

## Maternal Supplementation with *RRR*- $\alpha$ -Tocopherol (400 IU) and Its Relationship with Serum and Breast Milk Retinol

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### Abstract

Vitamin A and E are important during pregnancy, the neonatal period, and childhood. The objective of this study was to assess whether maternal *RRR*- $\alpha$ -tocopherol supplementation affects serum and breast milk retinol. Serum was collected at baseline and twenty days later, and breast milk, at baseline, and on days 1, 7, and 20 after delivery. After the baseline serum collection, the supplemented group (n=16) received a single 400 IU of *RRR*- $\alpha$ -tocopherol. The control group (n=18) was only performed collections. Retinol and alpha tocopherol levels were determined by liquid chromatography. Serum retinol and alpha tocopherol at baseline and 20 days after delivery indicated proper vitamin A (> 20  $\mu$ g/dL) and E (> 516  $\mu$ g/dL) statuses in the control and supplemented groups ( $p > 0.05$ ). Colostrum retinol levels on days 1 and 7 after delivery were significantly higher in the supplemented group ( $p = 0.018$  and  $p = 0.012$ , respectively). Maternal vitamin E supplementation increased colostrum retinol by 52.23% and 111.2%, 24 hours and 7 days, respectively. However, retinol in mature milk did not differ between the groups ( $p > 0.05$ ). In conclusion, the supplementation with 400 IU of *RRR*- $\alpha$ -tocopherol improved vitamin A bioavailability in breast milk.

Keywords: vitamin E; vitamin A; maternal serum; lactation; liquid chromatography.

### 1. Introduction

Vitamin A is an essential micronutrient for many metabolic processes, and it is especially important during times of rapid cell proliferation and differentiation, such as pregnancy, the neonatal period, and childhood. This vitamin acts as a carrier of mannosyl residues, in the synthesis of some glycoproteins, and in the production of mucus and resistance to infections mediated by the modulating action of cell growth and differentiation [1].

The term vitamin A is generically used for all beta-ionone derivatives that have the biological activity of all-trans retinol or that are structurally correlated to it, except retinoids, which are called provitamin A [2,3].

This vitamin is present in some foods in two forms: preformed vitamin A and provitamin A of the carotenoids. Preformed vitamin A is present in animal foods, such as eggs, liver, and dairy products. Provitamin A is present in green and yellow vegetables, and in some orange fruits [2-4].

Vitamin A deficiency can lead to xerophthalmia and night blindness in children, to anemia, and to low resistance to infections, which can increase the severity of infectious diseases and the risk of death [2,3]. Preschoolers and pregnant women are the populations at greatest risk of vitamin A deficiency [5].

Vitamin E also has an important role in the health of newborns. Like vitamin A, it is a fat-soluble vitamin, and its main function is its antioxidant activity in cell membranes as it can inhibit lipid peroxidation [6]. This vitamin has eight chemical compounds, namely  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocopherol, and tocotrienols, but these compounds have different structures and bioavailability. Alpha-tocopherol is the most active form. The other forms do not contribute to the nutritional requirements of vitamin E [4].

Alpha-tocopherol is an efficient inhibitor of lipid peroxidation *in vivo* as it donates a hydrogen to the peroxy radical, interrupting chain reactions and consequently, protecting the long-chain polyunsaturated fatty acids and lipoproteins in cell membranes from oxidation; it is the main fat-soluble antioxidant in tissues [6].

Preterm infants are born with sparse body fat and low body stores of fat-soluble vitamins, including vitamin E [7]. Newborns are considered a high-risk group for vitamin E deficiency. Insufficient consumption of vitamin E at this phase of life can compromise the immune system and affect lung development [8]. However, giving extra vitamin E to preterm babies can provide some benefits, but it increases the risk of life-threatening infections. Preterm babies (born before 37 weeks) can develop a range of problems because their organs are not mature. Vitamin E may be able to help prevent or limit some

of these problems, but it can potentially also have harmful effects [9]. The main source of alpha tocopherol for newborns is breast milk. Alpha tocopherol prevents possible damage from oxidative stress generated by the transition from a prenatal intrauterine environment relatively poor in oxygen to a postnatal extra uterine environment considerably richer in oxygen. Maternal supplementation with alpha tocopherol can be an efficient way to boost vitamin E levels in neonates [8]

Vitamins A and E have critical roles on the satisfactory health and development of newborns. Yet, fetal reserves of vitamins A and E are limited possibly because of placental barrier selectivity, which regulates the passage of both vitamins from the mother to the fetus, probably to avoid teratogenic effects. Hence, breast milk is the only source of these nutrients for exclusively breastfed children. Breast milk provides antioxidant protection and stimulates the newborn's immune system development [10].

Some mammal, including human, studies have found that vitamin A supplementation can reduce serum and/or breast milk  $\alpha$ -tocopherol because these vitamins share some tissue transport and distribution pathways [5,11,12]. On the other hand, [13] found that fat-soluble vitamins are absorbed in different sites in rats: vitamin A is more widely absorbed in the proximal region, and vitamin E, in the distal region of the small intestine. Moreover, the same study found that when vitamins A and E are taken together, some vitamin E is degraded, which reduces its absorption, and vitamin E also protects vitamin A from oxidation.

The purpose of this study was to assess whether maternal supplementation with RRR- $\alpha$ -tocopherol (400 IU) affects serum and breast milk retinol. The null hypothesis was that breast milk and serum vitamin A levels were not influenced by vitamin E supplementation.

## **2. Materials and Methods**

### **2.1 Participants**

This prospective, controlled, and randomized study included two groups of volunteers recruited at the School Maternity Hospital Januário Cicco and Unidade Mista das Quintas, both in Natal-RN, Brazil. These institutions were chosen because they provide obstetric services for women from Natal and other municipalities in the state of Rio Grande do Norte.

Data and samples were collected from March 2016 to March 2017, and the women were followed for 20 days after delivery. The study was approved in 07 March 2016 by the Research Ethics Committee of the Federal University of Rio Grande do Norte (UFRN) under protocol number 1.441.203 CAAE 43894615.4.0000.5537, complied with the Declaration of Helsinki guidelines, and was registered at ReBEC (Brazilian Registry of Clinical Assays) under code number RBR-9wch5m. The study is available at: <http://www.ensaiosclinicos.gov.br/rg/?q=RBR-9wch5m>.

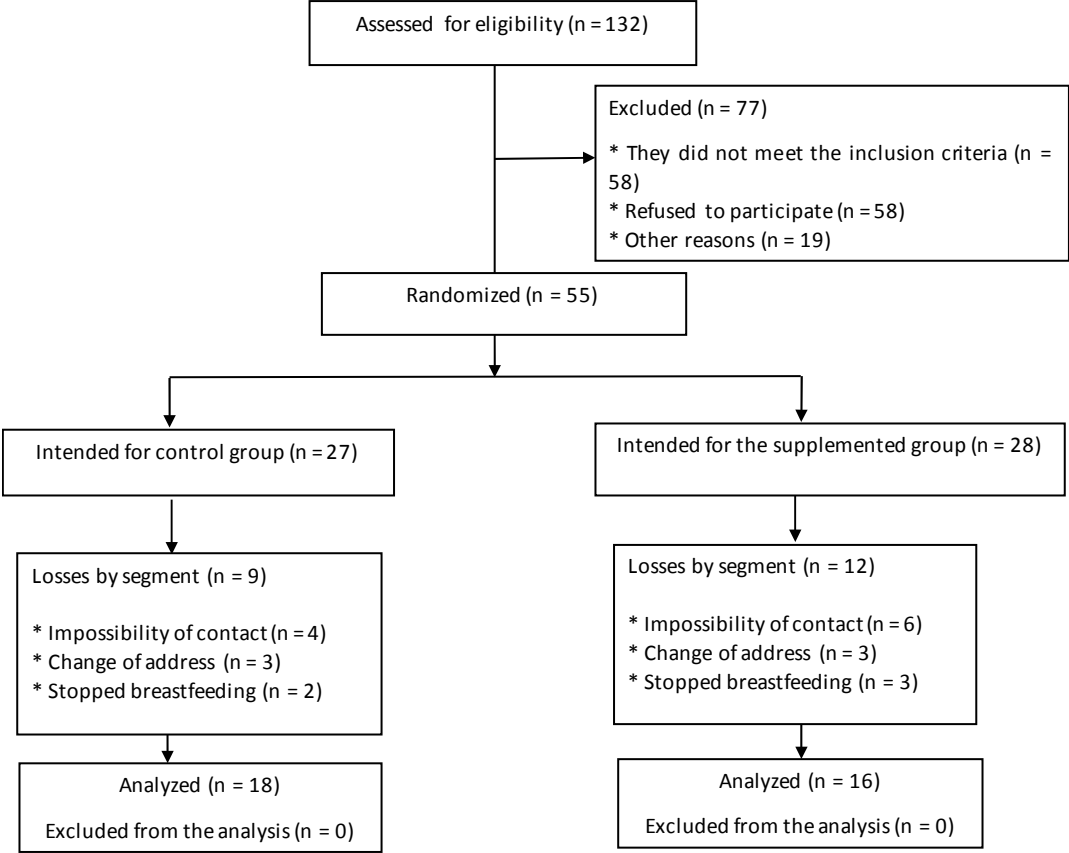
The sample size was calculated by the software GPower® version 3.1.9.2 [14] based on the following parameters:  $\alpha$  of 5%; power of 0.8; and expected effect of 0.40. Hence, each group should have at least twelve individuals.

The participants were informed about the objectives of the study and signed an informed consent form before enrollment. The inclusion criteria were age between 18 and 40 years, low obstetric risk, and term delivery (gestational age equal to or greater than 37 weeks). The exclusion criteria were: use of multivitamins during pregnancy and/or lactation, maternal complications or acute/chronic diseases, preterm delivery (gestational age < 37 weeks), fetal malformation, and/or multiple pregnancies.

The first woman to deliver was allocated to the control group (CG), the second to the supplemented group (SG), the third to the CG, and so on. This study was organized as recommended by the Consolidated Standards of Reporting Trials - CONSORT (Figure 1).

The CG did not receive any intervention. The supplemented group received a single 400 IU capsule of *RRR*- $\alpha$ -tocopherol (d- $\alpha$ -tocopherol acetate, gelatin (bovine), soybean oil, glycerin) on the first day after delivery and after collection of the biological material. The supplement was taken orally with water.

A 400 IU dosage of *RRR*- $\alpha$ -tocopherol corresponds to 268.5 mg of  $\alpha$ -tocopherol. This dosage was chosen because it has been considered safe by the Institute of Medicine [15] after analysis of long-term studies, which found no deleterious effects associated with this dosage.



**Figure 1** - Consolidated Reporting Trials (CONSORT) flow diagram.

**2.2 Collection of data and biological material**

Socioeconomic, prenatal, and delivery data, clinical history, and biochemical tests were collected from the patients’ medical records.

Blood and breast milk were also collected. On the first day after delivery, immediately after collection of five milliliters of blood and two milliliters of breast milk from all participants, the participants in the SG received a 400 IU capsule of *RRR-α*-tocopherol. No intervention was done on the CG. On the second day after delivery, two milliliters of breast milk were collected from all participants. On the seventh day after delivery, two milliliters of breast milk were collected from all participants, and on the twentieth days after delivery, five milliliters of blood were collected from all participants. All collections were done in the morning after an overnight fast. Of the 55 study women, 34 completed the study, 18 in the CG and 16 in the SG.

The biological materials were collected and stored in opaque polypropylene tubes, which were immediately refrigerated and delivered to the Laboratory of Food and

Nutrition Biochemistry of UFRN. The blood samples were centrifuged at 4000 rpm for 10 minutes to separate the serum. Maternal serum and milk were stored at -20 °C for zero to four days until analysis.

### 2.3 Biochemical analyses

Serum  $\alpha$ -tocopherol and retinol were extracted as recommended by Ortega et al. [16]. One mL of 95% ethanol (Merck, Darmstadt, Germany) and 2 mL of hexane (Merck) were added to 1 mL of serum.

Later, the samples were homogenized for one minute and centrifuged for ten minutes to separate the hexane phase. This stage was repeated three times, totaling 6 mL of hexane phase. Two mL of hexane phase were collected from each sample and the hexane was evaporated in a water bath at 37°C. The extract was then diluted with 250  $\mu$ L of absolute ethanol (Vetec, Speyer, Germany), and 20  $\mu$ L were injected in the high-performance liquid chromatography (HPLC) device.

Breast milk retinol was extracted as recommended by Giuliano et al. [17]. Five hundred mL of 95% ethanol and 500  $\mu$ L of 50% w/v potassium hydroxide were added to 500  $\mu$ L of breast milk. The mixture was vortexed for one minute, placed in a water bath at 60°C for one hour, and stirred at 10-minute intervals. Two mL of hexane was added to the mixture, and the mixture was again vortexed for one minute and centrifuged (500 g) for 10 minutes. The hexane phase was then transferred to another tube. This process was repeated three times. Aliquots of 3 mL of hexane were collected from the baseline and on days 1, 7 and 20 breast milk samples. These aliquots were evaporated in a water bath at 37 °C. The extract was diluted with 250  $\mu$ L of absolute ethanol, and 20  $\mu$ L were injected in the HPLC device.

Retinol was extracted from mature milk as recommended by Giuliano et al. [17], described above. However, only one mL of breast milk was extracted, so only one mL of 95% ethanol (Merck) and one mL of 50% w/v potassium hydroxide were added to it. The dry extract was then diluted with 250  $\mu$ L of absolute ethanol.

The amounts of  $\alpha$ -tocopherol and retinol in the biological samples were measured by the chromatographic device Shimadzu (Shimadzu Corp., Kyoto, Japan). This device contains the pump LC-10 AD Shimadzu coupled to the detector SPD-10A Shimadzu UV-VIS, the column CLC-ODS (M) 4.6 x 250 mm (Perkin Elmer, Boston, MO, USA), and the integrator Chromatopac C-R6A Shimadzu. The isocratic mobile phase in all analyses

consisted of 100% methanol flowing at 1.0 ml/min. The wavelengths used for quantifying  $\alpha$ -tocopherol and retinol were 292 nm and 325 nm, respectively.

Alpha-tocopherol and retinol in the samples were identified and quantified by comparing the retention time and area provided by the chromatographic profile with standard stock solutions of  $\alpha$ -tocopherol and retinol, respectively (Sigma, St Louis, MO, USA). The concentrations of the standards were confirmed by the specific extinction coefficients in absolute ethanol for  $\alpha$ -tocopherol ( $\epsilon$  1%, 1 cm = 75.8 at 292 nm) and retinol ( $\epsilon$  1%, 1 cm = 1,780 at 325 nm)[18,19].

Maternal vitamin E deficiency was defined as serum  $\alpha$ -tocopherol < 516  $\mu\text{g/dL}$  (12  $\mu\text{mol/L}$ ), the cut-off point for healthy adults [15]. A cut-off point for adequate vitamin E in breast milk does not exist.

Maternal vitamin A deficiency (VAD) was defined as serum retinol < 20  $\mu\text{g/dL}$  (0.70  $\mu\text{mol/L}$ ) [20], and low serum retinol was defined as serum retinol between 20 and 30  $\mu\text{g/dL}$  (0.70 and 1.05  $\mu\text{mol/L}$ ) [21].

Low colostrum retinol was defined as colostrum retinol  $\leq$  60  $\mu\text{g/dL}$  (2.1  $\mu\text{mol/L}$ ) [22], and low mature milk retinol was defined as mature milk retinol < 30  $\mu\text{g/dL}$  (1.05  $\mu\text{mol/L}$ ) [20].

## 2.4 Statistical analyses

The statistical analyses were performed by the software IBM SPSS version 21.0 for Windows (SPSS Inc., Chicago, IL, USA). Serum and breast milk  $\alpha$ -tocopherol and retinol were expressed as means, standard deviations, and percent frequency. The Shapiro-Wilk test confirmed that all numerical variables had normal distribution. Group homogeneity was verified by the chi-square test. The Student's t-test for independent samples compared the serum vitamins between the groups, and the Levene test verified intergroup variance homogeneity. The Student's t-test for paired samples tested the difference between intragroup vitamin levels. The significance level was set at 5% ( $p < 0.05$ ).

## 3. Results

Among 132 women recruited before delivery to participate in the study, 77 were excluded due to lack of milk or blood data, and 55 were included in the characterization, 27 of the control group and 28 of the supplemented group.

Table 1 presents the maternal, obstetric and neonatal characteristics. The participants had a mean educational level, 23 (46%) had monthly income between 1 and 3 minimum wages, 31 (56%) had normal birth and 24 (47%) were overweight or obese at the end of gestation. None of the characteristics mentioned above were associated with serum retinol ( $p > 0.05$ ), and the groups were homogeneous.

**Table 1** – Characterization of the study population based on maternal and newborn variables.

Characteristics	Control group		Supplemented group	
	% (n)	Mean (standard deviation)	% (n)	Mean (standard deviation)
<b>Age (years)</b>		25 (6)		24 (6)
<b>Marital status</b>				
Single	19 (5)		18 (5)	
Married/domestic partner	81 (22)		82 (23)	
<b>Education level</b>				
Incomplete elementary school	30 (8)		40 (11)	
Complete elementary school	6 (2)		0 (0)	
Incomplete high school	30 (8)		18 (5)	
Complete high school	30 (8)		31 (9)	
Incomplete higher education	4 (1)		7 (2)	
Higher education (n, %)	0 (0)		4 (1)	
<b>Monthly income (BMW)<sup>a</sup></b>				
No regular income	4 (1)		8 (2)	
< 1 minimum salary	40 (10)		36 (9)	
1 to 3 minimum salaries	48 (12)		44 (11)	
3 to 5 minimum salaries	8 (2)		12 (3)	
<b>Type of delivery</b>				
Vaginal	52 (14)		61 (17)	
Caesarean	48 (13)		39 (11)	
<b>BMI gestational (kg m<sup>-2</sup>)<sup>b</sup></b>		29 (5)		30 (5)
<b>Maternal nutritional status<sup>**</sup></b>				
Low weight	30 (7)		4 (1)	
Normal weight	35 (9)		40 (10)	
Excess weight / obesity	38 (10)		56 (14)	
<b>Birthweight (g)</b>	—	3000 (240)		3370 (390)
<b>Birth height (cm)</b>	—	49 (2)		49 (2)

n, number; <sup>a</sup>BMW, minimum Brazilian wage; One minimum Brazilian wage = \$US 372;

<sup>b</sup>BMI, body mass index; BMI / gestational week (Atalah et al., 1997).

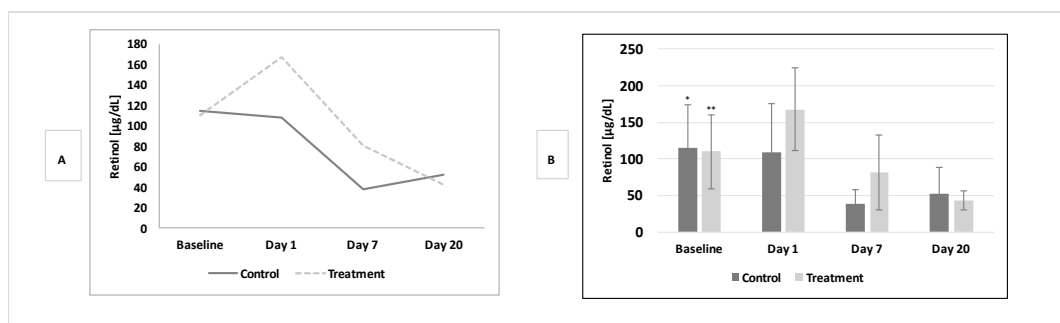


In the biochemical analysis 34 women participated in the study, 18 of the control group and 16 of the supplemented group. Baseline serum  $\alpha$ -tocopherol ( $\mu\text{g/dL}$ ) and retinol ( $\mu\text{g/dL}$ ) of the control group were  $1195 \pm 328$  and  $44 \pm 14$ , respectively, and on day 20,  $908 \pm 439$  and  $43 \pm 12$ , respectively. Baseline serum  $\alpha$ -tocopherol ( $\mu\text{g/dL}$ ) and retinol ( $\mu\text{g/dL}$ ) of the supplemented group were  $1184 \pm 411$  and  $42.5 \pm 13$ , respectively, and on day 20,  $956 \pm 381$  and  $42.2 \pm 12$ , respectively. Those levels did not differ between the groups on any occasion ( $p > 0.05$ ).

Both groups had appropriate serum levels of  $\alpha$ -tocopherol and retinol according to the established cut-off points.

Colostrum retinol levels in the control group were:  $114.3 \pm 60 \mu\text{g/dL}$  at baseline;  $108 \pm 66 \mu\text{g/dL}$  on day 01 after delivery;  $38.5 \pm 19.5 \mu\text{g/dL}$  on day 07 after delivery; and  $53 \pm 36.3 \mu\text{g/dL}$  in mature milk. Colostrum retinol levels in the supplemented group were:  $110 \pm 50.4 \mu\text{g/dL}$  at baseline;  $167.3 \pm 57 \mu\text{g/dL}$  on day 01 after delivery;  $81.3 \pm 51.3 \mu\text{g/dL}$  on day 07 after delivery; and  $43 \pm 13.2 \mu\text{g/dL}$  in mature milk.

The main significant effect appeared when breast milk retinol levels were compared over time ( $p < 0.01$ ,  $\eta^2 = 0.544$ ), but a significant effect was not found between the groups ( $p = 0.506$ ,  $\eta^2 = 0.032$ ). A significant effect was found when the interaction between the factors 'breastfeeding duration' and 'study groups' was analyzed ( $p < 0.01$ ,  $\eta^2 = 0.279$ ). Thus, the colostrum retinol of the supplemented group had increased by 52.23% 24 hours after the intervention. When the two groups were compared on day 07 after the intervention, the breast milk retinol of the supplemented group was 111.17% higher than that of the control group (Figure 2).



**Figure 2.** Retinol profile from baseline (immediately after delivery) to 20 days after delivery in the control and treatment groups. Natal/RN, 2016 – 2017.

A – postnatal effect of breast milk retinol. Split-plot ANOVA:  $p < 0.01$ ,  $\eta^2 = 0.544$ .

B - \* breast milk retinol difference between baseline and day 07 ( $p = 0.001$ ); between day 01 and day 07 ( $p = 0.008$ ); between baseline and day 20 ( $p < 0.01$ ); and between day 01 and day 20 ( $p = 0.003$ ).

\*\* breast milk retinol difference between baseline and day 01 ( $p = 0.008$ ); between baseline and day 20 ( $p < 0.01$ ); between day 01 and day 07 ( $p < 0.01$ ); between day 01 and day 20 ( $p < 0.01$ ); and between day 07 and day 20 ( $p = 0.006$ ). One-way ANOVA.

#### 4. Discussion

A balanced diet during pregnancy is necessary to guarantee the availability of nutrients to the fetus, thereby preparing the fetus for birth and breastfeeding. All nutrients are essential during this period, but vitamin A stands out because of its important role in the cell growth and proliferation that occurs during pregnancy, breastfeeding, and early childhood [23].

Micronutrient deficiency during pregnancy may cause numerous adversities for maternal and fetal health. During lactation, nutritional deficiencies may contribute to infants' low nutrient reserves, thus increasing the prevalence of nutritional deficiencies in the first years of life [24].

The two study groups consisted of adult women who were normal weight at conception and birthed normal birth weight infants, confirming other studies in similar populations [4,25].

Serum  $\alpha$ -tocopherol and retinol levels in both groups indicated that both groups had proper vitamin E and A statuses, respectively. The baseline serum alpha tocopherol levels of the study control and supplemented groups were similar to the levels reported by other studied similar populations [4,26,27]. However, the study results are higher than those reported by Rodriguez et al.[28], but the difference may stem from the population studied by the latter, namely, a population living in poverty with terrible health indicators. The alpha tocopherol levels of the study groups were lower than those reported by Garcia et al. [11] and Dimenstein et al. [29], which also may be justified by their samples, consisting of adult and adolescent women.

The baseline serum retinol levels of the study control and supplemented groups are similar to those reported by Fernandes et al. [30]; Lima et al. [10] and Grilo et al. [25], who studied women from the Brazilian northeast region. However, the baseline serum retinol levels of the study control and supplemented groups were higher than those found in India [31], Africa, [32,33], and Ethiopia [34] as these countries have high prevalence of vitamin A deficiency, and in Germany [35], probably because of the very different

sample size. On the other hand, Yang et al. [36] reported a higher level of serum retinol possibly because of different diets of the two samples and sampling methods.

Breast milk is the best food for newborns because of its ideal nutritional characteristics, high levels of fat-soluble vitamins [37], and ability to provide important immune and psychological factors that reduce infant morbidity and mortality [38]. Some studies have suggested that colostrum may be a biochemical indicator of vitamin A status; therefore, breast milk vitamin A can be used for monitoring the impact of vitamin A-related intervention programs as it is easy to obtain in a non-invasive manner [37,39].

The baseline colostrum retinol levels in the study control and supplemented groups were higher than those reported in Cuba [22], where women have very bad health, and in Brazil [25] possibly because of different sample sizes. The study retinol level was lower than that reported in Germany [37], where people have better access to food and counseling during pregnancy and puerperium.

Countries with high risk of vitamin A deficiency have a supplementation policy that consists of giving mothers a megadose of vitamin A (200,000 IU) at most six weeks after delivery to increase the level of this vitamin in breast milk (World Health Organization 2009). Study [25] have assessed the action of vitamin A on vitamin E, and the present study investigated for the first time whether maternal supplementation of vitamin E after delivery increases retinol, which it does as colostrum retinol 24 hours after vitamin E supplementation increased and remained high until the seventh day after supplementation.

Moore [40] found that vitamin E in the diet of rats increased vitamin A absorption and storage, corroborating the present study.

Gonçalves et al. [13] found that the fat-soluble vitamins are absorbed in different sites in rats, specifically, that vitamin A is mostly absorbed in the proximal region, and vitamin E, in the distal region of the intestine, and that vitamin E increased the absorption of vitamin A by 40%. This finding in rats is in agreement with the finding of the present study in humans.

Vitamin E supplementation did not affect mature milk retinol twenty days after the intervention. Because of its unique composition, colostrum is rich in vitamin A, but over time the composition of breast milk changes and its level of vitamin A decreases rapidly [37]. The present study found a significant decrease in breast milk retinol in both groups, confirming other literature reports [22,25,41].

Maternal supplementation with vitamin E was beneficial in the first days of breastfeeding but not later. A hypothesis to explain this finding would be that vitamin A and vitamin E may have supplied a maternal nutritional deficiency and from then on, the nutritional requirements of the newborn were not met. Based on the present study results, daily maternal supplementation with smaller doses of vitamins A and E after delivery could contribute to the proper statuses of these vitamins in the mother and newborn.

## 5. Conclusions

Maternal supplementation with vitamin E did not influence serum or mature milk retinol, but it increased colostrum vitamin A. Therefore, vitamin E improved the bioavailability of vitamin A in breast milk.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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