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# The Effect of Giving Casein on TLR-4, NF-κB and TNF-α Expression Chorioamnion Premature Rattus Norvegicus

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**Key words:** Casein, TLR-4, NF-κB, TNFα, *Rattus norvegicus*

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**Abstract:** During pregnancy, the fetus requires substantial elements including protein for growth and development. A pro-inflammatory cascade can cause TNFα expression through NF-κB due to the presence of casein which binds to the TLR. Knowing the effect of case in supplementation diet on chorioamniont issue of premature *Rattus norvegicus* pregnancies by evaluating the expression of TLR-4, NF-κB and TNF-α. The study was a real experimental laboratory with post-test only control group design. The sample using 32 *Rattus norvegicus* separated into two Groups (G). G1 who received a regular diet of case in 40 g $kg^{-1}$ BW/day (G1) and compared with G2 which case in supplementation diet 200 g $kg^{-1}$ BW/day (G2), since, the first day of pregnancy. Chorioamnion specimens taken on the 10, 12 and 14th days of gestation. Immunohistochemical examination was performed and examined using the Remmele Immuno Reactive Score index. The data was analyzed statistically by SPSS25. LR-4 among G1 control group) has an average value of 3.675±2.001 (95% CI: 0.40-7.00) whereas the treatment Group (G2) has 3.037±2.202 (95% CI: 0.20-7.20), there was no significant difference between both groups. NF-κB was 1.862±1.138 (95% CI: 0.60-5.60) in G1 and was not significantly difference (p = 0.055) against G2 (average 2.455±0.608; 95% CI:2.00-4.20). TNFα was also not significantly different between G11.912±0.655 (95% CI:0.00-2.80) against G-2, 3.644±2.795 (95% CI:1.00-11.40). The administration of casein supplemented nutrition to pregnant *Rattus norvegicus* was not a significant difference in the expression of TLR-4, NF-κB and TNFα in chorioamnion preterm pregnancy.

## INTRODUCTION

Pregnancy is believed to require the best nutrition to ensure maternal health and fetal growth. Therefore, protein components are needed for protein synthesis to fulfill the developmental needed form a ternal and fetalt issues. After that can reduce the risk of complications that may occur both in the mother and the fetus[1]. Pregnancy is expected to last until term have healthy offspring than can further develop and grow well.

The chemical formula of casein is C38H57N9O9, with have a molecular weight of 738.928 g $moL^{-1}$ (PubChem). Casein comes from the Latin "caseus" or cheese and the composition of casein is around 2.75% of cow's milk[2]. The proportion of proteins casein includes 34% α casein, 31% β-Casein, 16% K Casein, 2.1% α lactalbumin and 9.7% β lactalbumin[3]. Some studies state that the components of peptides in cow's milk contain natural substances as immuno modulators. Bovine Glyco-Macro-Peptides (BGMP) is a sizeable bioactive peptide in casein kappa which can directly bind to various microbial toxins of intestinal pathogens. Casein has a play a role in inducing monocytes through Mitogen-Activated Protein Kinase (MAPK) and NF-κB to express cytokines IL-1, IL-6, IL-8 and TNF-α through MAPK and NF-κB pathways. Lactoferrin, lactoperoxidase, lysozyme, α-casein, α-lactalbumin and β-lactoglobulin are substance incasein. There are known to prevent the effects of pathogenic bacteria[4, 5].

During pregnancy, TLR-4 will increase, especially during labor and chorioamnionitis and expressed more in the chorion membrane than amnion[6]. TLR-4 activation in the first trimester will cause a pro-inflammatory response. Needed to protect the implantation process, placentation and fetal growth as well as in the third trimester or at the end of pregnancy to plays a role in the labor process[7]. Immuno modulatory effects of casein can pass through the proinflammatory pathway. Cheng[8] in his study has reported that casein was able to inhibit the expression of cytokine cascade[8]. Casein influence TLR by inhibit the signaling of TLR-2, TLR-3, TLR-4, TLR-5 and TLR-9. Casein inhibits TLR-4/MyD88/NF-κB bond and TLR-4/MD-2 receptor bond then secretes pro-inflammatory cytokines[8]. The infection has an essential role in inducing prematurity, so that, casein supplementation during pregnancy is expected to reduce the risk of premature labor by overcoming the infection[9]. The purpose of this study was to analyze the role of casein, which could modulate the expression of TLR-4, NF-κB and TNF-α in the chorioamnion of *Rattus norvegicus* pre-term pregnancies.

## MATERIALS AND METHODS

This research has received an ethical clearance research animal care and use commission: 3. KE.042.03.2018 by Veterinary Faculty of Airlangga University. Thirty-two of female *Rattus norvegicus* aged 2-3 months with a weight of 100-180 g, healthy, never been pregnant were injected with pregnant mare hormone serum at a dose of 10 IU (gonadotropin). After that 48 h later, infused with Human Chorionic Gonadotropin (HCG) at a dose of 10 IU, after that the female *Rattus norvegicus* mated to male rats. About 17 h later, a vaginal plug examined. If there was a vaginal plug, the female rat then defined to have pregnant on day 0. Since, it was identified to be pregnant, 32 *Rattus norvegicus* were divided into two groups randomly. G1consisted of *Rattus norvegicus* and obtained are regular daily diet containing case in 40 g $kg^{-1}$ BW and G-2 received case in supplementation diet 200 g $kg^{-1}$ BW. In this study, casein sodium CAS 9005-46-3 used from Tokyo Chemical Industry Co., Ltd. Rats treated in 45×35 cm plastic cages, each cage contains six rats according to the standard Office of Laboratory Animal Care (OLAC).

Chorioamnion specimens were taken from groups of pregnant mice on days 12 and 14. The TLR-4, NFκB and TNF-α expressions examined by immuno histo chemistry and quantitative calculations performed. Slides of the chorioamnion specimen were carried out by deparaffination in different xylene, three times, 5 min each. Rehydration in 100% ethanol two times for 5 min, then dipped in succession of 90% ethanol, 80, 70% and 5 min of distilled water for each. Slides were unmasked in the citrate buffer solution at 90°C for 20 min then dipped in $H_2O_2$ solution in Phosphate-Buffered Saline (PBS) for 20 min at room temperature. Slides then were was hed in PB Satp H7.4 and were blocked by Fetal Bovine Serum (FBS) and Bovine Serum Albumin (BSA) in PBS for 60 min.

Subsequently, each slide labeled with TLR-4 primary antibody n FBS 5%, BSA 1% overnight temperature 40°C. The slides then washed by PBS pH 7.4. After that labeled with second ary antibodies for an hour at room temperature and removed by PBSp H7.4 once again. Slides poured with StreptAvidin-Horseradish Peroxidase (SA-HRP) 1: 500 for 40 min at room temperature. Than was hed by PBS pH 7.4, then given 3.3 chromogens substrate Di-Amino Benzidine tetra-hydrochloride (DAB) for 20 min and washed again with PBS pH 7.4 and rinsed by distilled water for minutes, three times. Counterstain was performed using methyl green at room temperature. Next, the slides were soaked and rinsed by $H_2O$ for 5 min, dried for 5 min over night at room temperature. Finally, mounting did with entellan.

Table 1: Composition of supplementation diet and a daily diet of casein

| Substance (g $Kg^{-1}$ BW) | Supplementation | Daily diet |
|---|---|---|
| Casein (>85%-protein) | 200.0 | 40.0 |
| Sucrose | 100.0 | 100.0 |
| Fiber | 10.0 | 10.0 |
| Corn oil | 80.0 | 80.0 |
| Mineral mixture | 40.0 | 40.0 |
| Vitamin mixture | 10.0 | 10.0 |
| L-methionine | 1.5 | 1.5 |
| Choline bitartrate | 2.5 | 2.5 |
| Corn starch | 556.5 | 716.5 |

*Iso-calorie 410 kcal 100 $g^{-1}$; American Institute nutrition recommendation for adult mice (Xavier, 2007)

Table 2: The semi-quantitative scale of the Immuno-Reactive Score (IRS)

| A (Percentage Scores of positive cells) | B (colour reaction intensity score) |
|---|---|
| Score 0: No positive cell | Score 0: No color reaction |
| Score 1: Positive cells <10% | Score 1: Weak color intensity |
| Score 2: Positive cells between 11-50% | Score 2: Medium color intensity |
| Score 3: Positive cells between 52-80% | Score 3: Vigorous color intensity |
| Score 4: Positive cells >80% | |

*The semi-quantitative scale of the Immuno-Reactive Score (IRS) defined by the multiplication result of A and B

**Method for calculating TLR-4, NF-κB and TNF-α:** By using immunohistochemical methods on chorioamniotic cells, TLR-4, NF-κB and TNF-α expressions can calculate. Sample data were assessed semi-quantitatively according to the Remmele modification method[10]. The Remmele scale index (immuno-reactive score/IRS) was the result of multiplying the percentage of reactive-immune cells cores with color intensity scores (Table1). The data for each sample is the average of IRS score observed in ten Fields of View (FV) at 100 and 400× magnification (Table 2).

**Statistical analysis:** The Shapiro-Wilk test is used to identify data normality. The Homogeneity Levene Variance was used to calculate TLR-4 data and TNF-α. The Mann-Whitney U test was used to determine whether there were differences in the mean values between the two groups of data.

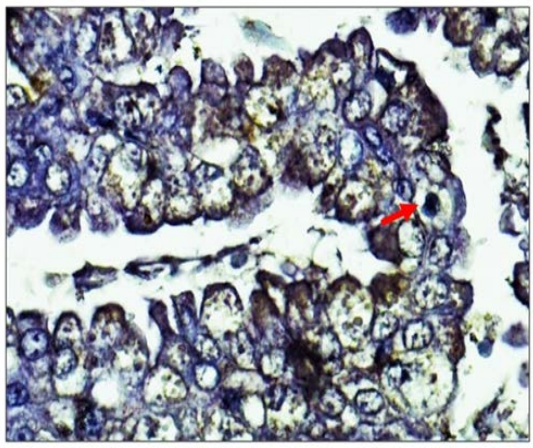

Fig. 1: Columnar cells in chorioamnion layer with positively immunoreactive (TLR-4) which indicated by brown chromogen color (arrow)

## RESULTS AND DISCUSSION

The Shapiro-Wilk normality testing carried out because the number of samples is the treatment group of TLR-4 a value was p = 0.254 means that the data usually distributed, TNF-α and NF-κB were 0.002 and 0.000, respectively, meaning those data not normally distributed.

**TLR-4 expression:** The expression of TLR-4 calculated on the *Rattus norvegicus* chorioamnion cells showed a normal distribution (p = 0.254 Shapiro-Wilk test>0.05) and had the same variant distribution (Levene p-test>0.05) (Fig. 1).

Mean value of TLR-4 in G-1 was 3.675±2.001 (95% CI: 0.40-7.00). While in G-2 was 3.037±2.202 (95% CI: 0.20-7.20). The TLR-4 expression in the second group was found to be lower; nevertheless, the difference between these two groups was not statistically significant (p-value of t-test = 0.398) (Fig. 2).

**NF-κB expression:** The Nuclear Factor of kappa-light-chain-enhancer of activated B cell (NF-κB) functions as the transcription controller of the DNA pro-inflammatory cascade. The results show that the NF-κB expression distributes the data is abnormal which is identified by the Shapiro-Wilktest with p = 0.000 (p<0.05). The Levene Homogeneity test of variance has p>0.05 (p = 0.168) its mean found no differentiation on the variance between 2 groups. Group-1 has to mean value 1.862±1.138 (95% CI: 0.60-5.60) whereas for G-2 has increased the mean value of NFκB to 2.455±0.608 (95% CI: 2.00-4.20). The Mann Whitney U test for both groups, found of p-0.006

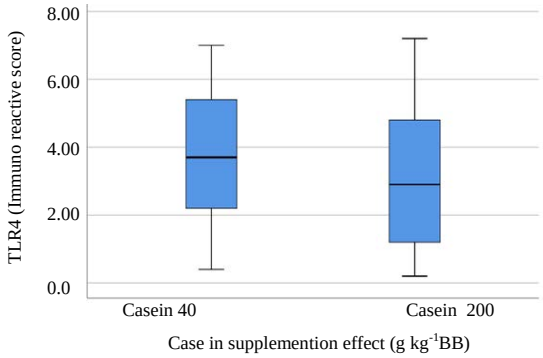


Fig. 2: Mean value of TLR-4 in group 1 with a regular daily diet of casein 40 g $kg^{-1}$ BW compared to group 2 with case in supplementation diet 200 g $kg^{-1}$ BW

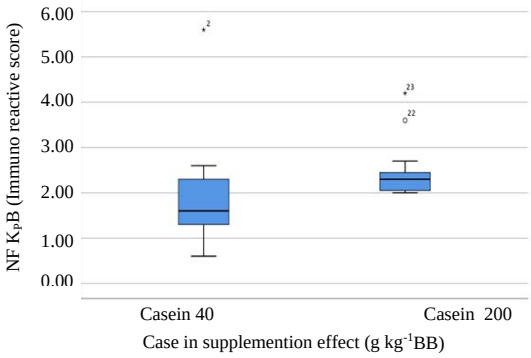


Fig. 3: Mean value of NFκB in group 1 with a regular daily diet of casein 40 g $kg^{-1}$ BW compared to group 2 with casein supplementation diet 200 g $kg^{-1}$ BW

($p<0.05$). It can be concluded that there was a significant difference in NFκB expression between groups (Fig. 3 and 4).

**TNFα expression:** The TNFα expression in G-1 and G-2 have a Mean value 1.912±0.655 (range 0.00-2.80, 95% CI) and 3.644±2.795 (range 1.00-11.40, 95% CI respectively. The Saphiro-Wilkho mogeneity of variance test of TNFα expression in both groups have obtained p value of 0.023 and 0.002 ($p<0.05$), means that the data was abnormally distributed. Mann-Whitney test of TNFα expression in both groups was performed and obtained p value of 0.012 ($p<0.05$), there was a significant difference of TNFα expressions between both groups (Fig. 5 and 6).

**Effect of casein on tlr-4expression:** The protein in cow's milk is around 3.2-3.8% of which 20% contains whey

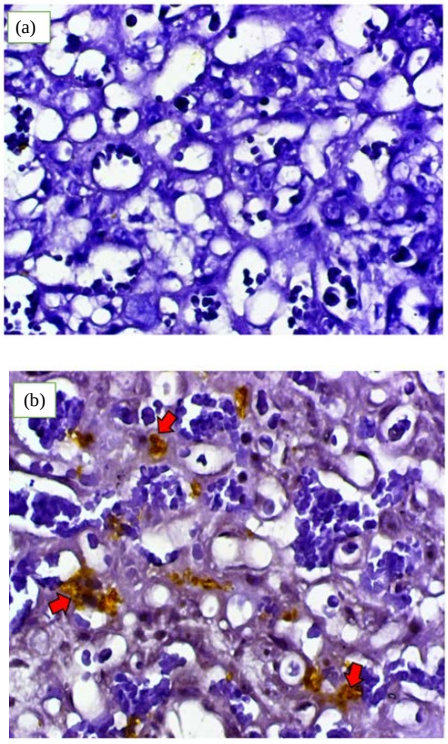


Fig. 4: NF-κB expression in chorioamnion layer, a) Showing no NF-κB expression and b) There is NF-κB expression indicated by brown chromogen color (arrow) (immunohisto chemical staining, 400× magnification; Nikon H600L microscope; DS Fi2 300 megapixel camera)

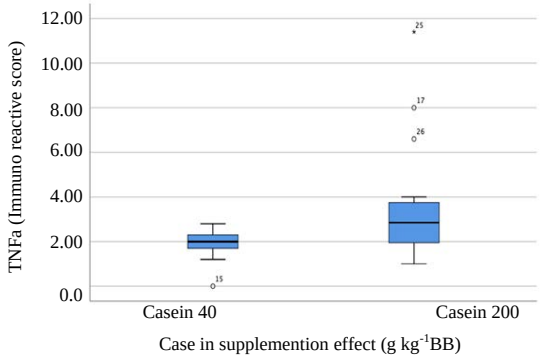


Fig. 5: Mean value of TNFκ in group 1 with a regular daily die to fcasein 40 g $kg^{-1}$ BW compared to group 2 with casein supplementation diet 200 g $kg^{-1}$ BW

protein and 80% casein or about 26 g $L^{-1}$[3, 11, 12]. Casein has bioactive properties which has an immunomodulatory effect and can involve itself in response to infection[3]. Casein-alpha can bind to TLR-2 and TLR-4 receptors on

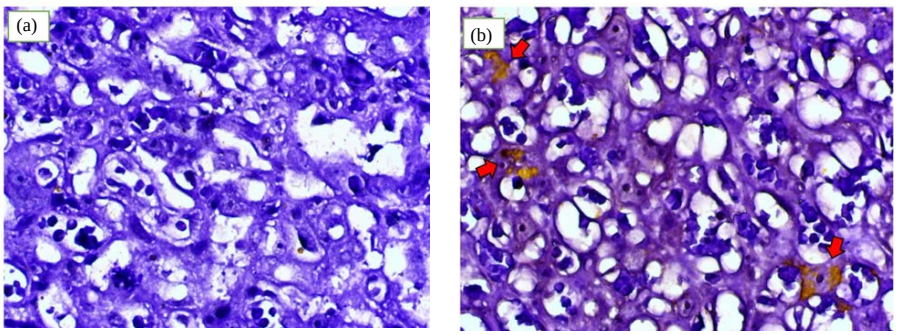


Fig. 6(a, b): TNF-α expression in chorioamnion layer, (a) Showing no TNF-α expression and (b) There is TNF-α expression indicated by brown chromogen color (arrow) (immunohisto chemical staining, 400× magnification; Nikon H600L microscope; DS Fi2 300 megapixel camera

macrophage cell walls. This bond can activate cytokine synthesis where this process does not depend on the presence of LPS[5]. Analysis of the results of the study was carried out based on various case in exposure doses. These results found a significant difference between G-1 and G-2 ($p>0.05$). At 40 g $kg^{-1}$ case in BB exposure, TLR-4 expression was found to be >200 g $kg^{-1}$ BW exposure. The 5-fold case in exposure dose can suppress TLR-4 expression, so that, the cytokine synthesis process can suppress. It has been reported that TLR-4 can produced by various female genital cells[14]. During menstruation or pregnancy, the production rate can be different[15]. TLR-4 is a Pattern Recognition Receptor (PRRs) and is a trans membrane protein that functions to recognize bacteria through molecular patterns related to pathogens (PAMP). It can also be a Damage receptor Associated with Molecular Patterns (DAMP)[16]. TLR is not only expressed by immune cells such as macrophages, monocytes, dendritic cells, neutrophils, B-lymphocytes and T-cells but as well as expressed by non-immune cells such as fibroblast cells, endothelial cells and genital epithelium[16].

In pregnancy, many cells express TLR such as endometrial, decidual, myometrial. It has found that cell life resistance to intrauterine infections is specifically associated with the presence of TLR-2 and TLR-4. TLR-2 is associated with gram-positive bacteria while TLR-4 has a relationship with gram-negative bacteria[17]. If the expression of TLR-4 during the pregnancy period decrease, it will cause resistance to LPS induction, decrease of monocyte mobilization and delay the production of genes responsible to uterine activation. Furthermore, it can avoid the risk of uterine contractions[18].

Intrauterine infections originating from a bacterial infection in the vagina are the leading causes of chorioamnionit is pre labour ruptured of membranes[19] and preterm labour[20]. Ligands of pathogenic bacterial bacteria (PAMP) will be known by TLR which will trigger activation to synthesize pro inflammatory mediators. This activation is also followed by prostaglandin production which causes uterine contractions[21]. The TLR on the surface, namely TLR-1, TLR-2, TLR- 4, TLR-5 and TLR-6 have the introduction of cellular responses to ligands composed of Lipopolysaccharide (LPS), Peptidoglycan (PG) and CpG microbial nucleotides that do not experience methylation[22, 23].

TLR-4 expression will increase during labor or during chorioamnionitis[6]. This high expression from TLR-4 is more obvious when compared to the amnion TLR-4. TLR-4 will decrease if gestational age increases chorioamnionitis[24]. The results of this study, TLR-4 chorion expression and amniotic membrane cannot be separated but calculated into one unit.

Vondenbaumen *et al*.[5] in his study used human kidneye mbryos293(HEK-293) which were known not to expressTLR-4[5]. If this cell exposed to casein-a, it is true that it does not secrete IL-1 cytokines. The conclusion is that TLR4 is a receptor for casein-a as its ligand. It can also be stated that TLR-4 expression is not affected by increased casein or LPS exposure 4[5]. Thus, a decrease in TLR-4 expression may not be due to case in exposure but because of an increase in gestational age. The reduction in TLR-4 expression is expected to reduce the secretion of pro-inflammatory cytokines, especially IL-6 and IL-8 and the synthesis of Matrix Metallo proteinases (MMPs) which can cause softening of cervix and chorioamnion[25, 26]. The synthesis of IL-10, IL-12 and CRH can also induce uterine contractions and premature rupture of membranes (PROM).

**NF-κB expression:** The mean value of NF-κB in group G-1 = 1.86, compared with G-2 = 2.45. Statistically, the

differences between the two groups were found to be significant ($p = 0.006$). Nuclear factor-kappa B is a factor responsible for the process of transcription and expression of pro-inflammatory mediators, the cytokine and chemokine which occur in innate and adaptive immunity. This factor is also a major factor in cell proliferation, differentiation and survival[27]. Liu[28] activation of NF-κB has three types, canonical, non-canonical and atypical pathways. In the canonical path, it is also called the classical pathway, NF-κB secretion is triggered by pro-inflammatory receptor signals such as the Interleukin-1 Receptor (IL-1R), Tumor Necrosis Factor Receptor (TNFR) and Toll Like Receptor (TLR)[28]. This receptor activates the IkB kinase complex through phosphorylation and ubiquitination. Furthermore, the p65/p50 and c-Rel/p50 dimer forms translocate into the nucleus to activate gene expression[29]. However, little is known about the activation of this pathway in chorioamnion. Lim[30] who examined the expression of NF-κB in amniotic membranes of pregnancy samples successfully found a positive correlation between activation through canonical and non-canonical path ways but not for atypical pathways Lim *et al*.[31].

TLR-4 can bind to casein which causes activation of the proinflammatory cascade through activation of phosphorylation and transcription of the family NF family, NF-κB-1, NF-κB-2, RelB, c-Rel, p65 (RelA). Expressions of NF-κB-1 and NF-κB-2 in the form of precursor proteins p105 and p100 will sequentially form dimers p50 and p52. Furthermore, p50 and p52 will bind to the trans-activation domain p65 or RelB; eventually, both are transcriptional activators[31, 32]. Although, casein can bind to TLR4, it does not cause phosphorylation activation, so it does not affect the expression of NF-κB.

**Effect of casein on TNFα expression:** Tumor Necrosis Factor (TNFα) is known as a cytokine which has the function of controlling the expression of cytokines, immune responses, proteases, growth factors, inflammatory regulation, survival, apoptosis, cell migration, proliferation and differentiation. A lot of data from the results of the research have been studied regarding the expression of TNFα, especially in the reproductive organs. Including the presence of alpha TNF in early pregnancy in the endometrium, myometrium and chorioamniotic membrane. This finding is related with choriomnionitis, early puerperal birth and Premature Rupture of Membranes (PROM). Cytokines IL-1b have an important role in the initiation of uterine contractions and preterm labor; this role is related to TNFα involvement whereas intra-amniotic IL-6 and IL-8 administration did not increase uterine contraction activity and did not trigger an increase in IL-1b and TNFα[33]. Research in mice induced by Shiga toxin-2 caused fetal death and activation of labor; this has been proven because of TNFα activity and is mediated by an increase in NOS production. Increased TNFα induces an increase in PGE2 which causess of tening of the cervix and causes an increase in PGF2a which will cause myometrium to be sensitive to oxytocin. Then what happens is the occurrence of preterm labor[34]. The mean TNFα in G-2 compared to G-1, there were differences of 3.64 and 1.91, respectively. The results of the statistical test showed that there were significant differences between the two groups ($p = 0.012$).

In this study, exposure to case in (200 g $kg^{-1}$ body weight) were five times higher turned out to have no significant effect on TLR-4 expression. Casein does not trigger TLR-4 expression but casein binds to extracellular TLR to trigger pro-inflammatory signaling and subsequently, expression of NF-κB, synthesis of IL-1B, TNFα, IL-6 and IL-8[35]. Using mono mac cell 6, casein-a exposure which binds to TLR-4, causes IL-1B synthesis. If the casein concentration is 10 times higher, IL-1B synthesis will be four times higher. Case in-a sand TLR-4 bonds involve proinflammatory mediators MD2 and CD14[5].

Protein in cow's milk is around 3.2-3.8% of which 20% contains whey protein and 80% casein or about 26 g $L^{-1}$[3, 12]. Casein has bioactive properties which has an effect as an immuno modulator and can involve it self in response to infection[13]. Casein-alpha can bind to TLR-2 and TLR-4 receptors on macrophage cell walls. This bond can activate the synthesis of cytokines where this process does not depend on the existence of LPS[5]. Analysis of the results of the study using various casein exposure doses. These results found a significant difference between G1 and G2 ($p>0.05$). At exposure to 40 g $kg^{-1}$ Bbcasein, TLR-4 expression was found to be >200 g $kg^{-1}$ exposure. The 5-fold case in exposure dose can suppress TLR-4 expression, so that, the cytokine synthesis process can be suppressed. It has been reported that TLR-4 can be produced by various female genital cells and is known to also have different levels of production during menstruation or pregnancy[14,15]. TLR-4 is a Pattern Recognition Receptor (PRRs) which is a transmembrane protein that functions to recognize bacteria through Pathogen-Associated Molecular Patterns (PAMP) or Damage-Associated Molecular Pattern (DAMP)[16]. TLR is not only expressed by immune cells such as macrophages, monocytes, dendritic cells, neutrophils, B-lymphocytes and t-cells but can also be found in non-immune cells such as fibroblast cells, endothelial cells and epithelium[16].

In pregnancy, there are many TLRs expressed by endometrial or decidual cells, myometrium and pregnancy products such a splacenta, chorion and amnion which are found to be associated with cell survival against intrauterine in fections, especially, TLR-2 and TLR-4. TLR-2 is associated with gram-positive bacteria while TLR-4 is associated with gram-negative bacteria[17]. Reduced TLR-4 expression during pregnancy causes resistance to LPS induction, decreased monocyte mobilization and delay inuterine activation gene

production, thus avoiding the risk of uterine contractions[18]. Intrauterine infection originating from increase dvaginal bacterial infection is the main cause of chorioamnionitis, prelabour membrane rupture and preterm labor[19].

Bacterial Pathogenic associated molecul arpatern (PAMP) ligands will be recognized by TLR and trigger the activation and synthesis of proinflammatory mediators and can be followed by the production of prostaglandins which induce uterine contractions[21]. Surface TLRs including TLR-1, TLR-2, TLR-4, TLR- 5 and TLR-6 have cellular responses to ligands in the form of Lipo Poly Saccharide (LPS), Peptidoglycan (PG) and non-methylated CpG microbial nucleotides. This receptoris activated by ligand activation signals[22, 23]. TLR-4 expression will increase during laboror during chorioamnionitis[6]. Higher expression of TLR-4 is found in the chorion membrane compared to a mnion and will decrease with increasing gestational age[16]. In this study, chorion and amniotic membrane expressions were not separated but were counted into one unit.

Vondenbaumen *et al*.[5] studied using embryonic Human Kidney 293 (HEK-293) that did not express TLR-4, in casein-a exposure, there was no secretion of IL-1 cytokines, it concluded that TLR4 had receptors for casein-a[5]. It also states that TLR-4 expression is not affected by increased casein or LPS exposure[5]. Thus, a decrease in TLR-4 expression may not be due to exposure to case in but because of gestational age. The decrease in expression of TLR-4 is expected to reduce the secretion of proinflammatory cytokines, especially, IL-6 and IL-8, by the synthesis of Matrix Metallo Proteinases (MMPs) which can causes of tening of the cervix and chorioamnion[25, 26]. The synthesis of IL-10, IL-12 and pCRH can also induce uterine contractions and Premature Rupture of Membranes (PROM).

## CONCLUSION

From the current research, it can conclude that the administration of casein supplementation diett opreterm pregnant *Rattus norvegicus* did not show a significant decrease in the expression of TLR-4 but show a significant increase in NF-κB and TNFα expressions in chorioamnion it is membrane. Further, research is still needed to determine the role of casein-a in suppressing the occurrence of cytokine release by involving various precursor proteins which may explain the role of the inhibitors of the emergence of uterine contraction mediators, so that, it is further expected to know the role of maintaining gestational age to the maximum extent aspossible.

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## REFERENCES

01. Marangoni, F., I. Cetin, E. Verduci, G. Canzone and M. Giovannini *et al.*, 2016. Maternal diet and nutrient requirements in pregnancy and breastfeeding. an Italian consensus document. Nutrients, Vol. 8, No. 10 10.3390/nu8100629.
02. Glab, T.K. and J. Boratynski, 2017. Potential of casein as a carrier for biologically active agents. Top. Curr. Chemi., Vol. 375, 10.1007/s41061-017-0158-z
03. Gellrich, K., H.H.D. Meyer and S. Wiedemann, 2014. Composition of major proteins in cow milk differing in mean protein concentration during the first 155 days of lactation and the influence of season as well as short-term restricted feeding in early and mid-lactation. Czech J. Anim. Sci., 59: 97-106.
04. Requena, P., A. Daddaoua, E. Guadix, A. Zarzuelo, M.D. Suarez, F.S. De Medina and O. Martinez-Augustin, 2009. Bovine glycomacropeptide induces cytokine production in human monocytes through the stimulation of the MAPK and the NF-κB signal transduction pathways. Br. J. Pharmacol., 157: 1232-1240.
05. Vordenbaumen, S., T. Saenger, A. Braukmann, T. Tahan, E. Bleck, J. Jose and M. Schneider, 2016. Human casein alpha s1 induces proinflammatory cytokine expression in monocytic cells by TLR4 signaling. Mol. Nutr. Food Res., 60: 1079-1089.
06. Kim, Y.M., R. Romero, T. Chaiworapongsa, G.J. Kim and M.R. Kim *et al*., 2004. Toll-like receptor-2 and-4 in the chorioamniotic membranes in spontaneous labor at term and in preterm parturition that are associated with chorioamnionitis. Am. J. Obstetrics Gynecol., 191: 1346-1355.
07. Mor, G., 2008. Inflammation and pregnancy: The role of toll-like receptors in Trophoblast-immune interaction. Ann. New York Acad. Sci., 1127: 121-128.
08. Cheng, X., D. Gao, B. Chen and X. Mao, 2015. Endotoxin-binding peptides derived from casein glycomacropeptide inhibit lipopolysaccharide-stimulated inflammatory responses via blockade of NF-κB activation in macrophages. Nutrients, 7: 3119-3137.
09. Kemp, M.W., 2014. Preterm birth, intrauterine infection and fetal inflammation. Frontiers Immunol., Vol. 5, 10.3389/fimmu.2014.00574.
10. Novak, M., J.A. Madej and P. Dziegeil, 2007. Intensity of Cox 2 expression in cell of soft tissue fibrosarcomas in dog as related to grade of tumor malignation. Bull. Vet. Inst. Pulawy, 51: 275-279.

11. Rodriquez, L.A., G. Mekonnen, C.J. Wilcox, F.J. Martin and W.A. Krienke, 1985. Effects of relative humidity, maximum and minimum temperature, pregnancy and stage of lactation on milk composition and yield. J. Dairy Sci., 68: 973-978.
12. Pereira, P.C., 2014. Milk nutritional composition and its role in human health. Nutrition, 30: 619-627.
13. Petrotos, K., E. Tsakali, P. Goulas and A.G. D'Alessandro, 2014. Casein and whey proteins in human health. Milk Dairy Prod. Funct. Foods, 1: 94-146.
14. Young, S.L., T.D. Lyddon, R.L. Jorgenson and M.L. Misfeldt, 2004. Expression of Toll-like receptors in human endometrial epithelial cells and cell lines. Am. J. Reprod. Immunol., 52: 67-73.
15. Aflatoonian, R., E. Tuckerman, S.L. Elliott, C. Bruce, A. Aflatoonian, T.C. Li and A. Fazeli, 2006. Menstrual cycle-dependent changes of Toll-like receptors in endometrium. Hum. Reprod., 22: 586-593.
16. Chin, P.Y., C.L. Dorian, M.R. Hutchinson, D.M. Olson, K.C. Rice, L.M. Moldenhauer and S.A. Robertson, 2016. Novel toll-like receptor-4 antagonist (+)-naloxone protects mice from inflammation-induced preterm birth. Sci. Rep., Vol. 6, 10.1038/srep36112.
17. Schatz, F., U.A. Kayisli, E. Vatandaslar, N. Ocak and S. Guller et al., 2012. Toll-like receptor 4 expression in decidual cells and interstitial trophoblasts across human pregnancy. Am. J. Reprod. Immunol., 68: 146-153.
18. Wahid, H.H., C.L. Dorian, P.Y. Chin, M.R. Hutchinson and K.C. Rice *et al*., 2015. Toll-like receptor 4 is an essential upstream regulator of on-time parturition and perinatal viability in mice. Endocrinol., 156: 3828-3841.
19. Helmo, F.R., E.A.R. Alves, R.A.D.A. Moreira, V.O. Severino and L.P. Rocha *et al.*, 2017. Intrauterine infection, immune system and premature birth. J. Maternal-Fetal Neonatal Med., 31: 1227-1233.
20. Klein, L.L. and R.S. Gibbs, 2005. Infection and preterm birth. Obstetrics Gynecol. Clin., 32: 397-410.
21. Witkin, S.S., I.M. Linhares, A.M. Bongiovanni, C. Herway and D. Skupski, 2010. Unique alterations in infection-induced immune activation during pregnancy. BJOG: Int. J. Obstetrics Gynaecol., 118: 145-153.
22. Eriksson, M., S.K. Meadows, S. Basu, T.F. Mselle, C.R. Wira and C.L. Sentman, 2014. TLRs mediate IFN-α production by human uterine NK cells in endometrium. J. Immunol., 176: 6219-6224.
23. Koga, K., P.B. Aldo and G. Mor, 2009. Toll-like receptors and pregnancy: trophoblast as modulators of the immune response. J Obstetrics Gynaecol. Res., 35: 191-202.
24. Choi, S.J., S.H. Jung, M. Eom, K.H. Han, I.B. Chung and S.K. Kim, 2011. Immunohistochemical distribution of toll-like receptor 4 in preterm human fetal membrane. J. Obstetrics Gynaecol. Res., 38: 108-112.
25. Stygar, D., H. Wang, Y.S. Vladic, G. Ekman, H. Eriksson and L. Sahlin, 2004. Increased level of matrix metalloproteinases 2 and 9 in the ripening process of the human cervix. Biol. Reprod., 67: 889-894.
26. Pustovrh, M.C., A. Jawerbaum, E. Capobianco, V. White, J.J. Lopez-Costa and E. Gonzalez, 2004. Increased matrix metalloproteinases 2 and 9 in placenta of diabetic rats at midgestation. Placenta, 26: 339-348.
27. Oeckinghaus, A. and S. Ghosh, 2009. The NF-κB family of transcription factors and its regulation. Cold Spring Harbor Perspect. Biol., Vol. 1, 10.1101/cshperspect.a000034
28. Liu, T., L. Zhang, D. Joo and S.C. Sun, 2017. NF-κB signaling in inflammation. Signal Transd. Targeted Ther., Vol. 2. 10.1038/sigtrans.2017.23.
29. Collins, P.E., I. Mitxitorena and R.J. Carmody, 2016. The ubiquitination of NF-κB subunits in the control of transcription. Cells, Vol. 5, 10.3390/cells5020023.
30. Lim, S., D.A. MacIntyre, Y.S. Lee, S. Khanjani, V. Terzidou, T.G. Teoh and P.R. Bennett, 2012. Nuclear factor kappa B activation occurs in the amnion prior to labour onset and modulates the expression of numerous labour associated genes. PloS One, Vol. 7, 10.1371/journal.pone.0034707.
31. Caamano, J. and C.A. Hunter, 2002. NF-κB family of transcription factors: Central regulators of innate and adaptive immune functions. Clin. Microbiol. Rev., 15: 414-429.
32. Pires, B.R., R.C. Silva, G.M. Ferreira and E. Abdelhay, 2018. NF-kappaB: Two sides of the same coin. Genes, Vol. 9, 10.3390/genes9010024.
33. Sadowsky, D.W., K.M. Adams, M.G. Gravett, S.S. Witkin and M.J. Novy, 2006. Preterm labor is induced by intraamniotic infusions of interleukin-1ß and tumor necrosis factor-a but not by interleukin-6 or interleukin-8 in a nonhuman primate model. Am. J. Obstetrics Gynecol., 195: 1578-1589.
34. Burdet, J., F. Sacerdoti, M. Cella, A.M. Franchi and C. Ibarra, 2012. Role of TNF-α in the mechanisms responsible for preterm delivery induced by Stx2 in rats. Br. J. Pharmacol., 168: 946-953.
35. Hsu, H.Y. and M.H. Wen, 2002. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. J. Biol. Chem., 277: 22131-22139.