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Original Research Article

Effect of casein supplementation on HBD-1, Th-17 and IL-22 in a preterm *Rattus norvegicus* chorioamnionitis model

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Abstract

Purpose: *To investigate casein's role in the expressions of human beta-defensin (HBD)-1, T helper subset (Th)-17, and interleukin (IL)-22 in preterm Rattus norvegicus (RNs).*

Methods: *Twenty-four healthy, first-time pregnant RNs were divided into three groups (G). Group 1 (G1) received regular casein (R) 0.4 g/RN/day, while G2 and G3 received casein supplementation (S) 2 g/RN/ day. Each RN was given 10 g of food and drank 10 mL. On day 10, a single intraperitoneal lipopolysaccharide (LPS) was injected with 100 g/kg body weight. Lipopolysaccharide was injected into G3 as a premature model with chorioamnionitis. Histopathological examination was carried out to measure the expression of HBD-1, Th-17, and IL-22 using methyl green counterstaining. Chorioamnion specimens were taken and fixed in 4 % paraformaldehyde, and then evaluated by immunohistochemical (IHC) procedures to obtain immunoreactive score (IMS).*

Results: *The IMS expression of HBD-1 in G1 was 1.187 ± 0.372, while G3 was 9.562 ± 2.351 ($p < 0.05$). Th-17 expression were G1: 2.062 ± 0.140, G2: 6.122 ± 1.347, and G3: 9.112 ± 2.019 ($p < 0.05$). Furthermore, IL-22 expressions were G1: 1.825 ± 0.468, G2: 4.700 ± 1.614, and G3: 7.800 ± 1.669 ($p < 0.05$).*

Conclusion: *Casein supplementation increases HBD-1, Th-17, and IL-22 expression in preterm RN and in the LPS-induced RN chorioamnionitis model. The increase in HBD-1, Th-17, and IL-22 indicate that casein plays a role in the protection and control of chorioamnionitis tissue inflammation.*

Keywords: *Casein, HBD-1, Th17, IL-22, Rattus norvigicus*

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INTRODUCTION

Prevalence of preterm birth from 184 of WHO member countries vary between 5 – 18 %, resulting in approximately 14.5 million premature births per year. Neonatal mortality is around 35 % while others have the potential to experience severe organ disorders, including cerebral palsy, neurocognitive disability, blindness, deafness, or severe disability [1].

The cause of prematurity in pregnancy is still unclear, but there are hypotheses, including maternal nutritional deficiency, cervical incompetence, and ascending infection [2]. Furthermore, the state of clinical chorioamnionitis showed several elevated proinflammatory

cytokines. These cytokines include interleukins, tumour necrosis factor, interferon-gamma [3], the chemokine CXCL10 in which this proinflammatory cytokine is a fetal rejection factor, and TLR-4 [4]. The increase in interleukin and tumour necrosis factor in preterm birth is associated with increased prostaglandins and matrix metalloproteinase (MMP), which in this condition will induce uterine contractions and cervical ripening [5].

The increased number of Th-17 cells in chorioamnionitis is a type of T-helper subset. Th-17 cells have a role in adaptive and innate immune responses to microbial lysis. Furthermore, in chorioamnionitis, there is also an increase in interleukin (IL)-22, which in this situation is more caused by an inflammatory process than a bacterial infection [5]. For example, it stimulates proliferation for cell repair and increases the production of antimicrobial peptides in tissues in the event of an infection or inflammatory reaction [6]. Interleukin (IL)-22 has been identified *in utero* without pregnancy, early pregnancy, and preterm pregnancy. However, in a study with pregnant mice, there was an increased response to IL-22 in chorioamnionitis if induced by LPS after IL-22 is closely associated with preventing preterm labour and suppressing the incidence of infection or inflammation [7].

Human beta-defensin (HBD) is a short peptide consisting of 29 - 34 amino acids. It is cationic and hydrophobic [8] and a part of the innate immune system. The benefit of HBD has the potential as a natural antimicrobial agent expressed by surface epithelial cells, in the mucosa of the respiratory tract, urogenital, large intestine, skin, and placental tissue [9]. There are two families of HBD (alpha and beta) with more than 20 sub-types. The HBD-1 sub-type is produced by microbial stimulation of interleukin [10]. Then, HBD-1 kills microbes by various mechanisms of actions including disrupting the microbe membrane function [9]. The chorioamnion is a source of HBD; in a state of chorioamnionitis, the levels increase up to 2.7 times [10]. Many pregnant women believe that consuming milk increases fetus growth, development, and wealth. Milk also has bioactive peptide components that act as immunomodulators, thus protecting against infection [11].

Protein hydrolyzate from casein or other products containing milk components can enhance proinflammatory immune response [12]. Cow's milk consists of lipids, minerals, and protein, where these components range from 3 to 3.9 %, while the content of whey protein is 20 %, and casein is 80 %. Casein in the form of colloidal particles binds to calcium phosphate to form casein micelles, consisting of α_{s1} 38 %, β 36 %, κ13 %, and α_{s2} 10 % caseins, respectively, and with a molecular size of 200 nm [13]. Casein hydrolyzate has been shown to act as an immunomodulator or an antibacterial. The role of bioactive against infection-causing bacteria or in inflammatory states depends on the size and sequence of the peptide. Casein hydrolysate has been found to have the ability to suppress IL-6 synthesis and does not affect IL-2, IL-10, and Interferon [14].

Few studies have been done to determine protein hydrolysates or peptide mechanisms that modulate the immune system. Furthermore, it is necessary to study which components of milk protein peptides have an immunomodulatory effect [14]. Then by observing the impact of HBD-1, Th-17, and IL-22 due to the consumption of casein as an immunomodulator, this study aims to reduce the risk of infection in prematurity.

EXPERIMENTAL

Materials

Sodium casein CAS 9005-46-3 was purchased from Tokyo Chemical Industries as casein supplementation. Co. Ltd. *E. coli* obtained from 0111.B4/Biological Laboratory Inc. Primary antibody HBD-1 (M4-146-H4:sc65501) and Th-17 (1900.Cat.sc 376374) were purchased from Santa Cruz Biotechnology Inc, USA, while IL-22 was obtained from Bs.2623R; Bioss, Inc, USA.

Ethical approval

This study was approved by the Research Commission on Animal Care and Use of Veterinary Medicines Universitas Airlangga Indonesia (approval no. 3.KE.042.03.2018), and followed international guidelines for animal studies.

Animals and treatments

Twenty-four *Rattus norvegicus* (RN) that were 2 - 3 months old and had a mean weight of 100 – 200 g with first-time pregnancy were used. As an indicator of pregnancy, a marker for the presence of a vaginal plug was used. The twenty-four (24) RN were randomly divided into three groups; group 1 (G1) received casein 0.4 g/RN/day, group 2 (G2), and group 3 (G3) 2 g/RN/day. Each RN received approximately 10 g of feed and drank 10 mL of water daily. The diet was based on the American Institute of Nutrition recommendation for adult rats [16].

Induction of chorioamnionitis

On day-10 of gestation, a single intraperitoneal injection of 100 g/kg lipopolysaccharide (LPS) was administered. The LPS was dissolved in phosphate buffered salt solution, which was then given orally to group G3 to make a model of chorioamnionitis in RN by injecting LPS 100 µg/BB intra peritoneal route [17]. Four RNs from each group were randomly dislocated on the 12th and 14th days after treatment. Chorioamnion specimens were taken and fixed in 4 % paraformaldehyde, and then immunohistochemical (IHC) procedures continued.

Determination of HBD-1 expression

To determine the presence of HBD-1 expression, Th-17 and IL-22 were labeled with the primary antibody HBD-1. Histopathological examination to measure the expression of HBD-1, Th-17, and IL-22 using methyl green counterstaining was then carried out.

Assessment of immunoreactive score

To determine the presence of immunoreactivity, a semi-quantitative calculation of the modified Remmele and Stegner method scale index as the immunoreactive score (IRS) was used. Immunoreactive score values were examined microscopically at a magnification of 400x/field of view and the mean of ten observations was calculated.

Statistical analysis

Statistical analysis was done using SPSS, version 25.0, while Shapiro-Wilk normality test, Levene of homogeneity variance test, and ANOVA used to measure HBD-1 and IL-22, while Kruskal-Wallis test was used to assess Th-17. Post Hoc of Games-Howell and Mann-Whitney test was employed to identify certainty differentiation of variance. Statistical significant was set at $p < 0.05$.

RESULTS

Rattus norvegicus body weight

At the beginning of the study, mean RN weight at G1, G2, and G3 was 129.6, 119.8, and 120.5 g, respectively, using the Shapiro-Wilk normality test in all groups with $p > 0.05$, and the Levene homogeneity test variance with $p = 0.128$. At the end of study, the mean body weight increased by 196.2, 186.2, and 178.7 g, respectively. No significant difference between RNs was observed ($p > 0.05$).

Human beta Defensin-1 expression

Results of HBD-1 expression showed a normal distribution using the Shapiro-Wilk test ($p = 0.075$). However, homogeneity variance is not normally distributed when tested with Levene's test, which produces a *p*-value of 0.000. The mean value of HBD-1 in G1 was 1.187 ± 0.372 (95 % CI: 0.876 - 1.498), G2 6.612 ± 1.311 (95 % CI: 5.515 - 7.709), while G3 9.562 2.351 (95 % CI: 7.596 - 11.528). Since ANOVA had a *p*-value of 0.000, it was followed up with post hoc Games-Howell to identify details on differentiation between groups. G1 to G2 had *p*-value of 0.000, G1 to G3 *p*-value of 0.000, and G2 to G3 *p*-value of 0.025, which means that casein supplementation had a significant difference compared to regular diet group. In chorioamnionitis group, casein supplementation differed significantly from the other groups (Figure 1).

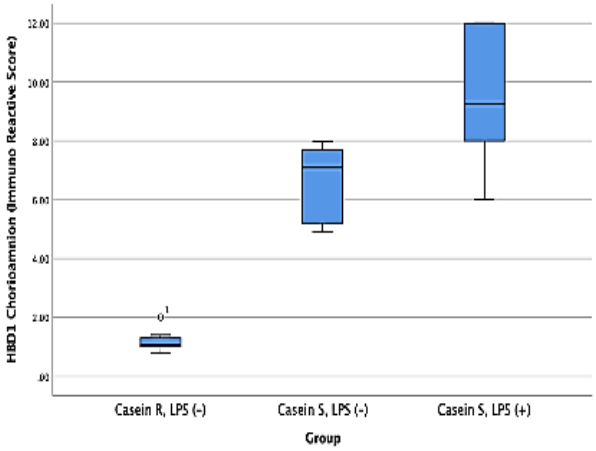

Figure 1: Relationship between HBD-1 in chorioamnionitis due to casein administration in groups with different doses. **Key:** Group Casein R, LPS (-), (G1): with casein regular 0.4 g/RN/day, Group Casein S, LPS (-), (G2): with casein supplementation 2 g/RN/days, LPS(-), and Group Casein S, LPS (+), (G3): with casein supplementation 2 g/RN/day, LPS (+)

T-helper 17 (Th-17) production

The mean value of Th-17 production in the 3 groups were: G1, G2 and G3: 2,062 ± 0.140 (95 % CI: 1,944 - 2,180), 6.122 ± 1,347 (95 % CI: 4,986 - 7,239), and 9,112 ± 2,019 (95 % CI: 7,424 - 10,800), respectively. The combined analysis results of G1, G2, and G3 showed a significant difference ($p < 0.05$) in the effect of casein supplementation on Th-17 production, especially in the chorioamnionitis group. There

was a significant increase in IL-17 production compared to G1 ($p < 0.05$; Figure 2).

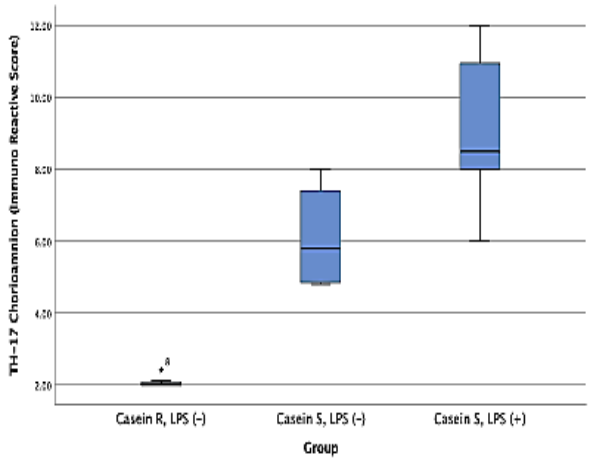

Figure 2: Relationship between Th-17 in chorioamnionitis is due to casein administration in different groups with different doses. ***Key:*** Group Casein R, LPS (-), (G1): with casein regular 0.4 g/RN/day, Group Casein S, LPS (-), (G2): with casein supplementation 2 g/RN/days, LPS(-), and Group Casein S, LPS (+), (G3): with casein supplementation 2 g/RN/day, LPS (+)

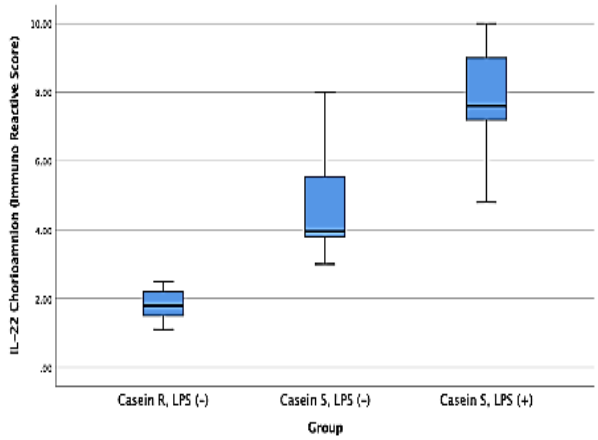

Figure 3: The relationship between IL-22 in chorioamnionitis is due to casein administration in different groups with different doses. ***Key:*** Group Casein R, LPS (-), (G1): with casein regular 0.4 g/RN/day, Group Casein S, LPS (-), (G2): with casein supplementation 2 g/RN/days, LPS(-), and Group Casein S, LPS (+), (G3): with casein supplementation 2 g/RN/day, LPS(+)

Interleukin-22 expression

Several roles of IL-22 are produced by the immune response, including stimulating tissue repair, cell surface barrier, synthesis of chemokines in inflammation, and natural antimicrobial peptides. Mean expression of IL-22 in chorioamnionitis specimens was G1 1.825 ± 0.468 (95 % CI: 1.827 - 1800), G2 4.700 ± 1.614 (95 % CI: 3.350 - 6.049) and G3 7.800 ± 1.669 (95 % CI: 6.403 - 9.196). Expression of IL-22 was normally distributed ($p = 0.113$). Results of further analysis showed significant differences between groups ($p < 0.05$) because the data distribution has the same variance. Bonferroni post hoc test was then continued to clarify IL-22 expression. Analysis of IL-22 expression in G1, G2, and G3 showed significant differences ($p < 0.05$). The expression of IL-22 in the chorioamnionitis group increased 4.2 times compared to control (G1) (Figure 3).

DISCUSSION

Apart from being a nutrient, casein has an immunomodulatory role in regulating normal immune function through activation or suppression. Various studies have proven the role of casein as an immunomodulator, which is indicated by triggering the expression of IL-1β, IL-6, and IL-8. Its mechanism as an immunomodulator is initiated by the ability to bind to TLR-4/MD2, although there is no association with LPS [18].

Casein hydrolysate suppresses the release of excess IL-6. Casein hydrolysate is proven to inhibit the proinflammatory activation process. The release of proinflammatory cytokines, especially IL-6, is triggered by the binding between LPS and TLR-4. This binding process is inhibited by casein. In addition, casein also inhibits the expression of prostaglandin-E through inhibition of mitogen-activated protein kinase (MAPK) activation. Proinflammatory cytokines, especially IL-6 and prostaglandin-E, affect uterine contractions [18].

One of the causes of preterm birth is an intra-amniotic infection. The route used ascending infection, including gram-negative bacteria, during the process, which will release endotoxin in LPS. The LPS will bind to a cell surface receptor known as TLR-4. This binding would involve MD2 and MyD88 providing a specific signal to NFκB and triggering the expression of proinflammatory cytokines, including IL-1β, Il-6, IL-8, TNFα, IL-17, IL-22, and Th-17 [19]. These are strongly affected to stimulate prostaglandin and uterine contraction.

Immunomodulators were HBD-1, TH-17, and IL-22 to determine the IMS expression of HBD-1 in the three groups. The development of prominent significant increase is shown in G2. This finding indicates that casein affect this increase in HBD-1 expression on the membrane chorioamnionitis RN in preterm pregnancy. In G3, there is also a significant increase in HBD-1 expression when

compared to G1 and G2. This increase in HBD-1 expression may be caused by either the response of chorioamnion membrane tissue due to preterm pregnancy, or exposure to LPS, or casein, and casein complexes that cause chorioamnionitis. These results suggest that preterm pregnancy and chorioamnionitis increase HBD-1. The increase in HBD-1 is stimulated by endotoxin-induced proinflammatory cytokines (LPS) [10]. High HBD-1 expression is helpful to lyse bacteria that mainly spread by ascending and cause chorioamnionitis. This bacterial lysis process occurs because HBD-1 has a positive cationic antimicrobial with a substantial cytotoxic/antimicrobial lysis effect and chemotactic properties, which is beneficial for host defence [20].

The second variable, Th-17 production in premature RN is due to 3 different treatments. Th-17 is produced due to the stimulation of cytokines IL-1, IL-6, and IL-23 as stimulators. Th-17 is derived from the differentiation of activated CD-4 T cells and depends on the transcription factors STAT3 and RORγt [21]. Th-17 is widely found under mucosal tissue and functions to overcome infection and protect the regeneration of cell damage caused by inflammation [8]. This study showed a statistically significant difference between groups ($p < 0.05$). The increase in Th-17 was significantly affected by casein supplementation in premature RN chorioamnionitis tissue. Significant differences also occurred between G1 and G2 and G2 and G3 ($p < 0.05$).

Chorioamnionitis is caused by LPS-induction which showed an increase in the mean value of 4.4 times compared to G1. This study also found that the production of Th-17 was high in premature infants, indicating the presence of chorioamnionitis, which was observed on histopathological examination. This increase is thought to be caused by a response to the ongoing inflammatory process. Another study found that an increase in Th-17 was accompanied by high neonatal morbidity and mortality. It is assumed that the rise in Th-17 may result from a response to infection, an inflammatory process, or the end state of septic conditions [22]. It is known that both LPS and Casein play an essential role; namely, they can bind to TLR-4 and NFkB as their receptors to trigger IL-17 expression. The high production of Th-17 may be due to LPS-induced inflammatory process and the indirect role of casein supplementation through the proinflammatory pathway.

Casein supplementation in preterm RN had a significant difference compared to the usual casein diet in RN feed. For chorioamnionitis, preterm RN had a high expression of IL-22, with a substantial difference in the treatment groups. Subsequently, regarding the effect of LPS and casein as pathogen-associated molecular patterns (PAMPS) on chorioamnion membrane, both preparations could not bind directly to TLRs as pattern recognition receptors (PRR). There must be another requirement, namely the need for an MD-2 adapter protein-ligand. This binding triggers signalling via activation of MyD88 and transcription of NFkB to produce most cytokines, particularly IL-1, IL-6, IL8, TNFα, and IFNγ [21]. This cytokine is responsible for activating the CD4+ Th-17 and Th-22 subsets to produce and release IL-22 by stimulating IL-22 receptor complex consisting of IL-22R1 and IL-10R2 [23]. IL-22 is a member of the IL-10 family, which is produced by lymphoid cells, epithelial cells, neutrophils, and macrophages.

Studies on lipopolysaccharide-induced preterm birth in mice suggest that IL-22 firmly controls infection-induced preterm birth [7]. IL-22 and IL-17 perform overlapping activities to maintain the integrity of host tissue cells. The mechanism increases the defence on the epithelial surface by producing antimicrobial peptides and recruiting neutrophils. This situation occurs as a result of the activity of various chemokines. Furthermore, the role of IL-17 is more proinflammatory and has pathological side effects when an excessive release occurs, while IL-22's primary function is to be regenerative and protective in inflammatory areas [24].

CONCLUSION

Casein supplementation increases the expression of HBD-1, Th-17, and IL-22 in preterm RN and a higher elevation in LPS-induced RN chorioamnionitis models. The increase in HBD-1, Th-17, and IL-22 indicate that casein plays a role in the protection and control of chorioamnionitis tissue inflammation.

DECLARATIONS

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Funding

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We certify that the work in question was performed by the author(s) identified in this article. All claims referring to claims related to the material in this paper will have to be borne by the writers.

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