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Original Article

Evaluation of Interleukin-10 and Pro-inflammatory Cytokine Profile in Calves Naturally Infected with Neonatal Calf Diarrhea Syndrome

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ABSTRACT

Multifactorial neonatal calf diarrhea (NCD) is one of the main causes of mortality in calves under 1 month of age. Studies have shown that pro-inflammatory cytokines (i.e., interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha [TNF-α]) can increase in many pathophysiological conditions. In addition, IL-10 as one of the most important anti-inflammatory cytokines contributes to the inhibition of pro-inflammatory cytokines. Few studies have addressed cytokine levels in calves with NCD. Therefore, the goal of this study was to evaluate the plasma concentrations of pro-inflammatory cytokines, as well as IL-10, in the calves naturally infected with NCD syndrome. For this purpose, 87 neonatal calves were monitored for 1 month. Out of this population, 10 cases were diagnosed with NCD and studied as the case group. In addition, a control group was considered that consisted of 10 age- and gender-matched neonatal calves without any diseases. From each calf, 5 ml blood sample was collected into EDTA tubes by jugular venipuncture. The samples were then centrifuged, and the extracted plasmas were aliquoted and stored at -20 °C. Finally, the plasma concentrations of pro-inflammatory cytokines (i.e., IL-1β, IL-6, and TNF-α) and anti-inflammatory cytokine IL-10 were measured using the ELISA kits based on the manufacturer's protocols. The results showed that the plasma concentrations of pro-inflammatory cytokines were significantly higher in the case group than in the control group. However, no significant difference was found between the two groups in terms of the plasma concentrations of IL-10. This study indicates that pro-inflammatory cytokines could be used to recognize the immune system response to NCD.

Keywords: Neonatal calf diarrhea, Cytokines, Pro-inflammatory, Anti-inflammatory

Évaluation du Profil de l'Interleukine-10 et des Cytokines Pro-inflammatoires chez les Veaux Naturellement Infectés par le Syndrome de La Diarrhée Néonatale des Veaux

Résumé: La diarrhée néonatale multifactorielle (DNM) est l'une des principales causes de mortalité chez les veaux de moins d'un mois. Des études ont montré que les taux de cytokines pro-inflammatoires (c'est-à-dire l'interleukine (IL) -1β, IL-6 et le facteur de nécrose tumorale alpha [TNF-α]) peuvent augmenter dans de nombreuses conditions physiopathologiques. De plus, l'IL-10 en tant que l'une des cytokines anti-inflammatoires les plus importantes contribue à l'inhibition des cytokines pro-inflammatoires. Peu d'études ont abordé les niveaux de cytokines chez les veaux atteints de DNM. Par conséquent, l'objectif de cette étude était d'évaluer les concentrations plasmatiques des cytokines pro-inflammatoires, ainsi que d'IL-10, chez les veaux naturellement infectés par le syndrome de DNM. À cette fin, 87 veaux nouveau-nés ont été suivis pendant 1 mois. Sur cette population, 10 cas ont été diagnostiqués avec une DNM et étudiés comme groupe de cas positifs. En outre, un groupe témoin, composé de 10 veaux nouveau-nés de même âge et sexe sans aucune maladie a été également considéré. De chaque veau, 5 ml d'échantillon de sang ont été prélevés dans des tubes EDTA par ponction

veineuse jugulaire. Les échantillons ont ensuite été centrifugés et les plasmas extraits ont été aliquotés et stockés à -20 °C. Enfin, les concentrations plasmatiques de cytokines pro-inflammatoires (c'est-à-dire IL-1β, IL-6 et TNF-α) et de cytokines anti-inflammatoires IL-10 ont été mesurées à l'aide des kits ELISA basés sur les protocoles du fabricant. Les résultats ont montré que les concentrations plasmatiques de cytokines pro-inflammatoires étaient significativement plus élevées dans le groupe de cas que dans le groupe témoin. Cependant, aucune différence significative n'a été trouvée entre les deux groupes en termes de concentrations plasmatiques d'IL-10. Cette étude indique que les cytokines pro-inflammatoires pourraient être utilisées pour reconnaître la réponse du système immunitaire à la DNM.
Mots-clés: Diarrhée néonatale des veaux; Cytokines; Pro-inflammatoire; Anti-inflammatoire

INTRODUCTION

Neonatal calf diarrhea (NCD) is a main factor threatening the health of calves under 1 month of age. This disease is associated with diarrhea, dehydration, poor growth, and acidosis as the most important clinical symptoms (Durel et al., 2017). The NCD poses a high therapeutic cost and leads to high mortality in case of the lack of response to treatment. It has been estimated that NCD causes a mortality rate of over 50% among pre-weaned calves (Kaveh et al., 2017). Accordingly, this disease imposes irreparable economic loss to livestock farming. The NCD is a multifactorial syndrome, which involves a series of bacterial, viral, and protozoal agents, transmitted by colostrum and environmental conditions, as well as passive immunity (Al Mawly et al., 2015). Therefore, the immune system of calves plays a pivotal role in protecting them against NCD syndrome. Cytokines, proteins of low-molecular weight, are the main components of the immune system, which contribute to signal transduction between cells and regulate the immune responses (Delirezh et al., 2016). Meanwhile, pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factors alpha (TNF-α), play a major role in this regard. These cytokines are mainly produced by mononuclear phagocytes (Murtaugh et al., 1996). According to the literature, pro-inflammatory cytokines can increase under many pathophysiological conditions (Kasimanickam et al., 2013). The other type of cytokine, known as anti-inflammatory cytokine owing to its function, is produced by the immune cells to regulate the secretion of pro-inflammatory cytokines and control the associated tissue damage (Opal & DePalo, 2000). The IL-10 is one of the anti-inflammatory cytokines released by many activated immune cells, controlling the inflammatory pathways in several diseases (Ouyang et al., 2011). Therefore, this cytokine can be considered an appropriate indicator to monitor the activity of the immune system.Studies on NCD have been mainly focused on passive maternal immunity (Arsenopoulos et al., 2017; Bok et al., 2018). Therefore, more studies are needed to recognize the immune system of calves with NCD syndrome. Regarding this, the present study was conducted to determine the plasma concentrations of IL-1β, IL-6, TNF-α, and IL-10 in the calves naturally infected with NCD in Sanandaj, west of Iran.

MATERIAL AND METHODS

Animals. This study was designed and implemented from April to October, 2018, according to the guidelines of the local ethical committee after obtaining written consent from the cattle owners. For the purpose of the study, 93 dairy cattle (Holstein Friesian) in the late stages of pregnancy were randomly selected from 6

villages of Sanandaj region, west of Iran. The cows were visited until the birth of calves after confirming their health based on history, vaccination card, and clinical examination. A total of 87 healthy calves (i.e., 48 females and 39 males) were enrolled in this study. The calves were fed with colostrum in two occasions (i.e., 2 and 12 h after birth). All 87 neonatal calves were monitored for 1 month without any communication with each other. During the study, they had access to clean water and starter grain ad libitum. The calves were examined every day for the symptoms of NCD syndrome, including diarrhea, dehydration, tachycardia, acidosis, weakness, and loss of appetite.

Grouping and sampling. During the study, a total of 10 neonatal calves (i.e., 7 females and 3 males, with the age of 12-17 days) were diagnosed with NCD and assigned to the case group based on the clinical symptoms and fecal consistency score of ≥ 3 (1: normal and solid, 2: pasty consistency, 3: aqueous consistency, 4: fluid consistency) (Soares et al., 2017). To assign the control group, 10 healthy neonatal calves were selected according to the clinical examination, laboratory findings, and fecal consistency (data not shown). The calves in the control group were of the same gender and age as those of the case group. For the purpose of the study, 5 ml blood sample from each calf was collected into EDTA tubes by jugular venipuncture. In the next stage, the samples were centrifuged at 1,000 g for 15 min. The extracted plasmas were then aliquoted and stored at -20°C for further analysis.

Pro-inflammatory cytokines and interleukin-10 assay. After deforesting the samples, the plasma concentrations of IL-1β (detection range: 15.6-1000 pg/ml), IL-6 (detection range: 7.8-500 pg/ml), TNF-α (detection range: 7.8-500 pg/ml), and IL-10 (detection range: 15.6-1000 pg/ml) were measured using the ELISA kits (USCN, Wuhan, China) based on manufacturer's protocols at 450 nm (microplate reader, Stat Fax 4200). The results were expressed in pg/ml.

Statistical analysis. The data were reported as mean±SD. After confirming the normality of the data by Shapiro-Wilk test, independent samples t-test was used to compare the mean values between the two groups. The statistical analyses were performed by GraphPad Prism software, version 7.03 (GraphPad Software, San Diego, California, USA). A *p*-value less than 0.05 was considered statistically significant.

RESULTS

As shown in Figure 1a, the mean plasma IL-1β concentrations in the control and experimental groups were respectively estimated at 81.78±40.164 and 506.92±150.496, indicating a statistically significant increase in the case group ($P<0.001$). Furthermore, the mean plasma levels of IL-6 in the neonatal calves of the control and case groups were obtained as 14.53±9.215 and 23.87±4.931, respectively, indicating a significant difference between the two groups ($P=0.0112$; Figure 1b). The results revealed a higher mean level of TNF-α in the case group (176.32±42.956), compared with that in the control group (23.51±7.436; $P<0.001$; Figure 1c). Although the mean plasma concentration of IL-10 was increased in the case group (26.55±7.309), compared to that in the control group (15.79±14.835), the difference was not statistically significant ($P=0.0601$; Figure 2).

DISCUSSION

Although IL-1β plays an important role in the regulation of heat and feeding in normal conditions, its increased production in many infectious diseases causes appetite suppression, temperature elevation, and inflammation (Kasimanickam et al., 2013). Contrary to the present research, many studies on viral diarrhea in cattle have reported a systemic and localized decrease in IL-1β levels (Murtaugh et al., 1996; Aich et al., 2007). The increase in plasma concentrations of IL-1β in the present study seems to be due to the presence of lipopolysaccharides (LPS) in the cell walls of Gram-negative bacteria, as well as the multivariate nature of NCD syndrome (Sohn et al., 2007; Durel et al., 2017). Bovine rotavirus (BRV) and coronavirus (BCV) are the

common viral agents of NCD (Al Mawly et al., 2015). In a study, IL-6 levels in the intestinal tissues of newborn calves with BRV and BCV were increased and decreased, respectively (Aich et al., 2007). In the current study, the increase in IL-6 levels can be justified in three ways, namely its role in the inflammation pathway and inducing the production of acute-phase proteins (Murtaugh et al., 1996), its anti-inflammatory function in inhibiting IL-1 and TNF-α, and the increase in its level induced by LPS and BRV (Opal and DePalo, 2000; Aich et al., 2007). In line with the present study, IL-6 has been shown to increase in NCD syndrome (Fischer et al., 2016). Through the expression of endothelial adhesion molecules and the increase in vascular permeability, TNF-α and IL-1β lead to the migration of leukocytes to the infection site (Murtaugh et al., 1996). Increased TNF-α production is associated with the suppression of appetite and loss of weight in inflammatory conditions (Kasimanickam et al., 2013). *Cryptosporidium parvum* is a common cause of NCD, the invasion of which is limited by increasing the concentration of TNF-α in an in vitro study (Lean et

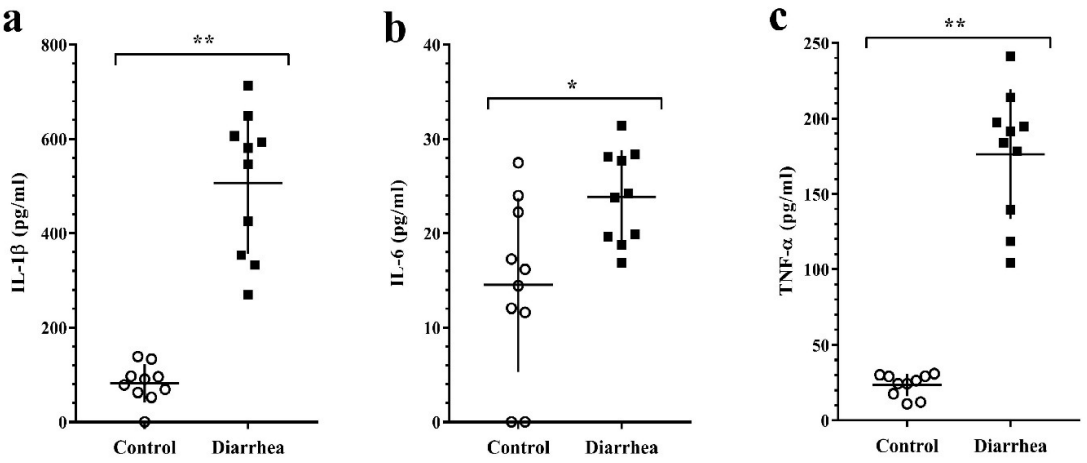

Figure 1. Plasma concentrations of interleukin-1β (a), interleukin-6 (b), and tumor necrosis factor-α (c) in the case and control groups (Data are presented as mean±SD [n=10]. In cases where the plasma concentrations of cytokines were not detectable, they were considered zero in statistical analysis [*P=0.0112, **P<0.001 compared with the control group]).

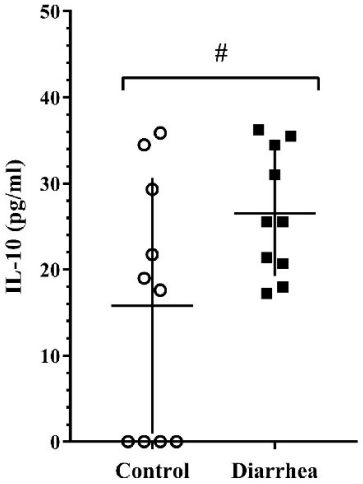

Figure 2. Plasma concentration of interleukin-10 in the case and control groups (Data are presented as mean±SD [n=10]. In cases where the plasma concentrations of interleukin-10 were not detectable, they were considered zero in statistical analysis. [$^{\#}P$=0.0601, compared with the control group]).

al., 2006; Al Mawly et al., 2015). In the present research, the increased plasma concentration of TNF-α is probably due to its antiviral properties, LPS-induced inflammatory response, and protozoal agents (Murtaugh et al., 1996; Lean et al., 2006; Sohn et al., 2007). Environmental conditions can play a role in the production of bovine IL-10. Several in vitro studies have shown that high temperature (42°C) and temperature-humidity index (THI) value of > 72 can reduce the proliferation of IL-10-secreting cells (e.g., monocytes and regulatory T cells), compared to low temperature (38.5°C) and a THI value of < 72 (Elvinger et al., 1991; Lacetera et al., 2005). Moreover, it is reported that the plasma level of IL-10 is lower in the tropical thermal condition than in the cold climate (Alhussien et al., 2018). It has been established that IL-10 has a regulatory role in inflammatory conditions (Ouyang et al., 2011). The IL-10 directly inhibits pro-inflammatory cytokines and reduces their damaging effects (Opal & DePalo, 2000). In the present study, TNF-α and IL-1β synergism seemed to inhibit the timely and adequate secretion of IL-10 (Murtaugh et al., 1996; Ouyang et al., 2011).

In conclusion, the findings of this study showed that pro-inflammatory cytokines (i.e., IL-1β, IL-6, and TNF-α) were increased in NCD syndrome. Therefore, these cytokines can be used to recognize the immune system response in the NCD. Another important finding of the present study was the negligible role of IL-10 as an important anti-inflammatory cytokine in controlling the pro-inflammatory cytokines.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contribution

Study concept and design: Raeeszadeh, M.
Acquisition of data: Beheshtipour, J.
Analysis and interpretation of data: Beheshtipour, J.
Drafting of the manuscript: Raeeszadeh, M.
Critical revision of the manuscript for important intellectual content: Beheshtipour, J.
Statistical analysis: Beheshtipour, J.
Administrative, technical, and material support: Beheshtipour, J.; Raeeszadeh, M.

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