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Omega-3 LCPUFA supplement: nutritional strategy to prevent maternal and neonatal oxidative stress.

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Short title: DHA and Oxidative Stress

Abbreviations: ABTS – 2,2´-Azinobis-di[3-ethylbenzthiazoline sulfonate]; AA – Arachidonic acid; CAT – Catalase; CT – Control group; DHA – Docosahexaenoic acid ; FO – Fish oil group; GPx – Glutathione peroxidase; HPLC – Highe performance liquid chromatograpy; IVA cPLA2 – Ca2+-dependent cytosolic phospholipase A2; NADPH – Nicotinamide adenine dinucleotide phosphate; SOD – superoxide dismutase; LC-PUFA – Long-chain polyunsaturated fatty acid; TAS – Total antioxidant status; TMB – 3,3´,5,5´-tetramethylbenzidine.

Key Words: Docosahexaenoic acid (DHA), n-3 fatty acids, pregnancy, infant development, oxidative stress, antioxidant defense.

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What's Known on This Subject

There is some controversy with several studies showing antioxidant protection of omega-3 LC-PUFA, while some others report an increase in oxidative damage after this supplementation.

What This Study Adds

Omega-3 LC-PUFA supplementation during pregnancy and lactation has a beneficial effect on evoked oxidative stress in mother at delivery and particularly in the neonate at birth and during the first two months of postnatal life.

Contributors' Statement Page

Each author has participated sufficiently in the work in order to take public responsibility for appropriate portions of the content and has approved the final version of the manuscript.
Naroa Kajarabille drafted the initial manuscript and participated in the analysis.
Jose A. Hurtado, and Javier Diaz-Castro, carried out the initial analyses, reviewed and revised the manuscript, and approved the final manuscript as submitted.
Luis Peña-Quintana, Manuela Peña, Josefina Ruiz, Yessica Rodríguez-Santana, Estefania Martin-Alvarez, Magdalena López-Frias, Olga Soldado and Federico Lara-Villoslada designed the data collection instruments, and coordinated and supervised data collection in the Obstetrics and Gynecology Department and Obstetrics and Gynecology Services, critically reviewed the manuscript, and approved the final manuscript as submitted.
Julio J. Ochoa: Dr. Ochoa conceptualized and designed the study, coordinated and supervised data collection at the Institute of Nutrition and Department of Physiology, and approved the final manuscript as submitted.

ABSTRACT

Background. There is controversy about fish-oil supplementation and oxidative damage. This ambiguity should be explored to elucidate its role as modulator of oxidative stress, especially during gestation and postnatal life. This is the objective of this study.

Methods. 110 pregnant women were divided in two groups: control group CT (400 ml/day of the control dairy drink); supplemented group FO (400 ml/day of the fish oil-enriched dairy drink (± 400 mg EPA-DHA/day)). Different biomarkers of oxidative damage were determined in the mother's at enrolment, at delivery and at 2.5 and 4 months postpartum and newborns at delivery and at 2.5 months postpartum.

Results. Omega-3 LC-PUFA supplementation during pregnancy and lactation decreased plasma hydroperoxides especially in newborn at delivery (p= 0.001) and 2.5 months (p=0.006), increased superoxide dismutase (SOD) and catalase (CAT) in mothers at delivery (p= 0.024 (SOD)) and after 2.5 months (p= 0.040 (CAT) and in newborns at 2.5 months (p= 0.035 (SOD); p= 0.021 (CAT)). Also, supplementation increased α-tocoferol in mothers at 2.5 months (p= 0.030) and in umbilical cord artery (p= 0.039). Higher levels of CoQ10 were found in mothers at delivery (p= 0.039) as well as in umbilical cord vein (p= 0.024) and artery (p= 0.036).

Conclusions. Our supplementation prevents the oxidative stress in the mother and neonate during the first months of postnatal life, being a potential preventive nutritional strategy to prevent functional alterations associated with oxidative stress that have an important repercussion for the neonate development in the early postnatal life.

INTRODUCTION

There are numerous studies available in the scientific literature revealing the importance of Docosahexaenoic acid (DHA) for a correct development of the neonates. DHA is a long-chain polyunsaturated fatty acid (LC-PUFA) which accumulates in brain and retina especially during the third trimester of pregnancy, contributing actively in neuron formation[1-3]. Several studies have shown that DHA deficiency during the pregnancy induces a delay in cognitive and visual development[4], thereby low maternal intake of DHA may cause a suboptimal fetal development.

The importance of DHA has been extensively documented, with many studies focused in mother´s DHA or fish oil consumption during pregnancy and the offspring's cognitive performance[4-8], but most of the studies performed have been focused on neural and visual functions. Nevertheless, there are other interesting effects of the dietary supplement with DHA that have not been deeply studied and could have a key role in the development of the newborn. In this sense, oxidative stress could be a crucial factor for newborn development, aspect that should be elucidated[9,10].

It is important to consider the fact that diverse *in vitro* studies show that a high degree of polyunsaturation is directly correlated with a higher susceptibility to oxidative damage or lipid peroxidation, therefore DHA is a fatty acid highly susceptible to this type of aggression[11]. Nevertheless, the studies in this regard show contradictory and controversial results. Diets rich in PUFA, particularly n-3 PUFA, are considered effective in the prevention of many chronic diseases due to its antioxidant effect[12,13], however, on the other hand, while others studies report that PUFA susceptibility to free radical oxidation increases oxidative stress[14,15]. In this sense, it has been suggested that its antioxidant and/or pro-oxidant effects, somehow depend on the dose and the evoked oxidative stress and/or antioxidant defense of the subject[11,16,17]. Therefore this dual behavior of DHA should be explored to elucidate its role as modulator of cellular antioxidant/oxidant status, especially in those situations in which oxidative stress and antioxidant defense are impaired, as

occurs during labor[10,18]. During pregnancy, oxygen consumption is increased and therefore there is an increase in mitochondrial respiration and in the formation of free radicals. In addition, there are other factors to considerer such as a rapid change from relative hypoxic intra-uterine to the extra-uterine environment, where alveolar pO2 is almost five times higher or the mediation of several physiologic processes involved in the finalization of the gestation and delivery enhancing the Fenton reaction and leading to the production of free radicals[18].

Nevertheless and in spite of all the commented previously, the studies about the effect of DHA supplementation on the oxidative stress evoked in the mother and neonates are really scarce and in the case of the neonates, virtually there are no studies available. In the scientific literature a study in cell cultures is available[16] and also in animals[19,20]. With regard to the mother supplementation, the few existing studies have been focused on the supplementation during the gestation[21,22,23,24] and the samples were of maternal or umbilcal cord blood[21], while those performed in neonates were focused on the effect of the administration with supplemented infantile formulae and its effect on the vitamin E[25]. Therefore the data available is limited and contradictory, provided that some of them reported absence of effects[22] or a slightly antioxidant effect in the mother[21,23] and even a pro-oxidant effect which is dose- dependent[16].

Therefore, considering the controversy existing with DHA supplementation and the importance of oxidative stress in neonate, it is essential to know how this supplementation can affect the oxidative/antioxidant status of both the mother and the neonate. Therefore, this study was designed to study for the first time the effect of a milkshake supplemented with DHA on antioxidant defense and oxidative stress in both, the mother and the newborn during the first stage of the life. These would lead to a deeper understanding of the effects of DHA, shedding some light to the controversies observed to date in the scientific literature.

METHODS

Study design and Subjects

This study involved a group of mothers and their healthy term neonates enrolled in a registered, double-blind, controlled, lasting form the sixth month gestation to fourth month of newborn´s life. 110 volunteers were recruited into the study from "Hospital Materno-Infantil" (Granada, Spain) and "Hospital Universitario Materno-Infantil" (Canary Islands, Spain). The inclusion criteria for the mother-child pair were: no presence of disease that affect the development of gestation or lactation, singleton gestation, body mass index of 18-30 Kg/m^2 at the start of pregnancy, weight gain of 8-12 Kg since pregnancy onset, normal course of pregnancy, no intake of DHA supplements, spontaneous vaginal delivery, gestational age at delivery of 38-42 weeks, an appropriate weight for gestational age, Apgar index ≥ 7, and breastfeeding of the neonate. The flowchart for participant enrolment and drop-outs is shown in figure 1. The study was approved by the Bioethical Committee on Research Involving Human Subjects at both Hospitals. Written informed consent was obtained from each participant after a complete explanation of the study details. The study is registered at www.clinicaltrials.gov (Clinical Trial Identifier NCT01947426).

Women were randomly assigned to one of the following intervention groups following an unpredictable sequence computer-generated: Fish oil group (FO): Consumption of 400 ml/day of fish oil enriched dairy drink; Control Group (CT): Consumption of 400 ml/day of the control dairy drink. Control and Fish oil-supplemented dairy milkshakes were identical in appearance. Detailed information on the composition of the dairy drink using during the intervention is given in Table 1. 100 ml of the fish oil- enriched dairy drink provided 18 mg of EPA and 80 mg of DHA (that means in the fish oil group a supplementation of approximately 400 mg LC-PUFA/day). The dairy products were distributed in white packing without any indication that reflects the type of product containing (double blind), therefore trial investigators and participants were unaware of the

treatment allocation. The dietary intervention began in the 28th week of pregnancy and concluded at the fourth month of lactation.

Ascertainment of mother´s dietary intake

An important confounder in this kind of trials is the diet and therefore, we supervised the mother´s diet during the intervention period. Maternal dietary intake was assessment using a 110-item food frequency questionnaire that included specific questions about consumption of sources of DHA such as freshwater fish, seafood, canned tuna and sardines, salmon, trout, and cod liver oil[26,23] together with a 72 hour diet record. The food frequency questionnaires were recorded in the moment of the recruitment, a month after beginning the study and at first month of lactation. Together with these questionnaires, nutritional recommendations adapted to the conditions of the mothers (gestation - lactation) were given and especially those related to the suitable consumption of fish (2/3 portions per week as daily sources of EPA+ DHA), something really important from an ethical point of view. The analysis of the nutritional intake was performed using a Nutriber v.1.1.1 software (Funiber, Barcelona (Spain)).

Blood sampling

Samples of mothers´ blood (5 mL) were obtained at the enrollment (28th week of pregnancy) (sample M0)), at delivery (sample M1), at 2.5 months of lactation (sample M2) and at the end of the dietetic intervention (four month postpartum) (sample M3). After delivery blood samples were collected from the umbilical vein and arteries (samples NHOV and NHOA, respectively) and at 2.5 months of life a sample of blood form all the neonates was obtained (sample NH1). The samples were centrifuged at 1750 g for 10 min at 4º C in a Beckman GS-6R refrigerated centrifuge (Beckman, Fullerton, CA, USA) to separate plasma from red blood cell pellets. Plasma samples were aliquoted and stored at -80 °C until analysis. Erythrocyte cytosolic and membrane

fractions were prepared by differential centrifugation according to the method of Hanahan and Ekholm[27]. The final fractions were aliquoted and stored at -80 °C until analysis.

Oxidative damage in plasma and erythrocyte membrane

Plasma hydroperoxides content was assessed using a commercial kit (Oxystat, Biomedica Gruppe, Vienna, Austria). It is a colorimetric assay for the quantitative determination of peroxides in plasma, serum and other biological fluids. Preparation of samples, reagents and standards was done by following the manufacturer's instructions. The peroxide concentration is determined by reaction of the biological peroxides and a subsequent color-reaction using TMB (3,3´,5,5´-tetramethylbenzidine) as the substrate. A calibrator is used to calculate the concentration of circulating biological peroxides in the sample (one-point calibration). The plate was measured at 450 nm on a Bio-tek microplate reader (Bio-tek, Vermont, USA).

Erythrocyte membranes were assessed according to the method previously described by Ochoa et al.[9], which is based on the rapid peroxide-mediated oxidation of Fe2+ to Fe3+ in acidic conditions. Fe3+ reacts with xylenol orange forming an orange complex that can be measured spectrophotometrically at 560 nm (Perkin Elmer UV-VIS Lambda-16, Norwalk, Connecticut, USA).

Plasma fat soluble antioxidants and total antioxidant capacity in plasma

Fat soluble antioxidants in plasma were also determined: Vitamin A (Retinol), Vitamin E (α-Tocoferol), β-Carotene, Coenzyme Q9 and Coenzyme Q10. Samples were mixed with ethanol in polypropylene tubes and left on ice for 10 minutes, then hexane was added, and we left them on ice for 5 minutes. Samples were centrifuged at 2200g for 10 minutes at 4 °C and finally dried in a speed-vacum). A mixture of ethanol: isopropanol (90:10, v/v) was added to the sample and then analyzed by Ultra performance liquid chromatography-tandem mass spectrometry, UPLC-MS / MS

system. The equipment used was an ACQUITY UPLC H-Class detector coupled to a triple quadrupole Xevo TQ-S (Waters Corporation, Milford, USA). Chromatographic separation was performed using an isocratic mobile phase gradient with 0.1% methanol (v/v) formic acid. The column used was an Acquity UPLC BEH C18 1.7 um, 2.1 x 100 μM (Waters Corporation, Milford, USA). Mass spectrometry in turn operated by atmospheric pressure chemical ionization (APCI) in positive ion mode. All the parameters studied were optimized individually using standard solutions from Sigma-Aldrich (minimum 98% purity, Grade HPLC) and quantified with standard curves. MassLynx 4.1. (Waters Corporation, Milford, USA) software was used to obtain all the data.

Total plasma antioxidant capacity in plasma samples was measured using a kit (TAS Randox® kit, Randox Laboratories Ltd., Crumlin, UK). The assay involves an incubation of ABTS (2,2´-Azinobis-di[3-ethylbenzthiazoline sulfonate]) with peroxidase and hydrogen peroxide, resulting in the generation of ABTS + radical cation. Plasmatic antioxidants can reduce the generation of the ABTS + radical cations and this decrease is proportional to their total antioxidant concentration. Results were expressed in μM of Trolox equivalents. A standard control was provided in the kit. The linearity of calibration extends to 2.5 mmol/L of Trolox. Duplicate measurements were used to calculate intra-assay variability.

Fat soluble antioxidants in erythrocyte membranes and antioxidants enzyme activity

Fat soluble antioxidants in erythrocyte membrane (Vitamin E and Coenzyme Q10) were also determined following the same procedure described above for fat soluble antioxidants in plasma. Determinations of antioxidant enzymes, Glutathione Peroxidase (GPx), Superoxide dismutase (SOD) and Catalase (CAT) in erythrocyte cytosol were measured as previously described Diaz-Castro et al.[18]. Briefly, Glutathione peroxidase (GPx) activity is based on the instantaneous formation of oxidized glutathione during the glutathione peroxidase catalyzed reaction and subsequent reduction by glutathione reductase y NADPH. The subsequent oxidation of NADPH to

NADP+ was monitored spectrophotometrically at 340 nm (Thermo Spectronic, Rochester, USA). SOD activity determination is based on the inhibition by SOD of the reduction of cytochrome C, measured by spectrophotometry at 550 nm. Finally, Catalase activity was assessed monitoring spectrophotometrically H_2O_2 descomposition by the catalitic activity of CAT at 240 nm during 1 minute at 25ºC.

Statistical analyses

All data are presented as the Mean ± SEM. Prior to statistical analysis all variables were tested to see if they followed the criteria of normality and homogeneity of variance using the Kolmogorov-Smirnoff´s and Levene´s tests, respectively. To compare categorical variables we used chi-squared test. U-Man Whitney non-parametric test was used to find differences between groups, when the DHA responses were not normally distributed and when the DHA response were normally distributed we used unpaired Student´s t test. In all cases a value of $p<0.05$ was considered significant. For data analysis we used the SPSS version 20.0 (SPSS Statistics for Windows, 20.0.0. SPSS INC. Chicago, IL, USA).

RESULTS

No statistically significant differences were found in the age, height, weight and biochemical parameters of the volunteers participating in the study (Table 2). Concerning Newborns, there were no statistically significant differences in anthropometric parameters between groups. The dropout percentage was similar in both groups (29% CT group and 32% FO group) (Figure 1).

The analysis of both food frequency questionnaire and 72-hour diet record revealed no differences between groups (data not shown). No statistical differences were observed in the three nutritional surveys performed to the mothers during the study.

Plasma and erythrocyte membrane hydroperoxides are shown in figure 2 (Plasma and hydroperoxides (fig. 2A) and erythrocyte membrane hydroperoxides (fig. 2B)). For both groups, the highest concentrations of plasma hydroperoxides (figure 2 (A)) were recorded during pregnancy (at 6th month) and at delivery. This concentration decreased markedly postpartum, at 2.5 months and 4 months. In umbilical cord samples, concentrations of peroxides are lower to those found in their mothers, whereas at 2.5 months of life peroxides concentration increases. We found significant differences between groups (supplemented *vs* control), with the lower concentrations in the experimental group in mothers at 2.5 months postpartum (329.02 ± 18.16 µmol/L *vs.* 417.58 ± 29.08 µmol/L; p= 0.006) in umbilical cord artery (51.70 ± 3.83 µmol/L *vs* 84.65 ± 7.04 µmol/L; p=0.001) and at 2.5 months of neonate´s life (246.16 ± 21.92 µmol/L *vs.* 333.33 ± 29.23 µmol/L; p= 0.006).

With regard to hydroperoxides in erythrocyte membrane (figure 2 (B)), in general, we found the highest values in the mothers at 6th month of pregnancy and at 2.5 months and 4 months after partum, as well as in neonates at 2.5 months of life. Regarding to differences between groups (supplemented *vs.* control), we found statistically significant differences only in mothers, at labor (M1) (14.63 ± 1.28 nmol/mg *vs* 18.18 ± 1.30 nmol/mg; p= 0.040) and at 4th month at postpartum (M3) (25.52 ± 2.44nmol/mg *vs* 37.68 ± 6.86 nmol/mg; p= 0.042).

In general, we found the highest antioxidant values in mothers at 6th month of pregnancy and during lactation (at 2.5 months postpartum and 4 months postpartum), whereas these values decreased at delivery. Regarding to neonates we observed less antioxidant capacity compared to mothers. In both groups (supplemented and control), antioxidant capacity is almost two times greater in mothers than in newborns. We found a significant difference between groups (supplemented *vs* control) at delivery (3.15 ± 0.14 nmol/ml *vs.*3.66 ± 0.14 nmol/ml; p=0.006) and at 2.5 months of life of newborn´s life (1.72 ± 0.11 nmol/ml *vs.* 1.28 ± 0.08 nmol/ml; p= 0.001).

Plasma fat soluble antioxidants in mothers and their neonates are shown in table 3. In both groups, we found higher retinol levels in mothers at 2.5 and 4 months postpartum. We observed significant differences between the control group and the DHA supplemented group, in mothers at 2.5 months postpartum (p=0.031) and in newborn´s umbilical cord vein (p=0.034).

α-Tocopherol concentrations found in mothers were higher than those found in newborns, especially compared with umbilical cord blood samples, and we also we observed an increase at 2.5 months of life in neonates compared with the values found in cord blood samples. Regarding to statistically significant differences between groups, we observed differences in mothers at 2.5 months postpartum (p= 0.017), as well as in newborn´s umbilical cord vein (p= 0.041) and artery (p= 0.035).

β-Carotene values followed a similar pattern than retinol with higher values in mothers at 2.5 and 4 months postpartum. We found really low values in neonate (umbilical cord blood), however, both groups showed and increase at 2.5 months of newborn´s life. We found significant differences only in mothers at postpartum, at 2.5 months (p= 0.042), as well as at 4 months postpartum (p= 0.039).

With regard to CoQ9 and CoQ10 content, the lower values were found in both groups at 4 month after birth. The values found in umbilical cord are much lower to those found in mother plasma, although in both cases an increase was observed at 2.5 month postpartum. We only found significant differences for CoQ9 in neonate´s umbilical cord artery samples (p= 0.010). With regard to CoQ10 in general, we observed greater values in the supplemented group, however statistically significant differences were only observed in mothers at 4th month postpartum (p= 0.037) and in neonate´s umbilical cord vein (p= 0.036) as well as at 2.5 months after birth (p= 0.045).

Fat soluble antioxidants in mother and neonate erythrocyte membrane are shown i n table 4. With regard to α-tocoferol we observed highest values in mothers at 2.5 months as well as at 4th month postpartum. The lowest values were found in newborn, especially at birth and were similar to

those found in mother at delivery. Significant differences between groups were found in mothers at 2.5 months postpartum (*p*= 0.030) and in neonate umbilical cord artery (*p*= 0.039).

In contrast, we found higher levels of CoQ10 in newborns, particularly in umbilical cord compared to those found in the mother. Significant differences between groups were found in mothers at delivery (*p*= 0.039) as well as in neonates at birth, in umbilical cord vein (*p*= 0.024) and artery (*p*= 0.036).

Cytosolic antioxidant enzymes are shown in table 5. With regard to the superoxide dismutase (SOD), in general, both groups showed similar values in mothers and neonates. Significant differences between groups were observed in mothers at delivery (*p*= 0.024) and in newborns at 2.5 months of life (*p*= 0.035).

Similarly to SOD, both groups showed similar values in mothers and for CAT activity. We found significant differences between groups in mothers after 2.5 months postpartum (*p*= 0.040), as well as in neonates at 2.5 months after birth (*p*= 0.021).

With regard to GPx we observed higher values in mothers at 2.5 months and at 4 months postpartum. No differences were found between the supplemented and control group.

DISCUSSION

As previously reported[28], mother supplementation during the gestation and lactation with a dairy milkshake supplemented with omega 3 LC-PUFA increased the percentage of DHA and other fatty acids of the omega 3 series in different cell compartments, both in the mother (placenta, plasma, erythrocyte membrane and milk) and in their neonates (plasma and erythrocyte membrane). This is the main reason why we have not included information about the mother or neonate fatty acids profile in the current study.

Maternal omega-3 LC-PUFA intake during pregnancy has a key impact on some birth outcomes, such as duration of gestation and infant´s weight[29]. In the current study, no differences

were found in pregnancy outcomes between groups regarding the newborn´s weight, cranial perimeter or perinatal development. Our results are in agreement with those reported by a systematic review of the available lierature[4].

A noteworthy aspect to consider during pregnancy, labor and postnatal life is the evoked oxidative stress, affecting both the mother and neonate[9,10,30]. Kankofer[31] reported a maximum peroxides output in mother plasma at delivery and during pregnancy, results which are in agreement with those found in the current study. We have found that plasma hydroperoxides were also elevated in neonates, being similar to those found in the mother during lactation. Omega-3 LC-PUFA supplementation showed a clear beneficial effect on hydroperoxides levels in the newborn, by decreasing them in umbilical cord artery, as well as in the postnatal life of the newborns at 2.5 months. Similarly, we have observed the effect of DHA supplementation in mothers at delivery and postpartum (2.5 and 4 months), and we found that peroxide levels in erythrocyte membrane were also significantly reduced. The decrease in plasma and erythrocyte membrane peroxides content induced by omega-3 LC-PUFA supplementation in our study may be due to the effect of the LC-PUFA on some key factors responsible of free radicals generation and the effect on the antioxidant system in the mother and the newborn.

As previously commented one of the possible protective mechanisms of the omega-3 LC-PUFA to oxidative stress would underlie in the generating mechanisms of free radicals, including the inflammatory signaling. DHA has an anti-inflammatory effect that appears to be correlatedwith its inhibiting action on the arachidonic acid (AA)[24]. AA is released via selective Ca^{2+}-dependent cytosolic phospholipase A2 (IVA cPLA2), however this mechanism also implies the conversion in eicosanoids, generating free radicals[13]. In this sense, we can postulate that a possible antioxidant role of DHA can be attributed to its inhibiting action on the AA/eicosanoids production and to the anti-inflammatory and protective properties of the protectins and resolvins[24]. In this sense, Gonzalez-Periz, et al.[32] demonstrated that DHA prevents DNA damage and oxidative stress in liver

cells, effect that was associated with a decrease in the hepatic synthesis of n-6-derived eicosanoids, as well as an increase in the generation of protective DHA-derived lipid mediators.

Another aspect to be taken into account is the effect on the antioxidant defense. We have analyzed the plasma antioxidant capacity. Our results showed that the highest antioxidant values were found in mothers during pregnancy and lactation, whereas these levels diminished at delivery. In both groups, the antioxidant capacity is almost double in mothers compared to their neonates. Thereby, the main differences between control and supplemented groups were found in mothers at parturition and in the newborn at 2.5 months of life, although with different behavior. The lower value of total antioxidant capacity found in the mothers supplemented with omega 3 LC-PUFA at the moment of childbirth, together with the lower peroxides level, especially in erythrocyte membrane could indicate a lower need of antioxidants in the supplemented group, probably due to a lower production of free radicals[13,16,19]. In this sense, we could also consider a lower transfer of antioxidants to the neonate, explaining the lower content in plasma hydroperoxides found in umbilical cord artery in the fish oil-supplemented group. Newborns are particularly susceptible to oxidative stress for several reasons[33]. Our results show that omega-3 LC-PUFA supplementation avoids the decrease of antioxidant capacity at 2.5 months of life in newborns and, in addition, this supplementation also reduces peroxide levels in plasma compared with the control (non-supplemented) group.

Regarding to the fat soluble antioxidants in plasma and erythrocyte membrane, in mothers we did not find any significant differences between the control and the fish oil-supplemented group during the last trimester of pregnancy, finding which is in agreement with the data from an study carried out with mothers who were supplemented with salmon during pregnancy[23]. In this study, the authors did not observed significant differences for α-tocopherol, β-carotene and CoQ10 in the supplemented group compared to control. However, retinol levels in plasma were different from those found in our study, since they observed significantly higher retinol concentrations in mothers

who were supplemented with two portions of salmon per week during gestation, fact that could be attributed to the great retinol concentration in salmon. Nevertheless, we found significant differences in plasma and membrane samples at 2.5 months postpartum as well as in umbilical cord artery, suggesting that fish oil-supplementation has positive effects on the neonate, enhancing the antioxidant capacity and reducing the evoked oxidative stress.

After parturition we observed higher of retinol, CoQ9 and α-tocopherol in plasma in the supplemented group at 2.5 months postpartum and -carotene and CoQ10 at 4th month postpartum. In erythrocyte membrane, higher values of CoQ10 were found at delivery and α-tocoferol at 2.5 months postpartum. These data suggest a higher fat soluble antioxidant defense in the supplemented mothers, especially after lactation. The result observed for α -tocoferol in plasma at 2.5 months postpartum is interesting. Florian et al.[34] reported a significant decrease in α-tocopherol levels in plasma at 2.5 months postpartum, which is in agreement with our results, although in the supplemented group this decrease was not observed.

With regard to the newborns, in general, a higher content in retinol, CoQ10 and α-tocopherol (in plasma and erythrocyte membrane) was observed in the samples obtained in umbilical cord of the supplemented group. This information indicates, once again, the higher antioxidant defense at the moment of the childbirth in the neonate and this is the moment when the maximum oxidative stress occurs[10,33]. In the same way, at 2.5 months of postnatal life, a higher CoQ10 content is observed in the supplemented group. All these findings can be correlated with the results found in plasma peroxides, because fish oil-supplemented group showed a decrease of peroxides in umbilical cord vein and artery, as well as at 2.5 months of newborn´s life.

To avoid induced oxidative damage in cells, the antioxidant defense system provides protection and the capacity of this defense system is determined by a dynamic interaction between individual components, which include fat soluble vitamins and several antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)). Regarding to our

data for antioxidant enzymes, in the supplemented group as well as in the control group, both mothers and their neonates showed similar activity. With regard to SOD we found the highest activity at 4 month postpartum in mothers and in umbilical cord artery in neonates. In general, the omega 3 LC-PUFA supplementation leads to an increase in the activity of this antioxidant enzyme, though it is only significant at the moment of the delivery in the mother and at 2.5 months of postnatal life in the newborn. Garcia-Rodriguez et al.[23] did not report any differences in the activity of this enzyme due to the maternal supplementation with two portions of salmon during gestation, though this study was only focused in the period before the childbirth and not during the labor, neither in the newborn child. On the other hand, in a study carried out by Garrel et al.[35], they observed a significant increase in SOD activity in brain of rats that were fed with a diet rich in DHA, during postnatal development.

With regard to CAT, in the fish oil-supplemented group, we observed higher values at 2.5 months postpartum in mothers, as well as at 2.5 months of newborn´s life. We did not found significant differences for CAT during pregnancy neither the birth, which is in agreement with the results found by Garrel et al.[35] and García-Rodríguez et al.[23].

Regarding to GPx, we found the highest values in both groups after delivery, particularly at 4 month postpartum and the lowest values were observed in the newborns at 2.5 months postpartum. Though a higher activity of this enzyme was observed in the supplemented group in umbilical cord artery and in the newborn at 2.5 months of life, it was just a trend and not statistically significant. García-Rodríguez et al.[23] found a higher activity GPx in DHA-supplemented mothers with salmon, though this increased activity was associated with the increase in the levels of selenium, due to the high content of this mineral in salmon and it is known the effect of selenium as a cofactor in the activity of this enzyme. In general, it is noteworthy the effect of the mother supplementation with omega 3 LC-PUFA in the newborn child at 2.5 months of life on the enzymatic activity of the complex SOD-CAT, a very interesting finding, due to the importance in

the development of the newborn child, because as it has been previously reported, a decrease in the activity of these enzymes promotes the oxidative damage in proteins and lipids[33,36].

CONCLUSIONS

Omega-3 LC-PUFA supplementation (approximately 400 mg/day DHA-EPA) during pregnancy and lactation has beneficial effects on the evoked oxidative stress in mothers, though the most noteworthy effect is recorded in the neonate at birth (artery of umbilical cord) and during first two first months of postnatal life. This finding can be explained due to the enhanced free radical scavenging ability, a higher plasma total antioxidant capacity in the neonate, the increase in fat soluble antioxidants (especially in umbilical cord artery) and the higher activity of the cytosolic antioxidant enzymes SOD and CAT at 2.5 months of postnatal life. In the mother those effects were also observed, though in a lower extent, being these effects more prominent during the stage of lactation. All these findings suggest a correlation between omega-3 LC-PUFA and the evoked oxidative stress in the mother during labor and in the neonate during the early postnatal life, leading to a renewed interest in the fish-oil supplementation as a potential preventive nutritional strategy to prevent several functional alterations associated with oxidative stress that have an important repercussion for the neonate development in the postnatal life.

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TABLE 1. Composition of the dairy drinks used during the study.

	Fish oil Drink	**Control Drink**
Energy (kcal-kJ/100 mL)	58-145	58-145
Protein (g/100 mL)	3.7	3.7
Carbohydrates (g/100 mL)	6.7	6.7
Fats (g/100 mL)	1.8	1.8
Saturated (g/100 mL)	0.32	0.36
Monounsaturated (g/100 mL)	1.06	1.10
Polyunsaturated (g/100 mL)	0.42	0.34
EPA (mg/100 mL)	18	-
DHA (mg/100mL)	80	-
Folic Acid (µg/100 mL)	80	80
Vitamin B_{12} (µg/100 mL)	0.4	0.4
Vitamin C (mg/100 mL)	9.0	9.0
Vitamin D (µg/100 mL)	0.75	0.75
Vitamin E (mg/100 mL)	1.5	1.5
Calcium (mg/100 mL)	160	160
Iron (mg/100mL)	2.2	2.2
Iodine (µg/100mL)	23.0	23.0

TABLE 2. Characteristics of the mothers and their neonates.

		FO group	CT group
Mothers			
Age (years)		30.5 ± 4.8	29.9 ± 4.7
Weight (kg)		71.7 ± 11.5	73.2 ± 12.3
Lenght (cm)		163.2 ± 6.3	164.2 ± 6.5
BMI		26.9 ± 4.1	27.2 ± 4.6
Parity	Uni (%)	64.4 %	68.2 %
	Multi (%)	35.6 %	31.8 %
Weight gaining		7.4 ± 2.8	7.0 ± 2.8
SBP (mm Hg)		107.2 ± 8.7	110.5 ± 12.3
DBP (mm Hg)		64.3 ± 6.5	65.5 ± 8.1
Hemoglobin (g/L)		11.8 ± 0.8	11.7 ± 1.0
Hematocrit (%)		34.4 ± 2.5	34.4 ± 2.5
Glucose (g/dL)		102.5 ± 34.0	101.2 ± 29.2
Cholesterol		253.2 ± 41.1	257.3 ± 39.2
Triglycerydes		157.7 ± 42.2	169.2 ± 72.5
Neonates			
Gestational age (weeks)		39.3 ± 1.8	39.6 ± 1.5
Gender	boys (%)	47,9 %	51.9 %
	girls (%)	52,1 %	48.1 %
Apgar 1		8.7 ± 0.7	8.6 ± 0.8
Apgar 2		9.0 ± 0.4	8.9 ± 0.5
Weight (kg)		3.3 ± 0.5	3.2 ± 0.5
Height (cm)		50.0 ± 2.5	50.2 ± 2.7
Craneal Perimeter (cm)		34.0 ± 1.5	34.1 ± 1.4
Weight gaining first year		6.3 ± 0.9	6.6 ± 0.9
Height increase first year		24.7 ± 2.4	25.3 ± 2.6
Craneal Perimeter increase (first year)		12.6 ± 6.3	11.92 ± 2.0

Values are means ± SD. SBP (Sistolic Blood Pressure); DBP (Distolic Blood Pressure).

TABLE 3. Plasma fat soluble antioxidants in mother and their neonates.

MOTHER				
	M0	M1	M2	M3
Retinol (μmol/L)				
FO Group	1.85 ± 0.10	1.93 ± 0.17	5.71 ± 0.29*	5.06 ± 0.21
CT Group	1.68 ± 0.12	1.89 ± 0.18	4.61 ± 0.30	4.71 ± 0.25
α-Tocopherol (μmol/L)				
FO Group	24.31 ± 0.97	26.07 ± 2.19	26.37 ± 0.91*	23.36 ± 0.84
CT Group	25.73 ± 1.46	27.00 ± 2.54	23.12 ± 1.22	22.38 ± 1.18
β-Caroteno (μmol/L)				
FO Group	0.59 ± 0.09	0.52 ± 0.06	1.29 ± 0.18*	1.40 ± 0.20*
CT Group	0.67 ± 0.11	0.56 ± 0.08	0.94 ± 0.12	0.92 ± 0.11
Coenzyme Q9 (nmol/L)				
FO Group	43.45 ± 3.19	39.81 ± 3.44	36.87 ± 3.27	30.51 ± 1.74
CT Group	43.11 ± 2.86	37.03 ± 2.85	37.18 ± 5.03	30.97 ± 2.25
Coenzyme Q10				
FO Group	0.92 ± 0.02	0.95 ± 0.09	0.69 ± 0.09	0.66 ± 0.04*
CT Group	0.92 ± 0.06	0.93 ± 0.06	0.63 ± 0.03	0.56 ± 0.03
NEONATE				
	NOV	NOA	N1	
Retinol (μmol/L)				
FO Group	1.84 ± 0.11*	1.71 ± 0.15	1.84 ± 0.16	
CT Group	1.55 ± 0.09	1.53 ± 0.18	1.68 ± 0.15	
α-Tocopherol (μmol/L)				
FO Group	6.09 ± 0.56*	6.29 ± 0.57*	15.39 ± 1.47	
CT Group	4.97 ± 0.24	4.75 ± 0.59	14.89 ± 1.95	
β-Caroteno (μmol/L)				
FO Group	0.14 ± 0.05	0.07 ± 0.01	0.61 ± 0.20	
CT Group	0.09 ± 0.01	0.08 ± 0.01	0.59 ± 0.26	
Coenzyme Q9 (nmol/L)				
FO Group	7.13 ± 0.74	8.16 ± 0.29*	15.11 ± 2.23	
CT Group	6.55 ± 0.26	7.07 ± 0.34	17.30 ± 2.91	
Coenzyme Q10				
FO Group	0.16 ± 0.02*	0.16 ± 0.03	0.71 ± 0.04*	
CT Group	0.12 ± 0.01	0.13 ± 0.01	0.59 ± 0.05	

Values are means ± S.E.M. * means statistically significant differences between groups ($P<0.05$). M0: at recruitment; M1: at delivery; M2: at 2.5 months postpartum,; M3: at 4 months postpartum; NOV: umbilical cord vein; NOA: umbilical cord artery; N1: at 2.5 months of life.

TABLE 4. Erythrocyte membrane fat soluble antioxidants in mother and their neonates.

	MOTHER			
	M0	**M1**	**M2**	**M3**
α-Tocopherol				
FO Group	0.98 ± 0.10	0.43 ± 0.06	1.66 ± 1.15*	1.98 ± 0.23
CT Group	1.06 ± 0.09	0.40 ± 0.05	1.25 ± 0.15	1.78 ± 0.13
Coenzyme Q10				
FO Group	12.31 ± 0.78	15.10 ± 1.26*	13.37 ± 1.29	11.95 ± 0.89
CT Group	13.01 ± 0.56	12.29 ± 0.91	12.30 ± 1.02	11.64 ± 0.71

	NEONATE		
	NOV	**NOA**	**N1**
α-Tocopherol			
FO Group	0.38 ± 0.06	0.42 ± 0.07*	0.69 ± 0.08
CT Group	0.42 ± 0.08	0.27 ± 0.02	0.58 ± 0.08
Coenzyme Q10			
FO Group	30.69 ± 3.96*	22.29 ± 1.76*	18.36 ± 1.45
CT Group	20.54 ± 3.05	17.91 ± 1.58	16.45 ± 1.77

Values are means ± S.E.M. * means statistically significant differences between groups (P<0.05). M0: at recruitment; M1: at delivery; M2: at 2.5 months postpartum,; M3: at 4 months postpartum; NOV: umbilical cord vein; NOA: umbilical cord artery; N1: at 2.5 months of life.

TABLE 5. Cytosolic antioxidant enzymes in mothers and their neonates.

MOTHER	M0	M1	M2	M3
SOD (U/mg)				
FO Group	118.72 ± 8.34	130.82 ± 6.78*	134.44 ± 8.13	151.55 ± 5.93
CT Group	114.67 ± 8.84	111.76 ± 6.50	124.21 ± 10.51	138.58 ± 7.04
Catalase (U/mg)				
FO Group	71.64 ± 3.85	69.32 ± 2.52	74.22 ± 3.43*	74.50 ± 8.09
CT Group	67.43 ± 3.63	74.31 ± 3.63	64.87 ± 3.96	68.67 ± 9.70
GPx (U/mg)				
FO Group	126.75 ± 6.98	139.31 ± 7.16	157.18 ± 7.74	174.96 ± 9.07
CT Group	133.71 ± 5.74	148.00 ± 6.88	166.09 ± 5.48	170.14 ± 11.90

NEONATE	NOV	NOA	N1
SOD (U/mg)			
FO Group	134.71 ± 9.18	150.00 ± 5.68	140.09 ± 6.98*
CT Group	128.16 ± 7.70	139.06 ± 10.42	121.40 ± 7.07
Catalase (U/mg)			
FO Group	70.12 ± 3.98	63.86 ± 6.22	70.02 ± 3.71*
CT Group	65.76 ± 3.16	63.53 ± 6.22	59.22 ± 3.49
GPx (U/mg)			
FO Group	127.08 ± 11.44	124.83 ± 10.98	115.50 ± 8.57
CT Group	121.92 ± 10.98	101.09 ± 8.77	109.87 ± 8.64

Values are means ± S.E.M. * means statistically significant differences between groups ($P<0.05$). M0: at recruitment; M1: at delivery; M2: at 2.5 months postpartum,; M3: at 4 months postpartum; NOV: umbilical cord vein; NOA: umbilical cord artery; N1: at 2.5 months of life.