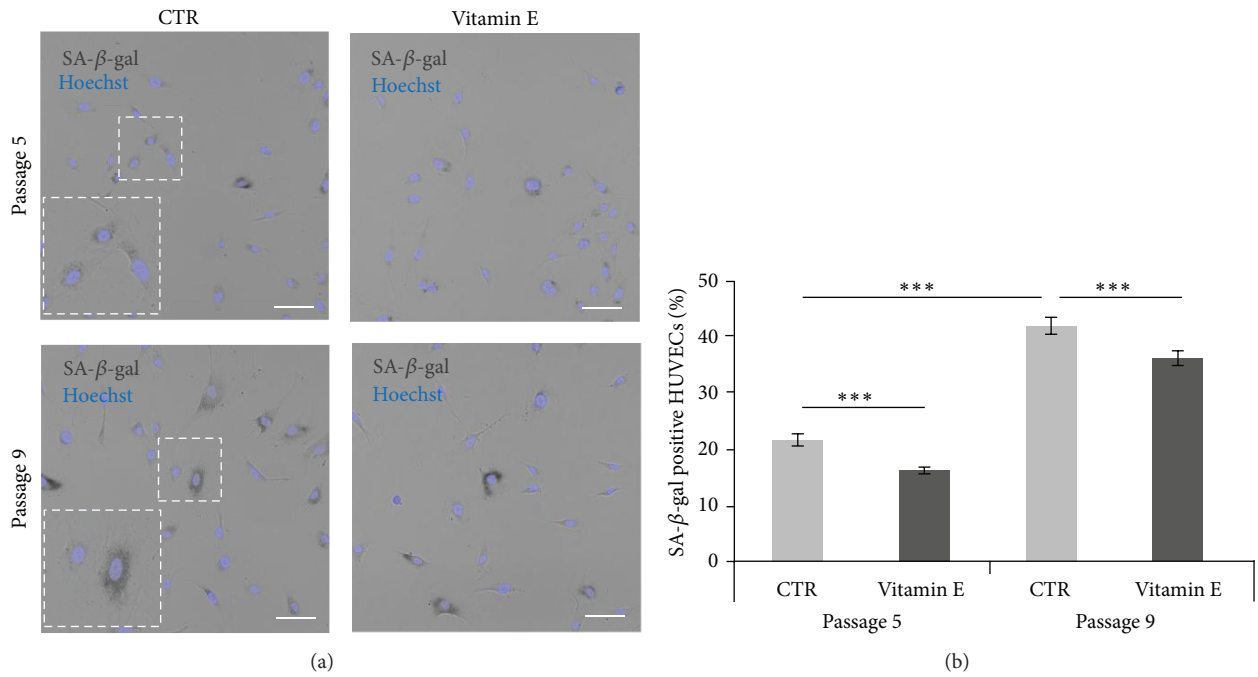

Figure 2: Acute vitamin E supplementation reduces the percentage of SA-β-gal positive HUVECs. (a) Representative images of the graph shown (b) comparing HUVEC cultures at passages 5 and 9 stained for the SA-β-gal (dark grey cells). Enlargements highlight the morphological differences observed comparing early and late passages. Nuclei are visualized in blue. Scale bars = 100 μm. (b) Graph showing the percentage of SA-β-gal positive cells measured in passage 5 and passage 9 HUVEC cultures. For all analyses $n = 8$. Passage 5: CTR = 22%, vitamin E = 16%. t test $p = 0.001$ (***); passage 9: CTR = 42%, vitamin E = 36%. t test, $p = 0.001$ (***); CTR (passage 5) versus CTR (passage 9) cultures: t test, $p < 0.001$ (***). Error bars represent the s.e.m.

Given the reduction in SA-β-gal positive cells following vitamin E treatment, we next examined whether the expression levels of genetic markers of senescence were also affected. Using RT-PCR we measured the expression levels of the P16 and P21 genes in HUVEC cultures (at passages 3 and 9) grown in optimal conditions and supplemented with 25 μM of vitamin E. In agreement with previous results (Figure 1), we measured a significant overexpression of the P16 ($p < 0.001$) and P21 ($p = 0.01$) genes when passage 9 cultures were compared to passage 3 cultures (Figures 4(a) and 4(b)). When the vitamin E effect was analyzed, we detected downregulation of P21 gene expression ($p = 0.015$) in passage 9 HUVEC cultures but not in early passage cultures (Figure 4(b)) but detected no effect regarding the P16 expression (Figure 4(a)). Similar results were also obtained in human fibroblasts where, surprisingly, an increased expression of the P21 gene (CTR passage 10 versus CTR passage 33) was observed over time (Figure 4(c)) but not overexpression of the P16 gene (Figure 4(d)). In this case instead, acute vitamin E treatment did not affect the expression of the analyzed genes.

Therefore, acute vitamin E supplementation reduces the number of SA-β-gal positive HUVECs without affecting the proliferation rate and furthermore vitamin E supplementation downregulates the overexpression of the P21 gene observed in older HUVEC cultures.

3.4. Effect of Chronic Vitamin E Supplementation in HUVECs. As acute vitamin E treatment reduces the percentage of SA-β-gal positive cells in both passage 5 and 9 HUVEC cultures (Figure 2) and P21 expression is downregulated following acute vitamin E treatment (Figure 4(b)), we decided to test if a prolonged (chronic) vitamin E supplementation was more effective in reducing the senescence parameters associated with ageing *in vitro*.

HUVECs were cultured in optimal conditions and supplemented with vitamin E starting from an early passage. Cultures were maintained *in vitro* until most of the cells were considered senescent due to the following criteria: decreased cell division rate and adaptation of an abnormal morphology typical of senescent cells (refer to Figure 1 and Supplementary Figure 1). As expected, we measured an increase in the number of SA-β-gal positive cells in the cultures over time (Figures 5(a) and 5(b) compare CTRs at different passages). In agreement with the acute treatment data, the long-term vitamin E supplementation showed a constant and significant reduction of the percentage of SA-β-gal positive cells over time (Figures 5(a) and 5(b)). Interestingly, age-dependent increase of SA-β-gal positive cells and a similar vitamin E effect were also measured in human fibroblasts (Supplementary Figure 5).

The proliferation rate (percentage of EdU positive cells) of these cultures exhibited no differences at early stages but significantly differed when the cultures were older (Figure 5(c)) suggesting that vitamin E chronic treatment reduced the number of SA-β-gal HUVECs by reducing their proliferative capacity and therefore delaying the senescence onset.

Finally we also measured the expression levels of the P21 and P16 genes and found that in agreement with our

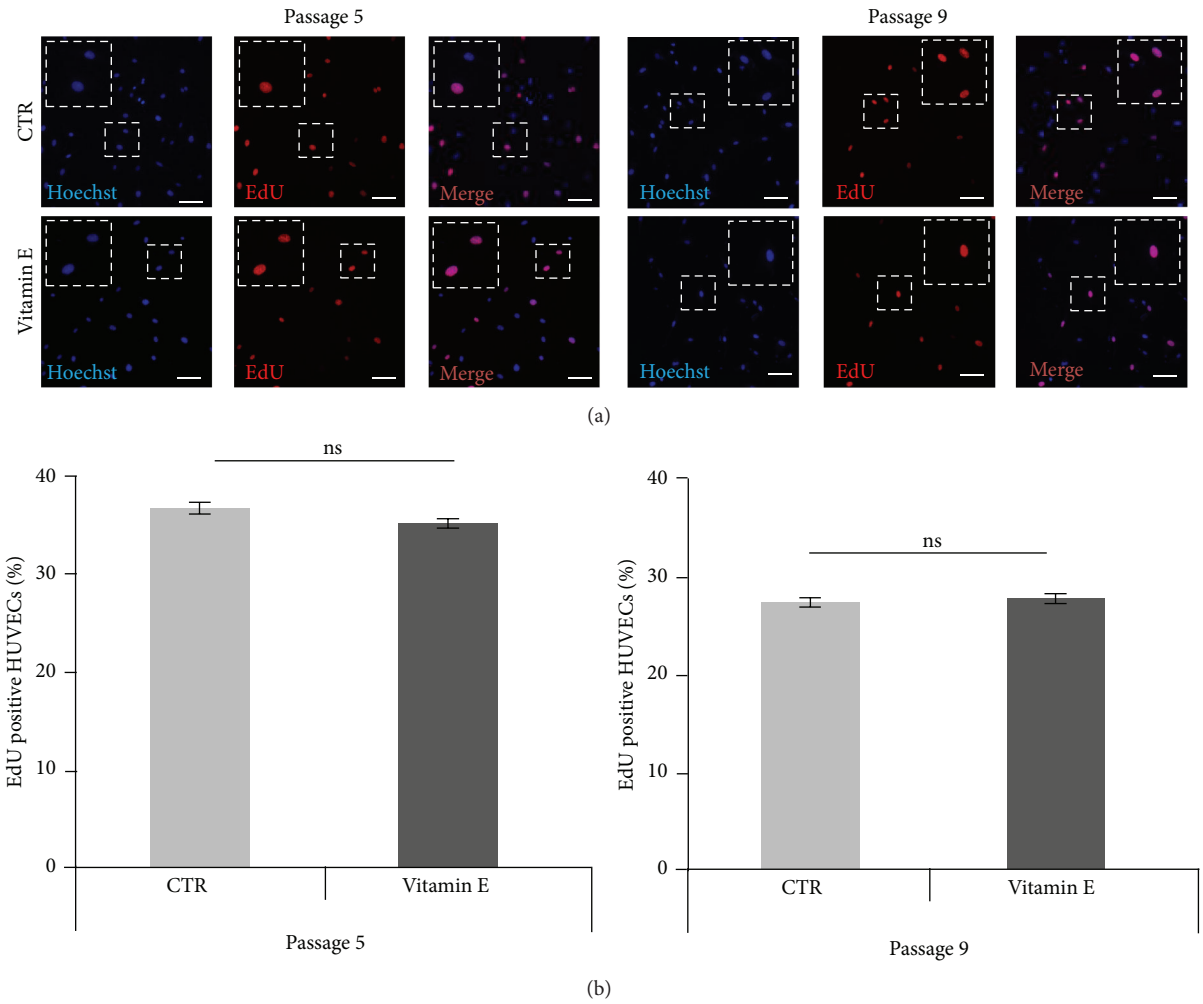

Figure 3: Vitamin E treatment does not alter the percentage of proliferating HUVECs. (a) Representative images showing passage 5 and passage 9 (CTR and vitamin E treatment) HUVEC cultures stained for the proliferation marker EdU (red). Nuclei are visualized by Hoechst staining (blue). Scale bars = 100 μm. (b) Graphs represent the percentage of EdU positive HUVECs. Error bars represent s.e.m. For all analyses $n = 8$. Passage 5: CTR = 37%, vitamin E = 35%. t test: $p > 0.05$ (ns). Passage 9: Ctrl = 27%, vitamin E = 27%. t test: $p > 0.05$ (ns).

previous data (refer to Figure 4) chronic vitamin E treatment decreased the expression levels of the P21 gene but not the expression of P16 (Figure 6). Of note, during this long-term treatment a less pronounced overexpression of the P16 gene was detected in later passages (Figure 6(b)) when compared to earlier ones (2.5-fold change versus 8-fold change observed previously: refer to Figure 4). Moreover, no age effect was in this case observed when the P21 gene was analyzed (Figure 6(a) compares CTRs at different passages). Such apparent inconsistencies may be justified by the experimental conditions inherent in chronic versus acute treatment regimes.

4. Discussion

The data presented here provide evidence for the antisenescence activity of vitamin E *in vitro*. Antioxidant vitamins are known to protect cells from oxidative stress, a major contributor to ageing [12, 21–23]. Here we modeled human ageing *in vitro* by continuously culturing primary cells until they reached a state of irreversible growth arrest, also known as cellular senescence.

The quantification of cellular senescence adopted in this study is based on the automatic detection of the SA-β-gal positive cells present in a culture at a given time. Despite the extensive utilization of the SA-β-gal staining to determine the senescent status of cells *in vitro* [9], limited studies report that this assay is not robust and reproducible enough for a quantitative analysis [24–26]. Therefore, we first tested whether the SA-β-gal quantification could be used as a reliable marker of cellular senescence in our two *in vitro* models at different passages. Human endothelial cells (HUVECs) were utilized in this report and compared with human fibroblasts, the most common cellular model to study senescence. In agreement with previous reports [4, 17, 19, 27, 28] we observe a significant increase of SA-β-gal positive cells, including altered cell morphology, reduced proliferation rate, and overexpression of the P16 and P21 genes in HUVEC cultures at late passages

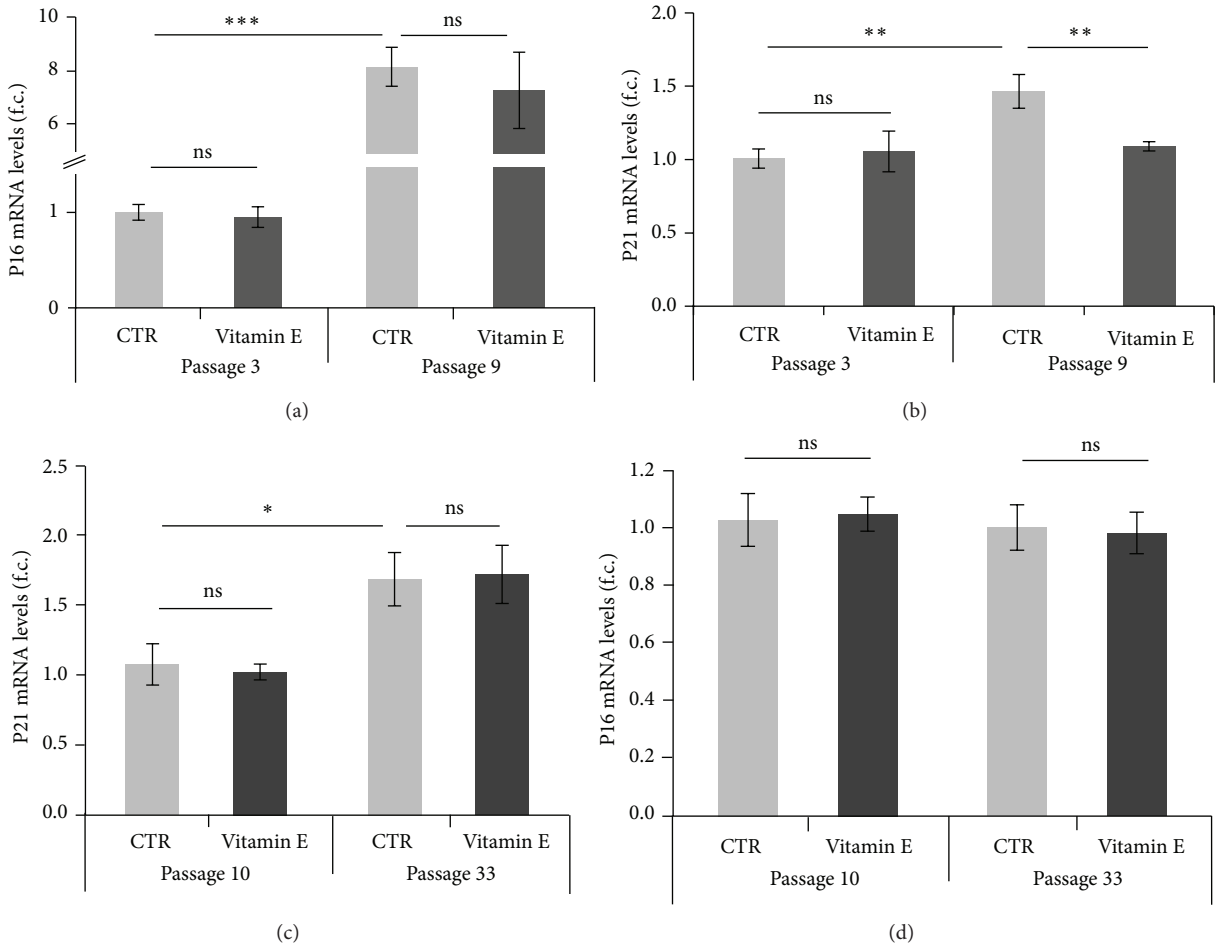

Figure 4: Acute vitamin E treatment downregulates the expression of the P21 gene in HUVEC cultures at passage 9. Gene expression analysis of HUVEC and human fibroblasts cultures at different passages and following vitamin E treatment. Error bars represent s.e.m. All values were normalized to the expression of the housekeeping gene 18S. Gene expression is indicated as fold change (f.c.). (a) P16 gene expression analysis in HUVECs. Passage 3: CTR = 1.0 $n = 3$, vitamin E = 1.0 $n = 4$; t test: $p > 0.05$ (ns). Passage 9: CTR = 8.5, $n = 4$, vitamin E = 7.7, $n = 4$; t test, $p > 0.05$ (ns). Passage 3 CTR versus passage 9 CTR: t test, $p < 0.001$ (***). (b) P21 gene expression in HUVECs. Passage 3: CTR = 1.0 ($n = 4$), vitamin E = 1.1 ($n = 4$); t test: $p > 0.05$ (ns). Passage 9: CTR = 1.5 ($n = 3$), vitamin E = 1.1 ($n = 4$); t test, $p = 0.015$ (**). Passage 3 CTR versus passage 9 CTR: t test, $p = 0.014$ (**). (c) P21 gene expression in human fibroblasts. For all analyses $n = 8$. CTRs were already reported and described in Figure 2. Passage 10: CTR = 1.1, vitamin E = 1.0; t test: $p > 0.05$ (ns). Passage 33: CTR = 1.7, vitamin E = 1.7; t test, $p > 0.05$ (ns). (d) P16 gene expression in human fibroblasts. For all analyses $n = 8$. CTRs were already reported and described in Figure 2. Passage 10: CTR = 1.0, vitamin E = 1.0; t test: $p > 0.05$ (ns). Passage 33: CTR = 1.0, vitamin E = 1.0; t test, $p > 0.05$ (ns).

(Figure 1 and Supplementary Figure 1). Although a combination of many biomarkers for senescence is preferred [6], our experimental method is quantitative and reliable enough to measure the senescence state *in vitro*. This conclusion is also supported by our data obtained in other human primary cells (human fibroblasts) where an increased number of SA-β-gal positive cells are measured in association with altered morphology and reduced proliferation rate (Supplementary Figure 2). Of note, in agreement with [4] and differently from what described in HUVECs [29, 30], human fibroblasts (analyzed at late passages) do not show an increased size of the nuclei (Supplementary Figure 2).

Considering that adequate vitamin E intake was previously associated with a reduction in cognitive decline over time [12, 14, 31, 32] as well as improvement of specific symptoms in individuals affected by Alzheimer's disease (AD) [12, 33, 34], we tested vitamin E effect on ageing *in vitro*. Using the quantification of SA-β-gal positive cells, we show here that acute vitamin E supplementation is sufficient to reduce the number of senescent HUVECs at early and late passages (Figure 2). These results are in agreement with Makpol et al. who reverted the senescence phenotype of human fibroblasts using specific vitamin E isomers (tocotrienols) [35]. To further validate this vitamin E effect, we supplemented HUVEC cultures with vitamin E starting from early passages and for the entire culturing time (chronic treatment). The experiment was concluded only when the cultures where dividing very slowly and showed a number of SA-β-gal positive cells over

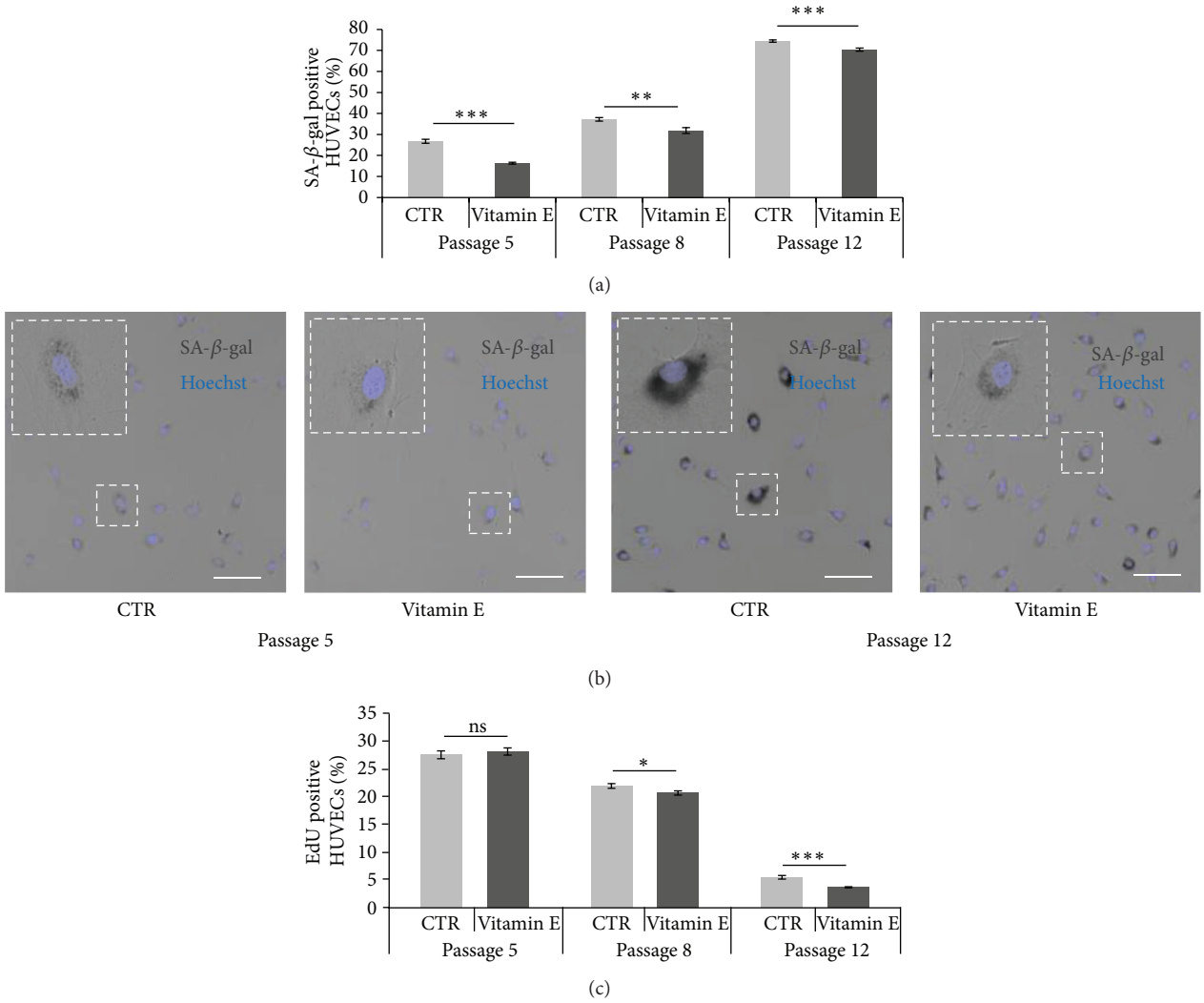

Figure 5: Chronic vitamin E supplementation reduces the percentage of SA-β-gal positive and proliferating HUVECs. (a) Graph showing the percentage of SA-β-gal positive cells measured in HUVEC cultures at passages 5, 8, and 12. For all conditions $n = 8$. Error bars represent the s.e.m. Passage 5: CTR = 27%, vitamin E = 16%; t test, $p < 0.001$ (***); passage 8: CTR = 37%, vitamin E = 32%; t test, $p = 0.003$ (**); passage 12: CTR = 74%, vitamin E = 70%; t test, $p < 0.001$ (***). (b) Representative images of the graph shown in (a) comparing passage 5 and passage 12 HUVEC cultures stained for the SA-β-gal (dark grey cells). Enlargements highlight the morphological differences. Nuclei are visualized in blue (Hoechst staining). Scale bars = 100 μm. (c) Graph showing the percentage of EdU positive cells measured in HUVEC cultures at passages 5, 8, and 12. Error bars represent the s.e.m. For all analyses $n = 10$. Passage 5: CTR = 27.6%, vitamin E = 28.2%; t test, $p > 0.05$ (ns); passage 8: CTR = 22%, vitamin E = 20.7%; t test, $p = 0.038$ (*); passage 12: CTR = 5.5%, vitamin E = 3.7%; t test, $p < 0.001$ (***).

60%. In this case vitamin E treatment resulted in a significant and consistent reduction of the number of SA-β-gal positive HUVECs (Figure 5). A similar phenotype was also described for human fibroblasts (Supplementary Figure 5). In both of our experimental systems, the progression of senescence was reduced but not arrested during "ageing" suggesting that vitamin E delays the onset of senescence.

Our data that vitamin E reduces senescence in aged cultures is in agreement with epidemiological human data. Nutrient intakes were assessed with a 146-item food-frequency questionnaire in a cross-sectional study (586 participants, aged 35–74 y), and relative telomere length of leukocyte DNA was measured. After adjustment for age and other potential confounders, multivitamin use was associated with longer telomere length. In the analysis of micronutrients, higher intakes of vitamins C and E from foods were each associated with longer telomeres, even after adjustment for multivitamin use [36]. Telomere length has been proposed as a marker of biological ageing because telomeres typically shorten by a few dozen to a couple hundred base pairs (bps) per cell division eventually leading to chromosomal instability, senescence, and cell death [37]. Therefore, these human data strongly suggest an antiageing effect of vitamins C and E in humans. Considering the existing inverse correlation between senescence and proliferation, we then verified whether vitamin E treatment altered the proliferation rate of cultures during

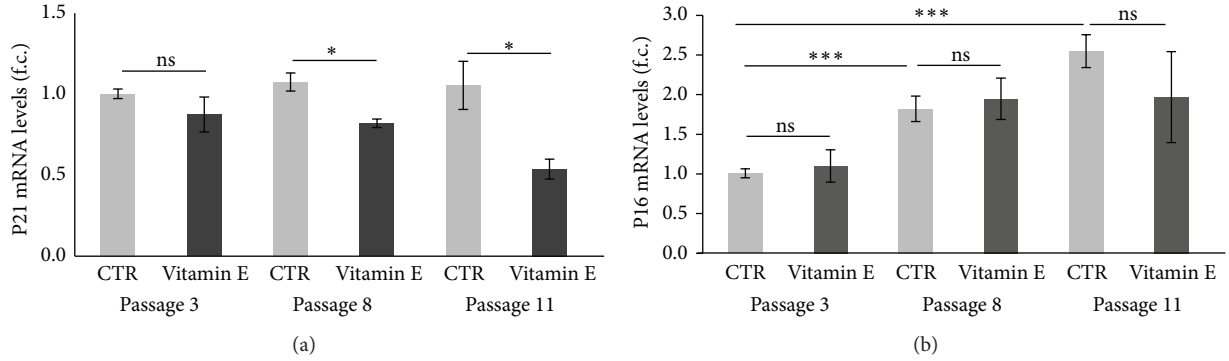

Figure 6: Chronic vitamin E supplementation reduces the expression levels of the P21 gene. P21 and P16 gene expression analysis in passage 5, 8, and 11 HUVEC cultures chronically treated with vitamin E. Error bars represent s.e.m. All values were normalized to the expression of the housekeeping gene 18S. Gene expression is indicated as f.c. Unless differently indicated, $n = 5$. (a) P21 gene expression. Passage 5: CTR = 1.0, vitamin E = 0.9; t test: $p > 0.05$ (ns). Passage 8: CTR = 1.1, vitamin E = 0.8; t test, $p = 0.03$ (*). Passage 11: CTR = 1.0; vitamin E = 0.5, $n = 3$; t test: $p = 0.04$ (*). (b) P16 gene expression. Passage 5: CTR = 1.0, vitamin E = 1.1; t test: $p > 0.05$ (ns). Passage 8: CTR = 1.8, vitamin E = 1.9; t test: $p > 0.05$ (ns). Passage 11: CTR = 2.5; vitamin E = 2.0, $n = 3$; t test: $p > 0.05$ (ns). CTR passage 5 versus passage 8 and passage 11: t test, $p < 0.001$ (***).

senescence. We measured the number of EdU and Ki67 positive cells as markers of proliferation [15, 16] and found that vitamin E does not influence this process in early or late passage cells after acute treatment (Figure 3 and Supplementary Figure 4). Conversely, chronic supplementation of HUVECs cultures showed differences in the proliferation rate following vitamin E treatment at later passages (Figure 5(c)). These data suggest that, under optimal conditions, prolonged vitamin E supplementation reduces senescence progression through mechanisms that alter cellular proliferation.

When the possible effects of vitamin E supplementation were monitored at molecular level, we found a vitamin E-dependent reduction of the elevated expression of the P21 gene in HUVEC cultures at late passages (Figure 4) (acute treatment). This effect was also conserved under chronic treatment conditions (Figure 6). A similar effect was not observed for P16 gene expression in both HUVECs and human fibroblasts. It is possible that the fibroblast cultures contained too few senescent cells to detect such an effect associated with vitamin E. Indeed, although overexpression of the P21 gene was measured in fibroblast at passage 33, similar overexpression was not detected for the P16 gene (Figures 4(c) and 4(d)); perhaps due to different kinetic these genes are subjected to during senescence progression [1]. Another possible reason may be associated with a difference in the senescence program that HUVECs and fibroblasts follow [4] and/or differences in intrinsic factors provided in the culturing media.

The P21-dependent effect of vitamin E observed in our study is in line with mechanistic data *in vivo* showing that long-term dietary vitamin E (alpha-tocopherol) supplementation increases the lifespan of wild-type mice [38]. The authors argue that the increase in lifespan, referred to as an antiageing effect, may reflect an anticancer process that occurs via the induction of the P21 signaling pathway [38].

In conclusion, our data demonstrate that through specific micronutrient supplementation it is possible to delay the onset of cellular senescence in two *in vitro* models: human endothelial cells and fibroblasts. Moreover, our data suggest that these compounds may act through the P21 pathway. Additional research is needed to identify the mechanisms that drive and regulate cellular senescence and to identify specific pathways that can be regulated by supplementation with particular micronutrients.

5. Conclusions

In this study we show that vitamin E supplementation reduces the number of SA-β-gal positive cells *in vitro*. Our data are in favor of reduced cellular senescence associated with the activity of this compound that is already used as preventive and curative measures for specific pathologies typical of old age. More research is needed to study the mechanisms by which vitamin E intake can ameliorate ageing and extend longevity.

Conflict of Interests

All authors are employed by DSM.

Authors' Contribution

All authors contributed to literature search, planning the experiments, and writing the paper. Giorgio La Fata and Nicole Seifert performed the experiments.

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Research Article

Intake of Meals Containing High Levels of Carbohydrates or High Levels of Unsaturated Fatty Acids Induces Postprandial Dysmetabolism in Young Overweight/Obese Men

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Postprandial metabolic response depends on the meals' components and can be different in normal weight and obese people. However, there are some discrepancies between various reports. The aim of this study was to determine the metabolic response after intake of standardised meals with various fat and carbohydrate contents and to determine the differences among normal weight and overweight/obese individuals. The study group comprised 46 healthy men. The participants were divided into two groups and study was carried out using a crossover method. Group I received high- and normal-carbohydrate meals, whereas group II received high-carbohydrate and high-fat meals. Glucose, insulin, triglyceride, and free fatty acids levels were measured at fasting state and at 30, 60, 120, 180, and 240 minutes after meal intake. Despite the lack of differences in glucose levels, insulin levels were higher among overweight/obese individuals after each meal. TG and FFA levels were higher after normal-carbohydrate and high-fat meals. Moreover, in overweight/obese young men after high-fat meal intake postprandial hypertriglyceridemia was observed, even if meals contained predominantly unsaturated fatty acids, and fasting triglycerides levels were in normal range. The conducted study showed that postprandial metabolic response depends not only on the meal macronutrient content but also on the current body mass index (BMI).

1. Introduction

Obesity is a chronic metabolic disease and a growing medical issue with a global reach. In the last 30 years, a dramatic increase in number of people suffering from obesity has been observed. The prognosis for the future is alarming, as it is believed that, with current trends, over a billion of people will have been obese by the year 2030, and another 2 billion will have been overweight [1]. The main causes of obesity are positive energy balance and unbalanced diet. Recently, the trends of diets have become more and more encouraged and understanding the health benefits resulting from complying with appropriately composed diet may help to reduce the progress of obesity and its consequences development. Considering that meals are usually consumed at least three times a day, where assimilation of nutrients usually takes around 5-6 hours, the human body remains in the postprandial state for the greater part of a day [2]. Meal ingestion causes a series of metabolic reactions, often referred to as "postprandial dysmetabolism," which is related to coronary artery diseases and cardiovascular incidents [3–5]. To reduce the risk of cardiovascular disease it is recommended to replace the saturated fatty acids (SFA) with unsaturated fats, and this action seems to be more important than reduction in total fat intake [6–8]. It has been demonstrated that high-monounsaturated fatty acids (MUFA) diet improves

postprandial metabolic response [9] and MUFA, as a replacement for SFA, provides a greater reduction in risk of coronary artery disease than carbohydrates [10]. Lozano et al. showed that people with BMI greater than 26.18 kg/m^2 benefit from the consumption of MUFA coming from oil of olives, which came down to lowering their levels of TG-rich lipoproteins at the postprandial state [11]. On the other hand, the test meal studies confirm that high-fat meals have an adverse effect on postprandial vascular function; however the evidence for effects of high-fat meals rich in unsaturated fats is limited and inconclusive [12]. Moreover, it is worth mentioning that the postprandial metabolic changes, which depend on the composition of a meal, can be also different in overweight/obese people [11, 13, 14]. Ingestion of meals, which may have clinical and metabolic benefits, is crucial in prophylaxis and treating of obesity and its clinical consequences.

The aim of this study was to evaluate the metabolic changes after intake of meals with various fat and carbohydrate contents, using a crossover method, and to analyse the differences between postprandial metabolic responses among normal weight and overweight/obese healthy men. It was expected to discover early postprandial metabolic disturbances in young overweight/obese men in postprandial state, even if the baseline results were in normal range.

Table 1: The characteristic of study groups.

Group I	NW (n = 11)	OO (n = 12)	P
Age, years	33 ± 2	40 ± 2	<0.02
BMI, kg/m^2	23.8 ± 0.5	31.4 ± 1.5	<0.001
Body fat content, %	17.9 ± 1.0	28.6 ± 1.7	<0.0001
Group II	NW (n = 12)	OO (n = 11)	P
Age, years	33 ± 3	36 ± 3	>0.05
BMI, kg/m^2	23.9 ± 0.2	33.7 ± 2.2	<0.00001
Body fat content, %	18.6 ± 1.5	31.9 ± 2.7	<0.001

Data are presented as a mean value ± SE.

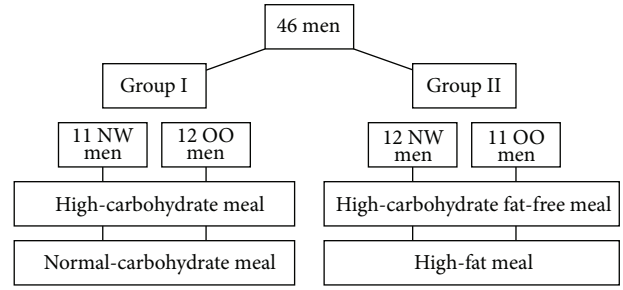

Figure 1: Study design. NW: normal weight; OO: overweight/obese.

2. Experimental Methods

2.1. Study Participants. 46 men participated in the study: 23 with normal weight (NW) and 23 were overweight/obese (OO) in the age range of 21–58 years. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving patients were approved by the local Ethics Committee of the Medical University of Bialystok (Poland) and a written informed consent was obtained from all participants. The participants of the study did not suffer from any glucose metabolism disorders, endocrine disorders, renal or liver failure, and digestive system diseases, did not undergo any gastroenterological and bariatric surgeries or procedures, and did not suffer from any other diseases which could influence the results. People who had received pharmacological treatment or had used any other products with undocumented or unknown influence on metabolism were excluded from the study. Taking into consideration the fact that the levels of some factors can be characterised by sexual dimorphism and its analysis was not the aim of the study, only male participants were selected for the study group, in order to obtain reliable results.

Based on BMI, the participants were divided into two groups: men with normal weight (NW, BMI < 25.0 kg/m^2) and men that were overweight/obese (OO, BMI ≥ 25.0 kg/m^2). Subsequently, the participants were randomly divided (randomisation using sampling without replacement) into two experimental groups (the study design is presented in Figure 1 and the characteristic of the groups is presented in Table 1). The study was carried out using the crossover method.

Group I (11 NW men and 12 OO men) received a high-carbohydrate (HC) meal (450 kcal), 89% of energy coming from carbohydrates, 11% coming from protein, and 0% coming from fat (Nutridrink Fat Free, Nutricia, Poland), and, then, after a period of 1-2 weeks received a meal with normal-carbohydrate (NC) content (450 kcal), 45% of energy coming from carbohydrates, 30% coming from protein, and 25% coming from fat: 12,4% SFA, 59,0% monounsaturated fatty acids (MUFA), 28.7% polyunsaturated fatty acids (PUFA), and n-6/n3 ratio 5,11 (Cubitan, Nutricia, Poland).

Group II (12 NW men and 11 OO men) received a HC (fat-free) meal (450 kcal), 89% of its energy coming from carbohydrates, 11% coming from protein, and 0% coming from fat (Nutridrink Fat Free, Nutricia, Poland), and, then, after a period of 1-2 weeks received a high-fat (HF) meal (450 kcal), 4% of energy coming from carbohydrates, 0% coming from protein, and 96% coming from fat: 10.7% SFA, 60.7% monounsaturated fatty acids (MUFA), 28.6% polyunsaturated fatty acids (PUFA), and n-6/n3 ratio 5,02 (Calogen, Nutricia, Poland).

The participants were asked not to change their diet and daily physical activities during the study.

2.2. Study Procedure. The participants were arriving at the lab at 08:00–08:30 in the morning, in the fasting state, with at least 12 hours since their last meal. The following procedures were carried out: height and weight measurements and body fat content measurement (using the bioimpedance method, InBody 220 Biospace, Korea). A peripheral venous catheter was placed in the elbow crook and venous blood was drawn

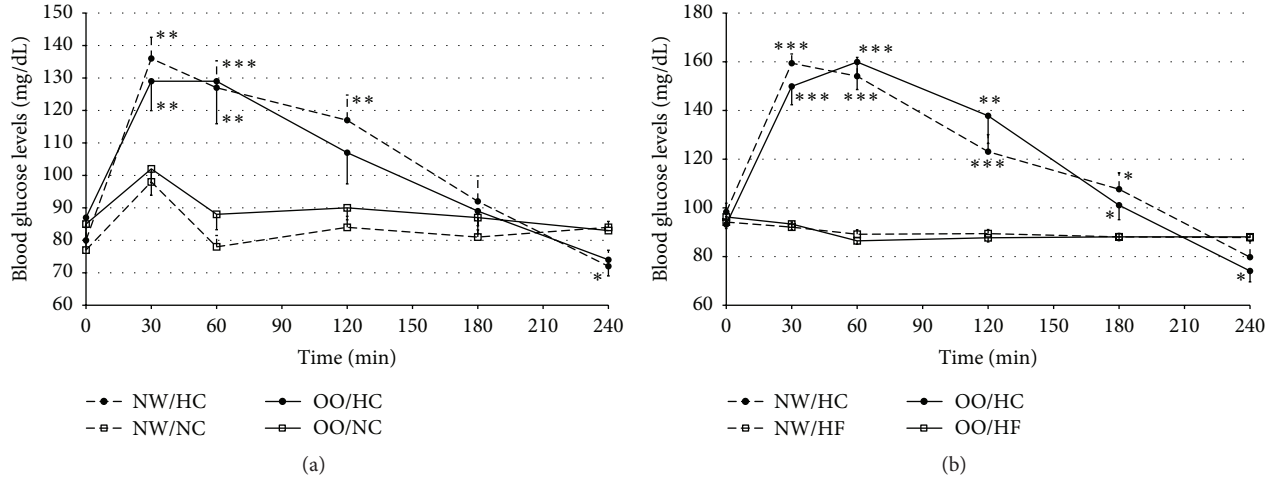

Figure 2: Blood glucose levels (mg/dL) in normal weight (NW) and overweight/obese (OO) men in fasting state (time 0 min) and after (time 30–240 min): (a) high-carbohydrate (HC, black circle) and normal-carbohydrate (NC, white square) meal intake. (b) High-carbohydrate (HC, black circle) and high-fat (HF, white triangle) meal intake. Data are presented as mean value ± SE. Comparison between different meals in NW or OO men: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Comparison between NW and OO men after the same meal intake: $^{A}P < 0.05$, $^{B}P < 0.01$, and $^{C}P < 0.001$.

in order to determine fasting blood glucose and insulin and free fatty acids (FFAs) and triglyceride (TG) levels. The participants then received a randomly selected meal (at room temperature) and were advised to consume it within 10 minutes. 30, 60, 120, 180, and 240 minutes after meal intake the venous blood was drawn in order to measure the above-mentioned factors levels again.

2.3. Laboratory Tests. The specimen was drawn and prepared for testing in accordance with the recommendations provided by the laboratory kit producers. The assay of particular levels was carried out immediately after drawing the specimen, while the remaining ones were determined after the specimen was obtained for the whole period of tests. The specimen was stored in accordance with the recommendations of the producers until it was tested, at temperature of −20°C/−80°C. The particular factors levels were determined using the following methods: glucose-hexokinase enzymatic colorimetric assay (Cobas c111, Roche Diagnostics Ltd., Switzerland), insulin-immunoradiometric assay (Insulin, IRMA, DiaSource, Belgium; Wallac Wizard 1470 Automatic Gamma Counter, PerkinElmer, Life Science, Turku, Finland), TG-enzymatic colorimetric assay (Cobas c111, Roche Diagnostics Ltd., Switzerland), FFAs-enzymatic assay (FFA ELISA, Zen-Bio, Czech Republic; μQuant, BioTek Instruments Inc., Winooski, Vermont, USA).

2.4. Statistical Analysis. In order to obtain the general characteristics of the data, arithmetic mean and standard error of the mean were calculated for all numerical features, which were treated as dependent variables in subsequent steps of the analysis. The aim of the study was to check whether there is a statistically significant postprandial metabolic response depending on meals' components. There were two main null hypotheses stated: (1) different types of meals (differentiated by main component of the meal) have no influence on postprandial metabolic response in normal weight and overweight/obese patients (analyzed separately); (2) there is no difference in postprandial metabolic response to a particular meal in normal weight and overweight/obese patients. The first hypothesis (1) was verified for two pairs of meals: HC versus NC and HC versus HF for normal weight and overweight/obese subjects. Since both meals were given to the same patients, tests for dependent variables were used: either one-way ANOVA or Wilcoxon signed-rank test (both for paired samples)—depending on fulfilling of the condition of the normality of the variables' distribution. The second hypothesis (2) was verified for each type of meal: HC, NC, and HF in order to check whether there are significant differences in postprandial metabolic response between normal weight and overweight/obese subjects. One-way ANOVA or Wilcoxon rank-sum test (both for unpaired samples)—depending on fulfilling of the condition of the normality of the variables' distribution—and the homogeneity of variances were used to test the stated hypothesis. To address the issue of multiple hypothesis testing, false discovery rate P value adjustment method was used. For all calculations, the alpha level was set at 0.05. The areas under the curve (AUCs) were calculated using the trapezoidal method and underwent the same analysis schema as the rest of the features.

3. Results

The blood glucose levels were significantly higher after the HC meal intake than after NC meal at 30 and 60 minutes in OO men and also at 120 minutes in NW individuals (Figure 2(a)). Similarly, the AUCs values were higher after the HC meal intake in comparison to the NC meal, both in NW (25577 ± 1264 versus 20088 ± 402, resp., $P < 0.01$) and in OO men (24994 ± 1687 versus 21345 ± 938, resp., $P < 0.01$).

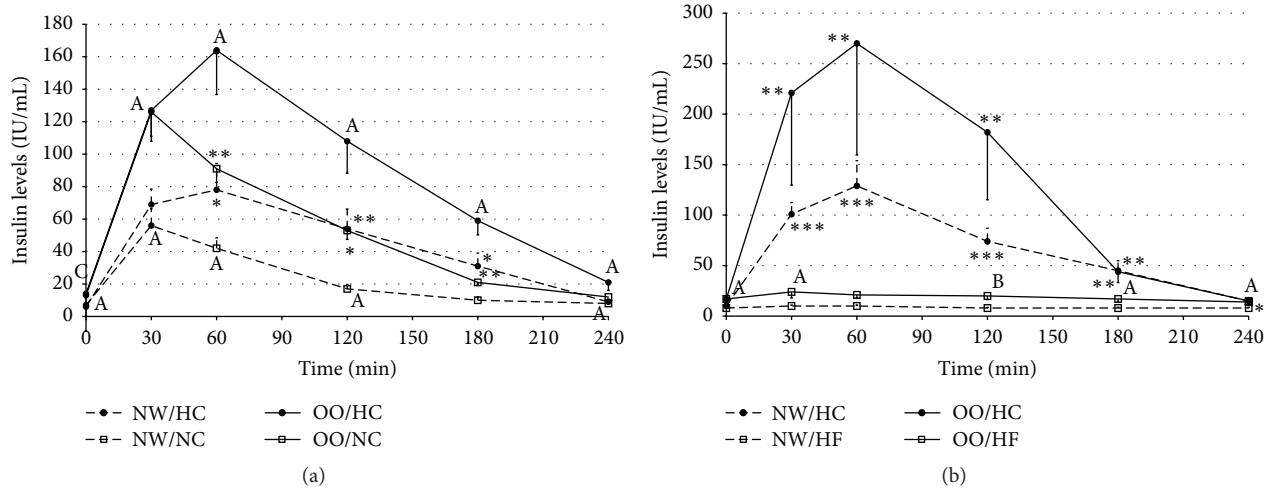

Figure 3: Insulin levels (IU/mL) in normal weight (NW) and overweight/obese (OO) men in fasting state (time 0 min) and after (time 30–240 min): (a) high-carbohydrate (HC, black circle) and normal-carbohydrate (NC, white square) meal intake. (b) High-carbohydrate (HC, black circle) and high-fat (HF, white triangle) meal intake. Data are presented as a mean value ± SE. Comparison between different meals in NW or OO men: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Comparison between NW and OO men after the same meal intake: $^{A}P < 0.05$, $^{B}P < 0.01$, and $^{C}P < 0.001$.

The blood glucose levels were significantly higher after HC meal in comparison to HF meal as well, both in NW and in OO men (Figure 2(b)). The AUCs for glucose levels were significantly higher after HC meal intake in comparison to the AUCs for glucose levels after HF meal (for NW men 29429 ± 1082 versus 21473 ± 381, resp., $P < 0.0001$, and 29648 ± 1378 versus 21327 ± 331, $P < 0.0001$, for OO men). 240 minutes after HC meal intake the blood glucose levels were significantly lower in comparison to the levels after the remaining meals and glycaemia ≤65 mg/dL was observed in 29% of the participants (6 NW men and 6 OO men; minimal noted glycaemia was 41 mg/dL).

At fasting and postprandial state (Figures 2(a) and 2(b)) blood glucose levels did not differ between NW and OO subjects after any of the meals. The AUCs for blood glucose levels did not differ between NW and OO men after HC meal ($P > 0.05$) and after NC meal ($P > 0.05$) in group I and after HC meal ($P > 0.05$) and HF meal ($P > 0.05$) in group II.

The insulin levels were significantly higher after HC meal in comparison to NC meal, both in NW and in OO men (Figure 3(a)). The insulin levels were significantly higher from 60 to 180 minutes in NW men and in OO men. The values of AUCs for insulin levels were significantly higher after HC meal in comparison to NC meal, both in NW (11047 ± 2162 versus 5694 ± 716, resp., $P < 0.03$) and in OO men (21999 ± 2917 versus 12845 ± 1712, resp., $P < 0.001$).

The insulin levels in NW and OO men were also significantly higher after HC meal intake, in comparison to HF meal (Figure 3(b)). In NW men, the insulin levels were significantly higher from 30 minutes until the end of the tests; however, in OO men insulin levels were significantly higher from 30 to 180 minutes after HC meal intake. The AUCs for insulin levels were higher after HC meal intake than after HF meal, both in NW and in OO men (for NW 16641 ± 2010 versus 2135 ± 149, resp., $P < 0.0001$, and 33075 ± 11070 versus 4533 ± 973, resp., $P < 0.01$, for OO men).

While comparing the insulin levels between NW and OO men, the OO men from group I had significantly higher insulin levels both at fasting state and after HC and NC meals intake (Figure 3(a)). Furthermore, the AUCs for insulin levels after HC and NC meals intake were significantly higher in OO than in NW men (after HC meal 21999 ± 2917 versus 11047 ± 2162, resp., $P < 0.01$; and after NC meal 12845 ± 1712 versus 5694 ± 716, resp., $P < 0.01$). In group II, both at fasting state and after HC meal intake, no differences between insulin levels in NW and OO men were observed (Figure 3(b)), which was most likely due to a high standard error. The insulin levels were significantly higher in OO men before and after HF meal intake (except 60 minutes). Moreover, the AUCs for insulin levels were significantly higher in OO men only after HF meal intake (4533 ± 973 versus 2135 ± 149, resp., $P < 0.01$).

In the OO men the TG levels were significantly higher after NC meal at 120, 180, and 240 minutes, in comparison to the levels after HC meal (Figure 4(a)) which was not observed in NW men. The AUCs for TG levels after HC and NC meals in NW men did not significantly differ and amounted to 27592 ± 4785 versus 28382 ± 4344, respectively ($P > 0.05$). In the OO men the AUCs for TG levels were significantly higher after NC meal than after HC meal and amounted to 38888 ± 4640 versus 27057 ± 1879, respectively ($P < 0.02$).

When comparing the TG levels after HC meal with the TG levels after HF meal intake, from 120 to 240 minutes in NW and from 180 to 240 minutes in OO men the TG levels were significantly higher after HF meal intake (Figure 4(b)). The AUCs for TG levels were significantly higher after HF meal in comparison to the AUCs for TG levels after HC meal in NW men (26506 ± 2668 versus 18158 ± 2669, resp., $P < 0.03$). In OO men a tendency for higher values of the AUCs

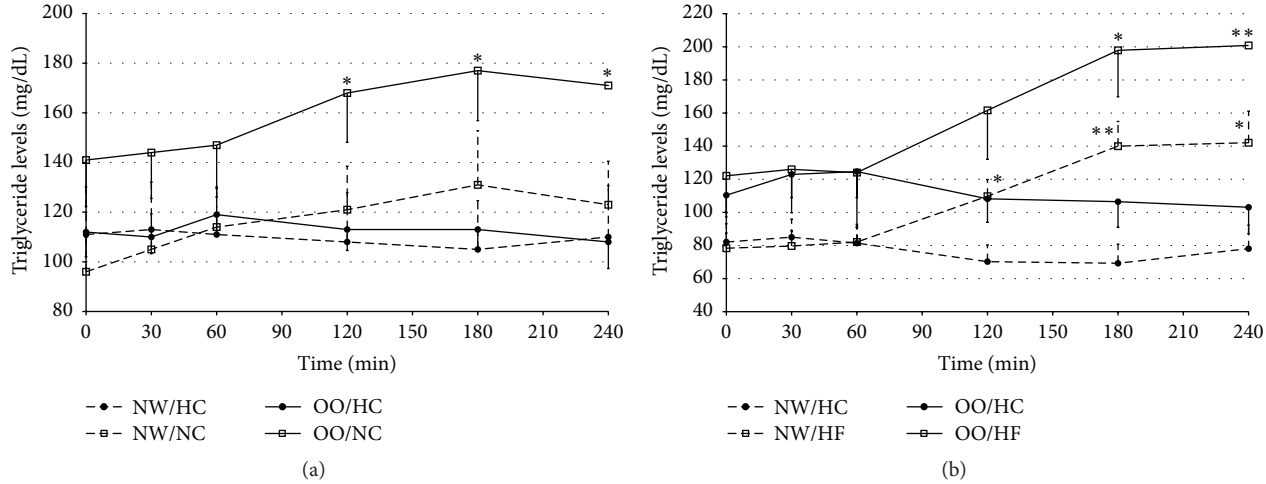

Figure 4: Triglycerides levels (mg/dL) in normal weight (NW) and overweight/obese (OO) men in fasting state (time 0 min) and after (time 30–240 min): (a) high-carbohydrate (HC, black circle) and normal-carbohydrate (NC, white square) meal intake. (b) High-carbohydrate (HC, black circle) and high-fat (HF, white triangle) meal intake. Data are presented as a mean value ± SE. Comparison between different meals in NW or OO men: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Comparison between NW and OO men after the same meal intake: $^{A}P < 0.05$, $^{B}P < 0.01$, and $^{C}P < 0.001$.

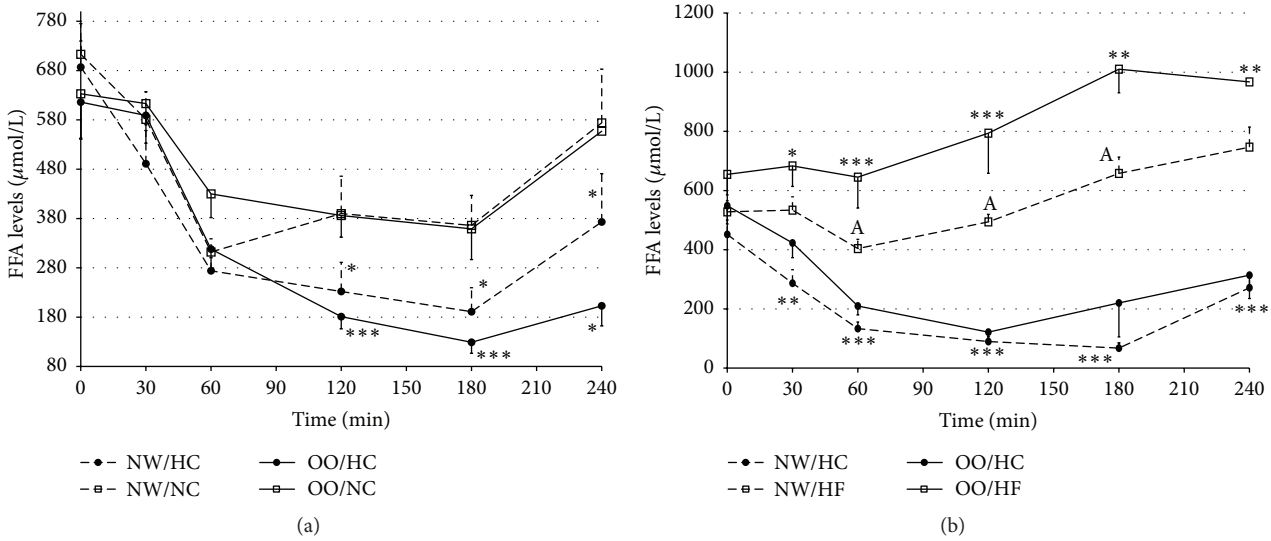

Figure 5: Free fatty acids (FFAs) levels (μmol/L) in normal weight (NW) and overweight/obese (OO) men in fasting state (time 0 min) and after (time 30–240 min): (a) high-carbohydrate (HC, black circle) and normal-carbohydrate (NC, white square) meal intake. (b) High-carbohydrate (HC, black circle) and high-fat (HF, white triangle) meal intake. Data are presented as a mean value ± SE. Comparison between different meals in NW or OO men: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Comparison between NW and OO men after the same meal intake: $^{A}P < 0.05$, $^{B}P < 0.01$, and $^{C}P < 0.001$.

for TG levels after HF meal intake, in comparison to HC meal, was noted (38789 ± 6476 versus 26922 ± 3439, resp., $P > 0.05$).

No differences between NW and OO men (Figures 4(a) and 4(b)) were observed in TG levels nor in the AUCs for TG levels after any of the investigated meals.

Both, in NW and in OO men, the FFAs levels were significantly higher from 120 to 240 minutes after NC meal intake, in comparison to the levels after HC meal (Figure 5(a)). Moreover, the AUCs for FFAs levels after NC meal were significantly higher than those after HC meal in NW (103435 ± 12984 versus 74272 ± 12380, resp., $P < 0.03$) and OO men (108645 ± 10686 versus 65846 ± 7378, resp., $P < 0.0001$). The differences were more distinct after HF meal intake, when for 30 minutes of testing the FFAs levels were higher in comparison to FFAs levels after HC meal intake, among NW and OO men (Figure 5(b)). The AUCs for FFAs levels were significantly higher after HF meal than after HC meal in NW (133630 ± 6336 versus 38960 ± 5120, resp., $P < 0.000001$) and OO individuals (196624 ± 18147 versus 60300 ± 10017, resp., $P < 0.0001$).

The FFAs levels were similar among NW and OO men, both at fasting and after HC and NC meals intake (Figures 5(a) and 5(b)). However the FFAs levels were significantly higher in OO men in comparison to NW men from 60 to 180 minutes after HF meal intake (Figure 5(b)). The AUCs for FFAs levels did not differ between NW and OO men after HC and NC meals, but significantly higher value of the AUCs for FFAs levels was noted for OO men in comparison to NW men after HF meal intake (196624 ± 18147 versus 133630 ± 6336, resp., $P < 0.01$).

4. Discussion

The study confirmed that metabolic response depends on the components of a meal and on the current body energy balance. Moreover, after HF meal intake postprandial hypertriglyceridemia was noted in OO men, even if fasting TG levels were within the normal range and the meal contained predominantly unsaturated fatty acids. It is worth noticing that the investigated study groups comprised healthy young men, and, in spite of this, we observed very early disturbances in postprandial metabolic response, which in long-term can lead to development or progress of metabolic disorders such as obesity, hypertriglyceridemia, metabolic syndrome, and type 2 diabetes.

The highest glucose levels after HC meal, observed in NW men and in OO men, were not surprising and are consistent with the results obtained by other researchers [15, 16]. However, 240 minutes after HC meal intake the glucose levels were significantly lower when compared with the glucose levels after other meals. At the same time, glycaemia ≤65 mg/dL (minimal glycaemia 41 mg/dL) was observed among 29% of the participants, in the same number of NW men and OO men. The mild symptoms typical for hypoglycaemia were observed in most of these people and did not require any medical intervention. On the other hand, both after NC meal and after HF meal intake, glycaemia ≤65 mg/dL was not observed in any of the participants. Taking into consideration the clinical consequences, hypoglycaemia is particularly dangerous for people suffering from diabetes. Hypoglycaemic symptoms are certainly alarming for the patient, and, taking energy homeostasis into consideration, hypoglycaemic episodes can result in weight increase, as sudden and intense hunger constitutes one of the prodromal symptoms. Reactive hypoglycaemia observed after HC meal was most likely related to increased secretion of insulin in response to the significant amount of ingested carbohydrates.

The study showed that glucose levels in general were not different for NW and OO people, both at fasting and after each of the meals. Other researchers also did not observe any significant differences in glucose levels between people with normal weight and those with obesity after high-carbohydrate and high-fat meals intake [14, 17]. However, Zwirska-Korczala et al. [18] observed higher glucose levels among obese people at fasting state, 60 and 120 minutes after a combined meal intake. In our study, increased fasting glucose levels among OO men were observed just once—before NC meal intake.

The insulin levels, both in NW men and in OO men, were significantly higher after HC meal intake than after NC or HF meal. The increased secretion of insulin was undoubtedly a result of higher glucose levels after high-carbohydrate meal, which was also confirmed in studies carried out by other authors [15, 16, 19]. Increased insulin secretion after HC meal intake was most likely the cause of reactive hypoglycaemia observed in some of the participants.

Despite the lack of significant differences in glucose levels, the insulin levels were significantly different both in fasting and in postprandial states. OO men had significantly higher fasting insulin levels after HC, NC, and HF meals intake (in comparison to NW men). Other researchers also observed higher insulin levels, at fasting state and after a meal intake in people with obesity, also with a lack of significant differences in glucose levels [14, 18, 20, 21]. On the other hand, Peake et al. [22] did not observe any differences in postprandial changes of insulin levels in people with normal weight and people with a family history of diabetes and whose BMI categorised them as overweight. In our study, no differences in insulin levels both at fasting state and after HC meal intake between NW and OO men were observed only in group II, but the lack of statistical significance was most likely caused by the high value of standard error, as the average insulin levels in OO people in the first 120 minutes of the test were noticeably higher.

When comparing meals with different composition, we have noticed that in OO men, after NC meal intake, the TG levels were significantly higher in comparison to the levels after HC meal intake. Also among OO men the AUC for TG levels was significantly higher after NC meal, whereas among NW men the AUCs for TG levels after HC and NC meal did not differ significantly. The TG levels were also significantly higher after HF meal in comparison to HC meal, both in NW and in OO men. Other researchers also proved that the high-fat meal intake is related to the increase of TG levels, where the maximum values were obtained 4 hours after ingesting the meal [23]. The higher TG levels in people with normal weight 75 minutes after a high-fat meal intake, in comparison to a high-carbohydrate meal, were observed also by Raben et al. [15]. We did not observe any differences in TG levels between NW and OO people. It is worth noticing that after NC and HF meals intake a postprandial hypertriglyceridemia was observed in OO people. The study conducted by Peake et al. [22] also did not present any differences in TG levels after a high-fat meal between people with normal weight and people with a family history of diabetes and whose BMI categorised them as overweight. In our study, after HF meal rich in unsaturated fatty acids, which are recommended due to their beneficial effect on the risk of coronary heart disease [6, 24], we have noted postprandial hypertriglyceridemia in OO men, even though the average fasting TG levels remained in a normal range. As the studies show, postprandial dyslipidaemia is related to the intensification of inflammatory disease processes, vascular endothelial dysfunction, impaired fibrinolysis, platelet instability, risk of coronary artery disease, and cardiovascular incidents such as myocardial infarction, ischemic stroke, and death [3, 25, 26]. Postprandial hypertriglyceridemia could be observed due to

a high total fat content in the meal but also due to a high content of MUFA, since in some studies there were observed benefits of MUFA on cardiovascular risk factors [27]. However, other studies showed that monounsaturated fatty acids appeared not to have cardioprotective effect and, what is more, they showed that MUFA intake may be associated with increased risk of fatal coronary heart disease [28, 29].

Also the high FFAs levels are responsible for elevated endothelial activation markers, vascular endothelial cell inflammation, and increased prothrombotic activity markers, which can cause vascular and atherosclerotic anomalies resulting in circulatory system diseases [30]. As a part of this study, the FFAs levels were evaluated and it was observed that in NW men, as well as in OO men, FFAs levels were significantly higher after NC and HF meals intake, in comparison to HC meal. FFAs levels were decreasing in relation to the fasting values after HC meals, and after NC meals they were decreasing to a lesser degree. Raben et al. [15] also observed lower FFAs levels after a high-carbohydrate meal intake, in comparison to other meals in people with normal weight. FFAs, if not metabolized for energy, can be deposited as triglycerides in adipose tissue which results in obesity [31]; they can also be accumulated ectopically in muscle tissue, heart muscle, or liver, as a possible defence mechanism against lipotoxicity, which can cause cell dysfunction and death [32]. The ability to store lipids by cells other than adipocytes may be limited and ectopic accumulation of lipids can result in insulin resistance and type 2 diabetes mellitus [33]. The correlation between increased FFAs availability and impaired glucose metabolism is well established and it seems that several mechanisms can be involved in this process. The excess FFAs availability may disrupt glucose metabolism through the competition between substrate utilization, through lipid induced changes in the phosphorylation and function of proteins in the pathway of insulin signalling for GLUT4 translocation, but also other mechanisms have been proposed and discussed [34].

The conducted analyses did not show any significant differences in fasting FFAs levels between NW and OO people, and the AUCs for FFAs levels after HC and NC meals were the same for NW and OO people as well. However, 60 minutes after HF meal intake higher FFAs levels were observed among OO people in comparison to NW men and remained significantly higher until 180 minutes of the test. Also the AUC of FFAs levels in postprandial state after a high-fat meal intake was significantly higher in OO than in NW men. Imbeault et al. [14] also observed an increase in FFAs levels after a high-fat meal and significantly higher FFAs levels in obese people. Other researchers [22] did not find any differences in FFAs levels after a high-fat meal intake between people with normal weight and overweight ones. The results are not unambiguous and require further studies on larger study groups.

The differences in postprandial metabolic effects, especially in lipids response, probably can be explained by the lower numbers of lipoprotein particles of hepatic origin that compete with chylomicrons for lipolysis [35], which result in improved postprandial TG metabolism in NW individuals. Another mechanism may be related to insulin sensitivity, which stimulates lipoprotein lipase activity and TG clearance, and, as it was shown, by the weight loss (which improves insulin sensitivity) the fat-induced postprandial TG clearance was improved as well [36]. Some previous studies have shown that insulin resistance is associated with increased fatty acids levels released by adipose tissue and elevated VLDL-TG secretion in the hepatocytes and lower adipose tissue lipoprotein lipase activity [37, 38]. Therefore, the higher postprandial insulin levels noted in OO men might be related to the lower postprandial insulin sensitivity, and it can be an explanation of observed higher TG response.

Moreover, the postprandial response can be dependent also on the other, for example, genetic factors, which we have observed in one of our previous studies [39].

In summary, the conducted study confirmed that postprandial metabolic response depends on the components of a meal and moreover it is different among NW and OO people.

The highest blood glucose levels were observed after HC meal intake; however the levels were the same for NW and OO people. Insulin levels were also the highest after HC meal intake, and, despite the lack of significant differences in blood glucose levels, higher insulin levels were observed in OO people, in comparison to the NW group. Triglyceride and FFAs levels were higher after NC and HF meals intake. In OO people, postprandial hypertriglyceridemia was observed after HF meal intake, even if it contained mostly unsaturated fatty acids. Further investigations on the effect of MUFA on the CHD risk are undoubtedly warranted. Nevertheless, our findings showed that metabolic response in OO people to high-fat meals, even if rich in unsaturated fatty acids, caused postprandial hypertriglyceridemia and should be avoided in diet. Even if the study sample size was quite small and our results need to be confirmed, for example, by increasing the sizes of study groups, the obtained results have crucial significance in a period of obesity plague and of the omnipresent trends of diets with modified amount of macronutrients, including high-fat diets.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Edyta Adamska, Lucyna Ostrowska, Adam Krętowski, and Maria Górska conceived the idea for the study, contributed to the design of the research, interpreted the results, and wrote the paper. Edyta Adamska and Magdalena Waszczeniuk were involved in the data collection. Joanna Gościk performed statistical analyses and contributed to the discussion. Maria Górska and Adam Krętowski coordinated funding for the project.

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Research Article

Influence of Sorghum Kafirin on Serum Lipid Profile and Antioxidant Activity in Hyperlipidemic Rats (*In Vitro* and *In Vivo* Studies)

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The aim of this study was to compare *in vitro* the antioxidant potential of sorghum kafirin and sorghum flour and their influence on lipids and antioxidant capacity in rats. The antioxidant activity in sorghum kafirin extract measured by the DPPH and TEAC methods was increased 30 and 65 times, respectively, compared to that of its counterpart, sorghum flour. According to electrophoresis assay, the kafirins *tert*-butanol extract showed a high proportion of α-kafirin monomers, and its amino acid composition revealed higher hydrophobic amino acid content such as alanine, isoleucine, leucine, tyrosine and phenylalanine than sorghum flour extract. Diets supplemented with sorghum kafirin extract have improved lipid metabolism and increased the serum antioxidant potential (67%) especially in rats fed with added cholesterol. The bioactive peptides generated from kafirin *in vivo* hydrolysis appear to be associated with the positive effect on serum lipids and antioxidant activity. According to these results, sorghum kafirin extract at the levels used in this study apparently could be used for prevention of atherosclerosis and other chronic diseases.

1. Introduction

Cardiovascular disease (CVD) is the most common cause of death in Mexico and atherosclerosis is the most prevalent CVD in the adult population, while coronary heart disease (CHD) is its most frequent and lethal form [1, 2]. The main risk factor of CVD is dyslipidemia characterized by elevated total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and triglyceride (TG) levels, and decreased high density lipoprotein cholesterol (HDL-C) levels [3]. Oxidative stress may play a critical role in the pathophysiology of CVD and several epidemiological studies have shown an association between circulating antioxidants and cardiovascular diseases [4–6]. Several investigations have found an association between whole grain intake and CVD reduction [7–9]. Phenolic compounds present in grains are responsible for this protecting effect, and also some proteins and their peptides have been recognized for their biological activity [10, 11].

Sorghum is studied because of its high content of fiber, protein, mineral, and polyphenol content, and several studies have shown its possible role in the cardiovascular disease prevention [12, 13]. *In vitro* hydrolysis of sorghum protein has been studied for to its peptides and their relationship

to antiviral [14] and antihypertensive activities [15]. These activities are attributed to fractions isolated from α-kafirin, the main storage protein in sorghum. The mechanisms by which kafirin can exert a protective effect start with hydrolysis and the absorption of biologically active peptides or amino acids, that can affect biological processes including the body function or health status [16].

Antioxidant activity is defined as the property that some chemicals have in quenching free radicals by donating a proton or hydrogen atom. Free radicals can oxidize biological structures such as lipids, which once oxidized can cause alterations in the cell membrane, while that their oxidation products can promote the development of atherogenic processes [17, 18]. It is possible that the enzymatic digestion of sorghum kafirins in an *in vivo* model may promote the production of bioactive peptides with antioxidant activity that can be absorbed and passed into the bloodstream, leading to a beneficial effect on its lipid profile. The aim of the study was to evaluate the *in vitro* potential antioxidant of sorghum kafirin extract as compared with sorghum flour and their effect on lipid profile and antioxidant activity in serum of hypercholesterolemic rats. The findings of this study are determinant in further understanding and development of this nonconventional cereal to be used in treatment of CVD.

2. Materials and Methods

2.1. Materials and Chemicals. Sorghum (*Sorghum bicolor* L. Moench) white variety UDG-110 was provided by Fundación Produce, Mexico. Cholesterol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), fluorescein (FL), and 2,2′-azo-bis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and triglyceride kits were acquired from RANDOX. Unless otherwise specified, all chemicals and solvents were of analytical grade. Sorghum flour (SF) (9.25% total protein dry matter basis) was obtained from whole grain using a laboratory mill (Laboratory Mill 1100), fitted with a 0.5 mm opening screen.

2.2. Extraction of Kafirins. The method of Mazhar et al. [19] was used for the extraction of kafirins. In brief, 100 g of defatted sorghum flour was mixed with 500 mL 60% *tert*-butanol/water at 37°C and vigorously stirred for 6 h. After centrifugation, the *tert*-butanol was evaporated to recover the supernatant. The aqueous supernatant containing kafirins was freeze-dried and named SK. The protein content of the SK was 40% ($N \times 6.25$, dry matter basis).

2.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. The SK was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis procedure (SDS-PAGE) on 4–12% acrylamide gradient gel under reducing conditions [20, 21]. All gels were stained with Coomassie brilliant blue stain R-250 at 0.125%. The low MW markers (BIO-RAD) for gel electrophoresis were albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), and lactalbumin (14.4 kDa). Gels were analyzed with a GS-800 Bio Rad Densitometer using Quantity One version 4.6.9 software.

2.4. Amino Acid Composition Analysis. Amino acid analysis of samples of SF and SK was based on the methodology previously reported [22]. Briefly, powered samples (3 mg) were hydrolyzed with HCl (6 N) at 150°C during 12 hours. After hydrolysis, the acid was removed by rotary evaporation and the sample was resuspended on 2 mL of sodium citrate buffer pH 2.2. The HPLC method precision and accuracy was evaluated using external and internal standards. The amino acid reference standard consisted of sixteen amino acids (0.05 μmoles mL^{-1} each amino acid) and was utilized to determine the retention times for each amino acid. Internal standard α-aminobutyric (0.05 μmoles mL^{-1}) was added to amino acid reference standard and each sample to normalize and quantify the amino acid content. A gradient mobile phase of sodium acetate 0.1 M pH 7.2 and methanol (9 : 1) elute sample for amino acid separation through C18 column reversed-phase octadecyl dimethylsilane particles (100 × 4.6 mm × 1/4″ Microsorb 100-3 C18). Fluorescence detection was performed using an excitation emission wavelength of 360 and 455 nm, respectively. Star Chromatography work station (Varian version 5.51) software was used to achieve amino acid peak integration. The results (amount of amino acids g/100 g of protein) listed in Table 2 are means of three replications and coefficient of variation was lower than 5%.

2.5. Trolox Equivalent Antioxidant Capacity (TEAC). This assay is based on the ability of antioxidants to scavenge the blue-green $ABTS^{\bullet+}$ radical cation, relative to the $ABTS^{\bullet+}$ scavenging ability of the water-soluble vitamin E analogue Trolox. The $ABTS^{\bullet+}$ radical cation was generated by the interaction of 5 mL of 7 mM ABTS solution and 88 μL of 140 mM $K_2S_2O_8$ solution. After the addition of 3.9 mL of $ABTS^{\bullet+}$ solution to 0.1 mL of methanolic (SF) or *tert*-butanol (SK) extracts or Trolox standards (0 to 20 μM range), the absorbance was monitored exactly 1 and 30 min after the initial mixing. The percentage of absorbance inhibition at 734 nm was calculated and plotted as a function obtained for the extracts and the standard reference (Trolox). The final TEAC values were calculated by using a regression equation between the Trolox concentration and the inhibition percentage and expressed as millimol of Trolox equivalents per g of dry weight [23].

2.6. DPPH Assay. This assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH relative to the DPPH scavenging ability of the water-soluble vitamin E analogue Trolox. Briefly, 3.9 mL aliquot of DPPH (0.0634 mM) solution was added to the test

Table 1: Percent composition of experimental diets.

Components	Con	Chol	Chol/SF5	Chol/SF10	Chol/SK0.25
Corn starch	69.1	68.1	63.1	58.1	67.85
Casein	15	15	15	15	15
Soybean oil	10	10	10	10	10
Cellulose	1	1	1	1	1
Mineral mixture	3.7	3.7	3.7	3.7	3.7
Vitamin mixture	1	1	1	1	1
Choline	0.2	0.2	0.2	0.2	0.2
Cholesterol	—	1	1	1	1
Sorghum flour (SF)	—	—	5	10	—
Sorghum kafirin (SK)	—	—	—	—	0.25

Con: control diet group; Chol: cholesterol diet group; Chol/SF5: 5% sorghum flour diet group; Chol/SF10: 10% sorghum flour; Chol/SK: 0.25% sorghum kafirin diet group.

tubes and 0.1 mL of methanolic (SF) or *tert*-butanol (SK) extracts or Trolox standards (0 to 20 μM range) was added and shaken vigorously. The tubes were allowed to stand at 27°C for 60 min. A control reaction was prepared as above without any extract, and methanol was used for the baseline correction. Changes in the absorbance of the samples were measured at 515 nm. Radical-scavenging activity was expressed as the inhibition percentage. The final DPPH values were calculated by using a regression equation between the Trolox concentration and the inhibition percentage and expressed as millimol of Trolox equivalents per g of dry weight [23].

2.7. Animals and Diets. All experimental procedures were approved on August 1, 2013, by the Ethics Committee of the Research Center in Food and Development (CIAD, A.C.), Hermosillo, Sonora, Mexico. The mean weight of Wistar rats ($n = 30$) used was 120 g and they were provided by the Experimental Animals Laboratory of Universidad de Sonora, Mexico. They were allowed to have free access to basal diet and tap water for 7 days before experiment. After one-week acclimatization, the rats were randomly divided into five groups ($n = 6$, each) and were given the five different dietary treatments. During 28 days period, two control groups were fed a basal diet (Con) or hypercholesterolemic diet (Chol) and the treatment groups were fed with the Chol diet plus 5% sorghum flour (Chol/SF5), Chol diet plus 10% sorghum flour (Chol/SF10), or Chol diet plus 0.25% sorghum kafirin (Chol/SK0.25). We used 0.25% SK in Chol diet with the objective that rats had a similar amount of kafirin to that of rats fed with the lowest sorghum flour added to diet (Chol/SF5). The calculation of this % was as follows: 100 g of SF contained 9.5% of total protein, where 60% is prolamin fraction (~5.7 g). From this amount, 80% are kafirins (~4.56 g) consequently; 5 g of sorghum flour has 0.25 g of kafirin. Table 1 shows the percent composition of the diets for each experimental group.

The cholesterol batches were mixed carefully with the basal diet just before the diets were offered to the rats. All rats were fed *ad libitum* once a day at 10 a.m. and the intake of the diet and body weight were monitored weekly. All rats had unrestricted access to drinking water. At the conclusion of the experiment (day 28), all groups of rats were anesthetized using diethyl ether, and blood samples were taken from the left atrium of the heart. Serum was prepared for analysis including TC, HDL-C, LDL-C, and TG as described by RANDOX Labs.

Table 2: Amino acid composition of sorghum flour (SF) and sorghum kafirin (SK)[1].

Amino acid	SF	SK
	g/100 g dry weight	
Asparagine	5.81	6.68
Threonine	2.92	2.32
Serine	3.25	3.29
Glutamic acid	14.84	23.12
Proline	7.21	3.03
Glycine	6.32	3.20
Alanine	9.24	14.08
Valine	4.83	4.29
Methionine	1.31	0.746
Isoleucine	3.92	4.01
Leucine	10.10	14.17
Tyrosine	5.31	5.51
Phenylalanine	5.05	5.79
Histidine	2.22	1.19
Lysine	3.06	1.01
Arginine	5.70	3.16

[1]Each value is the mean of three replications. The coefficient of variation was lower than 5%.

2.8. Serum Antioxidant Activity Measured by Using Oxygen Radical Absorbance Capacity (ORAC) Assay. This assay measured the effect of antioxidant components of foods or biological fluids on the decline in FL fluorescence induced by AAPH, a peroxyl radical generator. The reaction mixture contained 1.7 mL of 75 mM phosphate buffer (pH 7), 100 μL of 0.0102 mM FL, 100 μL of 320 mM AAPH, and 100 μL of each sample or several dilutions of the Trolox standard. FL, phosphate buffer, and samples were preincubated at 37°C for 15 min. The reaction was started by the addition of AAPH, and the fluorescence was measured and recorded every 5 min until the fluorescence of the last reading declined to <5% in respect to the initial reading (approximately 60 min). One blank and a maximum of 12 samples were analyzed at the same time. The excitation and emission wavelength was set as 484 and 515 nm, respectively. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the FL decay curve and were expressed as millimol of Trolox equivalent per L [23]. The area under the curve (AUC) was calculated according to the following equation:

$$\mathrm{AUC} = \left(0.5 + \frac{\int_5}{\int_0} + \frac{\int_{10}}{\int_0} + \frac{\int_{15}}{\int_0} + \frac{\int_{20}}{\int_0} + \frac{\int_{25}}{\int_0} + \frac{\int_{30}}{\int_0} + \cdots + \frac{\int_i}{\int_0}\right) \times 5, \quad (1)$$

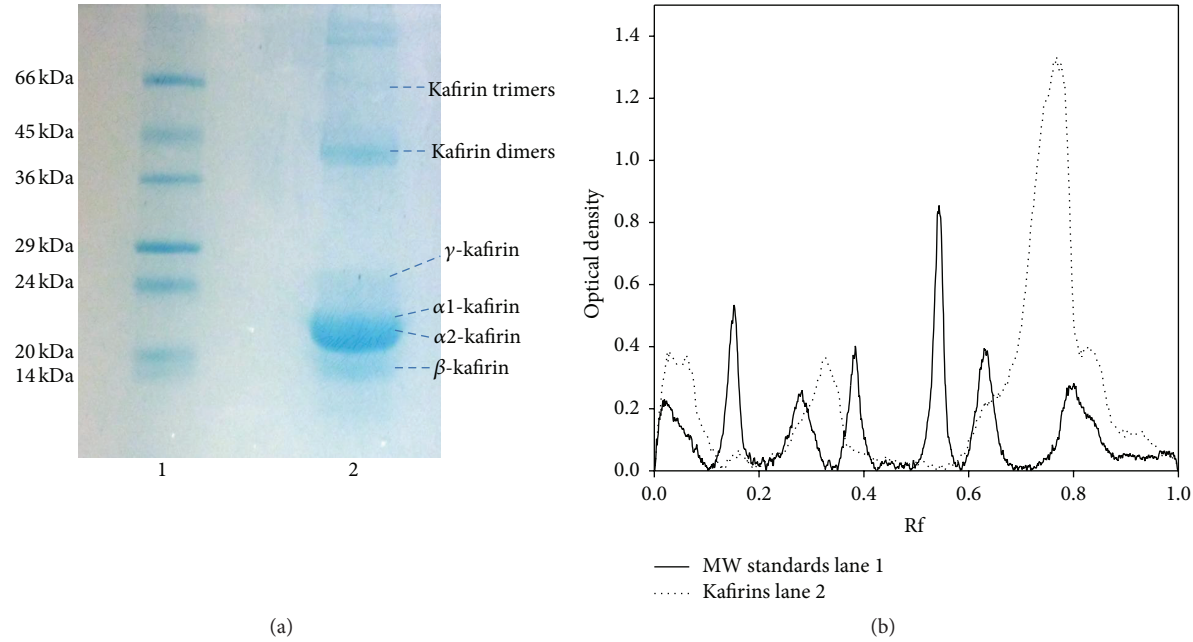

Figure 1: (a) SDS-PAGE pattern of kafirins from *Sorghum*. Lane 1: molecular weight standards; Lane 2: sorghum kafirin (SK) under reducing conditions. (b) Densitometry pattern of same 2 lanes.

where $\int_0$ is the initial fluorescence reading at 0 min and $\int_i$ is the fluorescence reading at time i. The data were analyzed by applying the equation in a Microsoft Excel spreadsheet to calculate AUC. The net AUC was obtained by subtracting the AUC of the blank from of the sample.

2.9. Statistical Analysis. The results of the *in vitro* study were analyzed by descriptive statistics, means ± SD, or coefficient of variance of three measurements. For the *in vivo* study, differences between groups were tested by two-way analysis of variance, followed by Tukey's multiple comparison test. The criterion for significance was $p < 0.05$.

3. Results and Discussion

3.1. Kafirin Extraction and Composition. Kafirins were extracted with 60% *tert*-butanol and analyzed under reducing conditions by SDS-PAGE. Figure 1(a) shows the SDS PAGE pattern of kafirin from sorghum extract. Major bands were observed at 20–24 kDa, indicating the presence of α1- and α2-kafirin monomers. Bands close to 19 kDa and 27 kDa were identified as β-kafirin and γ-kafirin polypeptides, which corresponds with previous reports [24]. In addition, two bands close to 45 and 66 kDa were observed, suggesting the presence of dimers and trimers, respectively. These results were in agreement with the findings by Correia et al. [25] and Mehlo et al. [26] who used similar reducing conditions for the assay. Densitometry analysis showed that the α-kafirins had the highest optical density (OD) at 1.4 which is more than threefold of the other kafirins present in the extract (Figure 1(b)). Prolamins are storage proteins in sorghum grain and constitute 60% of the total protein. Several authors have reported that α-kafirins comprise 80–84% and β- and γ-kafirins constitute 7-8% and 9–12% of the total monomers, respectively [27].

Among the different options that make kafirin extraction more efficient is the use of a reducing agent in addition to *tert*-butanol. This induces a breakage of disulfide bonds between proteins and promotes the release of more kafirin monomers. However, the use of reducing agents such as 2-mercaptoethanol carries a high risk of toxicity; thus, given the goals of this study, this procedure is not considered appropriate.

Table 2 shows the amino acid composition of SF and SK samples. The amino acid composition of SF was similar to that reported by Mosse et al. [28], who studied the amino acid profile of 10 *Sorghum* varieties. SK showed an amino acid composition different from that of SF, resulting in higher content of hydrophobic amino acids such as alanine, isoleucine, leucine, tyrosine, and phenylalanine. Our results agree with those reported by previous studies of kafirins obtained by similar extraction procedure [29]. Electrophoresis assay and the amino acid analysis of the sorghum kafirin extract were similar to those previously reported studies [27–29]. Therefore, protein obtained from *Sorghum* is referred to as kafirin. The extraction conditions were determinant on amino acid composition of kafirins. Nonreducing conditions and aqueous *tert*-butanol favored higher α-kafirin extraction rather than β- and γ-kafirin extraction. α-kafirins are monomers with a high affinity to aqueous *tert*-butanol and are characterized by high hydrophobic amino acid content but low proline and methionine content [27, 30–32].

It has been reported that the presence of hydrophobic amino acids is an indicator of antioxidant activity in several cereals or food plants extracts [33–35]. These authors

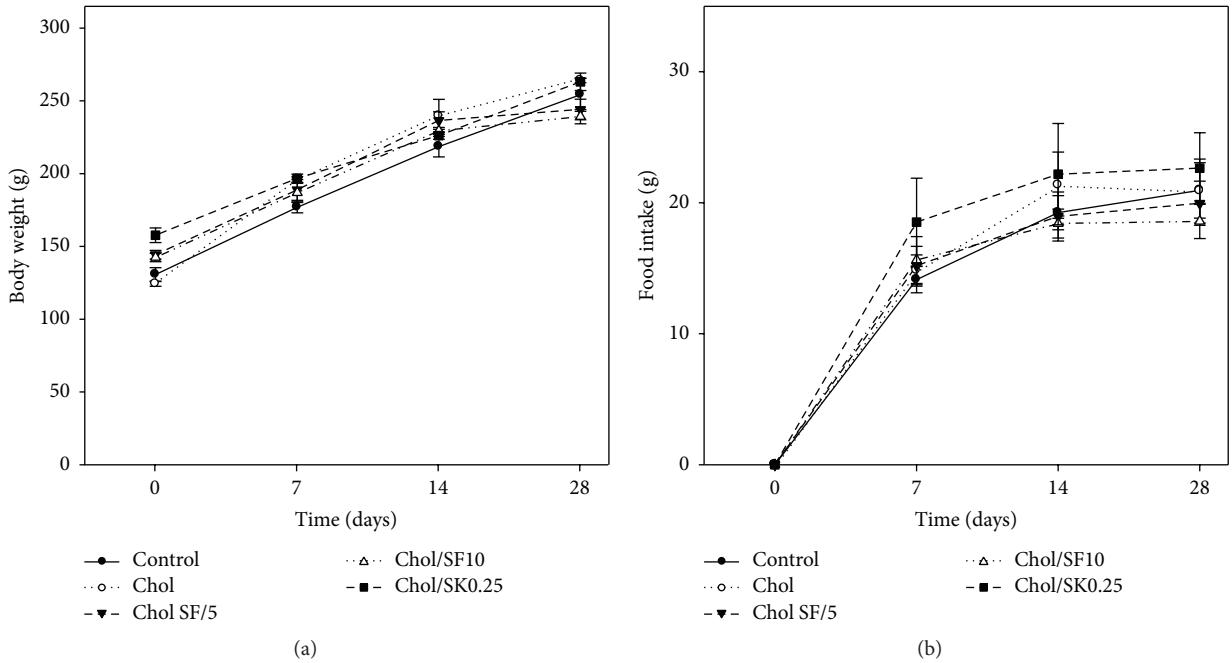

Figure 2: Body weight (a) and food intake (b) changes over the study period for each group of rats. Each value is the mean ± SD ($n = 6$) of each dietary group.

Table 3: Antioxidant activity in sorghum flour (SF) and sorghum kafirin (SK)[1].

Sample	DPPH	TEAC
	mmol TE/g dry weight	
SF	0.101 ± 0.001^{b}	2.74 ± 0.04^{b}
SK	3.03 ± 0.37^{a}	181.54 ± 1.76^{a}

[1]Each value is the mean ± SD of three replications. Means in columns not followed by common letters differ significantly ($p < 0.05$).

reported that hydrophobic amino acids can inhibit lipid oxidation by hydrogen transference or metal chelation. However, other studies have documented that antioxidant activity increases with peptide sequences when the amino acids proline, leucine, and histidine are the most abundant [36, 37].

In our study, we observed that the efficacy of the sorghum flour to scavenge DPPH and ABTS radicals was lower than that of sorghum kafirin (Table 3). Antioxidant activity in sorghum flour can be attributed mainly to the presence of phenolic compounds, which are located in different proportions in the endosperm and pericarp. However, we cannot discriminate the possible contribution of some proteins and fatty acids present in sorghum flour to the antioxidant activity.

In white *Sorghum*, Awika et al. [38] reported lower antioxidant activity values (6 μmolTE/g) in DPPH and TEAC assays. To our knowledge, there are no previous studies of the antioxidant activity of sorghum kafirin extract. Therefore, new information about the antioxidant properties of these proteins is being reported. Regarding the antioxidant activity of SK extract, this could be attributed essentially to the presence of hydrophobic amino acids, as seen above.

3.2. Lipid Profile and Antioxidant Evaluation. The data on body weight and food intake are shown in Figure 2. Figure 2(a) shows the overview of changes in body weight over the study period for the different rat groups; no significant differences in weight between the different groups were observed. Figure 2(b) shows that food intake was similar ($p > 0.05$) for each group. These observations indicate that the diet used in this study was well tolerated by rats.

SK supplemented diet in cholesterol fed groups after 4 weeks of feeding (Table 4) significantly hindered the rise of TC (1.74 ± 0.19 versus 2.12 ± 0.67 mmol/L) and LDL-C (0.84 ± 0.14 versus 1.33 ± 0.81 mmol/L). The same diets significantly increased HDL-C levels (0.96 ± 0.12 versus 0.68 ± 0.32 mmol/L).

A different behavior was observed in SF supplemented diets in the cholesterol fed groups after 4 weeks of feeding. The mean values of TC increased in both SF5% and SF10% (2.12 ± 0.67 to 2.75 ± 0.26 mmol/L, +29% and 2.12 ± 0.67 to 2.67 ± 0.26 mmol/L, and +26%, resp.). However, for the same diets, there was a significant increase in HDL-C content (0.68 ± 0.32 to 1.50 ± 0.17 and 1.36 ± 0.31, resp.).

At the end of the experiment, a significant increase in the serum antioxidant activity in the Chol/SK0.25 dietary group was observed with respect to Chol dietary group (16.22 ± 3.19 versus 27.10 ± 3.32 mmol/L). However, a decrease ($p > 0.05$) in the serum antioxidant activity after completion of the trial was registered in SF (5 and 10%) groups, with respect to Chol group diet (Figure 3).

Table 4: Changes in the serum lipid profile of rats fed with different diets: 1% cholesterol (Chol), 5% and 10% sorghum flour, and 0.25% sorghum kafirin[1].

Dietary group	TC	HDL-C	LDL-C	TG
		mmol/L		
Control	1.46 ± 0.10^{d}	0.71 ± 0.06^{c}	0.75 ± 0.06^{b}	0.99 ± 0.18^{a}
Chol	2.12 ± 0.67^{b}	0.68 ± 0.32^{c}	1.33 ± 0.81^{a}	0.75 ± 0.32^{ab}
Chol/SF5	2.75 ± 0.26^{a}	1.50 ± 0.17^{a}	1.17 ± 0.16^{a}	0.80 ± 0.09^{ab}
Chol/SF10	2.67 ± 0.26^{a}	1.36 ± 0.31^{a}	1.23 ± 0.21^{a}	0.67 ± 0.07^{b}
Chol/SK0.25	1.74 ± 0.19^{c}	0.96 ± 0.12^{b}	0.84 ± 0.14^{b}	0.61 ± 0.09^{b}

[1]Each value is the mean ± SD ($n = 6$) of each dietary group. Means in columns not followed by common letters differ significantly ($p < 0.05$).
TC: total cholesterol; HDL-C: HDL cholesterol; LDL-C: LDL cholesterol; TG: Triglycerides; Chol: nonoxidized cholesterol; SF: sorghum flour; SG: sorghum kafirin.

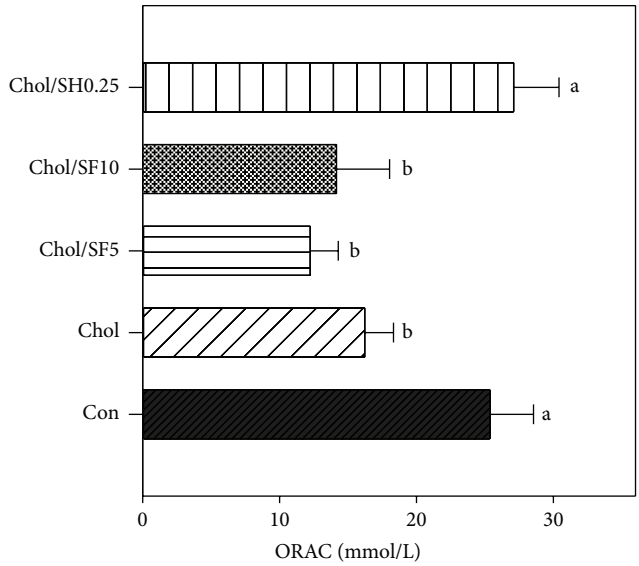

Figure 3: Oxygen Radical Absorption Capacity (ORAC) in rats fed with cholesterol. Each value is the mean ± SD ($n = 6$) of each dietary group. Bars with different letters are significantly different ($p < 0.05$).

This investigation has shown that sorghum kafirin positively influences the serum antioxidant activity in rats fed with added cholesterol. As far as we know, this is the first study that evaluates the *in vivo* changes in the lipid profile of the kafirin fractions of *Sorghum*. The mechanisms involved may explain that this favorable effect may be very different, ranging from a reduction in intestinal absorption of cholesterol and/or bile acids, a decrease in serum cholesterol favored by the activity of LDL receptors in the liver, and changes in the biotransformation of liver cholesterol [39].

With the results obtained in this study, we considered that kafirins could be partially hydrolyzed by digestive enzymes in the rat generating peptides that apparently are absorbed in the intestine resulting in different metabolic effects, especially in cholesterol metabolism.

Studies have shown that protein intake reduces cholesterol levels in circulation, and the mechanisms which have been attributed to this effect may be related to a reduction in total cholesterol synthesis [40]. Apparently, the bioactive peptides produced by an incomplete digestion of the protein affect cholesterol absorption in the digestive tract or have a direct effect on cholesterol synthesis and/or LDL receptor activity (LDLR) [41, 42].

Cereal grains are one of the most important sources of protein, and the storage proteins of these grains (prolamins) contain bioactive fragments. The bioactivity of these proteins has been extensively reviewed and it has been reported that prolamins are potential precursors of antihypertensive peptides [15]. Also, it has been found that the antioxidant activity of some peptides derived from whey or grains such as wheat may be attributed principally to hydrophobic amino acids such as leucine, proline, and also histidine [43, 44]. In the case of kafirin supplemented diets with cholesterol added, the antioxidant activity was kept at constant levels in circulation and even slightly higher than those achieved by the control group. This increase in serum antioxidant capacity may be related to the *in vivo* dietary ingested kafirin digestion, thus generating bioactive peptides with antioxidant activity that may be able to maintain serum levels of total cholesterol.

Another interesting result obtained in our study was the increase in TC values in the sorghum flour (5 and 10%) dietary groups fed with cholesterol, compared to the cholesterol group. Several authors have reported that this behavior had a positive influence on the inhibition of atherosclerosis [45, 46]. The mechanisms that could explain this are related to the inhibition of CETP (cholesteryl ester transfer protein), which is responsible for cholesterol esters of HDL transfer to other cholesterol fractions. When CETP is inhibited, there is accumulation of HDL-C, which has been recognized as antiatherogenic [47].

4. Conclusion

Our results demonstrated that sorghum kafirin extract had a good antioxidant potential in both *in vitro* and *in vivo* studies. The densitometric assay from electrophoresis pattern of kafirin fraction of sorghum flour showed that higher α-kafirins proportion in respect to other kafirins was found. Therefore, α-kafirins could be responsible of the antioxidant activity observed *in vitro*. The *in vivo* study confirmed that sorghum kafirin reduced the TC levels and increased the HDL-C levels in hyperlipidemic rats, suggesting that sorghum kafirin fraction can potentially reduce the risk of

CVD. We concluded that sorghum flour consumption at the levels studied apparently protect against an atherosclerotic event.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Effects of Dietary Supplementation of Oregano Essential Oil to Sows on Oxidative Stress Status, Lactation Feed Intake of Sows, and Piglet Performance

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Fifty-four multiparous large white sows were used to determine the effects of supplementing oregano essential oil (OEO) to the gestation and lactation diets on oxidative stress status, lactation feed intake, and their piglet performance. Two groups were fed diets with (OEO; $n = 28$) or without (Control; $n = 26$) supplemental 15 mg/kg OEO during gestation and lactation. The serum levels of reactive oxygen species (ROS) ($P < 0.05$), 8-hydroxy-deoxyguanosine (8-OHdG) ($P < 0.05$), and thiobarbituric acid reactive substances (TBARS) ($P < 0.05$) were higher during gestation (days 90 and 109) and lactation (days 1 and 3) than in early gestation (day 10). Compared with the control group, the OEO diet significantly reduced sows' serum concentrations of 8-OHdG ($P < 0.05$) and TBARS ($P < 0.01$) on day 1 of lactation. The OEO diet increased the sows' counts of faecal *lactobacillus* ($P < 0.001$) while reducing *Escherichia coli* ($P < 0.001$) and *Enterococcus* ($P < 0.001$). In the third week of lactation the treatment tended to increase sow's feed intake ($P = 0.07$), which resulted in higher average daily gain ($P < 0.01$) of piglets. Our results demonstrated that there is an increased systemic oxidative stress during late gestation and early lactation of sows. The OEO supplementation to sows' diet improved performance of their piglets, which may be attributed to the reduced oxidative stress.

1. Introduction

Oxidative stress results from increased production of reactive oxygen species (ROS) or a decrease in antioxidant defense. Oxidative damage is a strong indicator of health status and wellbeing of animals [1]. A recent study showed that pregnant sows had elevated oxidative stress during late gestation and lactation [2], which was responsible for impaired milk production, reproductive performance, and finally longevity of sows [3–5]. Accumulated evidence suggests that excessive ROS affect the insulin signaling cascade, which leads to insulin resistance [6, 7]. Insulin resistance during peripartal period was shown to have a negative effect on lactation feed intake of sows [8, 9]. Thus, dietary antioxidant concentrations need to be reevaluated for their sufficiency in sow diets especially to prevent excessive oxidative stress during gestation and lactation.

Oregano essential oil (OEO) is isolated from plants (*Origanum vulgare* L.) by steam distillation. Chemical analyses of these oils have shown the principal nutraceutical constituents to be carvacrol and thymol [10]. *In vitro*, OEO has been reported to possess antimicrobial [11, 12] and antioxidant activities [13, 14]. Although previous studies have reported that dietary supplementation of OEO to sows reduced the fat percentage in milk, did not affect growth pattern of suckling pigs, and increased reproductive performance of sows [15–17], its effect on sows' oxidative stress status during gestation and lactation remains unknown. Therefore, the

objective of this study was to examine the effects of dietary supplementation of sow diets with OEO during gestation and lactation on oxidative stress status, colostrum and milk composition, lactation feed intake, and piglet performance.

2. Materials and Methods

All experimental procedures were approved by the Animal Care and Use Committee of Huazhong Agricultural University.

2.1. Animals, Diet Treatments, and Management. Sixty large white sows were originally allotted to the study; the sows were divided into two groups (control group and OEO group) of 30 animals. After breeding, six sows (4 in control group and 2 in OEO group) were returned to estrus within the estrus cycle. Fifty-four large white sows and parities of 4.95 ± 1.12 were used in this trial. After breeding, the sows were randomly allotted to 1 of 2 experimental dietary treatments based on parity and body weight (BW): control sows were fed a basal diet (Table 1) with no supplementation (C, $n = 26$) and the treatment sows were fed a basal diet added with 15 mg/kg OEO through gestation and lactation (OEO, $n = 28$). The commercially available OEO is a mixture powder that contains 5% OEO of *Origanum vulgare* subsp. *hirtum* plants and 95% natural feed grade inert carrier. For each kilogram of sow feed we supplemented 300 mg Orego-Stim (Meriden Animal Health Ltd, UK), that is, 15 mg OEO. The components of OEO were shown in Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/525218. The OEO supplement contained carvacrol (81.92%) and thymol (3.50%). Sows from the two groups were restrict-fed with their respective diets during gestation. Sows were fed 2.0 kg/day from days 1 to 30 of gestation, 2.5 kg/day from days 31 to 90 of gestation, and 3.0 kg/day from day 91 of gestation to farrowing. The diets were supplied twice a day (07:00 and 14:30). During lactation, the diet was supplied three times a day (07:00, 11:00, and 17:30) to ensure sows *ad libitum* access to feed. Pregnant sows were housed individually in gestation stalls (2.2 m × 0.7 m × 1.1 m). Sows were moved from the gestation stalls to the farrowing rooms on day 107 ± 2 of gestation and then kept in individual farrowing crates with stalls (2.2 m × 0.7 m) in pens that provided space on both sides of the stall (2.2 m × 0.5 m) for the pigs after birth. Both sows and piglets had free access to water. Piglets were not offered creep feed. Sow milk was the only feed available to the piglets during lactation. During the experimental period, data from sows with illness, serious lameness, death, and reproductive failure were not included in the analyses (Table 2).

2.2. Performance Measurement. BW and backfat thickness of sows were measured on days 0 and 107 of pregnancy, within 24 h of farrowing and at weaning. Backfat thickness at 65 mm on each side of the dorsal midline at the last rib (P_2) was measured using ultrasound (PIGLOG105, SFAK-Technology). At farrowing, the numbers of total piglets born and piglets born alive were recorded. The piglets were cross-fostered within dietary treatment groups by 48 h after farrowing to adjust the litter size. The number of piglets per sow ranged from 9 to 12 piglets. At weaning, the numbers of weaned piglets were recorded. Piglets were weighed within 24 h of birth (day 1) and on days 7, 14, and 21. The daily feed intake of sows during lactation was recorded each morning by weighing daily feed refusals.

Table 1: Composition of the gestation and lactation diets (as-fed basis).

Item	Gestation	Lactation
Ingredient, %		
Corn	56.30	54.40
Soybean meal, 43% CP	10.00	26.00
Wheat bran	30.40	11.00
Calcium carbonate	1.20	1.51
Dicalcium phosphate	1.04	1.23
Salt	0.40	0.26
Mildewcide[1]	0.12	0.10
Choline chloride	0.14	1.00
Premix[2]	0.40	1.50
Nutrient composition		
Net energy, MJ/kg[3]	9.32	10.36
Crude protein, %	14.06	18.92
Lysine, %[3]	0.61	1.03
Calcium, %[3]	0.77	1.06
Available phosphorus, %[3]	0.33	0.45

[1]Mildewcide: ammonium propionate.
[2]Provided per kg of diet: Cu 30 mg; Fe 160 mg; Zn 160 mg; Mn 55 mg; I 0.5 mg; Se 0.5; Co 0.8 mg; Cr 0.2 mg; Vitamin A 14000 IU; Vitamin D_3 2900 IU; Vitamin E 120 mg; Vitamin K_3 6 mg; Vitamin B_1 2.4 mg; Vitamin B_2 8.5 mg; Vitamin B_6 4.5 mg; Vitamin B_{12} 0.03 mg; Vitamin H 0.55 mg; Pantothenic acid 30 mg; Folic acid 5 mg; Nicotinamide 50 mg.
[3]Calculated chemical concentrations using values for feed ingredients from the National Research Council (1998).

Table 2: The number of sows during the experimental periods.

Item	C[1]	OEO[1]
Breeding	26	28
Culled during gestation[2]	3	1
Parturition	23	27
Culled during lactation[2]	1	2
Weaning	22	25

[1]Dietary treatments: C = control diet; OEO = 15 mg/kg oregano essential oils diet.
[2]Data of sows that were ill, seriously lame, died during the study and had reproductive failure were not included.

2.3. Samples Collection. At 2 h after feeding on days 10, 60, 90, and 109 of gestation and on days 1, 3, 7, and 21 of lactation, before feeding on days 10 and 109 of gestation and on days 3 and 7 of lactation, blood samples were collected from sows (5 sows per diet group with the similar parity) by ear vein with a minimum amount of stress into heparinized tubes (5 mL) or in tubes containing no anticoagulant (5 mL). Fasting sows were selected for blood sampling after an overnight fasting period of 16 h during gestation and 12 h during lactation.

Table 3: Species and genus specific primers used for real time PCR to profile selected bacteria.

Target group	Sequence of primers (5′-3′)	Product size (bp)	Annealing temperature (°C)
Escherichia coli	CATGCCGCGTGTATGAAGAA CGGGTAACGTCAATGAGCAAA	96	60
Enterococcus	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	144	61
Lactobacillus	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	341	58

Samples collected for plasma assays (heparinized tubes) were kept on ice and centrifuged for 5 min at 8500 ×g at 4°C. Samples for serum assays (tubes containing no anticoagulant) were left at room temperature for 4 h and then centrifuged for 5 min at 5000 ×g at 4°C. Serum and plasma samples were stored at −80°C until they were assayed. Colostrum samples (30 mL) were collected from the third, fourth, and fifth pairs of mammary glands of sows (5 sows per diet group with the similar parity) within 4 h after the initiation of farrowing. Milk samples (30 mL) were also collected from the third, fourth, and fifth pairs of mammary glands of sows (5 sows per diet group with the similar parity) on day 18 after an intramuscular injection of 10 IU oxytocin behind an ear. The colostrum and milk samples were immediately frozen at −20°C until analysis. Fresh faecal samples were collected from the sows (5 sows per diet group with the similar parity) on day 109 of gestation into individual plastic containers and kept frozen at −20°C.

2.4. Quantification of Faecal Bacteria. Bacterial DNA was extracted and purified from faeces samples using a QIAamp DNA stool kit (Qiagen, Germany) in accordance with the manufacturer's instructions. Genomic DNA from faeces was pooled and amplified through routine PCR using species and genus specific primers (Table 4). After PCR amplification with a Taq DNA polymerase kit (Promega, USA) and electrophoresis on a 1.5% agarose gel, PCR products were purified according to the manufacturer's protocol (Omega, USA). The purified PCR products were linked to the pMD18-Tvector system (Takara Bio Inc) and then transferred to *Escherichia coli* DH5α (Qiagen, Germany) to clone. After checking the size of the cloned inserts with PCR amplification, the extracted plasmids of the positive clones were sequenced commercially, obtaining the positive plasmids.

Serial dilutions of these positive plasmids served to generate standard curves using quantitative real time PCR (BIO-RAD System, USA), permitting estimations of absolute quantification based on respective gene copies. After 10-fold dilution, microbial genomic DNA was performed to estimate absolute quantification. The reaction was performed in a total volume of 20 μL containing 4 μL template DNA, 1 μL forward and reverse primers, 10 μL iTaq SYBR Green PCR Master Mix (BIO-RAD, USA), and 5 μL nuclease-free water. The thermal cycling conditions involved an initial denaturation step at 95°C for 4 min followed by forty cycles of 95°C for 10 s, annealing temperature (Table 3) for 10 s, and 72°C for 30 s, followed by a product melting curve to confirm the specificity of amplification. The mean threshold cycle values from the triplicate of each sample were used for calculations. The data was calculated as gene copy numbers per gram of wet faeces and presented as Log_{10} CFU/g faeces for the convenience of data analysis.

Table 4: Effects of dietary supplementation of oregano essential oils to sows in gestation and lactation on the colostrum and milk composition and IgG and IgM concentrations in colostrum and serum of sows.

Item	C[1]	OEO[1]	SEM	*P* value
Number of sows	5	5		
Colostrum[2]				
Fat (%)	5.78	4.60	0.36	0.14
Lactose (%)	1.98	2.01	0.07	0.84
Protein (%)	15.02	15.88	0.60	0.51
Solid not fat (%)	20.50	21.34	0.53	0.47
Total solids (%)	27.10	26.58	0.73	0.75
IgG (mg/mL)	15.80	15.79	0.46	0.91
IgM (mg/mL)	2.86	2.85	0.14	0.97
Milk (%),[3] d 18 of lactation				
Fat	8.71	8.10	0.56	0.61
Lactose	4.84	4.79	0.12	0.85
Protein	4.56	4.60	0.06	0.77
Solid not fat	13.60	13.59	0.11	0.97
Total solids	22.18	21.64	0.40	0.53
Serum, d 109 of gestation				
IgG (mg/mL)	8.90	7.82	0.46	0.27
IgM (mg/mL)	2.69	2.82	0.15	0.69

SEM, standard error of means.
[1]Dietary treatments: C = control diet; OEO = 15 mg/kg oregano essential oils diet.
[2]Colostrum was collected within 4 h after the initiation of farrowing.
[3]Sows were injected with 10 IU of oxytocin intramuscularly behind the ear to induce milk ejection.

2.5. Analysis of Oxidative Stress Parameters. Serum samples were used to measure levels of thiobarbituric acid reactive substances (TBARS), 8-hydroxy-deoxyguanosine (8-OHdG), glutathione peroxidase (GSH-Px), and reactive oxygen species (ROS). An uncontrolled increase in ROS production leads to peroxidative damage of macromolecules, which, in turn, may cause disturbances in the metabolism and physiology [18]. TBARS is one of the most frequently used indicators of lipid peroxidation and was determined in the current study. The major marker for oxidative damage to nucleic acids, 8-OHdG, was chosen to determine the DNA

damage in the current study [19]. Serum samples were analyzed for activities of antioxidant enzymes including GSH-Px and for TBARS using the commercial kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) [20]. GSH-Px activity was determined based on quantifying the rate of oxidation of GSH to GSSG by H_2O_2 catalyzed by GSH-Px. GSH reacts with 5,5′-dithiobis-p-nitrobenzoic acid (DTNB) to produce yellow colored 5-thio-2-nitrobenzoic acid (TNB) that can be quantified spectrophotometrically at 412 nm. TBARS was analyzed based on the reaction with 2-thiobarbituric acid. The resulting pink product was measured spectrophotometrically at 535 nm. An ELISA kit (Dobio Biotech Co., LTD, Shanghai, China) that utilizes an anti-8-OHdG monoclonal antibody to recognize 8-OHdG was used to determine the concentration of 8-OHdG in the serum sample according to the method described by Pialoux et al. [21]. Levels of ROS were measured in serum by chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma) as probe. The measurements according to procedure were described in detail by Du et al. [22].

2.6. Laboratory Analyses. Crude protein was determined according to AOAC (1990). The milk composition was determined with a near infrared reflectance spectroscopy method by Milk-Scan 134A/B. Immunoglobulin concentrations were assessed in serum (IgG and IgM) and mammary (IgG and IgM) secretions by ELISA using pig polyclonal immunoglobulin-specific kits (Bethyl, Montgomery, USA). Prior to analysis, colostrum and milk were delipidated by centrifugation at 3000 ×g at 4°C for 20 min. Plasma concentrations of glucose and insulin were determined according to the glucose dehydrogenase activity colorimetric assay kit (BioVision Inc., CA, USA) and insulin ELISA kit (Biosource Inc., Sunnyvale, CA, USA) according to the manufacturer's instructions, respectively. All samples were analyzed in duplicate. The indirect methods were used to evaluate insulin sensitivity by homeostasis model assessment (HOMA); HOMA-IR (insulin resistance) = [(fasting insulin, mIU/L)] × (fasting glucose, mmol/L)]/22.5; HOMA-IS (insulin sensitivity) = 1/[(fasting insulin, mIU/L)] × (fasting glucose, mmol/L)] [23].

2.7. Statistical Analyses. An individual sow was considered the experimental unit in all statistical analyses. Results were analyzed by ANOVA using the general linear model procedure (SAS 8.0, Inst. Inc., Cary, NC). For sows and litter performances, the model included the effects of treatment and replicate and their interaction. The number of total piglets born was used as a covariate in the analysis of piglet birth weight and total litter weight at birth. The piglet weight and litter weight on day 21 of lactation were subjected to analysis of covariance with the piglet weight and litter weight after cross-foster as the covariate. Variations of oxidative stress parameters and HOMA values were analyzed by ANOVA using the procedure for repeated measurements of SAS. The model included the effects of treatment, physiological stage, and replicate. When an interaction was significant, this was specified in the text. Data were given as means and SEM. Differences between treatment means were significant at $P < 0.05$ and trends identified when $P > 0.05$ but <0.10.

3. Results

3.1. Oxidative Stress Parameters, Faecal Microbial Counts, and HOMA Values of Sows. Serum levels of GSH-Px, TBARS, 8-OHdG, and ROS on different days of gestation and lactation are shown in Figure 1. There was a treatment × sampling day interaction for serum TBARS concentrations ($P < 0.05$). The results showed that in both groups serum levels of ROS and TBARS were higher ($P < 0.05$) during late gestation (days 90 and 109) and lactation (days 1 and 3) than in early gestation (day 10). Additionally, in both groups serum concentrations of 8-OHdG were higher ($P < 0.05$) during gestation (days 60, 90, and 109) and lactation (days 1, 3, 7, and 21) than in early gestation (day 10). Compared with the C group, sows under OEO treatment had significantly lower serum concentrations of TBARS ($P < 0.01$) and 8-OHdG ($P < 0.05$) on day 1 of lactation. They tended to have higher serum concentrations of GSH-Px on day 60 of gestation ($P = 0.08$) and day 1 ($P = 0.07$) of lactation, lower serum concentrations of 8-OHdG on day 109 of gestation ($P = 0.09$) and day 3 of lactation ($P = 0.09$), and also lower serum levels of ROS on day 1 ($P = 0.09$) and day 3 ($P = 0.08$) of lactation than sows fed C diet. In addition, the OEO diet significantly increased the counts of faecal *Lactobacillus* ($P < 0.001$) whereas it reduced the counts of *Escherichia coli* ($P < 0.001$) and *Enterococcus* ($P < 0.001$) on day 109 of gestation (Figure 2). It was also found that the OEO diet tended to reduce the value of HOMA-IR ($P = 0.07$) but increased the value of HOMA-IS ($P = 0.06$) of the sows on day 109 of gestation (Figure 3).

3.2. Colostrum and Milk Composition and IgG and IgM Concentrations in Colostrum and Serum of Sows. Table 4 showed that the dietary treatments had no effect on the colostrum and milk composition, as well as IgG and IgM in serum and colostrum.

3.3. Sow Performance. OEO dietary supplementation of sows during gestation and lactation did not affect the BW and backfat gain during gestation, lactation weight, backfat loss, or weaning-to-estrus of sows (Table 5). Sows in the OEO treatment group tended to increase feed intake in the third week of lactation in comparison to the C group (6.46 versus 6.03 kg/day, $P = 0.07$) (Table 5).

3.4. Piglet's Performance. The effect of the dietary treatment on piglet performance is shown in Table 6. There were no differences in the numbers of total piglets born, live-born and weaned. However, sows fed the OEO diet significantly increased average piglet weights at birth (1.56 versus 1.44 kg, $P = 0.04$) and on day 21 of lactation (6.94 versus 6.49 kg, $P = 0.01$). Furthermore, average daily gain (ADG) of piglets during the third week (306.51 versus 273.12 g/d, $P < 0.01$) and on days 1–21 of lactation (252.36 versus 233.61 g/d, $P < 0.01$) were significantly increased for sows in the OEO diet group.

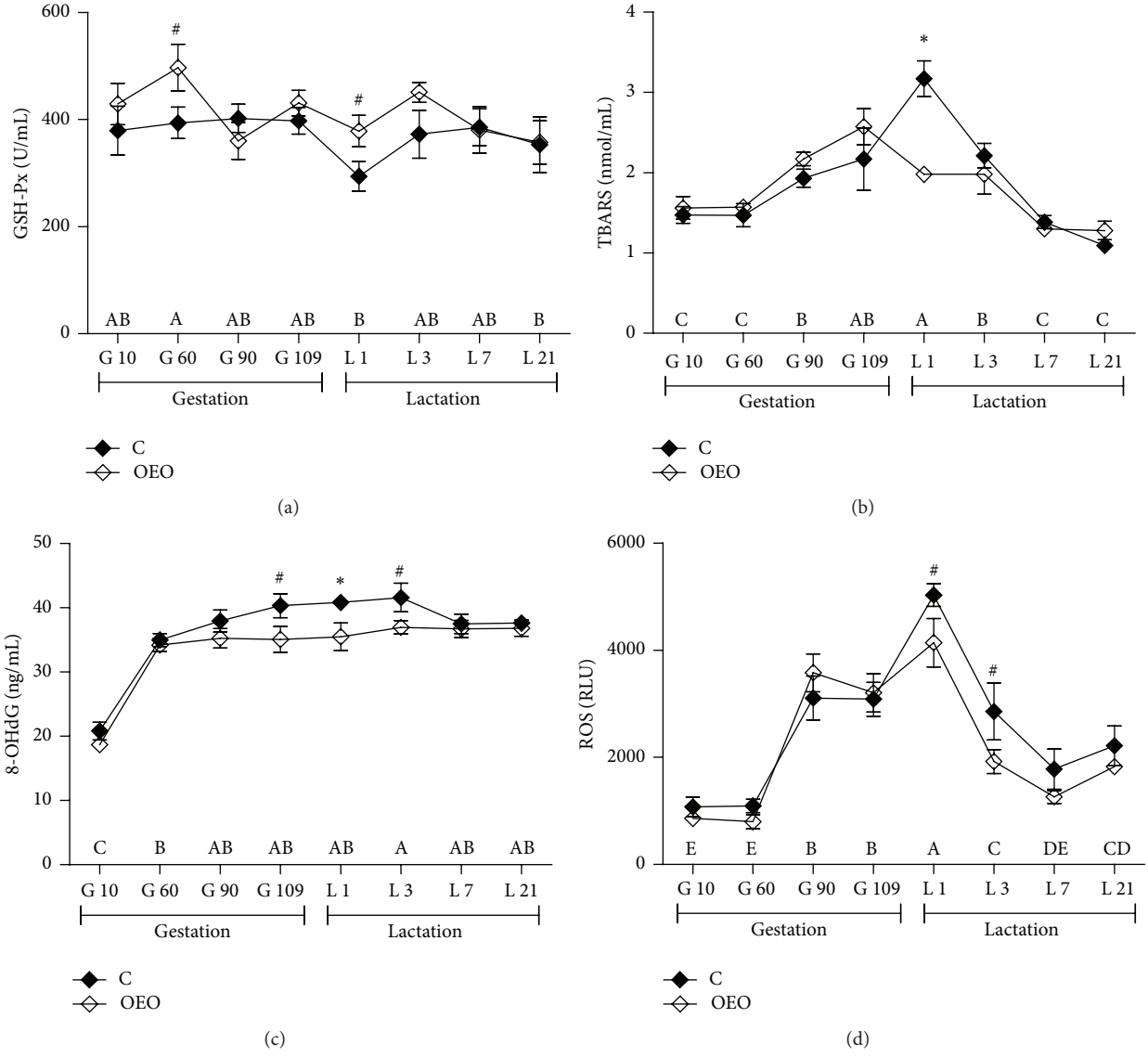

Figure 1: Diet effects on serum levels of GSH-Px (a), TBARS (b), 8-OHdG (c), and ROS (d) of sows (means ± SEM, $n = 5$). $^{A–E}$Effect of sampling day ($P < 0.05$). $^{\#}$Effect of dietary treatment ($P < 0.1$). *Effect of dietary treatment ($P < 0.05$). There was a treatment × sampling day interaction for serum TBARS concentrations ($P < 0.05$). C = control diet; OEO = 15 mg/kg oregano essential oils diet. GSH-Px, glutathione peroxidase; TBARS, thiobarbituric acid reactive substances; ROS, reactive oxygen species; 8-OHdG, 8-hydroxy-deoxyguanosine; RLU, relative light units.

4. Discussion

The peripartal period, particularly the delivery, is a critical time for maintaining a balance between the production of free radicals and the incompletely developed antioxidative protection of the fetus and the newborn [24]. Lipid peroxidation and antioxidant status are changed during delivery, and these changes affect the fetus by creating oxidative stress [25, 26]. Our study indicated that not only during delivery but also during late gestation and early lactation the sows suffer from increased oxidative stress indicated by their elevated ROS, 8-OHdG, and TBARS levels. These results were similar to a report from Berchieri-Ronchi et al. [2] which showed that there was an increased systemic oxidative stress during gestation and lactation and that the sows were not fully recovered until weaning.

In our study the OEO diet significantly reduced the concentrations of both TBARS and 8-OHdG on day 1 of lactation. One possible explanation is that sows suffer from the greatest oxidative stress then. In the other two parameters (GSH-Px and ROS), positive effects of supplementing OEO were also found. This is in line with the previous study [27] reports in weaned pigs. This positive effect of OEO could probably be attributed to its composition which mainly contained carvacrol (81.92%) and thymol (3.50%) (Table S1), because both carvacrol and thymol have been reported to scavenge superoxide radicals and hydrogen peroxide [28, 29].

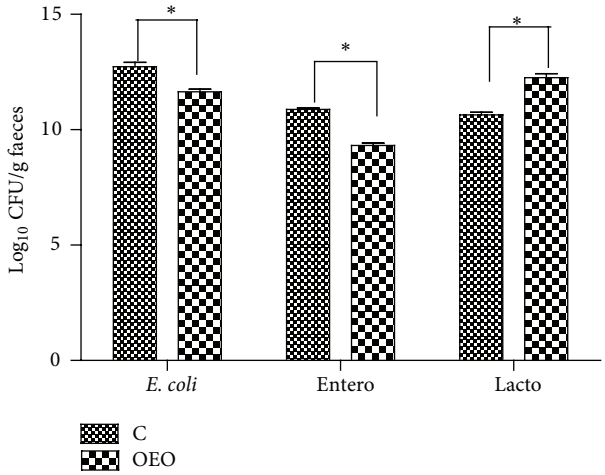

Figure 2: Diet effects on faecal bacterial counts (Log_{10}cfu/g) on day 109 gestation of sows. Values are means ± SEM ($n = 5$). *Significant difference between groups, $P < 0.001$. C = control diet; OEO = 15 mg/kg oregano essential oils diet. *E. coli*, *Escherichia coli*; Entero, *Enterococcus*; Lacto, *Lactobacillus*; CFU, colony forming unit.

Table 5: Effects of dietary supplementation of oregano essential oils to sows in gestation and lactation on sow performance.

Item	C[1]	OEO[1]	SEM	*P* value
Number of sows	22	25		
Daily allowances during gestation, kg/d	2.42	2.42	0.01	0.99
Sow BW, kg				
Breeding	232.7	234.0	4.01	0.59
Gestation, day 107	267.3	268.1	3.31	0.88
Gain	34.6	34.1	2.08	0.33
Parturition	246.3	247.3	3.30	0.98
Weaning	236.3	237.0	3.40	0.75
Loss	10.0	10.3	1.54	0.45
Sow backfat thickness, mm				
Breeding	16.2	16.0	0.43	0.78
Gestation, day 107	17.0	17.5	0.47	0.88
Gain	0.8	1.6	0.35	0.79
Parturition	16.3	17.0	0.49	0.46
Weaning	14.4	14.1	0.37	0.56
Loss	1.9	2.9	0.34	0.46
Average daily feed intake, kg				
1st week of lactation	4.21	4.17	0.15	0.88
2nd week of lactation	5.93	5.90	0.15	0.88
3rd week of lactation	6.03	6.46	0.17	0.07
Mean of 1st week to 3rd week	5.39	5.51	0.13	0.55
WEI, d	4.89	4.63	0.11	0.21

SEM, standard error of means; BW, body weight; WEI, weaning-to-estrus interval.
[1]Dietary treatments: C = control diet; OEO = 15 mg/kg oregano essential oils diet.

Table 6: Effects of dietary supplementation of oregano essential oils to sows in gestation and lactation on piglet performance.

Item	C[1]	OEO[1]	SEM	*P* value
Number of sows	22	25		
Litter size, number/litter				
Total born	11.59	11.28	0.51	0.65
Born alive	11.41	11.16	0.52	0.71
After cross-foster	10.00	9.76	0.17	0.50
Pigs weaned	9.45	9.60	0.18	0.70
Litter weight, kg				
At birth	16.29	17.24	0.68	0.36
After cross-foster	15.63	15.94	0.41	0.70
At day 7	26.99	27.77	0.72	0.59
At day 14	43.77	46.06	1.00	0.26
At day 21	61.17	66.51	1.42	0.06
Piglet mean BW, kg				
At birth	1.44	1.56	0.09	0.04
After cross-foster	1.57	1.63	0.03	0.30
At day 7	2.70	2.84	0.05	0.18
At day 14	4.57	4.78	0.07	0.12
At day 21	6.49	6.94	0.09	0.01
Piglet ADG, g/d				
Week 1	161.41	172.53	4.14	0.18
Week 2	264.12	276.53	4.10	0.13
Week 3	273.12	306.51	5.90	<0.01
Days 1–21	233.61	252.36	3.51	<0.01

SEM, standard error of means; BW, body weight; ADG, average daily gain.
[1]Dietary treatments: C = control diet; OEO = 15 mg/kg oregano essential oils diet.

Moreover, the OEO diet increased the counts of sows' faecal *Lactobacillus* but decreased the counts of *Enterococcus* and *Escherichia coli*. Actually, it has been demonstrated that dietary supplementation with essential oils containing carvacrol and thymol decreases populations of *Escherichia coli* in broiler chickens [30] and increases the proportions of *Lactobacillus* in cecum of broilers [31]. *Lactobacillus* has the ability to inhibit ROS production through fermentation of colon digesta and to inhibit the growth of *Enterococcus faecalis* and *Escherichia coli* [32]. The results of the present study indicated that sows fed the OEO diet shifted microbial ecology in favor of reducing ROS production that alleviated oxidative stress and oxidative damage of sows. The period of transition between late pregnancy and lactation represents an enormous metabolic challenge to the high-yielding sow. Alleviating oxidative stress could definitively benefit sow's health status.

We also found that supplementing the sow diet with OEO tended to increase lactation feed intake of sows. This observation is consistent with the work of Allan and Bilkei [16]. During pregnancy and lactation, the sow undergoes

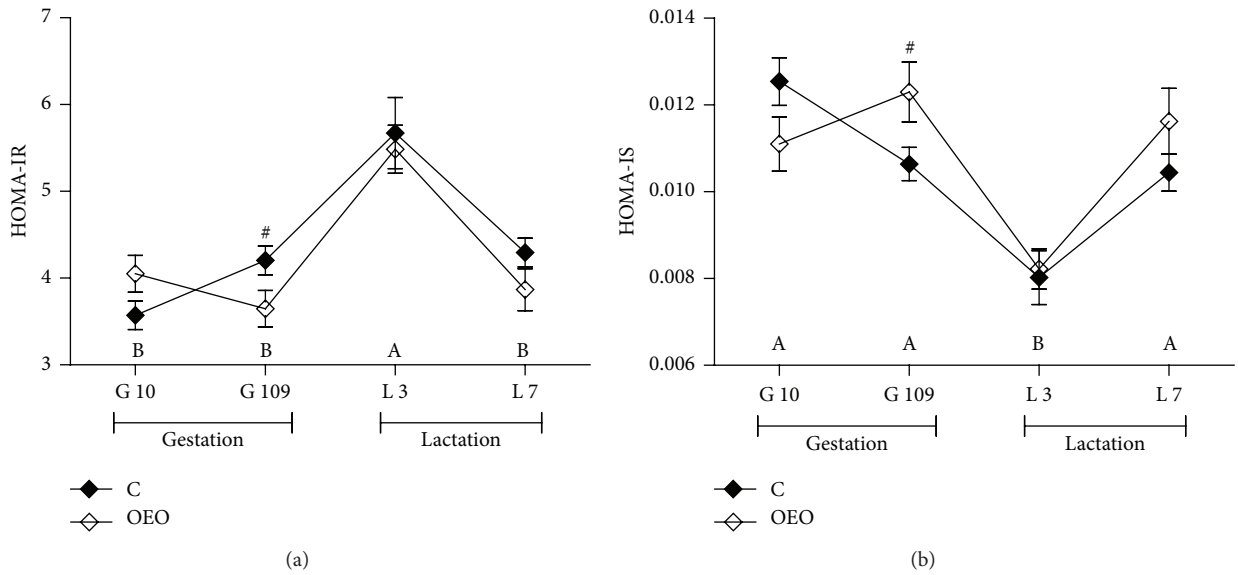

Figure 3: Diet effects on the value of HOMA-IR (a) and HOMA-IS (b) of sows. Plasma glucose and insulin concentrations before feeding were measured, the indirect methods to evaluate insulin sensitivity by homeostasis model assessment (HOMA); HOMA-IR = [(fasting insulin, mIU/L) × (fasting glucose, mmol/L)]/22.5; HOMA-IS = 1/[(fasting insulin, mIU/L) × (fasting glucose, mmol/L)]. Values are means ± SEM ($n = 5$). ^{A,B}Effect of sampling day ($P < 0.01$). $^{\#}$Effect of dietary treatment ($P < 0.1$). *Effect of dietary treatment ($P < 0.05$). There was a treatment × sampling day interaction for HOMA-IS value of sows ($P < 0.05$). C = control diet; OEO = 15 mg/kg oregano essential oils diet.

numerous physiologic and metabolic changes such as progressive and reversible insulin resistance corresponding to a decreased effectiveness of insulin to regulate blood glucose [33]. Moreover, insulin resistance during the peripartal period has negatively impacted the lactation feed intake of sows [8, 9]. Sows fed the OEO diet tended to improve their insulin sensitivity during late gestation (HOMA values, Figure 3). Excessive ROS has been shown to affect the insulin signaling cascade, and then the most common outcome of disrupted insulin signaling is insulin resistance [7]. Thus, we speculated that supplementation of OEO in sow diets may improve insulin sensitivity during late pregnancy by affecting ROS clearance in serum of sows.

In the present trial, sows fed the OEO diet exhibited significantly increased piglets ADG, which can usually indicate an improvement of the amount and/or quality of colostrum and milk, as they are major determinants of litter performance [34]. With regard to the quality of colostrum and milk, our results showed no differences among the dietary treatments in their nutrient compositions and immunoglobulin concentration, contradicting with previous finding of Ariza-Nieto et al. [17] who reported that OEO administered to lactating sows reduced fat percentage in milk on days 7 and 14. Discrepancies may be due to differences between the duration of treatments (gestation and lactation versus lactation) and the dose (15 mg/kg versus 250 mg/kg).

Since no differences of the quality of colostrum and milk were found, the improvement of piglet performance can only be explained by the increase of their amount. Actually we did find a tendency of increased sows' lactation feed intake with the supplementation diets, which resulted in the production of a higher amount of colostrum and milk [35, 36]. It was unexpected to note that sows fed the OEO diet showed more backfat thickness loss during lactation despite their increased lactation feed intake. This might be due to their higher litter weight, which might have pushed them to use their body reserves for milk production.

5. Conclusion

Our results demonstrated that there is an increased systemic oxidative stress during late gestation and early lactation of sows. The OEO supplementation to sows' diet during gestation and lactation improved performance of their piglets, which may be attributed to the reduced oxidative stress.

Conflict of Interests

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

Acknowledgments

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