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RESEARCH ARTICLE

Evaluation of Tumor Necrosis Factor-Alfa, Cholesterol, BHBA, NEFA and Acetylcholinesterase Levels in Cows with Ketosis

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ABSTRACT

In this study serum beta-hydroxybutyric acid, glucose, total protein, triglyceride, cholesterol, non-esterified fatty acids, acetylcholinesterase and tumor necrosis factor-α were investigated in cows with ketosis. The study material consisted of 10 control and 10 group with ketosis, total 20 holstein dairy cows of 10 control and 10 groups with ketosis, a total of 20 holstein dairy cows aged between 3 and 7 years. In this study serum concentration was measured for nonesterified fatty acids, beta-hydroxybutyric acid, aspartate aminotransferase, total protein, cholesterol, triglyceride, acetylcholinesterase and tumor necrosis factor-α in the healthy group and cows with ketosis. Serum glucose, triglyceride, nonesterified fatty acids, acetylcholinesterase and tumor necrosis factor-α did not differ significantly between the two groups. Aspartate aminotransferase and beta-hydroxybutyric acid increased, while cholesterol and total protein concentration decreased in ketotic cows compared with healthy cows. Consequently, acetylcholinesterase and tumor necrosis factor-α concentrations may prove beneficial biochemical findings in cows with ketosis.
Keywords: Acetylcholinesterase, Beta-hydroxybutyric acid, Cow, Ketosis, Tumor necrosis factor-alfa

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Ketozisli İneklerde Tümör Nekrosis Faktör-Alfa, Kolesterol, BHBA, NEFA ve Asetilkolinesteraz Düzeylerinin Değerlendirilmesi

ÖZ

Yapılan bu çalışmada ketozisli ineklerde serum beta hidroksi bütirik asit, glikoz, total protein, trigliserit, kolesterol, nonesterifie fatty acids, asetilkolinesteraz ve tümör nekroz faktör-α düzeyleri araştırıldı. Yapılan çalışmanın materyalini 3-7 yaş aralığında 10 adet ketozisli ve 10 adet sağlıklı olmak üzere toplam 20 adet holstein ırkı inek oluşturdu. Ketozis hastalığı bulunan ve sağlıklı gruptaki ineklerde serum nonesterifie fatty acids, beta hidroksi bütirik asit, aspartat aminotransferaz, total protein, glikoz, kolesterol, trigliserit, asetilkolinesteraz ve tümör nekroz faktör-α düzeyleri ölçüldü. Ketozisli ineklerde asetilkolinesteraz, glikoz, tümör nekroz faktör-α, nonesterifie fatty acids, trigliserit düzeyleri sağlıklı kontrol grubuna göre istatistiksel olarak önemli çıkmadığı, ketozisli ineklerde total protein ve kolesterol düzeyleri düşük çıkarken, beta hidroksi bütirik asit ve aspartat aminotransferaz seviyeleri sağlıklı ineklere göre yükseldiği belirlendi. Sonuç olarak ketozisli ineklerde asetilkolinesteraz ve tümör nekroz faktör-α düzeylerinin araştırılması hastalığın biyokimyasal bulgularına faydalı olabileceği kanaatine varıldı.
Anahtar kelimeler: Asetilkolinesteraz, Beta hidroksi bütirik asit, İnek, Ketozis, Tümör nekrosis faktör-alfa

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INTRODUCTION

Ketosis is a metabolic disorder in dairy cows, characterized by elevated levels of ketone bodies in the blood, urine and milk during early lactation. The source of ketone bodies can be either exogenous through dietary intake or endogenous from adipose tissue mobilisation (Zhang & Ametaj, 2020). During the transition period, a cow experiences a relative difference in energy demand between the day before parturition and the first day postpartum. Following these rapid metabolic changes, energy requirements double within a single day. Consequently, meeting these increased energy demands solely through feed intake becomes challenging after calving (Darckley, 2011). As a result, the animal compensates for the energy deficit by utilising body reserves. This metabolic adaptation leads to physiological changes in the organism, including an increase in somatotropin hormone levels and the activation of lipolysis in adipose tissue, which is mediated by norepinephrine signalling (Grum et al., 1996). Consequently, the rise in non-esterified fatty acids (NEFA) facilitates the conversion of these lipids into ketone bodies in the liver to meet the increased energy demand (Darckley, 2011).

The mobilisation of non-esterified fatty acids (NEFA) subsequently triggers glucose utilisation to compensate for the energy deficit. However, in response to glucose conservation, the organism converts NEFAs into acetyl-CoA and subsequently into ketone bodies (Zhang & Ametaj, 2020). In cows, the brain is entirely dependent on glucose. In ruminants, when ketone body concentrations increase, peripheral tissue utilization remains limited, leading to an accumulation of ketone bodies in the blood. Consequently, ketone bodies also appear in milk and urine. The elevation of ketone bodies in the blood results in decreased blood pH, reduced feed and water intake and suppressed immune system (Reece, 2008). In ketosis, fat depots in the body undergo breakdown due to glucose deficiency, resulting in elevated NEFA levels (Goff & Horst, 1997; Grummer, 1995).

Cholinesterase exists in two different forms in mammals: acetylcholinesterase and pseudocholinesterase. Acetylcholinesterase is produced in the liver, and its levels in the blood have been reported to decrease in liver diseases and hepatic degeneration (Stojevic et al., 2005). Pseudocholinesterase is primarily synthesized in the liver and found in serum, though its physiological function remains unclear (Kaplay, 1976). In humans, pseudocholinesterase is directly associated with insulin resistance, serum lipid profiles, and obesity (Iwasaki et al., 2007).

Tumor necrosis factor-alpha (TNF-α) is produced in response to various stimuli, including tumors, normal cells, bacteria, viruses, parasites, and cytokines, and exhibits biological activity in different cell types and tissues. It is crucial in systemic and local tumor-related effects, including cachexia and neoplastic tissue degradation (Çömez, 2006). Several studies have reported that cows with ketosis exhibit higher TNF-α, serum amyloid A, interleukin-6, and lactate concentrations during the prepartum period (4–8 weeks before calving) compared to healthy cows (Oetzel, 2007; Tehrani et al., 2011; Zhang & Ametaj, 2020).

This study aims to evaluate the relationship between β-hydroxybutyrate (BHBA), NEFA, TNF-α, acetylcholinesterase (AchE), cholesterol, glucose, total protein, and triglyceride levels in both ketotic and healthy cows.

MATERIALS and METHODS

Animal Material and Study Groups

This study was conducted on a dairy farm with a total of 70 lactating cows located in Bigadiç, Balıkesir Province. A total of 20 Holstein dairy cows, aged between 3 and 7 years and in the first four weeks of lactation, were included in the research group. Among these, 10 cows were diagnosed with clinical ketosis, while 10 healthy cows were assigned to the control group. All animals in the study and control groups were housed under the same feeding, management, and environmental conditions within the early lactation group of the farm. The cows included in the study were selected based on their metabolic health status, with an effort to ensure similarity in age averages and lactation stages (between 1 and 4 weeks postpartum). A detailed clinical examination was performed on all ketotic and healthy cows.

To prevent the inclusion of cows with secondary ketosis, animals diagnosed with metabolic disorders commonly observed in early lactation, such as milk fever, mastitis, abomasal displacement, metritis, retained placenta (RPT), or retained fetal membranes (retentio secundinarum), were excluded from the study. The study was ethically approved by the Animal Experiments Local Ethics Committee of Balıkesir University (Decision Number: 2021/8-5).

Diagnosis of Ketotic and Healthy Cows

Ketotic cows were identified based on a decreased body condition score (BCS) of more than 0.5 within the first four weeks postpartum. The milk yield of these cows was monitored, and those exhibiting a sudden drop in milk production and loss of appetite underwent clinical examination. Urinary ketone bodies were assessed using urine dipstick tests and blood sample collection to measure β-hydroxybutyrate (BHBA) levels. Cows with BHBA levels exceeding 1.5 mmol/L and positive ketonuria were diagnosed with ketosis and included in the study.

Healthy cows were selected based on their feed intake, stable milk production, and body condition score reductions of no more than 0.5. These cows underwent a comprehensive clinical examination to confirm the absence of any metabolic or systemic diseases. Following clinical assessments, blood BHBA levels were measured, and cows with BHBA concentrations below 1.0 mmol/L were classified as healthy and included in the control group.

Sample Collection

Blood samples were collected from the jugular vein following clinical examinations using sterile, single-use needles and 10 mL vacuum tubes. Samples for NEFA analysis were obtained before morning feeding, whereas samples for BHBA analysis were collected four hours after feeding. After collection, blood samples were left to stand briefly before being centrifuged at 5,000 rpm for 5 minutes to obtain serum. Three aliquots from each serum sample were transferred into Eppendorf tubes and stored at -20°C until analysis.

Laboratory Analyses

Serum samples were analysed in a specialised biochemical laboratory using an automated Randox analyser. The levels of TNF-α and AchE were measured using ELISA (Enzyme-Linked Immunosorbent Assay) kits (Bovine TNF-α, AchE SunRed ELISA Kit, Cat. No: E90440, Eastbiopharm, China) (Table 1).

Biochemical parameters were analysed using a Randox Daytona model automated analyser (United Kingdom). The biochemical parameters assessed in this study included BHBA, aspartate aminotransferase (AST), total cholesterol, glucose, NEFA, total protein, and triglycerides. The respective methodologies for these parameters were as follows: BHBA (Cat. No: RB1007) – Enzymatic kinetic method, AST (Cat. No: AS3804) – UV method, Total cholesterol (Cat. No: CH3810) – Enzymatic endpoint method, Glucose (Cat. No: GL3815) – Colorimetric method, NEFA (Cat. No: FA115) – Colorimetric method, Total protein (Cat. No: TP38669) – Biuret reagent endpoint method, Triglycerides (Cat. No: TR3823) – Lipase/GPO-PAP method. (Table 2)

Statistical analysis

Statistical analyses were performed using the SPSS 20 software package for Windows. The relationships between serum biochemical parameters were evaluated using an independent samples t-test, and a p-value of<0.05 was considered statistically significant.

RESULTS

The clinical parameters, including body temperature, heart rate, and respiratory rate, of the ketotic cows included in the study were within normal ranges. However, clinical signs observed in ketotic cows included anorexia, reduced milk yield, depression, constipation, teeth grinding, firm faecal consistency, reluctance to consume concentrate feed, reduced feed intake, acetone odour in the breath in some cases, and loss of body condition.

Biochemical analyses of serum samples revealed that although TNF-α and AchE levels were elevated in ketotic cows compared to healthy cows, the differences were not statistically significant (Table 1).

Table 1. AchE and TNF-α levels in ketotic and healthy cows.

Parameters	Healthy Cows (n=10)	Ketotic Cows (n=10)	p Value
TNF-α (µg/ml)	31.50±17.47	33.76±12.58	NS
AchE (ng/ml)	6.33±5.73	7.06±3.67	NS

NS- Not Significant

Biochemical analyses of serum samples revealed that BHBA levels were significantly higher in ketotic cows than healthy cows ($p<0.001$). Although serum glucose levels were lower in ketotic cows, the difference was insignificant compared to the control group. (Table 2)

The study also showed that serum AST levels were significantly higher in ketotic cows compared to the control group ($p<0.05$). Additionally, ketotic cows serum cholesterol levels were significantly lower than healthy cows ($p<0.01$). However, there was no significant difference in NEFA and triglyceride levels between the ketotic and healthy groups. (Table 2)

Table 2. Biochemical parameters in ketotic and healthy cows.

Parameters	Healthy Cows (n=10)	Ketotic Cows (n=10)	p Value
BHBA mmol/L	0.77±0.41	2.54±0.61	***
Glucose mg/dL	35.3±11.61	28.20±11.42	NS
Cholesterol mg/dL	61.80±13.51	44.80±8.57	**
Triglycerides mg/dL	12.10±7.63	11.50±5.21	NS
NEFA mmol/L	0.98±0.78	0.88±0.25	NS
Total protein g/dL	6.87±0.50	5.68±0.75	***
AST U/L	109.70±8.87	167.20±72.34	*

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, NS- Not Significant

DISCUSSION

Ketosis in dairy cows predisposes them to secondary diseases such as abomasal displacement, mastitis, metritis, and infertility (Civelek, 2011; Çatık, 2015; Radostits et al., 2006; Sevinç & Başoğlu, 2011). The diagnosis of ketosis is based on clinical and laboratory findings, including elevated BHBA levels in the blood and ketonuria detected using urine test strips. Clinical signs of ketosis in cows include anorexia, reduced milk yield, depression, decreased feed intake, reluctance to consume concentrate feed, increased roughage consumption, dry feces, reduced rumen motility, acetone odor in the breath, and body condition loss (Herdt, 2000; Herdt, 2005; Oetzel, 2007). In this study, cows were carefully examined, and those with secondary ketosis were excluded. Only cows in the first four weeks postpartum that exhibited primary ketosis symptoms were included. The clinical findings observed in ketotic cows in this study were consistent with previous reports, including anorexia, decreased milk production, depression, constipation, teeth grinding, firm faecal consistency, reluctance to consume concentrate feed, reduced feed intake, acetone odour in breath in some cows, and body condition loss.

Liver diseases, subclinical and clinical ketosis, hepatobiliary disorders, acute and chronic hepatitis, liver damage, hepatic lipidosis, biliary diseases, and muscle tissue injuries can lead to increased AST enzyme levels in animals (Civelek, 2011; Sevinç & Başoğlu, 2011; Steen, 2001; Zhang et al., 2018). Increased AST levels have been reported due to hepatic lipidosis (Bogin et al., 1988; Cebra et al., 1997), and elevated AST and GGT levels have been associated with liver damage (Civelek, 2011; Sevinç & Başoğlu, 2011; Steen, 2001). Several studies have found elevated AST, GGT, ALP, and ALT levels in clinically ketotic cows (Zhang et al., 2018), as well as increased AST enzyme levels in subclinical and clinical ketosis cases (Li et al., 2016; Cao et al., 2017). The findings of this study align with these previous reports, as AST levels in ketotic cows were significantly higher than those in healthy cows (Table 2).

It has been reported that total protein levels decrease in cows with hepatic lipidosis (Radostits et al., 2006; Turgut, 2000) and serum albumin levels decrease due to the utilization of body reserves in ketotic cows (Austin & Wilde, 1985). Some studies suggest that reduced urea levels in postpartum cows may be associated with decreased protein anabolism due to hepatic fat infiltration (Elitok et al., 2006). However, others have found that albumin and BUN levels remain within normal ranges in subclinical and clinical ketosis cases (Akgül et al., 2018). Additionally, no significant differences in total protein levels between healthy, subclinical, and clinical ketosis cows have been reported (Çatık, 2015; Li et al., 2016). Similarly, although total protein levels were slightly lower in ketotic cows compared to healthy cows in this study the difference was not statistically significant (Table 2).

In primary ketosis, decreased blood glucose levels lead to mobilising fat reserves. High-yielding dairy cows manage their negative energy balance primarily through glucose metabolism, followed by NEFA and ketone body utilization (Lean et al., 1992; Reynolds et al., 2003; Turgut, 2000). During early lactation, insulin production declines, reducing glucose utilization in fat and muscle tissues, necessitating alternative energy sources. As a result, body fat is mobilized, increasing NEFA levels (Drackley, 1999). Low postpartum blood glucose levels have been associated with negative energy balance or ketosis (LeBlanc, 2010). Several studies have reported increased BHBA levels coincide with decreased glucose concentrations in ketotic cows (Andre et al., 1987). However, blood glucose levels are considered less relevant than BHBA levels for ketosis diagnosis. Studies have found decreased glucose levels in cows with hepatic lipidosis and ketosis (Gilbert et al., 1998; Katoh, 2002; LeBlanc, 2010). Both subclinical and clinical ketosis cases show lower glucose levels than healthy cows (Li et al., 2016). However, some studies found no significant difference in glucose levels between clinically ketotic and healthy cows (Akgül et al., 2018; Çatık, 2015). Although glucose levels were lower in ketotic cows, the

difference was not statistically significant compared to healthy cows (Table 2).

Ketone bodies are intermediates of fat oxidation, with BHBA being a key diagnostic marker for ketosis due to its relative stability compared to acetoacetate and acetone (Herdt, 2005; Duffield, 2000; Ospina et al., 2010b). Studies have classified BHBA levels between 1.2–1.4 mmol/L as subclinical ketosis (Duffield, 2000; Walsh et al., 2007) and have used BHBA levels≥1.4 mmol/L to diagnose clinical ketosis. Several studies have found significantly higher BHBA levels in clinical ketosis cows compared to subclinical and healthy cows (Çatık, 2015; Sun et al., 2015; Xia et al., 2012; Shen et al., 2020). In agreement with these findings, this study also confirmed that BHBA levels were significantly higher in ketotic cows than healthy ones (Table 2).

Cows mobilize body fat during early lactation to compensate for energy deficits, leading to increased NEFA levels in circulation and accumulation in various tissues, including the liver (Ingvartsen, 2006; Oetzel, 2007). Excessive hepatic fat accumulation impairs liver function, contributing to hyperketonemia. Studies have reported that postpartum NEFA levels range between 0.70–1.0 mEq/L, with values above 0.7 mEq/L often associated with ketosis (Chapinal et al., 2011; LeBlanc, 2010; Ospina et al., 2010b). Several studies have reported increased NEFA levels in ketotic cows (Li et al., 2016; Cao et al., 2017; Sun et al., 2015; Xia et al., 2012). However, some studies found no significant difference between NEFA levels in subclinical and clinical ketosis cases (Çatık, 2015; Akgül et al., 2018). Similarly, no significant difference was observed in NEFA levels between ketotic and healthy cows (Table 2).

Serum cholesterol levels are reduced in chronic liver failure, cirrhosis, diabetes mellitus, hepatic lipidosis, and conditions involving hepatocellular damage (Turgut, 2000; Quiroz-Rocha et al., 2009; Nakagawa & Katoh, 1998). While some studies have reported elevated cholesterol levels in ketotic cows (Simonov & Vlizlo, 2015), others have found decreased cholesterol levels in clinical ketosis (Nakagawa & Katoh, 1998; Li et al., 2016). In agreement with the latter, this study found that cholesterol levels were significantly lower in ketotic cows than in healthy cows (Table 2).

Triglycerides are synthesized from dietary fats in the gastrointestinal tract and other liver lipids. Severe hepatic lipidosis impairs lipoprotein (VLDL) formation and fat mobilisation, leading to reduced triglyceride levels (Turgut, 2000; Katoh, 2002; Kennerman, 2011). Studies have found that triglyceride levels are lower in ketosis cases, particularly in early postpartum cows (Kessler et al., 2014). However, some reports found no significant difference in triglyceride levels between ketosis and healthy cows (Çatık, 2015; Li et al., 2016). Consistent with these findings, this study found no significant difference in triglyceride levels between ketotic and healthy cows (Table 2).

TNF-α, a key inflammatory cytokine, has been associated with metabolic disturbances in ketosis. Some studies found elevated TNF-α levels in ketotic cows (El-Deep & El-Bahr, 2017; Zhang et al., 2018), while others reported no significant difference (Brodzki et al., 2021). In this study, TNF-α levels were slightly higher in ketotic cows but not statistically significant (Table 1).

CONCLUSION

In conclusion, this study contributed to the biochemical diagnosis of ketosis by determining the levels of beta-hydroxybutyric acid (BHBA), non-esterified fatty acids (NEFA), aspartate aminotransferase (AST), tumor necrosis factor-alpha (TNF-α), acetylcholinesterase (AchE), glucose, total protein, triglycerides, and cholesterol in both ketotic and healthy cows. Given the limited research on the relationship between cholinesterase enzymes and TNF-α in ketotic cows, we believe further detailed studies are necessary to understand these associations better.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: YY and İA contributed to the article idea, design and execution the study. YY collected datas. YY and İA analyzed data. All outhors contributed to the critical revision of the manuscript and have read and approved the final version.

Ethical approval: This study was carried out at Balıkesir University Reserch Animals Application Center. This research was approved by The Ethics Committee of the Faculty of Veterinary Medicine, Balıkesir University (BAUNHADYEK, Ref No: 2021/8-5, Tarih: 30/09/2021).

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