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Evaluation of serum homocysteine and nitric oxide concentrations compared with other biochemical parameters in sheep naturally infected with *Fasciola hepatica*

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ABSTRACT: This study aims to determine the changes in serum homocysteine (Hcy) and nitric oxide (NO) concentrations in sheep naturally infected with *F. hepatica*. The animal material of the study consisted of a total of 50 sheep: 40 sheep with fascioliasis and 10 healthy sheep.

The statistical analysis indicated that serum homocysteine concentrations, folate and vitamin B_{12} levels of the sheep infected with *F. hepatica* were higher than those of the control group ($P<0.001$ $P<0.001$ and $P<0.05$, respectively), whereas the nitric oxide levels of the sheep infected with *F. hepatica* were significantly lower than those of healthy sheep ($P<0.001$).

In conclusion, it is thought that vitamin B_{12} and folate are not used sufficiently for the conversion of homocysteine to methionine in the remethylation cycle due to the damage in the liver tissue of sheep naturally infected with *F. hepatica.* This results in the increase of homocysteine which in turn inhibits the formation of nitric oxide.

Keywords: *F. hepatica*, sheep, homocysteine, nitric oxide

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INTRODUCTION

Turkey has about 31.507.934 sheep (Tuncer et al., 2017; Tuncer et al., 2018; Tuncer 2019) and is the 7th greatest sheep raiser country in the world (Tuncer, 2019). *F. hepatica* is a severe disease causing significant economic losses in the countries across the world where sheep, goats, buffalo (Alvarez et al., 2009), and cattle are raised (Yang et al., 1998). This parasitic disease may cause failure to thrive, decrease in meat and milk production, and even death in animals (Souza et al., 2002; Alcala-Canto et al., 2007; Hodžić et al., 2013). Young forms of the parasite destroy the parenchymal tissue, and mature forms destroy the bile ducts (Benzer and Ozan, 2003). Many researchers have focused on changes in serum enzyme activities to determine the level, prognosis, and diagnosis of liver damage caused by *F. hepatica* (Kozat and Denizhan, 2010).

Homocysteine is an intermediate in methionine metabolism, which takes place mainly in the liver (Still and McDowell, 1998; García-Tevijano et al., 2001; Kozat and Okman, 2017). Hcy is a sulfuric amino acid formed during the methionine metabolism and does not enter the primary structure of proteins (García-Tevijano et al., 2001; Fischer et al., 2003; Kozat and Okman, 2017). 70% of total homocysteine in plasma is bound to protein. One-fourth of it is present as disulfide homocysteine by binding to each other, the rest as cysteine-homocysteine or homocysteine thiolactone (Kozat and Okman, 2017). Hcy is metabolized in two major metabolic pathways: the remethylation and transsulfuration cycle (Blom and Smulders, 2011). Hcy is converted to methionine with the addition of a methyl group in a reaction catalized by vitamin B_{12}-dependent methionine synthase. The methyl donor can be either 5,10-methyltetrahydrofolate (MTHF) or betaine. The reaction of MTHF takes place in all tissues and is dependent on vitamin B_{12}. The reaction in which the betaine is the methyl donor takes place in the liver and kidney and is independent of vitamin B_{12}. Methionine is converted to S-adenosyl methionine (SAM) in the presence of ATPand S-adenosyl methionine synthetase. SAM acts as a methyl donor in many reactions. This methylation reaction results in S-adenosyl homocysteine (SAH). SAH is converted into Hcy by a reaction catalyzed by the enzyme hydrolase, and all reactions occur in the liver (Kozat and Okman, 2017). The liver plays a crucial role in sulfur amino acid metabolism. Therefore, Hcy metabolism may be impaired in chronic liver diseases (Ventura et al., 2005).The sulfur-containing amino acid methionine and its derivatives play a central role in the metabolism of homocysteine and cysteine. Chronic liver diseases and especially cirrhosis cause abnormalities in the methionine metabolism, which reflects disorders in multiple enzyme levels. Disorders in methionine demethylation, transsulfuration and remethylation of homocysteine, as well as cystathionine synthesis and hydrolysis, have been described both at the functional and genetic levels throughout the natural course of chronic liver diseases (Ventura et al., 2005; Kozat and Okman, 2017). Increases in total homocysteine levels, that is, the role of homocysteine in atherogenesis, atherosclerosis and thrombosis in hyperhomocysteinemia, cause cardiovascular system disease (Cayir and Kozat, 2016). Hyperhomocysteinemia leads directly to vascular endothelial damage; endothelin changes the anticoagulant effect to procoagulant and causes proliferation of smooth muscle cells (Temel and Ezerol, 2002). Some researchers have reported that the release of nitric oxide (NO), a potent vasodilator and platelet aggregation inhibitor, from bovine endothelium is inhibited by Hcy and inhibition and/or reduction of NO release may cause thrombotic events in hyperhomocysteinemia (Kerkeni et al., 2006).

This study was carried out to determine whether the Hcy remethylation mechanism was affected in sheep that were naturally infected with *F. hepatica*. Moreover, it aimed to determine whether there is a relationship between serum homocysteine values and NO changes in liver disorders.

MATERIALS AND METHODS

Animals

This study was conducted on all sheep that came from one farm: 40 sheep with fascioliasis and 10 healthy sheep. This research was approved (07/03/2017 and 27552122-604.01.02-E.16823) by the Animal Research Ethics Committee of Van Yuzuncu Yil University in Van, Turkey.

At the beginning of the study, all the animals were subjected to general clinical examination and the body temperature, respiratory rate, and heart rate of all sheep were recorded. At the end of the clinical examinations of sheep, approximately 30-50 g of faeces were taken from the rectum of each sheep and placed in the faeces collection containers. Samples were numbered according to the pedigree chart. Faecal samples were taken to the laboratory and kept at -20 °C until examinion. Benedek's sedimentation method

was used to examine the samples (Toparlak and Tuzer, 1994) and determine determine which sheep were infected with *F. hepatica* and which were healthy.

Homocysteine measurements

Sera from blood samples were collected and homocysteine levels in serum healthy sheep and the sheep infected with fascioliasis were determined by ELISA device (ELISA reader®-DAS).

Nitric oxide measurements

Suitable Nitrate/nitrite for sheep was determined by ELISA device (ELISA reader®-DAS) using the colourimetric assay kit (Cayman Chemical Company, catalogue No. 780001/USA).

Vitamin B_{12} and Folate measurements

Vitamin B_{12} and Folate levels in serum samples were determined by autoanalyser (Elecyc 2010 Roche Hitachi-Japan).

Statistical Analysis

Descriptive statistics for the measured parameters are expressed as Mean, Standard Deviation. To compare the groups, Student's t-test was performed. The statistical significance level was taken as 5% and SPSS (ver: 21) statistical package program was used for calculations.

RESULTS

Table 1 shows the statistical analysis of the measurements of serum Hcy, NO concentrations, folate and vitamin B_{12} levels of healthy sheep and sheep infected with *F. hepatica*. Homocysteine concentrations, folate and vitamin B_{12} levels of the sheep infected with *F. hepatica* were higher than those of the control group ($P < 0.001$, $P < 0.001$ and $P<0.05$, respectively), whereas the nitric oxide levels of the sheep infected with *F. hepatica* were significantly lower than those of healthy sheep ($P < 0.001$).

Table 1. Serum homocysteine, nitric oxide, folate and vitamin B12 levels in healthy sheep and the sheep infected with *F. hepatica*

Parameter	Control (n=10) (Mean±SD)	Infected with *F. hepatica* (n=40) (Mean±SD)	P<
Hcy (pg/ml)	5.83±0.93	9.99±2.99	.001
Vit B_{12} (pg/ml)	1227.25±431.16	1552.75±450,74	.05
Folate (ng/ml)	1.56±0.31	2.34±0.54	.001
NO (μmol/L)	53.19±7.20	34.15±15.82	.001

DISCUSSION

The purpose of this study was to investigate the changes in serum Hcy and NO concentrations of sheep infected with *F. hepatica.* The liver plays a key role in the synthesis and metabolism of Hcy, which is an essential intermediate metabolite of methionine metabolism. Methionine is largely metabolized in this organ. The liver has activated genes involved in the methionine and homocysteine metabolism. MAT (Methionine adenosyltransferases), which plays a role in the homocysteine metabolism is found only in the liver, whereas the majority of BHMT (Betaine-homocysteine methyltransferase) and CBS (Cystathionine β-synthetase) are synthesized in the liver ((Finkelstein, 1990; James et al., 1999). For these reasons, damage of the liver affects the metabolism of homocysteine (Paxton et al., 1986; Ciftci and Yuce, 2013). It has been reported that the mean plasma homocysteine levels in human patients with cirrhosis were much higher than in healthy controls, and there was a statistically significant difference between the homocysteine plasma values in patients with cirrhosis and healthy subjects (Ćulafić et al., 2013). In another study a disorder in the liver is thought to cause disorders in Hcy metabolism. Plasma Hcy levels in fatty liver patients are higher than in nonalcoholic and healthy individuals (de Carvalho et al., 2013). It is thought that basal hyperhomocysteinemia in cirrhosis is due to impaired transsulfuration and remethylation mechanisms (Duce et al., 1988; Look et al., 2000; Bosy-Westphal et al., 2001). In human studies it has also been reported that homocysteine metabolism is impaired in chronic liver diseases and leads to an increase in baseline hyperhomocysteinemia in hepatitis patients by 34%, in the fatty liver by 50%, in patients with cirrhosis by 54% and in 52% after orthotopic liver transplantation (Bosy-Westphal et al., 2003). When we examined the Hcy results from sheep infected with fascioliasis in this study (Table 1), serum homocysteine levels were significantly higher ($P < 0.01$) than in the control group. For these reasons, significant changes occur in the metabolism

of Hcy when the liver is damaged. These results are consistent with literature findings indicating that homocysteine levels are high in living organisms with liver damage (Paxton et al., 1986; Ćulafić et al., 2013; James et al., 1999; Ciftci and Yuce, 2013). There is a reported relationship between serum / plasma Hcy levels and folate and vitamin B_{12} levels. (Klee, 2000; Blom and Smulders, 2011; Kozat and Okman, 2017). Hcy is increased in the plasma of patients with deficiency in vitamin B_{12} or folate (Klee, 2000). In this study, Hcy concentrations, folate and vitamin B_{12} levels of the sheep infected with *F. hepatica* were higher than those of the control group ($P < 0.001$, $P < 0.001$ and $P<0.05$, respectively). High concentrations of Hcy are consistent with increases in serum folate and vitamin B_{12} levels. A significant increase in homocysteine concentrations of sheep infected with *F. hepatica* can be attributed to the deterioration of the mechanism of remethylation due to inadequate use of folate and Vitamin B_{12} as a result of liver damage

Some researchers have reported that the release of nitrite oxide (NO), a potent vasodilator and platelet aggregation inhibitor, from bovine endothelial cells is inhibited by homocysteine (Danishpajooh et al., 2001). Several studies have confirmed that the bioavailability of NO is decreased in hyperhomocysteinemia (Fisher et al., 2003; Stanger and Weger, 2003), which might be attributable to diminished NO production or to alternative mechanisms such oxidative stress or nitrosylation (Fisher et al., 2003; Stanger and Weger, 2003, Dayal et al., 2004; Kerkeni et al., 2006). Many studies have been conducted on changes in NO levels in liver disorders (Gupta et al., 1998; Cervi et al., 1998; Clemens, 1999; Benzer and Ozan, 2003; Chen et al., 2003). In a study investigating the functional role of vascular endothelium on increased vascular tone in intrahepatic microcirculation in rats with experimental cirrhosis; a decrease was reported in endothelial dysfunction and NO production in intrahepatic microcirculation of cirrhotic rats (Gupta et al., 1998). Another study investigated the proliferative responses of spleen cells against mitogens in *F. hepatica* -infected rats and reported a decrease in the amount of NO produced, and this decrease was partly associated with extra secretory antigens of *F. hepatica* (Cervi et al., 1998). A study examining lipid peroxidation, antioxidant enzymes and nitric oxide levels in the sheep infected with *F. hepatica* compared the results with those of healthy sheep and reported that serum NO levels of the sheep infected with *F. hepatica* were not affected but there was a significant decrease in NO levels in the liver disease (Benzer and Ozan, 2003). Some experimental and clinical studies have reported that hyperhomocysteinemia causes vascular oxidative stress and disrupts the vascular response to NO, indicating endothelial dysfunction in rats (Gupta et al., 1998). It has also been reported that homocysteine reacts with NO and inhibits not only the biological activity of endothelium-derived NO but also the biological activity of exogenously supplied NO (Nappo et al., 1999; Fu et al., 2002). In this study, serum NO levels of sheep with fascioliasis were found to be significantly lower than serum NO levels of healthy sheep. The decrease in NO levels in the infected sheep is consistent with the data reported by the researchers (Cervi et al., 1998; Gupta et al., 1998). Furthermore, in the sheep infected with *F. hepatica,* homocysteine concentrations were found to be significantly higher ($P < 0.001$) while nitric oxide levels were found to be lower than in healthy sheep ($P < 0.001$). Hyperhomocysteinemia and decreased NO concentration in the diseased group support the data of the researchers (Still and McDowell, 1998; Bosy-Westphal et al., 2003; Fisher et al., 2003; Stanger and Weger, 2003; Dayal et al., 2004; Kerkeni et al., 2006; Ćulafić et al., 2013).

In conclusion, homocysteine, folate and vitamin B_{12} changes can be caused by the following reasons: 1) It is thought that *F. hepatica* infection causes severe damage in the liver, which, in turn, leads to the deterioration of functions and disruption in the conversion of homocysteine to methionine, and, as a result, causes hyperhomocysteinemia, 2) Folate and Vitamin B_{12} levels were found to be high in the fascioliasis group because folate and Vitamin B_{12}, used as cofactors in the conversion of homocysteine to methionine due to functional disorders in the liver, are not used sufficiently, 3) Low NO level in the diseased group may be due to intrahepatic vascular endothelial disorder or may be due to the inhibition of NO synthesis due to the increase of Hcy.

The present study concludes that there is a negative correlation between serum Hcy values and NO values in liver disorders caused by *F. hepatica*. Besides, since the present study is the first study to present evidence with regards to hyperhomocysteinemia in liver damage, it is thought that it will promote future research on liver damage.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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