



Assessment of some inflammatory cytokines and immunologic factors in dairy cows with subclinical ketosis

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ABSTRACT

Altered cytokine profile and weakened immunity along with clinical or subclinical ketosis (SCK) are among the remarkable challenges around parturition. Therefore, the present study aimed to compare some inflammatory cytokines and immunologic factors between two groups of healthy and SCK cows. Serum specimens were collected from 30 clinically healthy dairy cows on the early dry period (EDP), one week before expected calving (-1w), and one week postpartum (+1W). The animals were divided into the two groups of healthy (N = 20) and SCK (N = 10) based on serum β -hydroxybutyrate cut-off of 1.2 mmol/L on +1W. The concentrations of immunoglobulin G (IgG), interleukin-4 (IL-4), IL-10, interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), and haptoglobin (Hp) were measured by enzyme-linked immunosorbent assay. The data were statistically analyzed by mixed analysis of variance and independent samples *t*-test using the SPSS software. The findings demonstrated that the overall levels of IL-4 ($p = 0.033$), IL-10 ($p = 0.049$), TNF- α ($p = 0.028$), and Hp ($p = 0.018$) were significantly higher in the SCK group than the control group. Furthermore, the interaction of time \times SCK had a significant influence on IL-4 ($p = 0.028$) and Hp ($p = 0.022$) levels. It was revealed that IL-4 ($p = 0.008$), IL-10 ($p = 0.009$), TNF- α ($p = 0.01$), and Hp ($p = 0.002$) were all significantly higher in the SCK group than the control group on +1W. In conclusion, SCK in dairy cattle might have a relationship with immunologic and inflammatory changes around calving.

Keywords

Immunologic factors, Inflammation, Ketosis, Subclinical ketosis

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Abbreviations

NEB: negative energy balance
BHB: β -hydroxybutyrate
SCK: subclinical ketosis
EDP: early dry period
IgG: immunoglobulin G
IL: interleukin

IFN: interferon
TNF: tumor necrosis factor
Hp: haptoglobin
ELISA: enzyme-linked immunosorbent assay
ANOVA: analysis of variance
NEFA: non-esterified fatty acids

Introduction

One of the most considerable challenges around calving is the debilitated immune responses, which have been reported to be correlated with the higher incidence of diseases during this period [1]. Furthermore, inflammatory markers have been reported to raise after calving [2, 3], and the postpartum inflammatory condition was revealed to have a relationship with the elevated risk of diseases and reduced milk production [4].

On the other hand, pregnancy and lactation are accompanied by remarkable metabolic demands around parturition. The NEB occurs at this period as the result of the maximum nutritional requirements of the fetus, lactation initiation, and decline in dry matter intake [5, 6]. A physiological adaptation to overcome NEB is the mobilization of fat from adipose tissue. However, an imbalance might take place in hepatic carbohydrate and fat metabolism in case of excessive fat mobilization. The consequence of this series of events is an augmentation in the blood concentrations of ketone bodies, namely BHB, acetoacetate, and acetone [7]. The SCK is known as hyperketonemia in the absence of the clinical manifestations of ketosis [8]. The gold standard for SCK diagnosis is BHB measurement in blood serum or plasma due to the stability of this marker [9].

Inflammation and disturbed immunity are among the highlights of the periparturient period in dairy cattle [1, 10]. On the other hand, high levels of ketone bodies have been shown to induce a proinflammatory state [11] and inflammatory responses might be more intense in ketotic cows than healthy animals [12]. Moreover, cows with ketosis were reported to experience immune suppression postpartum [13]. The SCK has a relatively high prevalence in different parts of the world, as well as Iran [14]. Therefore, the present study aimed to compare the levels of several immunologic and inflammatory variables between healthy and SCK cows. Furthermore, the alterations of these factors around calving, as the time of SCK occurrence and diagnosis, were compared between the two groups.

Results

The descriptive statistics of all variables in both groups and the p -values of ANOVA are presented in Table 1. The results of mixed ANOVA demonstrated that sampling time had a significant effect on IL-4 ($p = 0.02$) and IFN- γ ($p = 0.02$) with their highest levels found on +1W and EDP, respectively. It was observed that SCK as the grouping variable significantly influenced IL-4 ($p = 0.033$), IL-10 ($p = 0.049$), TNF- α ($p = 0.028$), and Hp ($p = 0.018$) as all these factors

were higher in the SCK group than the control group. Moreover, time \times SCK interaction imposed a significant impact on the levels of IL-4 ($p = 0.028$) and Hp ($p = 0.022$).

According to the results of the t -tests, the two groups had no significant difference on the EDP and -1W. However, the levels of IL-4 ($p = 0.008$), IL-10 ($p = 0.009$), TNF- α ($p = 0.01$), and Hp ($p = 0.002$) were all significantly higher in the SCK group than the control group on +1W (Figure 1). Furthermore, the difference in IgG ($p = 0.05$) and IFN- γ ($p = 0.05$) tended to be significant between the two groups at this time (Figure 2).

Discussion

Our findings indicated that overall, SCK cows had significantly higher levels of serum IL-4, IL-10, TNF- α , and Hp, compared to healthy cows. Furthermore, the alterations in the serum concentrations of IL-4 and Hp during the study period had a significant difference between the control and SCK groups. It was revealed that after calving, which is a critical time for SCK diagnosis, the levels of all the studied immunologic and inflammatory markers were higher in the subjects with SCK than in the healthy animals. The latter difference was significant for IL-4, IL-10, TNF- α , and Hp.

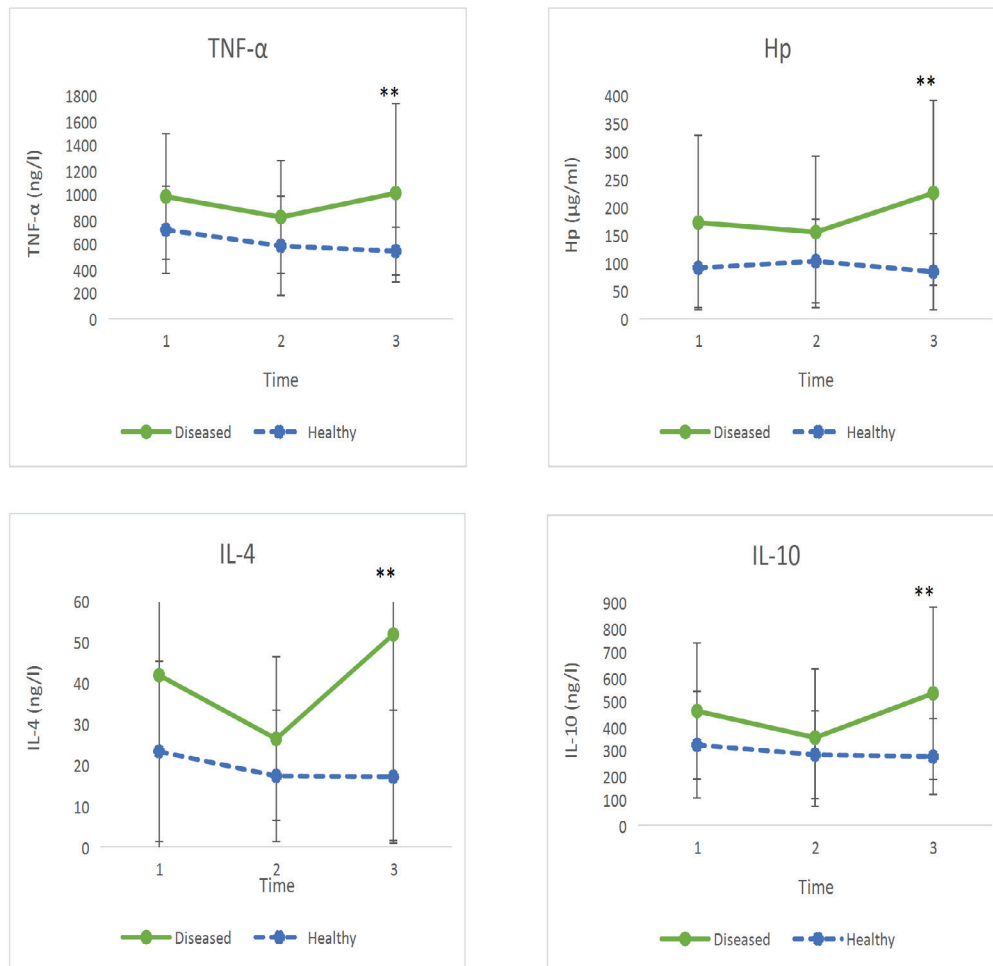
It is believed that the diminished viability of white blood cells in SCK results from the negative impacts of metabolic changes in this disease [15]. Moreover, white blood cells isolated from cows affected by ketosis had a weaker chemotaxis capacity, compared to healthy subjects [16]. In another study, exposure of bovine milk leukocytes to diverse BHB levels *in vitro* led to altered cell membranes, disturbed oxidative activity, reduced phagocytosis, and decreased chemotaxis of these cells [17]. Hoeben et al. found that butyric acid could inhibit respiratory burst in bovine blood polymorphonuclear leukocytes [18]. Some researchers suggest that controlling body condition score and lipid deposition in dry period resulting in lower mobilization of NEFA postpartum can diminish the dysfunction of leukocytes after calving [19].

Energy requirements for evoking the immune responses around calving may be involved in the exacerbation of NEB [15] coupled with increased lipid mobilization and subsequent hyperketonemia during this period. On the other hand, nutrients and metabolites are known to play role in distinct features of the immune system [20]. The main source of energy for leukocytes is glucose [21], which is also one of the key components of ketosis pathophysiology. In other words, the role of glucose in both metabolic and immune systems might lead to a relationship between

Table 1.Mean \pm SE of inflammatory cytokines and immunologic factors in subclinical ketosis and healthy cows

Variable	SCK	Healthy	<i>p</i> -value		
			Group	Time	Group \times Time
IL-4 (ng/L)	40.26 \pm 7.62	19.35 \pm 5.39	0.033	0.02	0.028
IL-10 (ng/L)	452.64 \pm 61.36	298.09 \pm 43.38	0.049	0.087	0.079
TNF- α (ng/L)	944.5 \pm 114.91	618.89 \pm 81.25	0.028	0.136	0.223
IFN- γ (pg/mL)	522.91 \pm 57.54	398.97 \pm 40.69	0.09	0.02	0.476
Hp (μ g/mL)	185.21 \pm 29.85	93 \pm 21.1	0.018	0.216	0.022
IgG (μ g/mL)	67.18 \pm 10.82	42.77 \pm 7.65	0.076	0.316	0.613

SCK, subclinical ketosis; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; Hp, haptoglobin; IgG, immunoglobulin G.

**Figure 1.**Concentration (mean \pm standard error of the mean) of serum IL-4, IL-10, TNF- α , and Hp in both healthy (line) and SCK (dash) groups on the EDP, -1W, and +1W.**, Significant difference ($p < 0.05$) between groups; SCK, subclinical ketosis; EDP, early dry period; -1W, one week before expected calving; +1W, one week postpartum; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; Hp, haptoglobin.

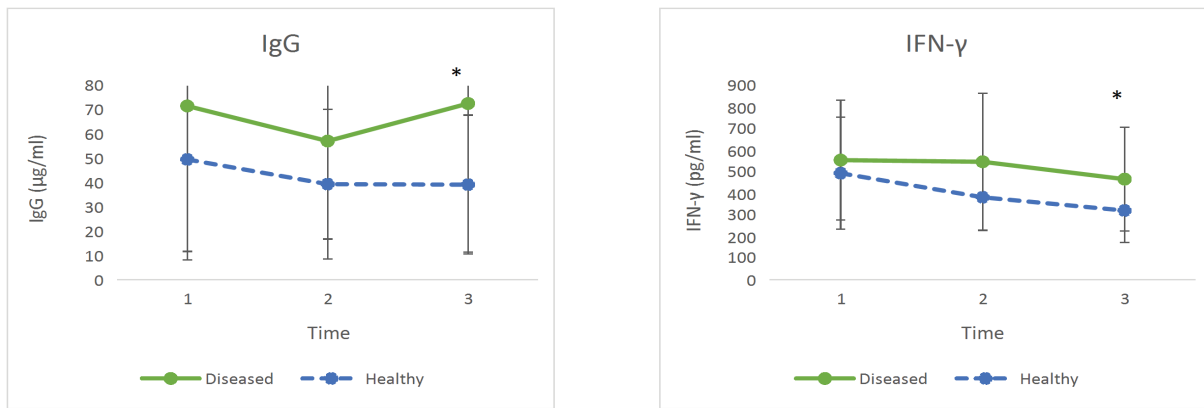


Figure 2.

Mean concentration (mean \pm standard error of the mean) of serum IgG and IFN- γ in both healthy (line) and SCK (dash) groups on the EDP, -1W, and +1W;

*, Differences that tend to be significant ($p = 0.05$) between the groups; SCK, subclinical ketosis; EDP, early dry period; -1W, one week before expected calving; +1W, one week postpartum; IFN, interferon; IgG, immunoglobulin G.

these two systems as changes in glucose due to metabolic alterations can cause changes in immune-related variables. As a result, the mentioned impacts may imply the role of SCK and clinical ketosis in the changes of the immune system and higher incidence of local and systemic infections around calving concomitant with clinical ketosis and SCK.

We found that the cows of the SCK group had a significantly higher TNF- α level than the healthy animals. It has been revealed that the high concentrations of acetoacetate in hepatocytes may upregulate and elevate the secretion of proinflammatory cytokines TNF- α , IL-1, and IL-6 [22]. Moreover, the exposure of hepatocytes to BHB has been shown to induce the NF- κ B signaling pathway and upregulate TNF- α [11]. In this regard, Sun et al. also reported greater NF- κ B activity, as well as more abundant mRNA of TNF- α , IL-1, and IL-6 in the mammary tissue of SCK cows than the animals with normal BHB [23]. These authors concluded that ketosis and overload of BHB may bring about oxidative stress that causes inflammation and immunologic dysfunction [23]. Some other researchers reported that high BHB and palmitic acid, as a component of NEFA, evoke inflammation in the bovine endometrial cells via triggering NF- κ B signaling [24]. In a study by Ohtsuka et al., TNF- α was significantly higher in cows with severe fatty liver, compared to mild cases. In addition, serum TNF- α was correlated with insulin resistance in cattle with fatty liver [25]. The TNF- α was reported to induce insulin resistance in the animal models of obesity through affecting insulin receptors [25] and insulin resistance is known to contribute to hyperketonemia and ketosis occurrence. Furthermore, hyperketonemia in humans could induce the secretion of

TNF- α by cultured monocytes and raise the concentration of this cytokine in the blood of patients with diabetes. It was justified by cellular oxidative stress caused by acetoacetate and cAMP deficiency in these patients [26].

The level of Hp, as one of the major acute phase proteins in cows, in the present study was significantly higher in SCK cows than healthy subjects during the study period and also postpartum. Moreover, the trend of changes in Hp concentration had a significant difference between the two groups. The period around parturition in dairy cattle is commonly characterized by proinflammatory status [27]. High BHB and acetoacetate levels, which are known as the markers of ketosis, cause inflammatory response through activating the NF- κ B signaling pathway [11]. Periparturient inflammation was observed in ketotic cows evident by the high levels of IL-6 [28]. In line with our results, Abuajamieh et al. found that the rise in both Hp and serum amyloid A, as acute phase proteins and markers of inflammation, was significantly higher in cows with ketosis than healthy animals. These authors concluded that the augmented concentrations of inflammatory markers in the peripheral blood of ketotic cases both pre- and postpartum might be suggestive of a relationship between ketosis development and inflammation [12]. Likewise, Mezzetti et al. (2019) stated that higher positive acute phase proteins in ketotic cattle after calving, in comparison with healthy animals indicate a more remarkable inflammation at this time in cows with ketosis [15]. It has been suggested that more severe NEB is associated with the more intense inflammatory condition around parturition [3].

Although our findings showed that time alone had a significant impact on IFN- γ , the effects of SCK

and SCK \times time on this variable were not significant. In contrast, Filar et al. and Kandefer et al. demonstrated that the leukocytes retrieved from the milk and blood of cows with ketosis produced significantly lower amounts of IFN- γ , compared to the control animals [29, 30]. These controversial findings might result from the differences between in vitro and in vivo conditions. The IFN- γ is believed to have the potential to induce SCK through downregulating peroxisome proliferation-activated receptor (PPAR)- γ [31], which plays role in adipocyte biology and contributes to keeping tissues, such as adipose tissue, sensitive to insulin [32, 33]. On the other hand, we observed that IL-4, which acts as one of the key regulators of humoral and adaptive immunity, was significantly higher in the SCK cases than in the healthy cows. Data concerning IL-4 alterations in ketosis are limited in the literature. While IFN- γ is mainly generated by Th1 cells (Cho et al. 2012), IL-4 is more produced by Th2 cells (Cho et al. 2012; Lastra et al. 2009). Consequently, the changes in IL-4 could to some extent indicate the possible impact of SCK on T cell populations.

We observed that the postpartum level of IL-10 was significantly higher in the SCK group than in the control animals. Similar results have been found in cows, as well as children and mice with diabetic ketoacidosis [34-36]. The IL-10 is regarded as an anti-inflammatory cytokine that has the potential to regulate both innate and acquired immune reactions by suppressing TNF- α , IFN- γ , IL-1, IL-4, IL-5, and IL-6 synthesis [34]. Consequently, it augments following inflammatory conditions and elevations in the concentrations of the mentioned cytokines.

In conclusion, the findings of the current study revealed that SCK might lead to some degrees of inflammatory conditions. This was evident by the higher concentrations of IL-4, IL-10, TNF- α , and Hp in cows with SCK than healthy subjects, especially after parturition as the critical time for SCK diagnosis. Considering the limited data about the impact of ketosis on IL-4, further evaluation of this cytokine in ketotic conditions is recommended. Furthermore, the effect of ketone bodies on T cell populations can be investigated in vitro.

Materials & Methods

Animals and Setting

The present investigation was approved by the Animal Welfare Committee of the Ferdowsi University of Mashhad with the code of 3/44869 under the institutional, national, and international guidelines. This cross-sectional study was carried out on 30 clinically healthy multiparous subjects selected from a commercial dairy herd of 3000 Holstein cows and 970 lactating animals in Neyshabur, Iran. The records of the herd showed an average milk production of 40 L. All cows were fed twice a day and had free access to water during the study. The animals received anionic salt during the far-off and close

up periods. Ingredients and nutritional composition of the dry and lactation periods diets are presented in Table 2. The investigated herd had loose pens and outside yards. The cows were dried approximately 80-60 days before the expected parturition and were transferred to separated pens at this time. All the studied subjects had normal easy calving without any clinical abnormalities during the study. Finally, 10 cows with serum BHB levels of >1.2 mmol/L on +1W were categorized as the SCK group [37] and the other 20 animals were considered as healthy controls.

Sample Collection and Laboratory Analyses

Blood specimens were taken on the EDP, one week before the expected calving (-1W), and one week postpartum (+1W). The samples were collected through coccygeal venipuncture into commercial evacuated tubes, which had a clot activator. The blood specimens were centrifuged at 1800 g for 15 min and the sera were collected instantly. All the serum samples were delivered to the laboratory on ice packs and were stored at -80°C until further analyses.

The serum specimens were thawed at room temperature prior to laboratory analyses. Serum BHB concentrations were measured by a colorimetric commercial kit (Randox Laboratories Ltd., Antrim, UK) using a biochemical autoanalyzer (Mindray, BS-200, Shenzhen, China). The immunologic variables, including IgG, IL-4, IL-10, IFN- γ , TNF- α , and Hp were evaluated in the serum specimens by ELISA (Bioassay Technology Laboratory, Shanghai, China) according to the instructions of the manufacturer. An ELISA automatic washer (BioTek, ELx-50, Winooski, USA) and an ELISA reader (BioTek, ELx-800, Winooski, USA) were utilized.

Statistical Analysis

All the data were statistically analyzed using SPSS software version 22 (IBM, USA). The cows in the present study were assigned to the two groups of SCK (N=10) and control (N=20) based on a serum BHB cut-point of 1.2 mmol/L. The normality of data distribution was evaluated based on the Shapiro-Wilk test, skewness, and kurtosis indicating that none of the variables had a normal distribution. Consequently, all the factors were modified by logarithmic transformation. A 3 (time) \times 2 (SCK) mixed ANOVA was carried out to evaluate the effects of time, SCK, and interactions between these factors on all immunologic markers. Pairwise comparisons for time and factor interactions were completed utilizing Bonferroni adjustment. In case a significant impact was observed for SCK or time \times SCK on a variable, the variable was compared between the two groups of SCK and control for all three times separately by the independent samples t-test. p -value < 0.05 and $0.05 \leq p$ -value < 0.1 were considered statistically significant and tending to be significant for all tests, respectively.

Authors' Contributions

NK contributed to study designing, sample preparation, laboratory experiments, data analysis, results interpretation, and manuscript drafting. MH conceived the study design and contributed to laboratory experiments, data analysis, and manuscript review. HAS contributed to study designing and manuscript review.

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Table 2.

Ingredients and nutritional composition (% DM unless noted) of diets fed to cows during dry and lactation periods

Item	Far-off	Close-up	Fresh cow
Ingredients			
Alfalfa hay	8.55	6.3	8.25
Corn silage	76.7	64.68	42.38
Sugar beet pulp	-	-	6.64
Wheat straw	3.54	2.45	0.92
Alfalfa silage	-	-	2.29
Sugar beet molasses	-	-	9.16
Barley grain	0.88	6.14	11.14
Corn grain, ground, dry	3.17	9.04	6.03
Soybean meal	0.98	4.3	4.97
Cottonseed	-	0.69	2.96
Wheat grain	1.88	-	-
Meat meal	3.36	-	-
Fish meal	-	2.45	3.3
Roasted whole soybean seeds	-	1.85	-
Urea	0.21	-	-
Salt	0.06	-	0.18
Calcium carbonate	0.26	0.54	0.23
Sodium bicarbonate	-	-	0.73
Dicalcium phosphate	-	-	0.09
Magnesium oxide	0.06	0.03	0.23
Anionic salt (Magnesium sulfate)	-	1.05	-
Mineral-vitamin supplement ¹	0.21	0.48	0.26
Toxin binder	0.14	-	0.14
Energy and nutrients			
Dry matter (kg)	11.9	12.5	20.4
Net energy for lactation (Mcal/kg)	1.32	1.5	1.61
Neutral detergent fiber	44.7	28.6	29.1
Non-fiber carbohydrates	30.2	38.5	37.5
Ether extract	2.1	3.6	4.3
Crude protein (%)	12.1	14.7	16.7
Rumen degradable protein (%)	7.5	9.5	10.7
Rumen un-degradable protein (%)	4.6	5.2	6
Calcium	0.75	0.97	0.86
Phosphorus	0.35	0.37	0.48

¹ Anionic pre-fresh supplement: 250,000 IU/Kg vit A, 40,000 IU/Kg vit D3, 40,000 IU/Kg vit E, 40 mg/Kg biotin, 12 g/Kg niacin, 168 g/Kg Ca, 65 g/Kg Mg, 1300 mg/Kg Mn, 2210 mg/Kg Zn, 600 mg/Kg Cu, 10 mg/Kg Co, 8 mg/Kg Se, 12 mg/Kg I, 52 g/Kg S, 120 g/Kg Cl

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Competing Interests

The authors declare no conflict of interests.

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