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Zinc, Beta-Carotene, and Vitamin D_3 Supplementation and Placental IL-1β in Spontaneous Preterm Labor

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Abstract Preterm birth (PTB) is one of the leading causes of neonatal death. Studies have shown that Interleukin-1 (IL-1β) critically contributes to inflammation-induced PTB, and micronutrients deficiencies might influence the inflammatory pathways. Since zinc, vitamin A, and vitamin D deficiencies are common in Indonesia, this study aims to evaluate those nutrients supplementation on placental IL-1β in PTB. A quasi-experimental study was conducted in pregnant women who underwent spontaneous delivery. They were divided into 3 groups; control-term group (n=25), control-preterm group (n=27), and experimental-preterm group (n=26). No intervention was given to the control-term and the control-preterm groups, while the experimental-preterm group was given zinc 50 mg/day, a single dose beta-carotene 25,000 IU, and vitamin D3 50,000 IU/week until delivery. Zinc, all-trans retinoic acid (AtRA) and 25-hydroxyvitamin D (25(OH)D) concentrations in maternal serum were measured before and after intervention, followed by assessment of placental concentration of those nutrients and IL-1β expression after delivery. One-way ANOVA and Kruskal Wallis tests were performed for comparison analysis and using Pearson or Spearman test for correlation analysis correlation test using Statistical Package for Social Sciences (SPSS). No significant difference in zinc, AtRA, and 25(OH)D concentrations was found in maternal serum, before and after the intervention. However, the experimental-preterm group had the lowest IL-1β concentration (7.17 pg/g (0.45–283.29), p=0.009). This study suggests that the administration of zinc, beta-carotene, and vitamin D3 in PTB was associated with lower placental IL-1β expressions, although it did not directly increase those nutrient concentrations in serum and placenta.

Keywords: *preterm birth, micronutrients, Indonesian women*

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1. Introduction

Indonesia ranked fifth out of ten countries for the highest rate of preterm birth (PTB) (15.5%), and ninth out of ten countries for the highest number of PTB per 100 live births. [1,2] The rates of PTB in Cipto Mangunkusumo Hospital as a tertiary referral hospital in 2015, 2016, and 2017 were 50.55%, 48.43%, and 48.35%, respectively. This high prevalence of PTB in Indonesia leads to an increase in neonatal morbidity and mortality rate. This is because preterm babies have increased risks of infections, respiratory and gastrointestinal complications in early life, in addition to neurodevelopmental, behavioral, cardiometabolic, and inflammatory disorders later in life. There are compelling shreds of evidence from basic science and clinical studies which demonstrate that intrauterine infection or inflammation is strongly associated with fetal inflammation and injuries that are commonly identified in preterm infants. [3,4] Studies have shown that IL-1β may cause fetal inflammatory response syndrome such as respiratory distress. [5]

The inflammatory process is an important physiological process in labor as pro-inflammatory cytokines play a pivotal role in the induction of uterine activation proteins. However, in PTB, this process was found to be pathological, marked by increased aberrant cytokines production. Excessive inflammation could occur early or later during gestation. Maternal stress, diet, endocrine dysfunction, metabolic dysregulation, microbiota composition, or infection could exacerbate the inflammatory burden, leading to PTB. Among all cytokines implicated, interleukin 1 β (IL-1β) has been the more largely studied one, revealing its central role in PTB. High IL-1β maternal plasma concentrations have been associated with preterm labor. Also, a high IL-1β level was found in the blood concentration of preterm neonates. These findings suggested the role of IL-1β as a new therapeutic target of PTB in numbers of studies. [3,6]

Furthermore, macronutrient and micronutrient malnutrition are also important risk factors in promoting intrauterine inflammation related to PTB. Deficiencies of folic acid, vitamin A, vitamin B_{12}, vitamin C, vitamin D, zinc, iron, copper, and docosahexaenoic acid (DHA) during

pregnancy can influence the inflammation signalling pathways and reduce placental oxidative damage, all of which increase the risk of preterm birth. Numbers of studies have risen more attention to nutritional intervention against pathogenic infections and inflammations. Evidence showed that periconceptional supplementation of these substances may reduce the risk of PTB by downregulating pro-inflammatory cytokines such as IL-6, IL-1β, TNF-a, and IFN-g. [7,8] Multiple nutrients such as branched-chain amino acids, short-chain fatty acids, zinc, vitamin A and vitamin D_3 increase antimicrobial peptides (AMPs)/host defense peptides (HDPs) expression, including in intestinal mucosa. HDPs can enhance innate and adaptive immunity functions to reduce inflammatory reactions and enhance antimicrobial capacity.[9]

Nutrients deficiencies such as energy, protein, zinc, vitamin A, and vitamin D remain a global challenge, especially in developing countries, including Indonesia. According to World Health Organization (WHO) data, the prevalence of vitamin A in Indonesia is estimated to be moderate (10 – 20%). Our previous study also found that 75% and 99% of first trimester pregnant women had deficiencies of zinc and vitamin D, respectively [10].

Insufficient amount of nutrition was suspected to highly contribute in an abnormal inflammatory response, especially in IL-1β, which could lead to PTB. If a short duration of multivitamin supplementation could reduce IL-1β expression in PTB, those multivitamins could become an important supplementation for pregnant women in preterm birth prevention in the future. Therefore, this study aims to investigate the effect of supplementation zinc, beta-carotene, and vitamin D in an inflammatory response in preterm birth by measuring serum and placental zinc, all-trans retinoic acid (AtRA), 25-hydroxyvitamin D (25(OH)D), as well as placental IL-1β.

2. Methods

2.1. Study Design and Workflow of Intervention

This was a quasi-experimental study conducted in Cipto Mangunkusumo National General Hospital and Budi Kemuliaan Hospital in Jakarta, within a period of 6 months. These are tertiary referral hospitals, which receive and treat referred cases from primary and secondary healthcare facilities. Thus, the obstetrical cases vary from normal pregnancy to pregnancy-related complications.

The sample size was calculated based on comparative-numeric analysis with more than 2 groups, with an effect size measured from the previous study of 30.[11] Thus, the minimum sample requirement was 24 for each group. Initially, there were 85 participants included in this study, who were divided into 3 groups; control-term group (n=25), control-preterm group (n=27), and experimental-preterm group (n=33). This study was restricted to pregnant women who underwent spontaneous delivery. Participants with spontaneous delivery in ≥ 37 weeks of gestational age were classified as the control-term group, whereas participants with spontaneous preterm delivery in 26 – 36 weeks of gestational age were classified into control-preterm and experimental-preterm groups. The only difference between control-preterm and experimental-preterm was the intervention given to the group. The exclusion criteria were subjects with drug hypersensitivity (for the experimental-preterm group), multiple pregnancies, intrauterine growth restriction (IUGR), fetal congenital anomaly, preterm premature rupture of membrane (PPROM), and other comorbidities (hypertension in pregnancy, preeclampsia, gestational diabetes mellitus, heart disease, autoimmune disease, etc.).

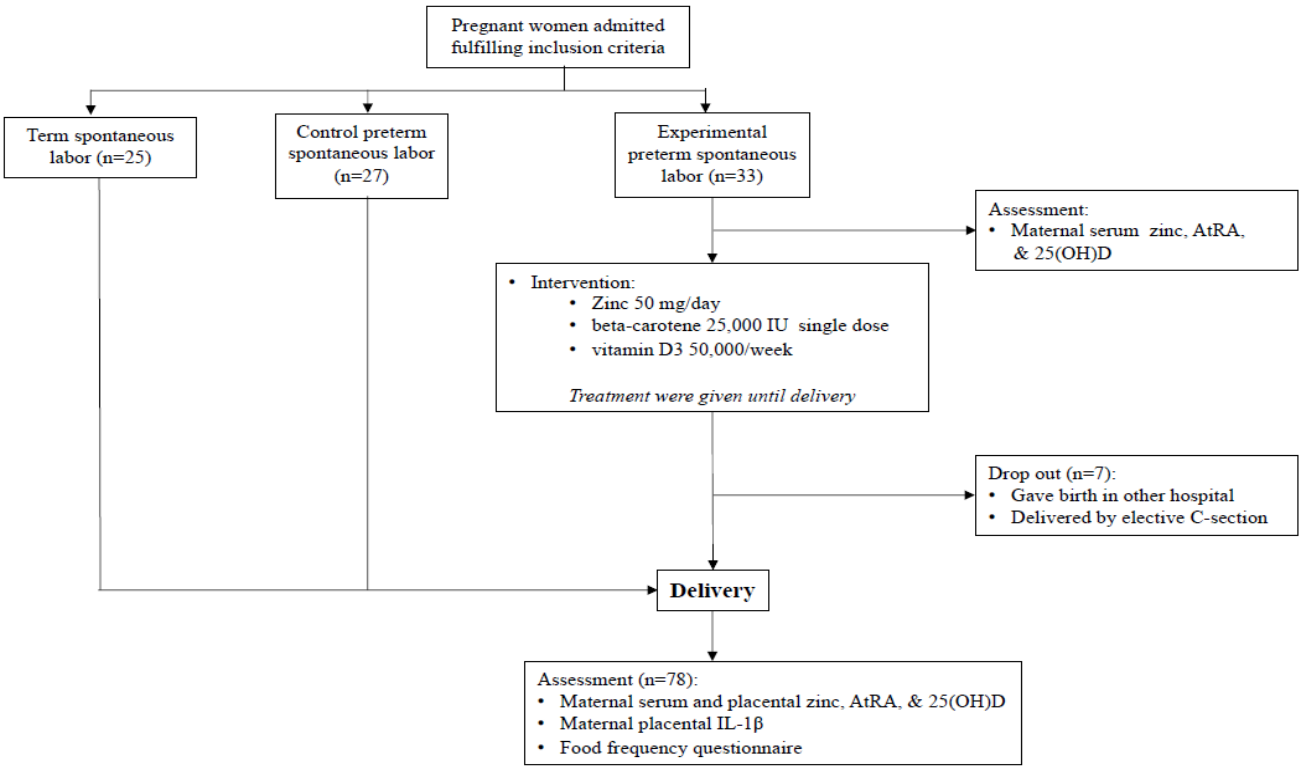

Figure 1. Workflow of The Study

The control-term and control-preterm groups had no intervention before delivery. For subjects in the experimental-preterm group, their serum concentration of zinc, AtRA, and 25(OH)D were evaluated prior the intervention. The interventions include a single dose of 25,000 IU oral beta-carotene, 50,000 IU of weekly oral vitamin D_3, and 50 mg of daily oral zinc from day 1 hospitalization until delivery. The presence of hypersensitivity and adverse effects were noted. During follow up, there were 7 drop-outs in the experimental-preterm group. Therefore, only 26 participants were included in the experimental-preterm group, with a total of 76 participants involved in this study. Following delivery, all participants had their serum and placenta examined as well as given the food frequency questionnaire. Figure 1 shows the workflow diagram.

Note that both spontaneous-preterm and experimental-preterm groups had been given preterm labor conservative management according to hospital protocol to ensure maternal and fetal well-being in preterm labor cases. This includes tocolytic with oral nifedipine 4 x 10 mg, and dexamethasone for lung maturation 2 x 6 mg for 48 hours.

This study was performed in accordance with the ethical guideline laid down in the Declaration of Helsinki. This study had been approved by Ethical Committee for Research in Humans from the Faculty of Medicine of Universitas Indonesia (LB.01.01/X.2/179/2016) and had been registered in clinicaltrials.gov. All of the participants agreed to participate and signed the informed consent prior to the study.

2.2. Nutrient Intake Assessment

Nutrient intake was assessed using the Semi-Quantitative – Food Frequency Questionnaire (SQ-FFQ) and was further processed in Nutrisurvey 2007 program. SQ-FFQ were also included multi-vitamins consumed by subjects of study. The interviews were managed by experienced nutritionists, while subjects underwent postpartum hospital care.

2.3. Serum Assays

Blood samples were taken soon after delivery for control-term and control-preterm groups. Blood samples from the experimental-preterm group were taken twice; before the intervention and soon after delivery. Up to 15 cc of venous blood was taken for sampling. Samples were put into serum separator tubes (SST) to measure AtRA and 25(OH)D concentrations and then placed into a trace element clot activator tube to measure zinc concentration.

2.3.1. Zinc

Briefly, 200 uL of a serum sample that collected with trace element tube and control material (ClinCheck Recipe, Germany) was transferred into a sample vial and 1800 uL of the diluent containing ammonia (Merck, Germany), EDTA Acid Trace Metal Basis (Aldrich, Germany), and Triton X-100 (Merck, Germany) in ultrapure water (ELGA Purelab Ultra, UK) was added and mix thoroughly. Diluted samples were injected into the Inductively coupled plasma mass spectrometry (ICPMS) system (Agilent 7700X, Agilent Technologies, USA) with an online addition of Indium internal standard. Analysis mode was set to Helium mode and the acquisition was set to Zinc as a selected element. The calibration curve was constructed from 8 levels of standard ranged from 0.02 – 250 ug/dL. Sample quantification was done using internal standard calculation with 10 times the total dilution factor.

2.3.2. AtRA

All trans-retinoic acid (AtRA) analysis was done through transferred 200 uL of serum to a microtube vial and then 200 uL of acetonitrile was added and mixed well. The mixture was then centrifuged at 14000 rpm for 10 minutes and the clear supernatant was transferred into an amber HPLC vial and injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) System (Agilent 6460 LCMS with 1290 binary pump, Agilent Technologies, USA). Liquid chromatography was developed as gradient elution consists of mobile phase A containing 0.1% formic acid in ultrapure water and mobile phase B containing 0.1% formic acid in acetonitrile gradient grade (Merck, Germany). Mass analyzer mode was set to multiple reaction monitoring (MRM). Calibration curve constructed from 7 levels of standard ranged 0.03 – 2 ng/mL and final concentration from samples were obtained with the external standard calculation.

2.3.3. 25(OH)D

25-OH Vitamin D3 analysis was done through transferred 50 uL of a serum sample, calibrator (ClinCal, Recipe, Germany), and Control material (ClinCheck Recipe, Germany) were added with 50 uL concentrated zinc sulfate and 10 uL 25 OH Vitamin D3 internal standard (ClinMass Recipe, Germany) followed by 100 uL acetonitrile and mix thoroughly. The mixture was then incubated for 10 minutes and centrifuged at 14000 rpm for 10 minutes. Clear supernatant transferred to HPLC vial and then injected to LC-MS/MS system (Agilent 6460 LCMS with 1290 binary pump, Agilent Technologies, USA). Liquid chromatograpy was developed as gradient elution consists of mobile phase A containing 0.1% formic acid in ultrapure water and mobile phase B containing 0.1% formic acid in methanol hyper grade (Merck, Germany). Mass analyzer mode was set to multiple reaction monitoring (MRM). Calibration curve constructed from 3 levels of standard ranged 2.25 – 74.4 ng/mL and final concentration from samples were obtained with the internal standard calculation.

2.4. Placental Examinations

Full-thickness placental samples were collected from the placental parenchyma. Each sample was put into phosphate buffer saline (PBS) to measure the concentrations of AtRA, 25(OH)D, zinc, and IL-1β. IL-1β measurement was performed using ELISA techniques with Quantikine ELISA human IL-1β HS (R & D Systems, Inc., Minneapolis, USA) Cat: HSLB00D, Lot: P145494). The blood and placental samples were examined in Prodia Laboratory, Jakarta. Investigators were blinded for the time of birth and supplementation intake.

2.5. Statistical Analysis

Data were processed with the Statistical Package for Social Sciences (SPSS) version 20.0 (IBM, United States). Each dependent variable was tested for homogeneity variance using the Shapiro Wilk normality test. Comparison between variables was tested using one-way ANOVA for normal distribution data and Kruskal Wallis for abnormal distribution data. Post Hoc analyses were used to identify significances among groups. Correlation between variables was tested using Pearson or Spearman correlation test. Data were supplemented with a 95% confidence interval (95% CI) with a significance limit of $p \leq 0.05$.

3. Results

3.1. Characteristics of the Subjects

Table 1 describes the characteristics of the subjects. Among the variables, no significance was found between groups. The median length of intervention in the experimental-preterm group was 22 days 18 hours. None of the patients complained about adverse reactions. The median gestational age at delivery in the experimental group was 37 weeks.

3.2. Nutrient Intake

Table 2 describes the nutrient intake of the subjects. Based on Recommended Daily Allowance (RDA) of Decree of Minister of Health of the Republic of Indonesia Number 75 the year 2013, most subjects had low consumption of energy, protein, zinc, iron, calcium, vitamin A, vitamin D, and folic acid.[12] No significant difference was found in nutrient intake for energy, protein, zinc, calcium, vitamin A, vitamin D, folic acid, and vitamin B_{12} among groups. Term group had significantly lower fat ($p = 0.038$) intake compared to the preterm groups. In addition, more subjects in control (n=16) and experimental preterm (n=14) had a low intake of vitamin A compared to the term group (n=6).

3.3. Concentrations of Maternal and Placental Zinc, AtRA, and 25(OH)D

The concentrations of zinc, AtRA, and 25(OH)D in maternal serum are shown in Table 3. Most subjects in all groups had serum zinc and 25(OH)D deficiency. Concentrations of maternal serum zinc, AtRA, and 25(OH)D before and after treatment in the experimental-preterm group were not significantly different. However, we found that there was a significant difference between term and preterm groups related to AtRA concentration ($p < 0.001$). Also, although there was no significant difference in serum 25(OH)D concentrations in control and experimental preterm groups, there were fewer vitamin D deficient subjects in the experimental groups (before (n=17), after (n=16)) compared to control-term (n=19) and control-preterm (n=19) groups.

The concentrations of zinc, AtRA, and 25(OH)D in the placenta are shown in Table 4. All the nutrient concentrations were found significant among groups, especially in between term and preterm groups (all groups; $p < 0.001$). However, placental zinc concentration was the only nutrient that showed a significant difference between experimental-preterm (16.25 ng/g (4.14–100.28)) and control-preterm groups (28.41 ng/g (1.46–137.69), $p < 0.05$).

In comparing those nutrient levels in maternal serum and placenta, AtRA was the only nutrient that consistently shows a higher concentration in term groups compared to preterm groups ($p<0.001$). With regards to zinc and 25(OH)D concentrations, the differences are only found in the placenta.

Furthermore, we performed a correlation analysis to evaluate the relationship between nutrient concentration in maternal serum and placenta. AtRA concentration in all subjects had positive correlation of serum and placental AtRA ($p < 0.001$; $r = 0.515$). Even though there were different amounts of zinc concentrations among groups in serum and placenta, we only found an insignificant negative correlation ($p=0.097$, $r = -0.149$). Also, no significant correlation was found for the 25(OH)D concentration.

Table 1. Characteristics of the subjects

Variables	Control-Term (n=25)	Control-Preterm (n=27)	Experimental-Preterm (n=26)	*p*
Age (y/o)	27.68 (6.47)	23 (18–41)	26.92 (6.63)	0.596[b]
Parity				
Nullipara	18	17	15	0.754[c]
Multipara	7	10	11	
History of preterm birth				
No	22	24	23	0.995[c]
Yes	3	3	3	
Body height (cm)	153.44 (5.79)	156.0 (150-175)	156.46 (6.20)	0.138[b]
BMI (kg/m^2)	21.74 (3.22)	21.87 (4.82)	21.04 (3.91)	0.723[a]
UAC	26.08 (3.45)	24.63 (3.08)	25.87 (3.62)	
< 23.5 cm	6	11	9	0.251[a]
≥ 23.5 cm	19	16	17	
GA on admission	39 (37 – 41)	32,22 (2,91)	32,58 (2,10)	< 0.001[b]
Methods of delivery				
Spontaneous	19	21	18	0.759[c]
Caesarean section	6	6	8	

Data presented as mean (SD) or median (min – max).
Statistical analysis; [a]: One way ANOVA, [b]: Kruskal Wallis, [c]: Chi-Square. Abbreviations: BMI-Body mass index; UAC-upper arm circumference; GA-gestational age.

Table 2. Nutrient intake of the subjects

Variable	Control-Term (n=25)	Control-Preterm (n=27)	Experimental-Preterm (n=26)	p
Energy (Kcal)	1329.6 (994.2–2927.4)	1350.3 (866.1–3040.6)	1618.5 (592.2)	
Low	20	24	24	0.680[a]
Normal	5	3	1	
High	0	0	1	
Protein (g)	41.8 (32.9–153.7)	46.7 (23.7–95.1)	53.8 (20.4)	
Low	21	25	24	0.715[a]
Normal	4	2	1	
High	0	0	1	
Carbohydrate	189.6 (115.5–389)	200.3 (107.6–433.8)	235 (81.1)	
Low	23	25	24	0.495[a]
Normal	2	2	2	
Fat	53.4 (29.8–136.9)	68.9 (20.1)[x]	67.1 (22.8)[x]	
Low	17	8	11	**0.038[a]**
Normal	8	19	15	
Zinc (mg)	4.1 (1.1–16.3)	2.2 (0.5–10.2)	2.6 (0.9–12.7)	0.208[a]
Low	25	27	26	
Iron (mg)	6.8 (2.7–24.6)	8.4 (2.7–140.4)	8.6 (3.8–26.3)	
Low	25	26	26	0.067[a]
Normal	0	1	0	
Calsium (mg)	336.2 (99.2–3.645.4)	434.2 (127.5–1234.5)	438.8 (143.6–1436)	
Low	22	26	24	0.17[a]
Normal	2	1	2	
High	1	0	0	
Vitamin A (mcg)	1398.2 (176.4–22,756)	773.2 (116.9–7947.2)	1011.25 (172.1–7303.5)	
Low	6	16	14	0.168[a]
Normal	4	3	3	
High	15	8	9	
Vitamin D (mcg)	2.2 (0–13.2)	1 (0.2–11.7)	1.95 (0.5–12.3)	0.288[a]
Low	25	27	26	
Folic acid (mcg)	122.8 (2.7–428.8)	100.1 (24.6–456.2)	143.75 (41.30–575)	0.362[a]
Low	25	27	26	
Vitamin B_{12}	1.7 (0.4–11.8)	2.3 (0.2–8.1)	2.45 (0.3–22.1)	
Low	19	18	15	0.532[a]
Normal	6	9	11	

Data presented as median (min – max)
Statistical analysis; [a] Kruskal Wallis;
[x] significant difference with term group.

Table 3. Concentrations of zinc, AtRA, and 25(OH)D in maternal serum

Variables	**Control-Term (n=25)**	**Control-Preterm (n=27)**	**Experimental-Preterm (n=26)**		**p**
			Before	**After***	
Zinc (µg/dL)	45.16 (9.32)	40.26 (13.96)	44.62 (11.10)	46.23 (12.73)	0.172[a]
Deficient (<50)	16	19	16	18	
Normal (50–150)	9	8	10	8	
AtRA (ng/mL)	0.22 (0.07)	0.12 (0.03)[x]	0.13 (0.02)[x]	0.13 (0.26)[x]	
25(OH)D (ng/mL)	15.42 (5.67)	14.00 (3–53)	16 (4–66)	15 (4–45)	**< 0.001[a]**
Deficient (<20)	19	19	17	16	0.76[b]
Normal (20–200)	6	8	9	10	

Data presented as mean (SD) or median (min–max).
Satatistical analysis; [a]: One way ANOVA, [b]: Kruskal Wallis
[x]: significant difference with term group ($p < 0.05$)
* samples were taken after delivery.

Table 4. Concentrations of zinc, AtRA, and 25(OH)D in placenta

Variables (ng/g tissue)	**Control-Term**	**Control-Preterm**	**Experimental-Preterm**	**p**
Zinc	58.34 (27.88 – 124.05)	28.41 (1.46–137.69)[x]	16.25 (4.14–100.28)[x,y]	< 0,001[a]
AtRA	21.7 (10.69)	0.70 (0.42–5.10) [x]	0.69 (0.45–2.29)[x]	< 0,001[a]
25 (OH)D	75.84 (45.12)	18.00 (5–88) [x]	17.58 (7.17)[x]	< 0,001[a]

Data presented as mean (SD) or median (min–max).
Satatistical analysis; [a]:Kruskal Wallis
[x]: significant difference with term group ($p < 0.05$)
[y]: significant difference with control preterm group ($p < 0.05$).

Table 5. Concentration of placental IL-1β

Variable	Control-Term	Control-Preterm	Experimental-Preterm	p
IL-1β (pg/g)	46.47 (0.89 – 269.96)	17.84 (0.34–465.83)	7.17 (0.45–283.29)[x,y]	0.009[a]

Data presented as median (min – max).
Satatistical analysis; [a]:Kruskal Wallis
[x]: significant difference with term group ($p < 0.05$)
[y]: significant difference with control preterm group ($p < 0.05$).

3.4. Placental IL-1β Expression

Placental IL-1β expression was found lower in the experimental-preterm group compared to the control-term group (7.17 vs 46.47 pg/g; p = 0.003) and control-preterm group (17.84 pg/g; p = 0.05) Table 5.

4. Discussion

Participants were dominated with pregnant mother who had low nutrient intake compared to the recommended dietary allowance by the Indonesian ministry of health. [12] This was consistent with our previous study which showed that pregnant women during the first trimester had insufficient nutrition intake. [13] Thus it might be suggested that there was no substantial improvement in nutritional consumption during pregnancy by Indonesian women. In comparing the three groups, fat intake was the only nutrition which we found significantly higher in preterm groups (p=0.038) compared to the term group. This finding was in line with previous study who explained that high fat intake was associated with shorter gestational age (regression coefficient -2.7, 95% CI -4.3, -1.1, p = 0.001). [14] High maternal fat consumption is associated with increased proinflammatory cytokines such as circulating TNF-α or IL-1β, which might cause PTB. [15] Lipids are essential substances for cells, as they not only supply energy but also compose cellular biomembrane structures. However, excessive lipid oxidation will trigger oxidative stress involving many biological processes such as apoptosis, autophagy, and even necrosis. Alterations in these processes are associated with increased proinflammatory cytokines/chemokines in both uterus and placenta, thus inducing preterm labor [16,17].

Zinc has antioxidant and anti-inflammation capacity. The decline in plasma zinc concentration during pregnancy is considered a physiological response to pregnancy. As pregnancy progresses, the fetus takes up zinc with the maximum concentration reached at the end of the third trimester. A previous study found that the maternal serum concentration of zinc was higher in women with preterm birth compared to term pregnancy, with a unit increase of zinc had a 7-fold increased risk of having a preterm birth. [18] Contrary to the findings of our study, we did not find any difference in serum zinc concentration between groups. It was suspected that the serum zinc concentration pre-pregnancy/early pregnancy in preterm groups in our study might be lower than in term groups. This is supported by zinc intake result in preterm was found lower than term group, albeit not statistically significant. Zinc administration (50 mg/day) did not increase serum zinc concentration in the experimental group. This could be explained by the zinc intake that was far below the RDA before treatment (10%) and low serum zinc concentration before treatment (61% of subjects were zinc deficient). Inadequate dosage, duration, and timing of administration during the study, or interactions between the treatments provided, might also influence the result. In this study, the treatment given in addition to zinc were beta-carotene and vitamin D. Previous research found a synergistic effect between zinc and vitamin A, meanwhile, studies about the interaction between zinc and vitamin D yielded different results. [19,20,21] A study also showed that administration of vitamin D_3 for 10 days increased the expression of the ZnT-10 gene. [22] Another randomized controlled trial showed that zinc supplementation in women decreased ZIP-4 and ZIP-8 mRNA in peripheral blood mononuclear cells after 23 days of zinc supplementation. [23] Zinc supplementation also increased ZnT-1. [24] This condition may cause zinc efflux, hence it decreased its concentration in cytoplasm. These data might explained the lower placental zinc concentration in the experimental-preterm group (16.25 ng/g) compared to control-term group (58.34 ng/g) and control-preterm group (28.41 ng/g) in this study.

Vitamin A serves as an endogenous antioxidant that counterbalances the oxidative stress, as well as regulators of both innate and adaptive immune response. [3,25] Previous studies showed lower serum maternal, placental, and cord blood AtRA concentrations in preterm group. [25] This study found a number of subjects with low intake vitamin A was lower in the control-term group (24%) than in the control-preterm group (59.3%) and experimental-preterm group (53.8%). The concentration of placental AtRA in the term group was also higher than the other groups, this might be attributable to higher serum AtRA in the term group. A moderate positive correlation was also found between serum and placental AtRA concentrations (p = <0.001; r = 0.515). There was no difference of serum AtRA concentration before and after treatment (0.13 ± 0.02 ng/mL vs 0.13 ± 0.26 ng/mL; p = 0.963). This might be caused by more than half of the subjects had vitamin A deficiency, inadequate dose, and duration of administration.

Serum 25(OH)D concentrations did not differ significantly between groups. The results showed that weekly 50,000 IU of vitamin D_3 did not increase the concentration of serum 25(OH)D in the experimental group. This might be due to two main factors. Firstly, low vitamin D intake, as only 11% of subjects had an adequate intake. Although Indonesia is a tropical country, exposure to sunlight is still low due to the use of sunscreen and a culture of avoiding sun exposure. This led to a high prevalence of vitamin D deficiency. A study of women in North Sumatra showed the presence of single nucleotide polymorphisms in the VDR gene causing low vitamin D concentrations. [26] In addition, several subjects also had vitamin D deficiency

before treatment such that the administration period was insufficient to enhance the serum levels.

Furthermore, the placental IL-1β level was analyzed to understand the inflammatory rate, as it is a major proinflammatory cytokine in human delivery. [3] Latest findings found that Zinc Oxide (ZnO) supplement was able to reduce the inflammatory response of decidual endothelial cells, in terms of vascular cell adhesion molecule-1 (VCAM-1), IL-8, IL-6, TNF- α, and monocyte chemoattractant protein-1 (MCP-1) expression induced by TNF-a stimulation. [27] Also, a study showed that beta-carotene inhibited NF-kB-dependent expression of inflammatory genes, such as TNF- α, IL-1β, iNOS, COX-2, and production of NO and PGE2 in LPS-stimulated macrophages. [28] Regarding vitamin D, a study in macrophage cells showed that LPS or TNF-α dan $1,25(OH)_2D_3$ downregulates mRNA IL-1, IL-6, dan TNF-α. [29] These studies have suggested that highly anti-inflammatory nutrient supplementation may cause inflammatory response reduction. Consistent with our findings, IL-1β concentration showed the lowest in the experimental-preterm group compared to others, suggesting that there was a shallow inflammatory reaction there. Nevertheless, other proteins related to inflammatory responses via other intracellular molecular patterns such as nuclear factor-kappa B (NF-kB), still need to be studied. [6] These would make a comprehensive information in nutrition related to inflammatory response pathways, especially in PTB.

To the best of our knowledge, this is the first study to analyze the effect of supplementation of zinc, beta-carotene, and vitamin D_3 on inflammatory cytokine in preterm birth placenta. However, the study was limited by the short duration of supplementation as well as no cord blood measurement. Also, the nutrients measured were total zinc, AtRA, and 25(OH)D, instead of its active form. Future research is required to evaluate the effect, exact dose, and duration of zinc, vitamin A, and vitamin D supplementation from early pregnancy in preventing preterm birth. Observational studies related to neonatal outcome in prenatal inflammation suppression treatment might also be needed.

5. Conclusion

In conclusion, the supplementation of zinc, beta-carotene, and vitamin D_3 in preterm birth was significantly associated with lower placental expression of IL-1β, even though it did not directly increase those nutrient concentrations in serum and placenta. This nutrition supplementation was proven to suppress inflammatory markers in placenta and might be clinically beneficial if it was started earlier in pregnancy or even before gestation, thus further studies are required.

Conflict Interests

All authors have confirmed no conflict of interest in this study.

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Ethical Approval and Consent to Participate

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and approved by Ethical Committee for Research in Human from Faculty of Medicine of Universitas Indonesia (LB.01.01/X.2/179/2016). It was also registered in clinicaltrials.gov. All of the participants agreed to participate and signed the informed consent prior to the study.

Trial Registration

Clinicaltrials.gov, NCT03005496. Registered 29th Dec 2016, https://clinicaltrials.gov/ct2/show/NCT03005496.

Authors Contributions

R.I, S.B, A.L, N.W, designed the study; R.I. conducted the study; R.I,S.B performed the analysis; R.I,N.W interpreted the analysis; R.I wrote the paper and had primary responsibility for the final content. All authors agreed to the published version of the manuscript.

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Abbreviations

AtRA:	All-trans retinoic acid
DHA:	Docosahexaenoic acid
ICP-MS:	Inductively coupled plasma-mass spectrometry
IL-1β:	Interleukin 1 β
IUGR:	Intrauterine growth restriction
LC:	Liquid chromatography
LC-MS/MS:	Liquid chromatography-tandem mass spectrometry
PBS:	Phosphate buffer saline
PPROM:	Preterm premature rupture of membrane
RDA:	Recommended daily allowance
SQ-FFQ:	Semi quantitative – food frequency questionnaire
SST:	Serum separator tubes
VDR:	Vitamin D receptors

WHO: World health organization
PGE2: Prostaglandin E_2
NO: Nitric Oxide

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