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## Investigation of serum homocysteine levels in relation to age and sex

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**ABSTRACT.** Homocysteine is a non-proteinogenic and a derived amino acid in the methionine metabolism and is a risk factor for cardiovascular and many other metabolic diseases. In this study, the purpose was to determine serum homocysteine levels in healthy sheep based on differences in age and gender. 220 healthy Akkaraman sheep, composed of both females (n=55 lambs and 55 ewes) and males (n=55 lambs and 55 rams), were used as animal samples. The measurements of serum homocysteine concentrations were performed with ELISA-HCY kit. The levels of serum homocysteine of sheep were detected in ewes, female lambs, rams and male lambs as  $2,91\pm 0,50$ ;  $2,99\pm 0,42$ ;  $11,22\pm 3,10$ ;  $6,43\pm 1,26$   $\mu\text{mol/L}$ , respectively. The primary intent of this study was to investigation and characterization the serum Hcy concentrations in healthy sheep broken down by different ages in both genders. As a result, the serum homocysteine values that can constitute a reference value for healthy breeds of sheep were determined in this study.

**Keywords:** *Sheep, homocysteine, gender differences, age differences*

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## INTRODUCTION

**H**omocysteine (Hcy), a sulphur-including amino acid that does not participate in protein construction and DNA structure, was first isolated from urine bag by Vincent du Vigneaud in 1933 and in 1955, for which he received the Nobel Prize in Chemistry (McCully 1969; Wijekoon et al., 2006; Graham et al., 1997; Ramakrishnan et al., 2006).

For the first time, McCully (1969) proposed a “homocysteine theory in atherosclerosis” and has developed the hypothesis that homocysteine may lead to vascular diseases (McCully 1969; Graham et al., 1997; Wijekoon et al., 2006).

Hcy is an intermediary product in methionine metabolism. Hcy, which mediates methionine metabolism, is catabolized in the reaction with the vitamin B6 cofactor of Cysteine (Cys). In most tissues, Hcy can be converted back to methionine by this re-methylation reaction, catalyzed by the enzyme “methionine synthase,” requiring 5-methylene tetrahydrofolate (THF4) in the form of reduced folate as the methyl donor with vitamin B12 cofactor (Finkelstein et al., 2000; Ravaglia et al., 2005; Wijekoon et al., 2006; Ramakrishnan et al., 2006; Martinez et al., 2017; Mohammad et al., 2010; Kumar et al., 2017; Škovierová et al., 2016).

The Hcy formed inside the cell is released into the blood and urine. Vitamin B12, folate and B6 are essential cofactors in Hcy re-methylation and trans-sulfation pathways. Plasma total Hcy levels are known and accepted to be important biochemical parameters as determinants of possible cardiovascular conditions and consequent mortality in patients with neuronal disorders, renal insufficiency, diabetes, systemic lupus erythematosus and venous thromboembolism, especially coronary-heart disease (Martinez et al., 2017; Ramakrishnan et al., 2006; Kumar et al., 2017). The conclusions of different studies clearly showed that insufficiencies of folate, vitamin B12, or methionin are affecting serum and cellular levels of S-adenosylmethionine (SAM), S-adenosylhomocysteine and homocysteine and are linked with molecular processes leading to oncogenesis and also carcinogenesis (Murin et al., 2017; Martinez et al., 2017).

Human studies have shown that plasma Hcy concentrations increase with age (higher in adults than in pediatric periods), and are higher in males than females. It is known that the most important factors affecting Hcy level are genetic and nutritional factors

(vitamin B6 and B12 or folate deficiencies), age, gender difference, physiological periods, especially the postmenopausal period and some metabolic disorders (Robinson et al., 1994; Mohammad et al., 2010; Piccione et al., 2008; Masuda et al., 2008; Škovierová et al., 2016).

While slight age-related increases are considered physiological, the high Hcy levels observed at younger ages are linked to genetic predisposition and environmental factors (smoking, toxic carcinogenic substances and drugs) (Corrales et al., 2002; Carmel and Jacobsen, 2001; Refsum 1998; Wijekoon et al., 2006; Varga 2005; Richards 2008; Hankey and Eikelboom 1999; Graham et al., 1997; Martinez et al. 2017; Ramakrishnan et al., 2006; Piccione et al., 2008). An increase in the homocysteine level is an indicator of lower intake of foods containing vitamin B12, or malfunction of renal functions or possibly a low enzyme activity in the metabolism of homocysteine (Martinez et al., 2017; Furlong et al., 2010; Murin et al., 2017).

With this current preliminary research, is aimed that, to reveal and compare the presence of homocysteine in sheep, depending on age and gender differences.

## MATERIALS AND METHODS

### Ethical scope

This study was conducted in accordance with the principles of the Local Ethics Committee in the framework of the ethics confirmed by the Bahri Daggas International Agricultural Research Institute, Directorate of Local Ethics Committee of Animal Experiments (14.01.2015 / 35 and 0088).

### Sample collection

The sample collection was done during May 2016. The sheep were on pasture free-ranging and normal-fed, and the ambient temperature was 1.5 °C above the normal seasonal average for May. In this study, blood samples were collected from animals in private enterprises located in Aksaray, Turkey. The animal material in this study consisted of 220 healthy Akkaraman sheep. The sheep originated from Aksaray and its vicinity. All animals were found healthy by clinical examination and general clinical perspective. The animals were separated into four groups of 55 sheep each, based on sex and age. Two hundred and twenty totally healthy sheep, both females (n=55 lambs and 55 ewes) and males (n=55 lambs and 55 rams), were used as animal material.

Blood samples were simultaneously collected during morning hours from each animal. 15 mL of animal blood was taken from the jugular vein and placed into ice-bed-cold vacuum serum-separating tubes as appropriate for the blood-collection procedure. Blood samples, which were collected into anticoagulant-free serum tubes, were centrifuged 10 minutes at 3000 rpm (Coles, 1986) in biochemistry laboratory. Hemolysis-free sera obtained after centrifugation were separated into the micro (Eppendorf) tubes and labeled and stored at  $-25^{\circ}\text{C}$  deep freeze until analyzed. These samples were used for homocysteine analysis.

### Hcy assays

For the Hcy assay, previously frozen serum samples were dissolved (at RT) and assayed at without wasting time. They were analyzed in the same experimental set and against the blind.

Levels of serum peptides were measured blindly by using commercially available enzyme-linked immunosorbent assay (ELISA) kits to detect peptides in the biological fluids and were read with using the ELISA plate reader.

In the scope of the study quantities of Hcy peptides in sera were studied by commercial quantification (Shangai Sunred, Biological Tech., China) using the ELISA method. HCY-ELISA-Plates were read on the ELISA-Plate Reader (450 nm) (ELx800 Absorbance Microplate Reader-Biotek). Homocysteine concentrations were calculated from standard curves.

**Assay range:**  $0.6\mu\text{mol/L}\rightarrow 100\mu\text{mol/L}$ .

**Sensitivity:**  $0.31\mu\text{mol/L}$ .

CV (%) =  $\text{SD}/\text{mean}\times 100$

Intra-Assay: CV < 10%

Inter-Assay: CV < 12%

### Statistical analysis

Descriptive statistics for the properties studied; Mean, Standard Deviation, Standard Error, Minimum and Maximum values.

Data were analyzed using the SPSS 15.0 for Windows™ statistical software (SPSS Inc., Chicago, IL, USA). Differences among the groups were analyzed by Student t-test. "One-way ANOVA" was performed to compare the group averages in terms of continuous variables. The Duncan multiple comparison test

was used to identify the different groups following the analysis of variance. The data are given as the means  $\pm$  standard error ( $X \pm \text{SH}$ ). Statistical significance was accepted as  $p < 0.05$  level.

### RESULTS

The serum Hcy levels of the four groups, which differs based on age and sex, were presented in table 1. In the current study, serum homocysteine levels (table 1) were found to be higher in males than in females when examined without regard to age (figure 3, 4). It was found that in females, age difference in Hcy values was not found statistically significant (Hcy values were found to be close to each other in female lambs and ewes, figure 1). In males, it was much lower in lambs than in rams (figure 2). These differences were significant compared to rams ( $p < 0.05$ ) (table 1).

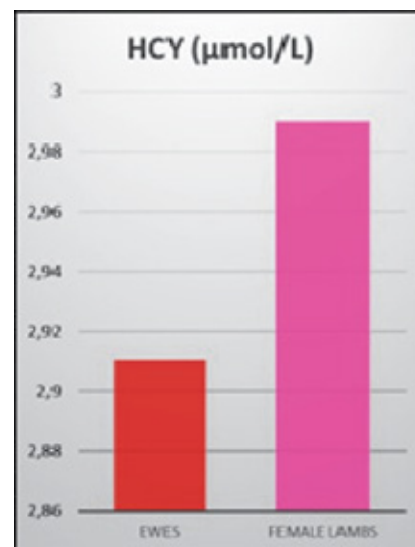


Fig. 1. Levels of serum Hcy in ewes and female lambs

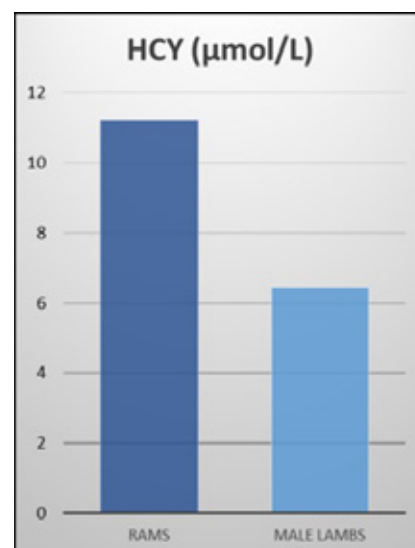


Fig. 2. Levels of serum Hcy in rams and male lambs

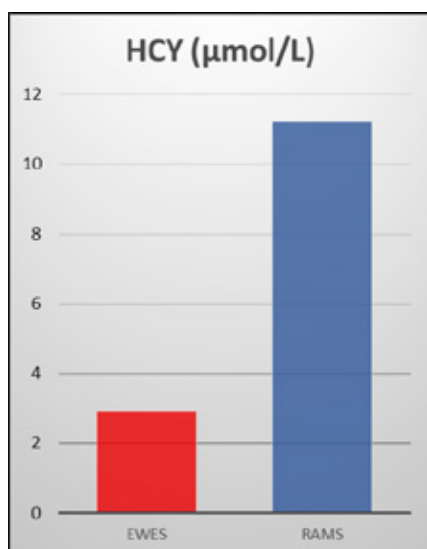


Fig. 3. Comparing the serum Hcy levels of ewes and rams

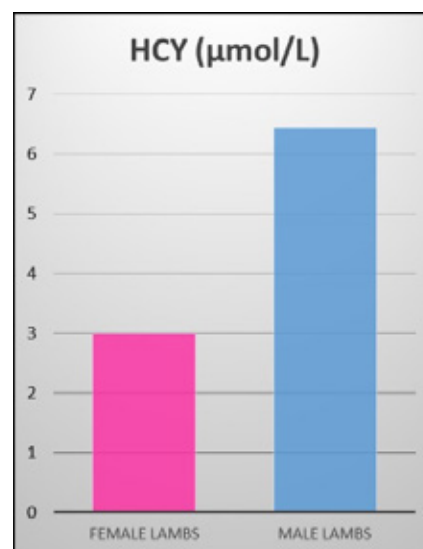


Fig. 4. Comparing the serum Hcy levels of female and male lambs

Table 1. Levels of serum Homocysteine of Akkaraman sheep

Polypeptide (Unit)	Ewes (n=55)	Female lambs (n=55)	Rams (n=55)	Male lambs (n=55)
Homocysteine (µmol/L)	2,91±0,50a	2,99±0,42a	11,22±3,10ab	6,43±1,26ab

## DISCUSSION

From 1969 until today, a variety of research was performed based on different viewpoints on Hcy. These first studies have been carried out in especially humans (Carson et al., 1963; Gibson et al., 1964; McCully 1969; Graham et al., 1997).

Hcy is known to be an important biochemical parameter, primarily as a cardiovascular marker, as a determinant of neuronal disorders, renal health, renal failure, diabetes and venous thromboembolism, and even carcinogenesis (Finkelstein et al., 2000; Ravaglia et al., 2005; Wijekoon et al., 2006; Martinez et al., 2017; Ramakrishnan et al., 2006; Kumar et al., 2017). Levels of the advanced biochemical research marker Hcy are a current and reliable tool for the evaluation of the health conditions of humans and animals in different metabolic and endocrinal periods in which the homeostatic continuum of organisms is altered. The analysis of Hcy amounts is a valuable parameter for the assessment of the health status of an animal in different endocrinal periods such as the pre-menopausal, post-menopausal or lactation period, etc (Piccione et al., 2008). Also in terms of homocysteine, gene, breed, age and sex differences influences the biochemical, physiologi-

cal, endocrinal and all metabolic variables of organisms. Therefore, it is important to consider the homocysteine levels as a trustworthy marker of human and animal welfare in order to demonstrate possible preventive changes of the healthy states and the beginning of the diseases and metabolic disorders.

The main goal of this research was to analyze and qualify the serum Hcy concentrations in healthy sheep broken down by different ages and genders. It could not find many research reference amounts for small ruminants (especially for sheep) on this subject. The current research was performed to find out normal physiological values of serum Hcy concentrations in the healthy Akkaraman breed sheep.

In human studies have shown that concentrations of plasma Hcy increase with age and are higher in males than females. This result shows that homocysteine levels are determined by both genetic and nutritional factors and gender differences are important (Robinson et al., 1994; Graham et al., 1997; Hankey and Eikelboom 1999; Corrales et al., 2002; Varga 2005; Wijekoon et al 2006; Richards 2008; Piccione et al., 2008; Masuda et al., 2008; Moham-

mad et al., 2010; Škovierová et al., 2016).

In the present study, results were obtained in a similar framework. Compared to the submitted work, homocysteine levels were found to be higher in males than females without regard to age. Levels were much lower in male lambs than in rams and it was observed that these results were statistically significant compared to rams ( $p < 0.05$ ). It was detected that for Hcy values, age differences were not very important in females (levels were found to be close to each other in female lambs and ewes).

In the literature, related research on the topic is sparse.

A study conducted by Furlong and colleagues in 2010 aimed to assess the possible diagnostic role of plasma Hcy in relation to methylmalonic acid and vit B12, in pregnant Romney ewes (3 years). They reported the mean concentrations of Hcy remained within the range of 1.5–3.0  $\mu\text{mol/L}$  (Furlong et al., 2010). It is seen that, this result is very close to our Hcy levels in 1-4 years old ewes ( $2,91 \pm 0,50 \mu\text{mol/L}$ ).

In other research Rezaei and Dalir-Naghadeh specified that, homocysteine amounts in lambs with the acute cardiac disease were higher than in lambs with the muscular dystrophy. Also they proved that Hcy concentrations in healthy lambs were lower than in with the cardiac form of acute selenium deficiency ones. The researchers declared that in sheep, increased plasma Hcy amounts would be a risk factor for myocardial disease (Rezaei and Dalir-Naghadeh, 2009). In another study, Kozat and coworkers had compared amounts of Hcy between healthy lambs and subclinical lambs with white muscle disease. They found that patient lambs have higher values of Hcy than healthy lambs ( $p < 0.05$ ). Compared with the present study (particularly male lambs, Hcy ( $6,43 \pm 1,26 \mu\text{mol/L}$ ), the Hcy levels of healthy lambs (without sex discrimination) is very similar and values may be close (healthy lambs control group Hcy= $5.10 \pm 3.33 \mu\text{mol/L}$ ) (Kozat et al., 2011).

In a study managed by Piccione and his colleagues intended to investigate the effect of Hcy levels and the antioxidant stress of lactation in sheep, considering the lactation periods, only female sheep were selected (Piccione et al., 2008). In this study, serum Hcy was  $2.91 \pm 0.50 \mu\text{mol/L}$  in female sheep (ewes) between 1-4 years old. It is

observed that in Piccione's study, the level of serum Hcy determined on the first day of lactation was slightly higher ( $3.40 \pm 0.17 \mu\text{mol/L}$ ) than the level in the current study ( $2.91 \pm 0.50 \mu\text{mol/L}$ ). In a research study conducted in 2015, (Razavi et al., 2015), serum levels measured in relation to a parasitic infections (Malignant Ovine Theileriosis) in sheep were found to be lower in controls than in patient samples (control animal hcy level:  $7.29 \pm 0.54$ ; non-infected animal hcy level:  $12.18 \pm 0.50$ ; infected animal Hcy level:  $11.16 \pm 0.57 \mu\text{mol/L}$ ). In 2010, Mohammad and co-workers analyzed levels of serum homocysteine in male and female subjects below and above 50 years of age who have coronary heart disease with or without diabetes mellitus (Mohammad et al, 2010). Serum homocysteine levels were higher in patients with coronary heart disease and diabetes than in healthy controls. Further, serum homocysteine levels were lower in women than in men. This study additionally proved that the level of Hcy increases with age. But interestingly, in women over 50 years of age (post-menopausal period), the increase in Hcy level was slightly higher than in men.

## CONCLUSIONS

If we support our research with these and similar studies, measuring quantities of Hcy in plasma or serum as a metabolic indicator in sheep, especially the methylation capacity, and as a general health parameter will provide vital information on animal health.

Considering all conditions, differences in region, breeds, sex, age, season and nutritional sources could affect homocysteine levels and cause changes in the present study.

Consequently, it is intended that in presented study Hcy variables of age and gender of clinically healthy sheep were based on the reference values for healthy sheep. Amounts of blood Hcy get on the basis of breed, age and gender of sheep should meet the requirement for diagnosis of healthy conditions. This value might be a reference value for serum Hcy levels in healthy Akkaraman sheep.

The purpose of current work is to identify and detect the serum Hcy values that are a biochemical, physiological, endocrinological, cardiological parameter and even carcinogenic biomarker of healthy sheep. These results will provide valuable contributions to the literature, for both academicians and veterinarians.

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## CONFLICTS OF INTEREST

The author has nothing to disclose. No conflicts of interest exist. The author has no financial or personal relationship that could inappropriately influence or bias the content of this paper.

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