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Evaluation of Protective Proteins and Cytokines in Milk and Serum of Healthy Camels During the First Two Months Postpartum

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ABSTRACT

The current study sought to investigate changes in the secretion of immuno-protective proteins, as well as inflammatory and regulatory cytokines in camel milk and serum during the first two months postpartum. Using commercially available ELISA kits, the concentrations of immunoglobulin G (IgG), lactoferrin (LTF), lactoperoxidase (LPO), tumor necrosis factor-alpha (TNF-a), interleukin-6 (IL-6), and interleukin-10 (IL-10) in milk samples collected on days 4, 10, 30, and 60 postpartum were measured. IgG, LTF, and LPO were also measured in the serum samples from the dams and their calves obtained at the same time points. There were no significant variations in the levels of the protective proteins LTF and LPO in the milk on day 4 compared to the other sampling days. However, IgG concentrations significantly decreased from day 4 to days 10, 30, and 60 ($P \leq 0.05$, $P \leq 0.001$, and $P \leq 0.01$, respectively). TNF-α secretions significantly increased on days 10, 30, and 60 compared to day 4 ($P \leq 0.001$). Only days 10 and 60 exhibited significant decreases in IL-6 secretion compared to day 4 ($P \leq 0.001$). The regulatory cytokine IL-10 levels were stable for the first three sampling times before significantly declining at day 60 ($P \leq 0.001$). Analysis of the dam's serum revealed a significant decline in LTF concentrations on days 10 and 60 compared to day 4 ($P \leq 0.05$) and a significant increase in serum LPO on day 10 only ($P \leq 0.01$). Serum IgG decreased in all the sampling days compared to day 4, however, these decreases were significant only on day 10 ($P \leq 0.05$). A significant decline in calf's serum IgG was observed on day 30 of age as compared to day 4. The results of the present study further our knowledge regarding the biologically active compounds found in milk that can be used to distinguish between healthy and diseased camel udders.

Key words: Tumor necrosis factor-alpha, Interleukin-6, Lactoferrin, Lactoperoxidase, Camel milk.

INTRODUCTION

Immuno-protective proteins that can be present in camel milk include immunoglobulin G (IgG), lactoferrin (LTF), and lactoperoxidase (LPO) (Mohamed et al. 2022; Albarrak 2023). Immunoglobulins, in particular immunoglobulin G (IgG), are part of the immune system that helps the body remove substances that are foreign to it when combined with other protective proteins. Immunoglobulins are essential to protect against newborn infections in many mammalian species that are born without an effective immune system (Shao et al. 2018; Pou et al. 2019). The camel serum contains both the traditional heterotetrameric antibodies (IgG1) and the distinct functional single-chain antibodies (IgG2 and IgG3). Camelid IgG2 and IgG3 antibodies are known as heavy chain antibodies (HCAbs) because they lack light chains (Azwai et al. 1996; Muyldermans 2013; Hussen and Schuberth 2021). IgG1 and HCAbs have been found to be released in camels in milk, with camel milk having higher quantities of immunoglobulin G compared to levels in goat, cow, sheep, buffalo, and human milk (Salhi et al. 2015; Kowalczyk et al. 2022).

LTF is prevalent in certain leukocytes and animal fluids such as milk, tears, semen, and saliva (Hao et al. 2019; Kell and Heyden 2020). First found in cow's milk, then in human milk, it is believed to have a number of biological activities, including having antibacterial and anti-inflammatory characteristics (Hao et al. 2019; Gruden and Poklar Ulrih 2021). LTF is one of numerous molecules released by the immune system that bind transient metals to prevent bacterial infections since iron is necessary for many vital biological processes, including DNA and ATP synthesis (Rainard 1993; Gruden and Poklar Ulrih 2021; Sienkiewicz et al. 2022). In addition to its anti-bacterial and antifungal activities, LTF can promote innate immunity to aid in the eradication of pathogens. According to one study, bovine LTF can effectively replace antibodies in initiating

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the classical pathway of the complement system and causing the opsonization of unencapsulated *S. agalactiae* (Fernandes and Carter 2017; Silva et al. 2020).

Another protective glycoprotein is lactoperoxidase (LPO), which is present in milk and other bodily fluids of both humans and animals (Amiri et al. 2021). The lactoperoxidase system (LPS), which LPO uses to exert its antibacterial activity, depends on hydrogen peroxide (H_2O_2) and thiocyanate (SCN-) (Tonoyan et al. 2020). The host's natural defensive mechanism is assumed to contain the LPS as a key element (Tonoyan et al. 2020). Gram-positive and Gram-negative bacteria are both vulnerable to LPS to varying degrees, and LPS has also been found to have antiviral properties (El-Fakharany et al. 2017; Yousefi et al. 2022). For the purpose of removing pathogenic microorganisms and ensuring milk safety, activation of LPS has been researched as a potential alternative approach.

Cytokines can be released differently in milk as a response to physiological or pathological changes, making them excellent indicators of the health of the udder (Akhtar et al. 2020; Vitenberga-Verza et al. 2022). Proinflammatory cytokines including TNF-a and IL-6 are essential for triggering the innate immune system in epithelial cells, macrophages, and neutrophils, which are the key players in the immunological response against intramammary infection. TNF-a and IL-6 levels have been found to be much higher in infected mammary glands in both clinical and subclinical mastitis (Akhtar et al. 2020; Serdal et al. 2021). These cytokines are produced locally and released into the bloodstream and have been suggested as indicators of early mastitis (Serdal et al. 2021). The anti-inflammatory cytokine IL-10 is another cytokine found in milk and known to restrict the immune response to infections, protecting the host tissues (Šerstņova et al. 2022). IL-10 is a multifunctional cytokine that has the ability to suppress the production of proinflammatory cytokines, as well as the activation and effector functions of T cells, and macrophages (Couper et al. 2008). It also has a variety of other effects on hemopoietic cells.

The current study aimed to investigate changes in the milk levels of immune-protective proteins, proinflammatory, and regulatory cytokines in healthy camels during the first two months post-parturition. The data of the current study contribute to a better understanding of milk bioactive molecules that can be used to differentiate between healthy and diseased udders in camels.

MATERIALS AND METHODS

Ethical Approval

The Institutional Animal Ethics Committee's approval was not needed because there were no invasive methods that would harm animals.

Animals and Samples Collection

Six lactating apparently healthy Asail camels (Abdallah and Faye 2012), aged between 6 and 8 years, participated in the study. Animals were sampled at days 4, 10, 30, and 60 postpartum. Typically, a single teat from each nursing animal was used to collect a 100mL milk sample. For serum collection, 10mL of blood was drawn from the dams and their corresponding calves without the use of anticoagulants.

Skim Milk Preparation

For 10 minutes at 4°C, milk samples were centrifuged at 13,000rpm to separate the skim milk from the whole milk. Assurance of fat-droplets-free Skim milk samples were done using a light microscope and then kept at -20°C until they were required for additional analysis.

Quantification of Bioactive Proteins

The concentrations of IgG, LTF, LPO, TNF-a, and IL-6 were determined using commercially available ELISA kits (Sunlog Biotech, Hangzhou, Zhejiang, China; kits, Cat. Nos.SL0039cm, SL0050cm, SL0051cm, SL0030Cm, and SL0032Cm, respectively). IL-10 concentrations were also quantified using a commercial ELISA kit per the manufacturer's specifications (Wuhan Fine Biotech Co., Ltd, Optics Valley Biomedical Industrial Park, Wuhan, China; Cat No. ECM0010).

Statistical Analysis

Statistical analysis and curve drafting were done using GraphPad Prism version 9 (Bangalore, 560035 Karnataka, India). Statistically significant differences between the means of sampling day 4 and the means of the other sampling days were determined using the t-test.

RESULTS

Lactoferrin (LTF), lactoperoxidase (LPO), immunoglobulin G (IgG), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-a), and interleukin-10 (IL-10) levels in milk samples obtained on days 4, 10, 30, and 60 postpartum were measured using commercial ELISA kits. Fig. 1 shows no significant differences between day 4 and the other sampling days regarding the milk levels of protective proteins LTF and LPO. In contrast, the concentrations of the immunoprotective protein, IgG, significantly declined on days 10, 30, and 60 as compared to day 4 ($P \leq 0.05$, ≤ 0.001, and ≤ 0.01, respectively). As shown in Fig. 2, quantification of inflammatory cytokines in the milk samples revealed significant increases in TNF-a secretions on days 10, 30, and 60 compared to day 4 ($P \leq 0.001$). Secretion of IL-6 significantly declined only on days 10 and 60 compared to day 4 ($P \leq 0.001$). The levels of the regulatory cytokine IL-10 remained constant during the first three sampling time points and significantly decreased at day 60 ($P \leq 0.001$).

The levels of LTF, LPO, and IgG in serum samples collected from dams 4-, 10-, 30-, and 60-days post-parturition were determined (Fig. 3). The serum LTF significantly declined on days 10 and 60 compared to day 4 ($P \leq 0.05$) with LTF levels on day 30 being similar to day 4. As compared to the samples obtained on day 4, a significant increase in serum LPO was observed on day 10 only ($P \leq 0.01$). A general decrease in the serum IgG was observed on all the sampling days compared to day 4, however, these decreases were significant only on day 10 ($P \leq 0.05$).

The serum levels of these immunoprotective proteins in the calf's sera were measured. Fig. 4 shows no significant differences among the sampling time points regarding calf serum levels of LTF and LPO. However, a significant decrease in the IgG levels was noted on day 30 ($P \leq 0.05$).

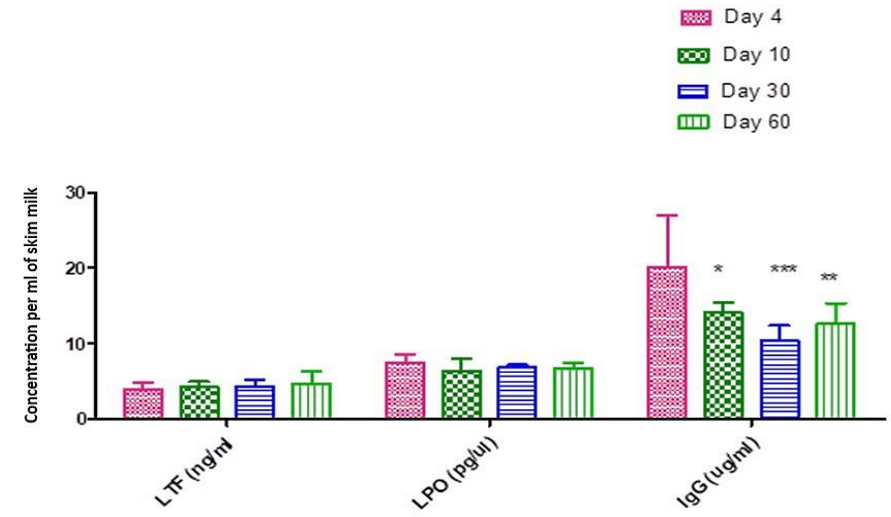

Fig. 1: Lactoferrin (LTF), lactoperoxidase (LPO), and immunoglobulin G (IgG) levels in milk samples collected 4-, 10-, 30-, and 60-days post parturition. Bars bearing *, **, and *** are significantly different from the mean value of day 4 at P≤0.05, ≤0.01, and ≤0.001, respectively.

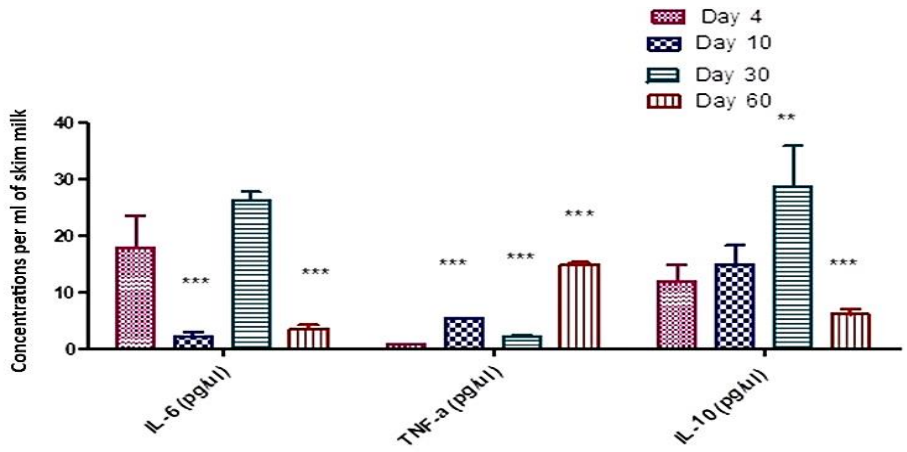

Fig. 2: Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-a), and interleukin-10 (IL-10) levels in milk samples collected 4-, 10-, 30-, and 60-days post parturition. Bars bearing *** significantly (P≤0.001) different from the mean value of day 4.

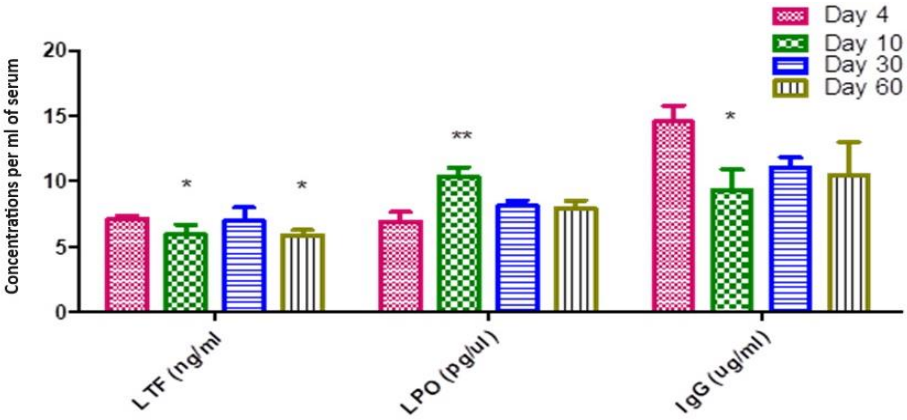

Fig. 3: Lactoferrin (LTF), lactoperoxidase (LPO), and immunoglobulin G (IgG) levels in serum samples collected from the dams 4-, 14-, 30-, and 60-days post parturition. Bars bearing *, and ** are significantly different from the mean value of day 4 at P≤0.05, and ≤0.01, respectively.

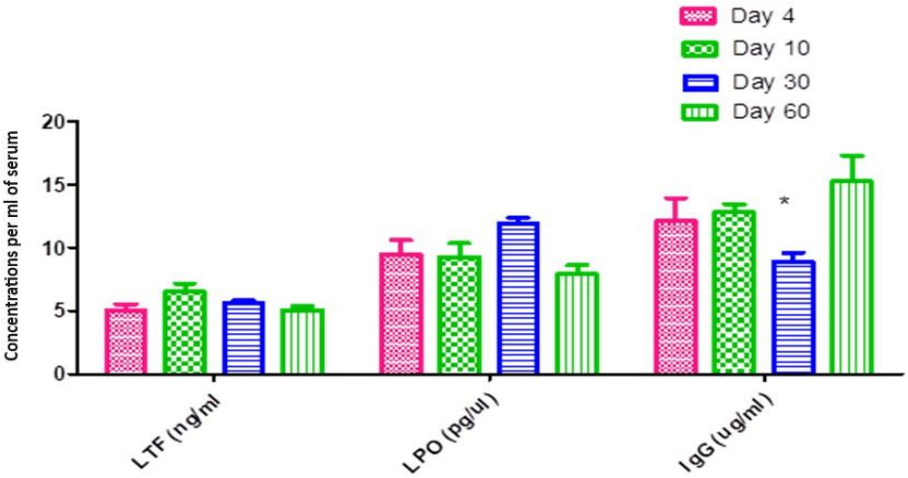

Fig. 4: Lactoferrin (LTF), lactoperoxidase (LPO), and immunoglobulin G (IgG) levels in serum samples collected from the calves at 4 -, 10 -, 30 -, and 60 -days of age. The bar bearing * is significantly different from the mean value of day 4 at P≤0.05.

DISCUSSION

The current study sought to investigate changes in the secretion of immunoprotective proteins, as well as inflammatory and regulatory cytokines in milk samples taken from healthy camels during the first two months postpartum.

Concerning the milk levels of immunoprotective proteins, our data indicated significant decreases in the IgG levels on days 10, 30, and 60 compared to day 4. Similar observations were noted when this immunoprotective protein was quantified in the serum. This observation corresponds to previous studies indicating gradual decreases in IgG secretion in milk postpartum. A study by El-Hatmi et al. (2006) quantified immunoreactive proteins in the camel colostrum during the first 14 milking times and reported gradual declines in the IgG levels. Another study by Merin et al. (2001) reported that total whey proteins steadily decreased until three months after delivery, at which point they returned to the milk's normal concentration. IgG and serum albumin make up the majority of the whey in camel colostrum with the IgG concentrations dropping rapidly. Camel milk has been shown to contain greater levels of IgG than cattle, goat, sheep, buffalo, and human milk (El-Agamy and Nawar 2000; El-Agamy et al. 2009). However, the camel milk contents of IgG have been shown to vary based on multiple factors including age, breed, parity, and management system (Mostafa et al. 2018; Albarrak 2023). Because blood IgG concentrations after birth exhibit a linear rise in relation to abnormal colostrum, colostrum quality evaluation based on immunoglobulin concentrations is not sufficient. Therefore, it is necessary to propose a classification of colostrum quality based on IgG levels (Al-Majali et al. 2007). The specific characteristics of the camel milk IgG described here, as well as their dynamic changes during postpartum, are crucial for understanding not only how the calf's immune system develops but also how camel milk and its bioactive components relate to human nutrition (Puppel et al. 2021).

LTF and LPO secretions were evaluated in the present study. Our data indicated no significant changes regarding the LTF and LPO concentrations in the milk samples taken during the first two months postpartum. However, the serum LTF was significantly lower on days 10 and 60 compared to day 4, while the serum LPO was significantly elevated on day 10 only. These observations correspond to a previous study in camels indicating that LTF concentrations were not affected by the lactation stage (Gazi et al. 2023). A study by Skarżyńska et al. (2018) reported no significant differences in the serum LTF concentrations during normal human pregnancy and postpartum period. LTF concentrations in human milk have been shown to significantly decline as time postpartum increases (Goldsmith et al. 1983). In lactating cattle, Hiss et al. (2009) reported a significant increase in milk concentrations of LTF in the 1st week postpartum compared to the 4th week. The early increase in LTF secretion in milk postpartum could be attributed to the general elevated production of acute phase proteins such as LTF due to the temporary immunosuppressive state associated with increased susceptibility to microbial infections (Molenaar et al. 1996; Eckersall et al. 2006). Many significant roles have been assigned to this protein over the years (Wang et al. 2019; Kowalczyk et al. 2022). By denying bacteria access to iron, lactoferrin's ability to bind iron has been associated with bactericidal effects (Vagge et al. 2020). Additionally, lactoferrin possesses strong antiviral and antifungal capabilities to defend against infections and protect newborns (Habib et al. 2021).

While IL-6 secretion was significantly higher on days 10 and 60 compared to day 4, TNF-α secretions were significantly elevated on days 10, 30, and 60. Changes in the secretion of proinflammatory cytokines like TNF-a and IL-6 in milk have not been studied in camels. An early study by Hagiwara et al. (2000) found that TNF-α levels in bovine colostrum are significantly higher than in mature milk. Expression of proinflammatory cytokines such as TNF-α and Il-6 has been shown to be upregulated in bovine mastitis (Akhtar et al. 2020). However, the milk contents of bioactive molecules including proinflammatory cytokines can be influenced by several factors including diets, environment, and exposure to infectious diseases. As a result of endocrine and metabolic changes, it has been demonstrated that immunological responses, which include the release of proinflammatory cytokines, are diminished during the first postpartum week (Mallard et al. 1998; Hiss et al. 2009). These findings support results reported by Bränn et al. (2019) that the immune system undergoes a significant alteration during the postpartum period.

According to the calves' serum analysis, the concentrations of three investigated protective proteins in the mother and its calf did not appear to be correlated. One interesting observation was that the calves' IgG levels were significantly reduced in the serum samples obtained on day 30 compared to day 4. This observation was accompanied by a significant decline in IgG secretion in milk on day 30 post-partum. This transient period could be critical for calf's survival as passive transfer of IgG decreases, and the calf's immature immune system relies more on its own B cells for immunoglobulins production, making the calf more vulnerable to infections. Thus, supporting the calves at this early age with immunostimulatory supplements such as vitamin E may help promote the calves' survival rates and reduces the high mortality rates which is considered a major problem in camel reproduction (Kamber et al. 2001; Lewis et al. 2019).

The present study used milk samples obtained from camels with healthy udders to investigate changes in milk content of immunoprotective, proinflammatory, and regulatory proteins during the first two months postpartum. Data from the current study further our knowledge regarding the camel milk bioactive molecules and their potential use in the early detection of mastitis in camels.

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Conflicts of Interests: None.

Author's Contribution

S. M. Albarrak and F. A. Al-sobayil design the study. F. A. Al-sobayil collected the samples. S. M. Albarrak

analyzed the samples, collected the data and drafted the manuscript.

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