Assessment of TNF-α and leptin gene expression by RT-PCR in blood of cows with left abomasal displacement

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SUMMARY

The aims of this study are to evaluate the TNF-α and leptin gene expression in blood from Holstein cows with left abomasal displacement and to correlate it with induced liver injury. The TNF-α and leptin expression in blood samples was determined by RT-PCR after normalisation using the constant expression of the housekeeping GAPDH gene in cows with left abomasal displacement (LAD) (n = 20) before surgery and 7 days after as well as in healthy controls (n = 10). Plasma hepatic enzyme (AST: aspartate aminotransferase, ALT: alanine aminotransferase and ALP: alkaline phosphatase) activities were measured in parallel. Plasma AST and ALP activities dramatically increased in diseased cows during the preoperative period and then declined. Although not significantly, the leptin expression tended to decrease in LAD affected cows while the TNF-α expression tended to increase during the postoperative period. These results suggest that TNF-α may be associated with liver damage during abomasal displacement and that leptin was inversely correlated.

Keywords: Cow, TNF-α, leptin, left abomasal displacement, RT-PCR, liver enzymes.

RÉSUMÉ

Détermination de l'expression des gènes codant pour le TNF-α et la leptine par RT-PCR dans le sang de vaches présentant un déplacement de la caillette à gauche

Les objectifs de cette étude ont été d'évaluer l'expression des gènes codant pour le TNF-α et la leptine dans le sang de vaches Holstein présentant un déplacement à gauche de la caillette et de la corréler avec les lésions hépatiques induites. L'expression du TNF-α et de la leptine a été déterminée par RT-PCR après normalisation en considérant l'expression du gène de ménage GAPDH comme constante dans les échantillons sanguins provenant de vaches atteintes d'un déplacement à gauche de la caillette (n = 20) avant et 7 jours après traitement chirurgical ou provenant de vaches saines (témoins, n = 10). Les activités plasmatiques des enzymes hépatiques (AST : aspartate aminotransférase, ALT ; alanine aminotransférase et PAL : phosphatase alcaline) ont été mesurées en parallèle. Les activités plasmatiques de l'AST et de la PAL étaient considérablement augmentées chez les vaches malades avant la chirurgie puis elles ont diminué durant la période postopératoire. Bien que les variations n'aient pas été significatives, l'expression de la leptine chez les animaux malades a tendu à diminuer alors que celle du TNF-α a augmenté durant la période postopératoire. Ces résultats suggèrent que le TNF-α pourrait être associé aux lésions hépatiques associées à un déplacement de la caillette alors que la leptine serait inversement corrélée.

Mots clés : Vache, TNF-α, leptine, déplacement à gauche de la caillette, RT-PCR, enzymes hépatiques.

Introduction

Abomasal displacement (AD) has a complex aetiology and composes the most popular reason of surgical gastrointestinal disorders in dairy cattle [44]. Left abomasal displacement (LAD) is more frequent than right abomasal displacement (RAD), accounting for 85 to 95.8% of all cases [44] but still yet the aetiology of this disease remains unclear. Many theories have been presented in an attempt to explain its cause and such factors as diet, genetic pre-disposition, housing, mechanical aspects of parturition and concurrent disease have been associated with LAD [24]. One theory, the endotoxin theory, states that endotoxins may play a significant role for the development of AD [3].

Feed grains are very rich in starch and associated with a shift of rumen bacterial populations from cellulolytic (cellulose-digesting) dominating species to amylolytic (starch-digesting) dominating species of bacteria [9]. Most of the known starch-digesting bacteria are Gram-negative [9], so the growth and reproduction of them is enhanced in the rumen resulting of feeding with high levels of grains to support high milk production. These population changes have been reported to associate with significant rises in endotoxin production in the rumen [4, 31, 32].

Endotoxin, also known as lipopolysaccharide (LPS), is a component of the membrane of all Gram-negative bacteria and is suggested by some investigators to play a role in development of grain-related metabolic diseases such as fatty liver, LAD, and laminitis [1, 4, 10]. It is a potent inducer of inflammation and activates the innate immune pathway via stimulation of toll-like receptors (TLRs) and induces the expression of inflammatory mediators (adipocytokines) such as leptin, tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6), amongst

others [18, 30]. However, the certain role of endotoxin in these important diseases of dairy cattle has not yet been cleared. TNF-α is a cytokine which is secreted by macrophages and can be effective on all nucleated cells that mediates these responses [2]. It has been noted that endotoxins may activate macrophages leading to an elevation of serum TNF-α [13, 43, 45]. Additionally, an increase in the TNF-α content in fatty liver has been reported [2, 25, 26]. Leptin is a peptide hormone with the tertiary structure of a cytokine which is largely conserved among mammalian species [16], regulates food intake and modulates immunity and inflammation [11, 14, 35]. A positive feedback mechanism has been described between TNF and leptin, and it has been suggested that leptin potentiates inflammation [22]. The present study aims to investigate TNF-α and leptin gene expressions in cows with AD during pre and post operative periods and to determine whether surgical treatment improves liver function.

Material and Methods

ANIMALS

Thirty Holstein cows (median age: 4.5 year) in postpartum period, which were presented to the clinics of Mehmet Akif Ersoy University Veterinary Faculty constituted the material of the present study. Among them, 20 exhibited a left abomasal displacement (LAD) whereas the others were considered as healthy controls. Administrations on the animal material were carried out by the acceptance of Ethical Committee of Kafkas University on Animal Experiments (Decision No.: 14.03.2009/02).

LAD was diagnosed by hearing a characteristic "ping" under the left rib cage upon simultaneous percussion and auscultation [38]. Additionally LAD diagnosis was confirmed by laparoscopic surgery in the beginning of the treatment. Treatment of the animals was performed by laparoscopic abomasopexy which is a two-stage technique and described previously [17]. Prior to the operation and seven days after the surgery, blood samples (8 ± 2 mL) from *vena jugularis* were collected into EDTA sterile tubes. Plasma samples were obtained after immediate centrifugation (2000 g, 15 minutes at room temperature) whereas total RNA isolation was performed from whole blood samples.

BIOCHEMICAL ANALYSIS

Laboratory tests were performed in İzmir Instıtute of Technology, Biotechnology and Bioengineering Application and Research Center.

Plasma activities of amino-transferases (ALT and AST) [37] and alkaline phosphatase (ALP) [20] were assessed colorimetrically (Thermo, Finland) using commercial kits (Emapol, Poland). Average levels of the biochemical parameters were checked against the corresponding laboratory references of MISCHLER *et al*. [29].

Total RNA was isolated using a single-step RNA isolation kit (TRI-reagent, Biobasic Inc., USA) based on the method of CHOMCZYNSKI and SACCHI [7]. Concentrations and purity of RNA samples were determined using optical densities at 260/280 nm by Nanodrop (Thermo). The integrities of the isolated RNA and cDNA were verified by 1.5% agarose (Vivantis, USA) gel electrophoresis. Master mix of Moloney Murine Leukemia Virus (MMLV) enzyme was used for synthesis of cDNAs and prepared as below; for a total volume of 25 µL: 8 µL MMLV enzyme buffer (Vivantis, USA), 8 µL dNTPs (Fermentas, Lithuania), 1 µL rRNAse inhibitor (Vivantis, USA), 1.6 µL (320 U) MMLV-RT enzyme (Vivantis, USA) and 6.4 µL nuclease free water. The mixture added to extracted RNAs was then incubated at 37°C for 1 hour, 95°C for 5 minutes and 4°C for 5 minutes. GAPDH gene as the housekeeping gene, and two other primers (Table I) were used for the preparation of the *Taq* DNA polymerase master mix (containing 5 µL of 10x reaction buffer, 1 µL of dNTPs, 1 µL of forward primer (10 mM), 1 µL of reverse primer (10 mM), 1 µL of *Taq* DNA polymerase (5 U/µL, Promega Co., USA) and 39 µL of nuclease free water). This mixture (48 µL) was added to cDNA (2 µL) and DNA amplification process was performed at 94°C for 1 minute, 55°C for 1 minute, 72°C for 1.5 minute for 35 cycles and finally at 72°C for 7 minutes, then the samples were kept at 4°C. Amplification products were resolved on 1.5% TAE agarose gel electrophoresis for 75 minutes at 100 V. After electrophoresis the gels were stained with ethidium bromide (0.5 µg/ml), visualized by VersaDoc® 4000 MP imaging system (Bio-Rad). The intensities of obtained bands were analysed by QuantityOne software (Bio-Rad).

STATISTICAL ANALYSIS

MINITAB® 12.1 program (Minitab Inc., USA) was used for the statistical analyses and the confidence interval was determined as 0.05. Data were evaluated according to normal distribution ($P > 0.05$) and possible differences were determined by the use of paired t-test and ANOVA.

Results

Significant increases in plasma AST and ALP activities were recorded in cows with LAD before surgery compared to healthy

Target genes	Sense primers (5'-3')	Anti-sense primers (5'-3')	Amplicon size (bp)
GAPDH	CTGGCAAAGTGGACATTGTCGCC	CTTGGCAGCGCCGGTAGAAGC	571
Leptin	GTGCCCATCCGCAAGGTCC	TCAGCACCCGGGACTGAGG	441
TNF-α	CAGAGGGAAGAGTCCCCAGG	CTTTGGTCTGGTAGGAGACT	325

TABLE I: Sequences of the oligonucleotide primers and amplicon sizes used for the RT-PCR.

cows ($P < 0.05$). In the postoperative period (7 days after surgery), the enzyme activities declined and reached values closely related to controls (Table II). By contrast, plasma ALT activities remained similar between diseased and control cows in pre and postoperative periods.

As the expression of the control housekeeping GAPDH gene remained similar between LAD affected and healthy animals as expected, the TNF-α or leptin mRNA / GAPDH mRNA ratio was calculated for comparing polypeptide expression between cows with LAD and controls. As shown in Table III, the TNF-α expression in blood tended to decrease in diseased cows before surgery compared to the healthy animals whereas 7 days after surgery it was similar to the control value. Additionally, cows with LAD exhibited a slightly lower expression of leptin gene than control cows during the 2 periods. However, differences in TNF-α and leptin expression were not statistically significant between diseased and healthy cows and between the 2 experimental periods.

Discussion

AD has been reported as the one of the most important diseases of metabolic origin in cows [6] and it is etiologically associated with fatty liver [2, 15, 25, 26]. Recent studies show that fatty liver disease causes the immune system activation in cows and endotoxin exposure has been recently involved in the development of fatty liver disease [2]. In this way, OHTSUKA *et al*. [34] have reported increases in serum TNF-α concentrations in cows with fatty liver. Post-operative period is often associated with gradual recovery for disorders in energy metabolism and fatty liver disease [36, 42, 44]. REHAGE *et al*. [36] have reported that surgical intervention is the key point for prevention of fatty liver, ketosis and excessive lipomobilisation in AD cases. Although not significantly, the slight increase in TNF-α expression and the decrease in leptin expression observed in the present study in LAD affected cows, particularly during the post-operative period, are consistent with the recovery state. It is known for a long time that cytokines, especially TNF-α, affect serum leptin concentrations [16] and direct [8, 19] or inverse [27, 28] correlations could be reported between the circulating concentrations of leptin and TNF, depending on the type of inflammation [23]. Among the aetiological factors of AD cases, long term endotoxin production in the rumen [1, 41] and chronic inflammations such as mastitis, metritis, retentio secundinarum and abomasal ulcers [39] stand out. Additionally, because of having more slightly symptoms as compared to RAD cases, LAD cases are presented to the clinics belatedly, and inflammation becomes more chronic [40]. It is reported that the acute immune response and release of TNF-α results in a prompt short term release and increase in plasma concentrations of leptin. Even so, chronic inflammation and its concomitant constitutive up-regulation of pro-inflammatory cytokines will cause suppression of leptin [23], and this is possibly what we have observed. In the present study, TNF-α and leptin gene expressions in blood were inversely associated probably because of the endotoxin exposure and of the convalescence state. Presumably by the effect of convalescence state of the scar tissues after the surgery as an acute inflammatory marker, TNF-α unsurprisingly increased. In parallel, with the opinion that states the fallen concentrations of leptin as the indicator of chronic inflammation, in our LAD group, both before and after surgery leptin concentrations were low. It is supposed that in the future studies by developing a thirty day after surgery group, normal leptin expression concentrations would be determined. Moreover, these findings are further strengthened by a study which was aimed to determine the association between diabetes and inflammation in clinically diagnosed diabetes patients, elevated concentrations of TNF-α and low concentrations of leptin were reported and related to existing chronic inflammation in the subjects [28]. Given this relationship of leptin with chronic inflammation, leptin could

Enzyme activity	Healthy cows	Cows with LAD (n = 20)		*P*
	(n = 10)	Before surgery	After surgery	
AST (U/L)	105.00 ± 8.15^{ab}	125.49 ± 10.51^{b}	92.74 ± 6.28^{a}	< 0.05
ALP (U/L)	93.00 ± 4.32^{a}	513.30 ± 140.85^{b}	123.80 ± 20.80^{a}	< 0.05
ALT (U/L)	27.00 ± 2.85	18.09 ± 3.95	19.23 ± 4.39	NS

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: alkaline phosphatase; NS: not significant.
Different superscripts ^{a,b} in the same row indicate significant differences among group ($P < 0.05$).

TABLE II: Hepatic enzyme activities in plasma from healthy cows (n = 10) and from cows with LAD (left abomasal displacement) (n = 20) during pre-operative and post-operative (7 days after surgery) periods. Results are expressed as mean ± standard deviations.

Enzyme activity	Healthy cows	Cows with LAD (n = 20)		*P*
	(n = 10)	Before surgery	After surgery	
TNF-α	1.515 ± 0.72	1.140 ± 0.32	1.850 ± 0.54	NS
Leptin	3.472 ± 0.84	2.212 ± 0.59	1.996 ± 0.47	NS

(Normalized gene expression: TNF-α or leptin mRNA (densitometry units) / GAPDH mRNA (densitometry units).

TABLE III: TNF-α and leptin gene expression in whole blood samples from cows with LAD (left abomasal displacement) (n = 20) before surgery (preoperative period) and 7 days after (postoperative period) and from healthy cows (n = 10). Results are normalized according to the expression of the GAPDH gene and are expressed as mean ± standard errors.

serve as a valuable marker for predicting the progression of chronic inflammation in cows with abomasal displacement.

Energy requirements usually exceed energy intake in periparturient period, so the required energy is provided by lipolysis, which is the cause of fat accumulation in liver [46]. The severity of fatty liver degeneration is variable and can cause hepatic damage [34]. In the present study, marked and significant increases in plasma AST and ALP activities recorded in LAD affected cows before surgery are similar to previous reports [5, 21, 34] and could be attributed to hepatic lipidosis, endotoxemia and hepatocyte damage [46]. AVKI *et al*. [5] have previously reported strong increases in serum TNF-α concentrations in dairy cows with AD during the postoperative period compared to the healthy controls which were significantly associated with increases in serum GGT activity and decrease in albuminaemia, these biochemical parameters being considered as markers for liver injury. In the current study, alterations in AST and ALP activities were also concomitant to the slight increase in TNF-α mRNA in blood from LAD affected cows.

Until now, the cytokine and leptin expressions have been explored using ELISA assays which allow determination of the peptide concentrations whereas no information about changes in mRNA expression in AD cases was available in the literature to our knowledge. However, RT-PCR has been stated as a more sensitive technique to determine variations in TNF-α and leptin expression [12]. In this regard, the present study constitutes the first work that explores the relation between TNF-α, leptin using RT-PCR and AD. However, the variations in the normalized expression of TNF-α and leptin were not statistically significant between diseased and healthy controls and between pre-and postoperative periods in the present study. Several factors can explain the lack of a significant effect: firstly, the translation of mRNAs into peptides leading to increases in blood peptide concentrations may exacerbate differences between groups and secondly it would be probable that increasing sample numbers in the future studies may allow detection of significant differences.

It is concluded that measurement of TNF and leptin mRNA expressions may help to explain links between AD and related diseases (fatty liver) and may also improve the ability to predict and prevent metabolic problems in post-partum period of dairy cows. In this regard anti-cytokine therapy [33] developed in human medicine might also play a potential therapeutic role in fatty liver and AD in cattle [5]. Consequently, further studies will be conducted in order to explore the TNF-α induced hepatic effects during AD eventually leading to hepatic coma after surgical treatment [36] and the potential interest of anti-cytokine therapy in metabolic disorders in cows.

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