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Original article

The effect of tilmicosin and diclofenac sodium combination on cardiac biomarkers in sheep

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Abstract

The aim of this study was to investigate the cardiotoxic effect of the combination of tilmicosin and diclofenac sodium in sheep. Thirty-two sheep were used and were randomly divided into four equal groups as tilmicosin (T), diclofenac sodium (D), tilmicosin+diclofenac sodium (TD) and control (C) group. Group T received a single dose of tilmicosin, Group D was administered diclofenac sodium once a day for 3 days, and group TD was administered diclofenac and tilmicosin at the same doses as group T and D. Group C received NaCl in a similar way. The blood samples were taken before dosing and at 4th, 8th, 24th and 72nd hour post-dosing for measurement of cardiac markers such as H-FABP, cTn-I, CK-MB. H-FABP level of group TD was found to be significantly ($p<0.05$) higher than of group C at the 8th, 24th and 72nd hour and group D and T at the 72nd hour. cTn-I and CK-MB levels of group TD were found significantly ($p<0.05$) higher compared with other groups. In conclusion, the combined use of tilmicosin and diclofenac in sheep causes an increase in cardiac biomarkers and it can be stated that this combination of drugs may cause cardiac damage.

Key words: cardiotoxicity, diclofenac, H-FABP, sheep, tilmicosin

Introduction

Macrolide antibiotics such as tilmicosin, a bacteriostatic agent, penetrate into lung tissue at a high rate for long time event at a single dose, thus making them drug of choice in the treatment of respiratory infections. Tilmicosin causes more cardiac side effects compared to other macrolides used in veterinary medicine (Gokce et al. 1997, Er et al. 2011). Tilmicosin has a cardiotoxic effect at different administration ways and doses (Christodoulopoulos 2009) but the mechanism is not fully known. Many side effects such as increased heart rate and decreased contractility (Jordan et al. 1993), increasing in plasma cardiac markers (Yazar et al. 2001, Yazar et al. 2002a, Yapar et al. 2006), and decreased cardiac antioxidant enzyme activities (Yazar et al.

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2002b) and antioxidant capacity abnormalities resulting in death were reported after tilmicosin use in animals or in exposed humans (National Institute for Occupational Safety and Health 2007). The cause of cardiac toxicity of tilmicosin has been associated with increased reactive oxygen species (ROS) production and disruption of antioxidant enzymes system in myocytes (Yapar et al. 2006) and calcium channel antagonist properties (Main et al. 1996). Heart tissue is more susceptible to free radical-mediated damage because it has low levels of antioxidant enzymes (Abou-El-Hassan et al. 2003). It has been stated that the increased production of ROS due to tilmicosin may cause damage to the cell membrane and intracellular cytoplasmic organelles by reacting with the phospholipid parts of the polyunsaturated fatty acids in the cell membrane (Mishra et al. 2007). In addition, a previous study (Tamargo et al. 1982), it was reported that the mechanism underlying the adverse effect of tilmicosin on the heart may be that it may cause direct toxicity in cardiac cells by contributing to cardiovascular overload due to an increase in epinephrine release.

Non-steroidal anti-inflammatory drugs (NSAIDs) have wide range of use in animals and human (Hunter et al. 2011). Diclofenac, a NSAID and potent inhibitor of cyclooxygenase (COX) 1 and 2 enzymes, is usually used for symptomatic management of pain, inflammation, and fever (Gan 2010, Schjerning et al. 2011). Several harmful effects of diclofenac such as gastrointestinal bleeding, nephrotoxic, hepatotoxic and cardiotoxic effect have been shown (Kearney et al. 2006). The cause of cardiac toxicity of diclofenac has been related to increased ROS production and reduced antioxidant defense system in myocytes (Abdulmajeed et al. 2015). Diclofenac has an effect on cardiac cells by inhibiting mitochondrial complex III activity, which leads to excessive ROS generation in cardiomyocytes, followed by decreased proteasome function and impaired mitochondrial function leading to apoptosis (Brandolini at al. 2020). In addition, diclofenac was considered the main cause of increased cardiovascular risk, as the imbalance between thrombogenic and anti-thrombogenic factors due to COX-2 inhibition may promote thrombotic events that can trigger and exacerbate cardiovascular disease (McGettigan and Henry 2013). Congestive heart failure may proceed into more severe injuries such as myocardial infarction and stroke by long-term treatment with diclofenac (Waksman et al. 2007).

Novel biomarkers such as heart-type fatty acid-binding protein (H-FABP) (Fuster et al. 2011), cardiac-specific conventional markers such as creatine kinase-myocardial band (CK-MB) and cardiac troponin-I (cTn-I), and non-specific traditional indicators such as lactate dehydrogenase (LDH), creatine kinase (CK), and aspartate aminotransferase (AST) (Yazar et al. 2002a) are in use to determine cardiac injury in experimental studies and clinical cases. H-FABP is a low molecular weight protein and is found in the cytoplasm of cardiomyocytes. H-FABP, constitute 10% of cytosolic proteins in all cardiac myocytes and regulates mitochondrial beta-oxidative systems in the heart cells (Fuster et al. 2011, Başar et al. 2013). H-FABP is a more reliable biomarker than myoglobin and cardiac troponins, and used for the diagnosis of the acute coronary syndrome (Kleine et al. 1992, Ishii et al. 1997). It is released from the damaged myocardial tissue leading to a rising in its level in serum (Fuster et al. 2011). Troponin I and CK-MB are accepted as biochemical markers of cardiac myocyte injury (Yazar et al. 2002a). Cardiac troponins are proteins involved in myocardial contraction by regulating the binding of actin and myosin to calcium. Cardiac troponins consist of three subunits, cardiac troponin-C (cTn-C), troponin-I (cTn-I) and troponin-T (cTn-T). cTn-I is responsible for the binding of myosin to actin filaments, while cTn-T acts as the bridging protein towards tropomyosin (Strauss et al. 2010). CK-MB, an isoenzyme of CK, is especially present in the cardiac tissue and is considered as an indicator of myocyte damage (Yazar et al. 2002a).

The combined use of antibiotics and NSAIDs is recommended to control pathogen-associated inflammatory process such as respiratory diseases complex in the veterinary field (Lekeux 2007, Guzel et al. 2010, Brentnall et al. 2013). However, in a study in albino rats (Oda and Derbalah 2018), the combination of tilmicosin and diclofenac sodium has been reported to cause acute cardiotoxicity. No studies on the cardiotoxic risks of this combination have been found in sheep and other ruminants. We hypothesized that this combination, which is used in the veterinary field, especially in the treatment of respiratory diseases, may cause cardiotoxic effects in sheep. The aim of this research was to investigate the cardiotoxic effect of combined use of tilmicosin and diclofenac based on plasma cardiac markers such as H-FABP, CK-MB and cTn-I and to determine the changes of conventional biochemical parameters in sheep.

Materials and Methods

Animals

Thirty-two female Akkaraman breed sheep, weighting 36-48 kg and aged between 2-3 years were included. Animals were considered healthy based on a general clinical and hematological examination and

Table 1. Biochemical and hematologic parameters of sheep groups (mean ± SEM).

Groups	Hours											
	0				24				72			
	C	T	D	TD	C	T	D	TD	C	T	D	TD
CK (U/L)	188±18	202±19	210±26	202±21	180±20.7^{b}	277±20.5^{ab}	335±31.7^{a}	342±60.4^{a}	192±20.1^{c}	361±26.8^{b}	347±20.7^{b}	572±78.5^{a}
LDH (U/L)	193±13	185±15	163±25	182±11	257±19.9^{b}	520±29.3^{a}	475±49.6^{a}	514±21.1^{a}	267±17.9^{b}	487±20.2^{a}	508±33.5^{a}	548±18.0^{a}
AST (U/L)	89±4.5	76±4.8	80±3.7	86±6.2	96.6±5.50^{b}	127±9.54^{a}	135±7.42^{a}	147±12.2^{a}	102.9±7.30^{b}	109.9±7.07^{b}	131.5±5.39^{b}	144.0±11.8^{a}
GGT (U/L)	40±3.0	41±3.8	40±4.3	44±5.2	39.1±2.71	50.1±3.72	44.9±3.75	47.6±4.81	41.4±3.10	44.1±4.02	39.6±2.24	49.9±4.16
ALT (U/L)	16±1.6	17±1.7	17±1.6	18±2.0	20.5±1.68	22.3±1.18	26.0±1.52	26.8±3.10	16.9±1.90^{b}	21.8±2.72^{b}	21.8±2.29^{b}	33.1±2.22^{a}
TP (g/dL)	6.4±0.1	6.5±0.1	6.3±0.1	6.3±0.2	6.53±0.13	6.64±0.15	6.58±0.14	6.33±0.18	6.36±0.06	6.09±0.08	6.21±0.18	6.01±0.16
ALB (g/dL)	2.9±0.2	3.0±0.1	2.8±0.1	2.9±0.1	2.63±0.06	2.78±0.05	2.66±0.05	2.73±0.06	2.65±0.05	2.58±0.04	2.63±0.04	2.63±0.04
BUN (mg/dL)	13±0.84	14±1.4	14±1.1	16±1.5	17.5±1.48	20.3±2.84	19.6±2.10	24.0±2.80	16.3±1.21	17.1±2.88	18.9±2.30	18.9±2.37
CR (mg/dL)	0.74±0.03	0.70±0.03	0.73±0.03	0.71±0.04	0.74±0.02	0.67±0.02	0.78±0.03	0.78±0.06	0.73±0.02	0.67±0.04	0.75±0.05	0.72±0.04
COL (mg/dL)	48±3.8	42±3.4	46±4.4	43±3.6	52.8±2.03	55.5±2.99	54.1±2.97	49.1±2.65	57.6±2.49	55.5±2.38	54.9±1.57	53.6±1.60
TG (mg/dL)	28±1.5	27±1.0	28±2.8	31±2.3	41.0±7.77	27.3±0.96	35.5±5.24	40.4±4.11	43.6±8.50	30.4±3.38	37.9±3.64	36.1±3.00
WBC (x10^{9}/L)	9.01±0.36	8.80±0.44	8.71±0.41	9.60±0.46	8.25±0.37	9.63±0.84	8.81±0.52	10.0±0.62	8.65±0.47	8.45±0.45	7.81±0.48	8.5±0.56
RBC (x10^{12}/L)	9.21±0.24	9.00±0.23	8.98±0.21	8.17±0.32	8.86±0.22	9.16±0.32	8.65±0.27	8.86±0.26	8.96±0.39	8.96±0.36	8.62±0.26	8.74±0.37
HGB (g/dL)	8.43±0.15	8.53±0.28	8.14±0.15	8.35±0.48	7.85±0.19	8.63±0.35	7.95±0.17	8.58±0.27	8.01±0.24	7.89±0.22	7.51±0.19	7.81±0.2
HCT%	32.8±0.75	32.3±0.89	31.5±0.58	32.1±1.77	31.6±0.71	32.5±0.69	32.2±1.2	32.1±0.85	32.0±0.93	31.1±0.92	30.1±0.93	30.8±0.91
PLT (x10^{9}/L)	245±18.5	264±28.9	273±26.5	312±39.7	251±39.2	260±52.8	252±29.6	332±61.9	233±32.6	287±41.8	249±30.1	296±29.7

$^{a, b, c}$ Different letters in the row are statistically significant ($p<0.05$).

Control group (C), diclofenac sodium group (D), tilmicosin group (T), tilmicosin+diclofenac sodium group (TD), heart-type fatty acid-binding protein (H-FABP), creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), alanine aminotransferase (ALT), total protein (TP), albumin (ALB), blood urea nitrogen (BUN), creatinine (CR), cholesterol (COL), triglyceride (TG), white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), platelet count (PLT).

did not receive any medication for 2 months prior to beginning of the study. Throughout the experimental procedures, all sheep were maintained together in the same paddock. Water and feed were available ad libitum. The animals were checked clinically and hematologically at the beginning and the end of the adaptation period (one week). Animals have undergone a check-up test [CK, blood urea nitrogen (BUN), creatinine (CR), LDH, total protein (TP), albumin (ALB), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), AST, cholesterol (COL), and triglyceride (TG)] and those with normal values were randomly divided into four equal groups as control group (C), T: tilmicosin group (T), diclofenac sodium group (D), tilmicosin combination with diclofenac sodium group (TD).

This study was approved by the Ethics Committee Directorate of Selcuk University Faculty of Veterinary Medicine (decision no: 2018/35p).

Experimental design

Group T was subcutaneously (on the chest) given 10 mg/kg of tilmicosin (Ekotil, Ekomed, Turkey) once. Group D received 2.5 mg/kg of diclofenac sodium (Diclovet, Vetas, Turkey) for 3 days once a day intramuscularly, and group TD were administered diclofenac sodium and tilmicosin as stated above. The control group (C) received only 0.9% NaCl in a similar way. Blood samples (3 mL) were taken from the right jugular vein into tubes treated with potassium ethylenediaminetetraacetic acid (K_3-EDTA) before (0 hr), at 24th and 72nd and into heparin treated tubes before (0 hr), at 8th, 24th and 72nd hour after drug administration. Samples were centrifuged at 5000 rpm for 10 minutes and the plasma was stored at -80°C until analysis.

Laboratory analysis

The routine biochemical parameters were determined using autoanalyzer device (ILab-300 plus, Instrument. Lab., Milano, Italy). Haemotological parameters [leukocyte (WBC), erythrocyte (RBC), hematocrit (HCT), hemoglobin (HGB), thrombocytes (PLT)] were measured using hematology analyzer device (BC-2800 Auto Hematology Analyzer Mindray Bio-Med. Electronics, Shenzen, China). Plasma concentrations of H-FABP were determined using commercially available sheep-specific ELISA test kits (Shanghai Sunred Biological Technology Co., Ltd, Shangai) on microplate reader (ELX800, BIOTEK®, USA) as instructed by manufacturer. Immunoassay System (Siemens Advia Centaur XP, Erlangen, Germany) device was used for the detection of plasma cTn-I and CK-MB levels.

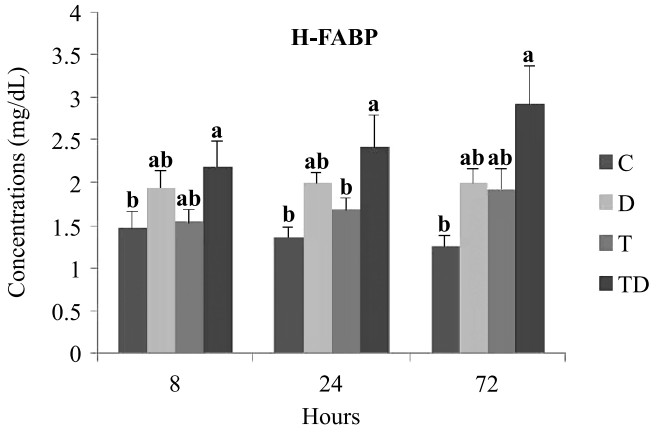

Fig. 1. Plasma heart-type fatty acid-binding protein concentrations in sheep groups (mean±SEM). Different letters (a, b, c, d) in the column are statistically significant ($p<0.05$). Control group (C), diclofenac sodium group (D), tilmicosin group (T), tilmicosin+diclofenac sodium group (TD), heart-type fatty acid-binding protein (H-FABP).

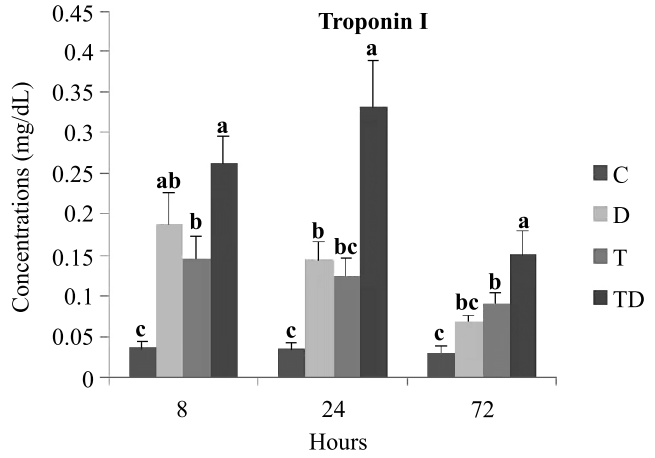

Fig. 2. Troponin-I levels in sheep groups (mean±SEM). Different letters (a, b, c, d) in the column are statistically significant ($p<0.05$). Control group (C), diclofenac sodium group (D), tilmicosin group (T), tilmicosin+diclofenac sodium group (TD).

Statistical analysis

All data were presented as mean and standard errors (mean±SEM). The data were evaluated by Kolmogorov-Smirnov test for normal distribution preconditions. One-way Anova (Posthoc Duncan) was used for statistical analyses of parameters between groups using the SPSS 22.0 software (USA). The Pearson correlation coefficient was used to quantify the relationship between H-FABP, cTnI, and CK-MB. The value of $p<0.05$ was set as statistically significant.

Results

Clinical and biochemical parameters

Biochemical and hematologic parameters of groups were presented in Table 1. No clinical and hematologic abnormalities were observed between groups during the study period. CK level of group D and TD was found to be significantly ($p<0.05$) higher than of group C at 24th hour. Moreover, CK levels of TD group were significantly ($p<0.05$) higher than T and D groups at 72nd hour. LDH and AST levels of experimental groups at 24th hour and CK and LDH levels at 72nd hour were found to be significantly ($p<0.05$) higher than group C. Also, AST and ALT levels of group TD were found to be significantly higher ($p<0.05$) than the rest of the groups at the 72nd hour. There were no significant changes in the levels of GGT, COL, TG, BUN, CR, TP, and ALB at 24th and 72nd hour.

Cardiac markers

H-FABP level of group TD was found to be significantly ($p<0.05$) higher than the group C at 8th, 24th and 72nd hour and group D and T at 72nd hour. H-FABP level of group T and D was higher than the group C but this difference was not statistically significant (Fig. 1).

The significant ($p<0.05$) changes of level of cTn-I (Fig. 2) and CK-MB (Fig. 3) in the experimental groups were presented for different sampling times. Also, cTn-I

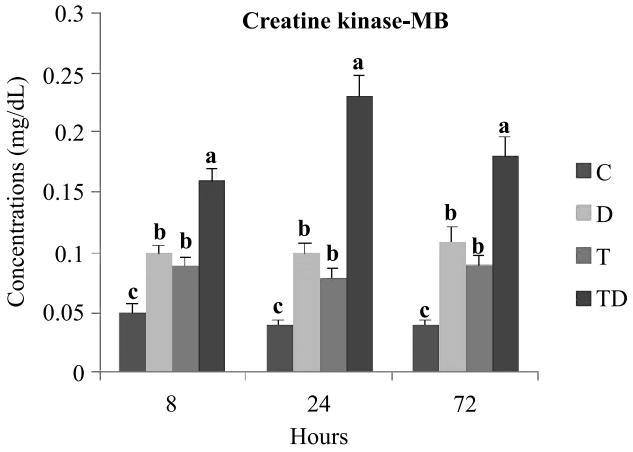

Fig. 3. Creatine kinase-myocardial band levels in sheep groups (mean±SEM). Different letters (a, b, c, d) in the column are statistically significant ($p<0.05$). Control group (C), diclofenac sodium group (D), tilmicosin group (T), tilmicosin+diclofenac sodium group (TD).

Table 2. Pearson correlation coefficient between cTnI, CK-MB, and H-FABP at 8. hour in sheep (n=32).

Parameters (n=32)	cTn-I (mg/dL)	CK-MB (mg/dL)	H-FABP (mg/dL)
cTn-I (mg/dL)	1	.714**	.165
CK-MB (mg/dL)	.714**	1	.445*
H-FABP (mg/dL)	.165	.445*	1

Cardiac troponin-I (cTn-I), Creatine kinase myocardial band (CK-MB), Heart-type fatty acid-binding protein (H-FABP),
* Significant correlation at $p<0.05$, ** Significant correlation at $p<0.01$.

Table 3. Pearson correlation coefficient between cTnI, CK-MB, and H-FABP at 24. hour in sheep (n=32).

Parameters (n=32)	cTn-I (mg/dL)	CK-MB (mg/dL)	H-FABP (mg/dL)
cTn-I (mg/dL)	1	,677**	.500**
CK-MB (mg/dL)	.677**	1	.392*
H-FABP (mg/dL)	.500**	.392*	1

Cardiac troponin-I (cTn-I), Creatine kinase myocardial band (CK-MB), Heart-type fatty acid-binding protein (H-FABP),
* Significant correlation at $p<0.05$, ** Significant correlation at $p<0.01$.

and CK-MB level of group TD was found to be significantly higher ($p<0.05$) than the rest of the groups at 24th and 72nd hour (Fig. 2). The serum levels of CK-MB were significantly correlated with H-FABP and cTn-I, with a Pearson correlation coefficient of 0.445 ($p<0.05$; Table 2) and 0.714 ($p<0.01$; Table 2), respectively, at 8th hour. The serum levels of cTn-I were significantly correlated with H-FABP and CK-MB, with a Pearson correlation coefficient of 0.500 and 0.677, respectively ($p<0.01$; Table 3) and CK-MB levels were significantly correlated with H-FABP, a Pearson correlation coefficient of 0.392 ($p<0.05$; Table 3), at 24th hour. The serum levels of cTn-I were significantly correlated with H-FABP and CK-MB, with a Pearson correlation coefficient of 0.618 and 0.650, respectively ($p<0.01$, Table 4) and CK-MB levels were significantly correlated with H-FABP, a Pearson correlation coefficient of 0.620 ($p<0.01$, Table 4), at 72nd hour.

Discussion

The present study is the first to assess the levels of H-FABP, cTn-I and CK-MB in sheep after the administration of combination of tilmicosin and diclofenac sodium. We demonstrated that the use of tilmicosin and diclofenac causes increase in cardiac biomarkers but their combination has more severe effects in sheep.

The cardiotoxic effect of tilmicosin has been reported in different animal species (Jordan et al. 1993, Main et al. 1996). It is stated that single dose of tilmicosin (10 mg/kg/SC) has no effect on cardiac monitoring such as heart rate and electrocardiogram in sheep (Modric et al. 1998) but it has been reported that tilmicosin has a cardiotoxic effect in a lamb with multiple ventricular septal defects (Christodoulopoulos 2009). Tilmicosin was detected up to 96 hours when administered subcu-

Table 4. Pearson correlation coefficient between cTnI, CK-MB, and H-FABP at 72. hour in sheep (n=32).

Parameters (n=32)	cTn-I (mg/dL)	CK-MB (mg/dL)	H-FABP (mg/dL)
cTn-I (mg/dL)	1	.650**	.618**
CK-MB (mg/dL)	.650**	1	.620**
H-FABP (mg/dL)	.618**	.620**	1

Cardiac troponin-I (cTn-I), Creatine kinase myocardial band (CK-MB), Heart-type fatty acid-binding protein (H-FABP),
** Significant correlation at $p<0.01$.

taneously to sheep at a dose of 10 mg/kg (Ibrahim et al. 2011), while it was detected for 24 hours when administered at a dose of 20 mg/kg to rats (Modric et al. 1999). Intramuscular single dose (2.5 mg/kg) of diclofenac shows a slight cardiotoxicity, muscular damage, and potent hepatic effects in sheep (Er et al. 2013) and extensive use of diclofenac substantially increases the risk of acute myocardial infarction (Jick et al. 2007). Our study confirmed that cardiac injury markers, especially cTn-I and CK-MB increased by single subcutaneous injection of tilmicosin and diclofenac sodium in sheep. In some studies, the relationship of cardiac damage due to the use of tilmicosin (Guo et al. 2010) and diclofenac (Singh et al. 2014, Abdulmajeed et al. 2015) with oxidative stress has been reported. Although it has been reported that long-term high-dose use of these two drugs in different species have cardiovascular side effects (Jordan et al. 1993, Main et al. 1996, Yarishkin et al. 2009), the cardiovascular effects of concomitant at normal dosing are not known. Cardiac specific biomarkers such as H-FABP, cTn-I and CK-MB levels and three of conventional non-specific cardiac injury markers have significantly increased in group TD. H-FABP peaks at 6-8 hours 1.5 hours after the onset of myocardial infarction symptoms and completely disappears within 24-36 hours (Glatz et al. 1988, Offner et al. 1998, Lindholm et al. 2017). However, in our study, H-FABP level of group TD was found to be significantly ($p<0.05$) higher than that group C at 8th, 24th and 72nd hour and group D and T at 72nd hour. We think that the consistently high H-FABP levels obtained in our study may be related to the use of long-acting tilmicosin (İbrahim et al. 2011) and repeated administration of diclofenac sodium (Jick et al. 2007). In addition, consistently high H-FAP levels may be associated with different animal species (sheep).

The data obtained in our study shows that diclofenac and tilmicosin may have a cardiac damage effect but the use of combination of these drugs shows stronger potential cardiac damage effect on the heart of sheep. Cardiac damage of diclofenac sodium is probably related with the increased production of ROS and decrease in antioxidant defense system in heart (Abdulmajeed et al. 2015), and myocardial apoptosis is the possible mechanisms of tilmicosin cardiotoxicity (Oda and Derbalah 2018) or cardiotoxic effect due to induced oxidative stress (Guo et al. 2010). The data of aggravated cardiotoxic effect of tilmicosin with diclofenac sodium treatment (Oda and Derbalah 2018) was supported with our results. The combined use of antibiotics and NSAIDs is recommended to treat the respiratory diseases complex (Lekeux 2007, Guzel et al. 2010) but some studies (Mellanby et al. 2009, Moammar et al. 2010, Hanedan et al. 2015) show that also pneumonia may effect in cardiac damage. Hanedan et al. (2015) have reported that pneumonia increased the serum cTn-I concentration. Chronic suppurative pneumonia in cattle had significantly increased serum cTn-I concentration than the healthy groups (Mellanby et al. 2009). Moammar et al. (2010) have reported that patients with community-acquired pneumonia have low blood oxygen levels and their data suggest that lowering of the oxygen level in the blood may lead to the development of acute myocardial damage and cTn-I may be a useful biomarker to determine this condition. Chang et al. (2013) have reported that cardiac dysfunction may be a considerable determining factor for mortality. Considering previous studies (Mellanby et al. 2009, Moammar et al. 2010, Hanedan et al. 2015), we think that studies evaluating cardiac damage are needed in the use of tilmicosin and diclofenac on animals with pneumonia.

The limitation of our study are the lack of histopathological examination indicating cardiac damage and other novel biomarkers such as high sensitive troponin I and oxidative stress [total anti-oxidant (TAS), total oxidant (TOS) status] cannot be evaluated due to financial constraints. Although the results of this study are promising, further immunohistochemistry-based studies need to be done to reveal association between evaluated serum biomarkers and cardiac injury in different ruminant species.

In conclusion, the use of tilmicosin and diclofenac causes increase in cardiac damage in sheep. The combination of tilmicosin and diclofenac in the veterinary field should be used with caution, as they may have a risk for cardiac damage.

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