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The Influence of Casein Supplementation on CD-4, IL-17, and TNF-α in a Preterm *Rattus norvegicus* Chorioamnionitis Model

Hari Paraton[1], Sanarto Santoso[2], Aulanni'am Aulanni'am[3], Erry Gumilar Dachlan[1], I W. Arsana Wiyasa[4], Alifah Wahyu Hariani[1], Widjiati[5], Sumarno Reto Prawiro[2]

[1]Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
[2]Department of Microbiology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia
[3] Department of Chemistry, Faculty of Sciences, Universitas Brawijaya, Malang, Indonesia.
[4] Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.
[5]Department of Embryology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya Indonesia.

ABSTRACT

PROPOSE: This study aims to prove the role of casein supplementation as an immune modulator in response to the CD-4, IL-7, and TNF-α Rattus norvegicus (RN) chorioamnionitis models. METHODS: Thirty-two pregnant RNs divide into four groups. Groups 1 and 2 were given a diet with casein 40g/kg (casein dose 0.4g/day), while groups 3 and 4 a casein diet 200g/kg diet (casein dose 2g/day). Meanwhile, groups 2 and 4 received 100µg/kg body weight lipopolysaccharide (LPS) on day 10th to induce chorioamnionitis. Terminated RN from each group randomly selected under general anesthesia on day 12 and day 14. The assessed CD-4, IL-17, and TNF-α expression semi-quantitatively. The data obtained were analyzed using the ANOVA test.

RESULTS: The concentration of CD-4 was not significantly different (p-value 0.380). The results show that casein supplementation in the preterm RN chorioamnionitis model did not affect the increase in CD-4. However, IL-17 and TNF-α levels differed significantly in the chorioamnionitis group treated with casein supplementation (p-value 0.000).

CONCLUSION: Casein supplementation acts as an immune-modulating response to chorioamnionitis

Keywords: Casein, CD-4, IL-17, LPS, *Rattus norvegicus*, and TNF-α.

Correspondence:
Sumarno Reto Prawiro
Department of Microbiology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia
Email: retroprawiros@yahoo.com

INTRODUCTION

Intra-uterine bacterial exposure activates the innate immunity cascade bypassing toll-like receptors (TLRs) [1]. In this state, the TLRs were present on the chorioamnionitis surface as pattern recognition receptors (PRRs). These receptors recognize lipopolysaccharides (LPS), which are part of the bacteria that have been lysed and are called pathogen-associated molecular patterns (PAMP). The presence of these receptors can also be found in cellular, for example, viruses. The binding of TLR and LPS will cause a primary response, namely, the differentiation of myeloid 88 (MyD88). This substance signals the adapter protein that causes activation of NFκB [2]. The next cascade will trigger the release of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-8, IL-17, and IL-2 [3]. Some of these pro-inflammatory cytokines cause CD-4 to proliferate. Several other cytokines will produce, which will carry leukocytes and stimulate the production of anti-microbial substances in phagocytic cells. The activation of this pathway plays a role in helping phagocyte cells kill pathogens [1]. CD-4 T cells also appear to play an essential immunosuppressive role in maintaining pregnancy [4]. After that can associated prematurity that occurs with underweight and malnourished mothers during pregnancy [5]. Mammalian milk, especially cow's milk, is a protein-rich food used as a multipurpose food ingredient.

Cow's milk protein has peptides that can inhibit microbial growth found in the alpha, beta, and kappa fractions of casein [6]. The type of hydrolyzate from casein can affect both innate and adaptive immune cells [7]. Many studies have not distinguished the advantages of casein and whey protein in the role of immune modulators. The role of casein as an immune modulator is not fully understood. It is not clear that casein plays a role in preventing preterm birth, especially CD-4, IL-17, and TNF-α.

METHODS

Subject

This research received proper permission from the Research Animal Care and Use Commission (No: 3.KE.042.03.2018) Veterinary Medicine, Airlangga University, Indonesia.

Work procedures

Group care

Thirty-two pregnant RNs were randomly selected and divided into four groups (each group contained 8 RNs). The first group (G1) and the second group (G2) were given a regular diet and gave dietary casein of 40g/kg (daily dose 0.4g/RN/day). Group III (G3) and group IV (G4) gave regular feed 200g/kg of feed (daily dose 2g/RN/day). Feed (approx. 10mg/RN/day) and drink (approx. 10ml / RN/day) regularly gave once daily. The diet base on the American Institute of Nutrition recommendations for adult RN. This study used pure casein sodium CAS 9005-46-3 from the Tokyo Chemical Industry. Co. Ltd as casein supplementation.

On day 10 of pregnancy, a single intraperitoneal injection of 100µg/kgBW lipopolysaccharide (LPS: *E.coli* 0111:B4/Biological Laboratories Inc.). LPS dissolved in a phosphate buffer salt solution (PBS) injected into G2 and G4. Four RNs randomly selected from each group terminated with general anesthesia on day 12 and day 14. Chorioamnion then took specimens that then fixe in 4%. We carried out the paraformaldehyde solution and immune-histochemical processes.

Measurement of CD-4, IL-17, and TNF-α

Expression of CD-4, IL-17, moreover TNF-α measures with immunohistochemistry method. Initially, the sample preparation of chorioamnionitis membrane specimen slides using the deparaffination. Then the samples rehydrate with different ethanol concentrations the

preparation blocks with serum bovine albumin. They subsequently labeled with primary antibodies CD-4 (MT310: sc-19641), IL-17 (G-4: sc-374218), and TNF-α (4E1: SC-130349). The next day, the slides wash with PBS 7.4. Labeling using secondary antibody streptavidin horseradish peroxidase (SA-HRP). After that, I added a slide chromogen substrate (3,3 diaminobenzidine tetrahydrochloride / DAB). As a counterstaining using methyl green color material.

Histopathological observation using immunohistochemical methods. The aim to determine the expression of CD-4, IL-17, and TNF-α on the chorioamnionitis membrane. Each sample assesses semi-quantitatively according to the modified Remmele and Stegner methods. Simultaneously, the Remmele and Stegner scale index, immune-reactive score (IRS), multiplied the percentage of immunoreactive with the color intensity score. The data for each sample is the mean of the observed IRS values in the ten fields of view at 400x magnification.

Statistical analysis

SPSS Statistics Version 25.0 used for analysis, including Shapiro-Wilk normality tests (with significant level α = 0,05), Levene's test (with significant level α = 0,05) homogeneity of variance of data for CD-4, IL-17, and TNF-α. Analysis of Variance (ANOVA) (with significant level α = 0,05) and post hoc Bonferroni and Gomes-Howell test was applied to identify detailed differentiation of data variances.

RESULT

At the start of the study, the average body weight was 124g with a Shapiro-Wilk normality test in all groups p-value >0.05, and Levene's homogeneity tests were p = 0.268, Anova test has p = 0.426. At the final of the study, the average body weight was 210g with normality test. All groups with p-value >0.05 and homogeneity tests were p = 0.409, and the ANOVA test was p = 0.199; there were no significant differences between RN in this study.

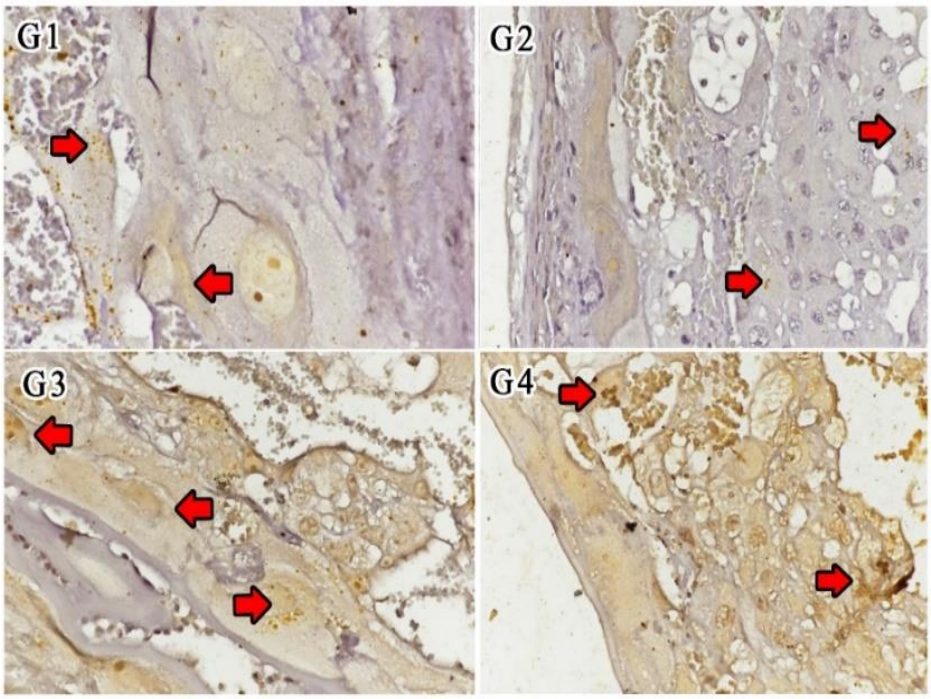

Fig. 1 Observation of CD-4 presented by immunohistochemistry. The arrows indicated the presence of Immunostaining for CD-4 expression on the chorioamnionitis membrane. Treatment groups: G 1 with casein 40g/kg diet + LPS (-); G2 with casein 40g/kg diet + LPS (+); G3 with casein 200g/kg diet + LPS (-); and G4 with casein 200g/kg diet + LPS (+) (use of magnification 400x).

The effect of casein if rat induces the LPS in the chorioamnionitis rat model. CD-4 concentrations were measured and expressed as mean of the square root: G1 1.4960 (95% confidence interval, CI, 0.8682-2.1237), G2 1.0504 (95% CI 0.6870-1.4138), G3 1.2973 (95% CI 1.1015-1.8866), and G4 1.3332 (95% CI 0.7838-1.8008). Conducted the homogeneity of variance test of CD-4, by getting mean p-value 0.739 (p>0.05), it means CD-4 has the same variance distribution. Indeed, there was an increase in CD-4 concentration in the LPS-induced group. However, the ANOVA test had a p-value 0.380, indicating no significant difference of CD-4 between all groups, whether or not LPS induced them (Fig. 1).

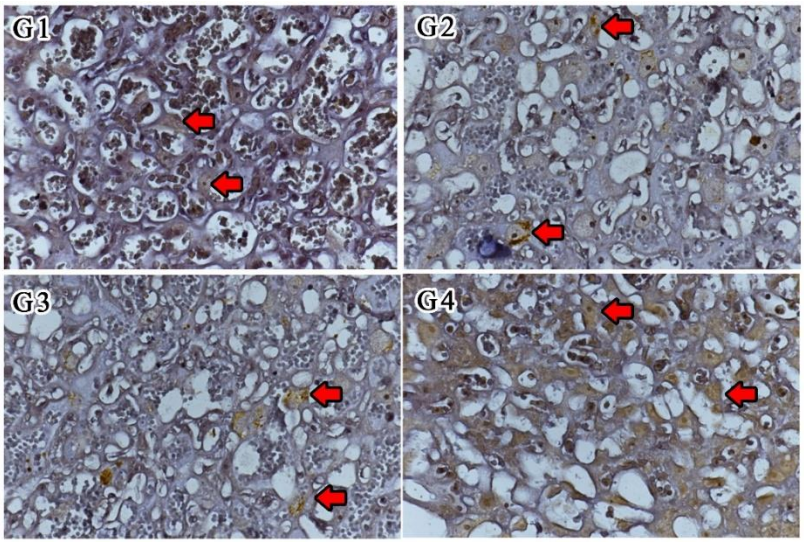

Fig. 2. Observation of IL-17 presented by immunohistochemistry. The arrows stain indicates the presence of Immunostaining for IL-17 expression on the chorioamnionitis membrane. Treatment groups: G1 with casein 40g/kg diet + LPS (-); G2 with casein 40g/kg diet + LPS (+); G3 with casein 200g/kg diet + LPS (-); and G4 with casein 200g/kg diet + LPS (+) (use of magnification 400x).

The mean expression levels of IL-17 in G1 were 3.1000 (95% CI 1.9177-4.2823), G2 2.9500 (95% CI 2.0137-3.8863), G3 3.7500 (95% CI 3.2082-4.2918), and G4 8.2750 (95% CI 7.4660-9.0480). Homogeneity of variance testing had a p-value 0.065, indicating that IL-17 showed the same variance between the groups. ANOVA test has p-value = 0.000, and the homogeneity test p = 0.065 (p>0.05). The statistical review proceeds to the post hoc Bonferroni multiple comparisons tests, G4 has p-value 0.000 (p<0.05) to G1, G2, and G3, showed a significant difference in the expression of IL-17 in the chorioamnionitis group that received casein supplementation. While among G1 to G2 (p = 1.000), G1 to G3 (p = 1.000) and G2 to G3 (p = 0.884) all had p-value >0.05, which means that there was no significant difference in IL-17 expression between these groups (Fig. 2).

The mean value of TNF-α concentration were: G1 2.1625 (95% CI 1.9349-2.3901), G2 3.0500 (95% CI 1.0843-5.0157), G3 4.2375 (95% CI 1.4297-7.0453), and G4 9.6875 (95% CI 8.1130-11.2620). ANOVA test has p-value = 0.000. The homogeneity of variance test based on means has p-value = 0.045, indicating TNF-α was not homogenous, then continued with the post hoc Gomes-Howell test to determine the differences between groups. G1 to G2 and G3 have p-value 0.722 and 0.371 respectively, while G2 to G3 have p-value 0.844. All have p-value >0.05, which means no significant difference in TNF-α expression between these groups, while G4 to G1 and G2 have p-value 0.000. To G3, the p-value was 0.010, which means that the chorioamnionitis group that received casein supplementation had a significant difference compared to the other groups (Fig. 3).

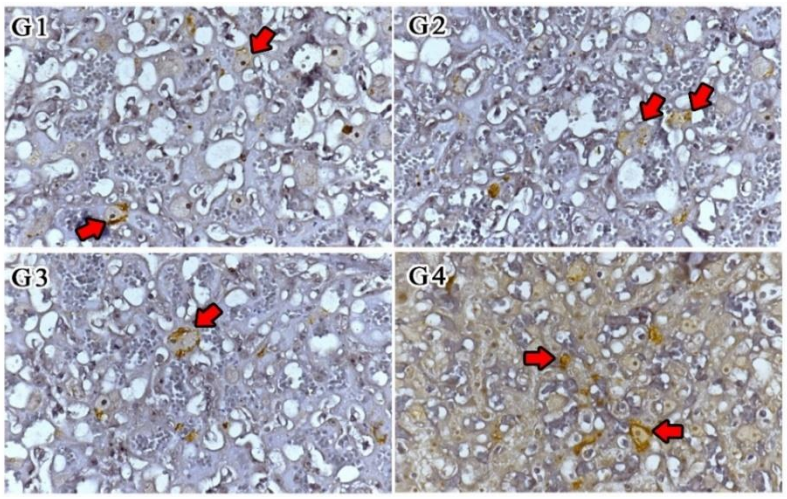

Fig. 3. Observation of TNF-α presented by immunohistochemistry. The arrows show the presence of Immunostaining for TNF-α expression on the chorioamnionitis membrane. Treatment groups: G1 with casein 40g/kg diet + LPS (-); G2 with casein 40g/kg diet + LPS (+); G3 with casein 200g/kg diet + LPS (-); and G4 with casein 200g/kg diet + LPS (+) (use of magnification 400x).

DISCUSSION

Milk is a daily food that women drink for nutrition and which is often consumed by pregnant women. Casein is the largest milk protein component and acts as an immune modulator in inflammation [8]. This compound is a phosphoprotein that can be precipitated at 20^0 C from skim milk at a pH of 4.6. Casein has a composition of 80% milk protein consisting of α, β, and κ subgroups, the components are about 50%, 45% and 15% respectively [9]. Casein has potential as a bioactive peptide depending on the type of chain, bond, and amino acid content. Casein's bioactive function is an immune modulator of the innate and adaptive immune response [10]. The serious problem of prematurity causes efforts to reduce the prevalence of preterm birth in various ways. One way is the use of casein as an immune modulator that plays a role in suppressing the inflammatory response. The inflammatory response to chorioamnion can provoke preterm birth.

Associated the selected variables in the response immunity with casein as a modulator for CD-4, IL-17, and TNF-α. The results discuss as follows: The first variable can see in Fig 1 and shows that casein affects the CD-4 cellular immune response. CD-4 expression sees on all chorioamnionitis RN membranes; statistically, there was no significant difference in the effect of casein for all treatments. CD-4 effector T cells in the decidua are involved in preterm birth, at term, and during labor. This condition also accompanies the cytokines IL-1β, TNF-α, and MMP-9 [11]. TLRs found on the cell surface recognize LPS and then trigger the release of pro-inflammatory cytokines. Macrophage cells have shown to express TLR4, which is a receptor for LPS [12]. LPS will bind to TLR4 and continue the pro-inflammatory cascade process by producing at least IL-1β, IL-6, IL-8, and TNF-α [13]. The results of this study are similar to previous studies on chorioamnionitis exposed to LPS in rhesus monkeys. There was an increase in CD-4, but not significantly. But CD-4 in chorioamnionitis was markedly higher than preterm and term delivery [14]. Nonetheless, activated CD-4 cells reproduce and primarily mediate secreted cytokines. Secreted cytokines can stimulate phagocytes to produce microbiocidal substances. The results of this study are descriptively different, but statistical tests are not proven. So, it still requires more detailed research on the effect of casein on preterm birth CD-4 cells.

A second variable finds in Fig 2 for the effect of casein on IL-17 concentrations in a model of preterm mice with chorioamnionitis. One of the Th-17 cell products is IL-17, where Th-17 cells are a subset of CD-4 cells that will increase due to exposure to bacteria. The relationship between casein and IL-17 in this study can be analyzed by comparing G1 and G3. Statistically not significantly different, p-value 0.000 with ANOVA between groups. Furthermore, the post hoc test obtained a p-value of 1,000. This statistical analysis concluded that there was no increase in IL-17 expression on the chorioamnionitis membrane. Obtained inversion results for chorioamnionitis between G2 and G4, where the ANOVA result was a p-value of 0.000, and the Bonferroni post hoc test p = 0.000. This study's products are not different from previous studies conducted by Rueda et al. (2016). LPS-induced pregnant macaques also had elevated IL-17 [15]. From the above findings, it feigned that casein supplementation promotes the release of IL-17 during chorioamnionitis first. This state is to prepare phagocytic cells to control bacteria. The second assumption is that casein supplementation leads to accelerated adaptive immunity during chorioamnionitis. Whereas the third, casein supplementation directly stimulated a subset of Th-17 cells as IL-17 producers. There is ample evidence to suggest that IL-17 and IL-22 can protect against infection, mostly by two mechanisms. The first involves producing an anti-microbial peptide, which is highly dependent on the synergistic action of IL-17 and IL-22 on epithelial cells. The second mechanism involves IL-17 and IL-22, which induce intestinal and pulmonary epithelial cells to express chemokines that attract granulocytes, especially neutrophils, to the site of infection [16]. Heterogeneity of CD-4 can identify three main cell subsets, namely Th-1, Th-2, and Th-17. Its primary function is protection against various pathogens [17]. The naming of the Th-17 subset is due to one of the cytokine products IL-17. These cytokines have several functions, such as establishing a link between T cell-mediated adaptive immunity and the acute inflammatory response. Besides, it plays a role in increasing monocytes to the site of inflammation and increasing neutrophils' production. As an initiator of anti-microbial peptides' output, as a means of defense from various superficial cells. The most prominent function of IL-17 is the recruitment of neutrophils to the site of infection, which is necessary to clear microorganisms. Neutrophil recruitment requires a rapid response to pathogenic bacteria and fungi. There were different results regarding IL-17 in decidua humans with chorioamnionitis compared with those without chorioamnionitis [18]. Similar results were shown in preterm birth with chorioamnionitis and caused IL-17, IL-8, and TNF-α production[19]. On the other hand, preterm without chorioamnionitis found low IL-17 levels [20]. The results there was no relationship between pro-inflammatory adaptive immune cells chorioamnionitis with preterm birth. Casein appears to play a role in increasing cytokine activity in acute inflammatory or infectious responses.

The third variable regarding the effect of casein on TNF-α concentration in a model of preterm mice with chorioamnionitis sees in Fig 3. All have p-value >0.05, which means no significant difference in TNF-α expression between them, while G4 to G1 and G2 have a p-value 0.000. To G3, the p-value was 0.010, which means that the chorioamnionitis group that received casein supplementation had significant difference compared to the other groups. As an acute pro-inflammatory cytokine, TNF-α plays an essential role in body defense and immune regulation. Macrophage manly produces cytokines mainly, monocytes, and surface cells that respond to bacterial signals, such as PAMP. Another study found that releasing large amounts of TNF-α in response to LPS. It can also find in other bacterial products, *Staphylococcus enterotoxin B* and bacterial superantigen toxins. TNFα overproduction and secretion are highly influential in acute inflammation [21]. The bacteria can reach the membranes and trigger a series of immune responses, including premature uterine contractions. The mechanism activated by the release of TNF-α, IL-1β, IL-6, IL-8, and IL-10 responds to LPS bacteria [22]. TNF-α causes an increase in prostaglandin E2 (PGE2), which is involved in cervical softening. The release of prostaglandin F2alpha (PGF2α) which causes the myometrium to become more sensitive to oxytocin. Both of these processes can lead to preterm labor [23]. Studies on mice induced by Shiga tox4n-2 (Stx2) showed a significant increase in serum TNF-α observed after 2 hours. These results are in line with previous studies

reported on human and animal models [20]. Elevated TNF-α levels follow intra-amniotic infection and chorioamnionitis and mid-trimester preterm delivery [24].

In contrast, some data found no increase in TNF-α during labor. These data link the rise in PGF2α with a role in inhibiting TNF-α production [9]. There was no significant increase in TNF-α in mothers and neonates at the time of infection or inflammation. The expression of these cytokines is closely related to the induction of LPS as PAMP [1]. There is an increase in TNF-α cases in preterm birth. However, preterm labor persisted [25]. TNF-α, a mediator of acute infection, has no significant relationship between the incidence of preterm birth, especially in response to chorioamnionitis. One can consider the high TNF-α expression in the chorioamnionitis group caused by casein supplementation. In other words, it can serve as an acute immunological response to treat the infection. It estimates that 925g protein intake during term pregnancy dominates fetus 42%, uterus 17%, blood 14%, placenta 19%, and breast 8% [26].

CONCLUSIONS

Casein supplementation has played a role in increasing the production of the CD-4, IL-7, and TNF-α. This finding in response to infections that cause labor. Further research could do to explore the role of casein in preventing chorioamnionitis or preterm birth. More detailed analysis needs regarding the types of peptides and the dosages required as supporting elements and immune modulators.

CONFLICT OF INTEREST

There was no conflict of interest in this study.

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AUTHOR CONTRIBUTIONS

Sumarno RP and Erry GD are for the conceptual framework. Hari P, IWA Wiyasa checks casein on CD-4 expression, casein on LI-17 expression, and casein on TNF-α expression. Aulanni'am A, Widjiati W preparation, maintenance, pregnant mice, histochemical Immuno examination. I W. A Wiyasa, Sumarno RP and Sanarto S reviews the manuscript before submitting it. Statistical analysis Alifah WH and Widjiati W. All authors contributed to the writing of the document.

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