IJAS Iranian Journal of Applied Animal Science

Effect of Protein Levels and Rumen Protected Glutamine Supplementation on Blood Metabolites, Thyroid Hormones, and Redox Status of Heat Stressed Fattening Lambs

Research Article

M. Feyz[1*], A. Teimouri Yansari[2], Y. Chashnidel[1] and E. Dirandeh[1]

[1] Department of Animal Science, Faculty of Animal Science and Fishery, Sari Agricultural Science and Natural Resources University, Sari, Iran

Received on: 15 Jul 2020
Revised on: 26 Mar 2021
Accepted on: 1 May 2021
Online Published on: Sep 2021

*Correspondence E-mail: m.feiz@stu.sanru.ac.ir

Online version is available on: www.ijas.ir

ABSTRACT

High ambient temperatures have a negative effect on the production and health of animals. Severe heat stress may lead to changes in the secretion of hypothalamus and pituitary gland hormones, which alter hormonal responses and metabolism. The aim of this study was to determine the effect of protein levels and rumen protected glutamine supplementation on blood metabolites, thyroid hormones concentrations, and redox status of fattening heat stressed lambs. Using sixteen Afshari male lambs (aged 3-4 months) over 45 days, a 2 × 2 factorial were designed to study the effects of two levels of protein (equal and 10% higher than requirement) and glutamine (0.0 and 0.2 g/kg body weight) in four experimental rations. Results showed the glutamine supplementation increased triiodothyronine and thyroxine concentrations; and conversely decreased aspartate transaminase, non-esterified fatty acids, and cortisol concentrations. Increased protein levels at the beginning of fattening did not affect triiodothyronine, thyroxine, aspartate transaminase, alanine transaminase, and non-esterified fatty acids concentrations. Interaction of glutamine and protein levels significantly decreased cortisol concentrations. Glutamine supplementation increased levels of superoxide dismutase and glutathione peroxidase, and decreased nitric oxide. The greater protein levels significantly decreased superoxide dismutase and glutathione peroxidase on day 30, total antioxidant status concentration on day 15 and 45, and increased nitric oxide concentration on day 15 of fattening. It can be concluded that glutamine supplementation improves redox status of heat stressed lambs during the fattening period but increase protein levels alone did not have this effect.

KEY WORDS antioxidant enzyme, blood metabolite, temperature humidity index, triiodothyronine.

INTRODUCTION

Sheep and goats have several advantages in the livestock industry due to their short gestation period, high fertility, rapid growth rate, high feed conversion efficiency, high disease resistance capacity, as well as easy marketing (Adams and Ohene-Yankyera, 2014). Among climate variables, environmental temperature appears to play a key role in negatively affecting the production. Heat stress leads to the excessive production of radicals and reactive oxygen species (ROS) which results in oxidative stress, an imbalance in the oxidant/antioxidant system. The production of free radicals, including nitric oxide (NO), is an integral feature of normal cellular function. In contrast, overproduction or inadequate removal of free radicals can lead to destructive and irreversible damage to the cell. The increase of NO concentration may play an important role in the heat stress. Enzymes with important antioxidant functions in-

clude superoxide dismutase (SOD) which catalysis the dismutation of superoxide radical to hydrogen peroxide, and water; glutathione peroxidase (GPX) and catalase which facilitates the destruction of both hydrogen peroxide and organic peroxides. Glutathione peroxidase can reduce lipid peroxidase and other organic hydroperoxides that are highly cytotoxic products. Severe stress may also lead to changes in the secretion of all the hypothalamus and pituitary gland hormones, which alter metabolism, immune response, and behavior other than changes in reproductive function. The main hormones that control animal adaptation include thyroid hormones, glucocorticoids, catecholamine, anti-urinary hormones, and growth hormone. In general, the endocrine regulation of the endocrine glands is the main regulator of all adaptive mechanisms that help animals survive stress. The hypothalamic-pituitary-adrenal axis (HPA) and the hypothalamic-pituitary-thyroid (HPT) axis play an important role in the release of several neurotransmitters and hormones that regulate thermal mechanisms in animals (Niyas *et al*. 2015). Corticotrophin-releasing hormone (CRH), adrenergic hormone (ACTH) and glucocorticoids are the main products of the HPA axis, ultimately controlling the stress response pathways in animals (Sejian *et al*. 2010). In addition, ACTH is an important regulator that helps produce and secrete cortisol (Sejian *et al*. 2010). Previous research has shown that hormones produced by the adrenal and thyroid glands play an important role in the metabolic response in livestock (Joy *et al*. 2016; Shaji *et al*. 2017). The HPA axis also regulates energy partitioning to support survival activities through the process of hepatic gluconeogenesis.

Activation of the HPA axis may lead to an increase in glucocorticoids produced such as cortisol, which is shown to be the main stress-reducing hormone and is also known as a reliable marker for assessing the severity of stress among species (Shaji *et al*. 2017). Increased cortisol levels also cause hepatic gluconeogenesis, which helps produce glucose from non-carbohydrate sources and keeps energy metabolism alive to support activity. Several authors have stated that the secretion of glucocorticoids is the main response of endocrine glands to heat stress conditions (Joy *et al*. 2016; Afsal *et al*. 2018). The HPT axis is responsible for the synthesis and release of thyroid-releasing hormone (TRH) in the pituitary gland (Fekete and Lechan, 2013). The TRH triggers the release of thyrotropin (TSH) from the anterior pituitary gland, which in turn stimulates the synthesis and secretion of thyroid hormones. Thyroid hormones T_3 and T_4 are very important for maintaining control over metabolism. Shortages or excesses of T_3 or T_4 have very pronounced effects on the affected animal. Thyrotropin-releasing hormone (TRH), thyroid-stimulating hormone (TSH) and thyroid hormones (T_3 and T_4) are components of the metabolic pathway in regulating body heat production. In general, cortisol and thyroid hormones are considered important biological markers of neuroendocrine pathway regulation of adaptive mechanisms during heat stress in livestock (Afsal *et al*. 2018). Determination of biochemical indices thyroid hormone concentration and enzyme activity in animal blood gives us a clear picture of nutritional and health status. There is little information concerning the oxidative stress enzymes and cortisol and thyroid hormones in sheep. Nutritional strategies such as adding protected sources of ruminal amino acids such as glutamine may reduce the negative effects of heat stress. The aim of this study was to determine the effect of dietary protected-glutamine (Gln) supplementation and protein levels on the concentrations of cortisol and thyroid hormones and antioxidant in the blood of Afshari lambs during fattening period.

MATERIALS AND METHODS

Experimental design

The research was carried out in the north of Iran (Latitude: 36˚ 33' 47.95" N and Longitude: 53˚ 03' 36.32" E) during heat stress (THI: 82.26). Sixteen male lambs of the Afshari breed (aged 3-4 months) with average weight 31.5 ± 0.22 kg were randomly selected. Prior to the experiments, all animals were treated against internal and external parasites. Water was offered *ad libitum* and were fed with TMR ration (Table 1) for 45 days. Four experimental rations including two levels of protein (equal and 10% higher than requirement) and rumen protected glutamine (0.0 and 0.2 g/kg body weight; Table 1). The degree of HS experienced by animals is estimated by the temperature-humidity index (THI) that includes both ambient temperature and relative humidity (Table 2). Information, consisting of daily maximum temperatures and relative humidity, was used to calculate the temperature-humidity index (THI; highest daily temperature in Celsius degrees; RH refers to maximum relative humidity) for each day using the following equation:

$$THI = (0.8 \times temperature) + [(\% RH/100) \times (temperature - 14.4)] + 46.4$$

Blood analysis

Blood samples were collected weekly in heparinized and serum clot activator vacutainers from the jugular vein of the lambs. Serum was separated by centrifugation at 3000 rpm for 10 min and stored at −20 ˚C until analyzed, whereas plasma was separated from the sample collected in heparinized vacutainers by centrifugation at 3000 rpm for 10 min and stored at −80 ˚C. After plasma separating, erythrocyte lysate was prepared.

Table 1 Ingredients and chemical composition of experimental rations

Ingredients (%)	Basal ration with protein at requirement levels	Ration with 10% protein higher than requirement levels
Barley grain	27.00	28.94
Corn grain	21.00	17.95
Alfalfa hay	22.90	22.46
Wheat straw	6.00	6.29
Beet pulp	5.00	5.49
Wheat bran	12.00	10.48
Soybean meal	4.00	5.99
Salt	0.40	0.40
Premix[1]	1.00	1.00
Calcium carbonate	0.70	0.80
Urea	0.00	0.20
Chemical composition (%)		
Dry matter	89.20	89.32
Crude protein	13.40	14.50
Neutral detergent fiber (NDF)	36.20	35.07
Acid detergent fiber (ADF)	16.17	15.90
Crude fat	3.07	3.40
Ash	10.11	9.83
Non fiber carbohydrate (NFC)	37.33	37.20
Metabolizable energy (Mcal/kg)	2.40	2.41

[1] 1 kg of control premix contained: vitamin A: 80 KIU/kg; vitamin D_3: 20 KIU/kg; vitamin E: 200 mg/kg; Fe: 640 mg/kg; Mn: 640 mg/kg; Cu: 120 mg/kg; Zn: 640 mg/kg; Co: 2.5 mg/kg; I: 10.5 mg/kg and Se: 2.5 mg/kg.

Table 2 Temperature, relative humidity and temperature humidity index (THI) of the environment during the experimental periods

Days of fatting	Average temperature (˚C)	Average Relative humidity (%)	THI
1-15	31.26	72.66	83.61
15-30	31.53	72.73	84.03
30-45	28.06	76.46	79.16

After triple washing of erythrocyte mass with physiological solution, 0.5 mL of cell suspension was dissolved in 2 mL cold water for lysis of erythrocytes. Hemoglobin was then precipitated by adding 1.8 mL water and 0.2 mL ethanol/chloroform (3:5/v:v) to 0.2 mL lysate. The tubes were shaken for 5 min and centrifuged at 10000 rpm for 20 min. The supernatant was used for the determination of enzyme activities (SOD, GPx and total antioxidant status (TAS)). Plasma samples were tested for aspartate amino transferase (using Pars Azmoon kits, Iran), non-esterified fatty acids (NEFA) (using Randox Company's kits, England) by colorimetric methods. Alanine transaminase (ALT) and aspartate transaminase (AST) were measured using Pars Azmon kits (Pars Azmun Laboratory, Tehran, Iran) in biochemical analyzer (Mindray BS-120).

Redox status

Glutathione peroxidase activity was evaluated by Paglia and Valentine's method (1967), using RANSEL Kit (RANSEL Kit, Randox, UK). Glutathione peroxidase catalysis the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was measured. Superoxide dismutase activity was measured by an indophenol nitrophenol phenyltetrazolium chloride modified method (RANSOD Kit, Randox, UK). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4- iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity measured by the degree of inhibition of this reaction.

One SOD unit was considered as that which caused a 50% inhibition of the reduction rate of INT under the assay condition. The total antioxidant status (TAS) activity was then measured by the TAS kit Randox, UK. ABTS (2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^{*+}$.

This has a relatively stable blue-green color, which is measured at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree which is proportional to their concentration. The NO level was established by the method of Cortas and Wakid (1990) by using nitric oxide assay kit (Navand salamat Company, Urmia, Iran).

Determination of thyroid hormones and cortisol

Triiodothyronine (T_3) and thyroxine (T_4) were estimated by ELISA techniques (stat fax 3200 (Awareness) USA ELISA) using assay kits manufactured by Pishtaz Teb (Pishtaz Teb Co., Tehran, Iran). Serum Cortisol hormone concentration was determined by using commercially available enzyme linked immunosorbent assay (ELISA) (Monobind Inc, Lake Forest, CA, USA).

Statistical analysis

The experiment was conducted as a factorial 2 × 2 in completely randomized design. Research results were processed by the SAS (SAS, 2004) software. Effects of treatment on the concentration of biochemical indicators, thyroid hormone concentration in blood of lambs, were analyzed by two way repeated measures ANOVA. Results are presented as least square means with standard error of the means (SEM) and P-value. Statistical differences were declared at $P < 0.05$.

RESULTS AND DISCUSSION

Cortisol and thyroid hormones

Serum concentrations of cortisol was increased in lambs fed higher protein levels and was decreased in those fed glutamine ($P<0.05$) during different periods (Table 3). Higher protein levels significantly increased cortisol and decreased T_3 on 15 day of fattening but did not have a significant effect on these parameters on days 30 and 45. Interaction effect of glutamine-protein levels decreased cortisol but was not significant on T_3 and T_4 concentrations. The mean concentrations of serum T_3 and T_4 showed a significant increase from the second week until the end of experiment. The greatest levels of T_3 and T_4 were observed in lambs that received glutamine and the lowest in lambs fed with higher protein levels. In the second week of the experiment, the highest levels of T_4 were observed in lambs fed glutamine alone, but in the subsequent periods (30^{th} and 45^{th} days) the levels of T_4 in the blood decreased with increasing protein levels, although this decrease was not statistically significant. Serum concentrations of cortisol and thyroid hormones can be used as a marker to evaluate the metabolic status. These changes are not necessarily indicative of disease but may reflect the physiological status of animal.

Cortisol is a corticosteroid, in which influence is varied depending on its concentration. The concentration of cortisol, as a catabolic hormone, stays in dynamic equilibrium with anabolic hormones. Through its multiple action, it mobilizes organism to fight stress, by ensuring a stable level of glucose, stimulating tissue's regeneration and inhibiting inflammation processes (Stachowicz and Lebiedzinska, 2016).

It regulates the usage of diet nutrients through increasing gluconeogenesis and increasing lipolysis in adipose tissue. In addition, it increases protein synthesis in the liver and restricts their formation in muscles and skin cells. (Stachowicz and Lebiedzinska, 2016).

Increased cortisol concentrations in lambs fed higher protein levels, indicating that more protein exacerbates stress conditions in livestock. Several studies show that cortisol increased in plasma and serum in sheep and goats under stress (McManus *et al*. 2009; Okoruwa, 2014). Higher temperature and THI cause heat stress and activation of the HPA axis, therefore, in response to this, the level of cortisol may increase. Adrenergic hormone (ACTH) and glucocorticoids are the main products of the HPA axis, ultimately controlling the stress response pathways in animals (Sejian *et al*. 2010). In addition, ACTH is an important regulator that helps produce and secrete cortisol (Sejian *et al*. 2010).

Increased cortisol levels also cause hepatic gluconeogenesis, which helps produce glucose from non-carbohydrate sources and keeps energy metabolism alive to support activity. High levels of cortisol can lower your body's stores of glutamine (Karami *et al*. 2014). Serum concentrations of cortisol was decreased in lambs fed glutamine during different periods. Glutamine has various anabolic and stimulatory roles such as regulating and modifying glucose from gluconeogenesis pathway, also improved the sensitivity of adipose tissue to insulin, decreased lipolysis, and subsequently improved glucose metabolism. Glutamine may reduce cortisol synthesis in the adrenal cortex due to decreased activity of key enzymes or possibly NADPH access. In addition to, glucocorticoids such as cortisol reduce the activity of the hypothalamic-pituitary-thyroid (HPT) axis. Long-term stress is associated with decreased HPT activity and decreased metabolic hormones in farm animals in order to reduce metabolic heat production during heat stress (Shaji *et al*. 2017). Similar results of inhibited thyroid hormone concentration level have been reported in various livestock species such as cattle, sheep, and goats were exposed to heat stress (Joy *et al*. 2016). The lower concentrations of T_3 and T_4 observed during the summer season, that may be due to the direct effect of heat stress on the thyroid gland activity as well as due to reduced feed intake to avoid extra metabolic heat load (Banerjee *et al*. 2015). Temperature with its direct effect on TRH and subsequently plasma T_4 and its indirect effect on decreased appetite can lower thyroid hormone levels.

Thyroid hormones, T_3 and T_4 play an important role in metabolic compatibility and animal growth function. Thyroid hormone stimulates lipolysis and lipogenesis.

Table 3 Concentration of cortisol and thyroid hormones in blood serum of fattened lambs by adding glutamine and different protein levels

Items	Experimental treatments				SEM	P-value			
	C	G	P	GP		Treat	Protein	Glutamine	Protein × glutamine
Cortisol, µg/dL									
Day 15	3.10^{cb}	1.93^{c}	6.06^{a}	4.00^{b}	0.208	0.0007	0.0003	0.0048	0.3131
Day 30	3.03^{ab}	3.00^{ab}	4.20^{a}	2.06^{b}	0.191	0.0271	0.7676	0.0218	0.0250
Day 45	4.60^{a}	3.83^{ab}	5.20^{a}	2.33^{b}	0.199	0.0049	0.2916	0.0019	0.0300
T_3, µmol/L									
Day 15	1.83	2.06	1.63	1.76	0.051	0.0838	0.0390	0.1082	0.6351
Day 30	2.13^{ab}	2.76^{a}	1.83^{b}	2.36^{ab}	0.080	0.0197	0.0622	0.0069	0.7649
Day 45	1.96	2.53	1.86	2.46	0.084	0.0505	0.6355	0.0087	0.9239
T_4, µmol/L									
Day 15	6.70	7.43	6.50	6.86	0.116	0.0988	0. 1399	0.0466	0.4558
Day 30	7.06^{b}	8.36^{a}	6.80^{b}	7.73^{ab}	0.120	0.0070	0.0974	0.0016	0.4665
Day 45	6.30^{b}	7.13^{a}	6.16^{b}	6.86^{ab}	0.107	0.0375	0.3775	0.0072	0.7635
T_4 / T_3									
Day 15	3.67	3.74	3.99	3.91	0.086	0.5453	0. 1853	0.9774	0.6732
Day 30	3.39	3.04	3.74	3.01	0.148	0.3251	0.6113	0.1040	0.5393
Day 45	3.26	2.86	3.33	2.78	0.134	0.4198	0.9760	0.1169	0.7870

C: basal diet; G: basal diet supplemented with glutamine at the rate of 0.2 g/kg of body weight; CP: basal diet with 10% higher protein and GP: basal diet with 10% higher protein and glutamine.
T_3: triiodothyronine and T_4: thyroxine.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

The fatty acids from lipolysis caused by thyroid hormone increase thermogenesis (Mullur *et al*. 2014). Several studies have demonstrated the direct regulation of hormone secretion by glutamic acid in primary anterior pituitary cells (Aizawa *et al*. 2012). It has been shown that there is a closely relationship between glutamate and γ-aminobutyric acid (GABA) neurotransmitter cycles and thyroid hormone metabolism (Ou *et al*. 2019). Glutamate and GABA, the most predominant excitatory and inhibitory neurotransmitters in the central nervous system (CNS), respectively, play a critical role in maintaining the normal movement performance. It is well known that glutamine is a precursor to glutamate and GABA (Cichosz and Czeczot, 2013). In addition, interruption of the serum Glu/Gln-GABA cycle was observed in Mn-exposed rats, as well as thyroid hormone disorder in the serum via increasing serum glutamate levels and decreasing glutamine, GABA and T_4 and T_3 serum levels (Ou *et al*. 2019). The above data show that the Glu/Gln-GABA cycle plays a potential role in modulating the balance of the thyroid hormone. Therefore, the role of glutamine in the regulation of thyroid hormones in accordance with the results of the present experiment seems reasonable.

Nitric oxide (NO) and antioxidant enzymes

The levels of NO were significantly lower in the groups G and GP (with glutamine) than groups C and P (P<0.05). Glutamine increased levels of SOD, GPx and TAS, but reduced the NO concentration. Higher protein levels significantly increased NO concentration on day 15, and decreased SOD and GPx on the 30th day and TAS concentration on the 15th and 45th day of fattening. But the effect of protein levels on the SOD values on days 15th and 45th was not significant. The interaction of glutamine-protein levels significantly decreased NO and increased GPx and TAS concentration at the end of fattening (Table 4). In the second week of the experiment, the highest levels of GPx were observed in treatments with glutamine, but on the 30th day, the levels of GPx increased with both of increasing protein levels and glutamine.

Numerous studies showed that heat stress induced oxidative stress can cause DNA damage, cell apoptosis, and inflammation (Nisar *et al*. 2013; Cong *et al*. 2017). The SOD is the first line of defense against the ROS and is active in catalyzing detoxification of superoxide radical (Gonzales *et al*. 1984). The hydrogen peroxide generated in this reaction is restored to water in the presence of CAT and GPX. The higher concentration of the SOD and GPx observed during summer season may be due to the heat stress conditions (Chaudhary *et al*. 2015).

The drop in SOD activity could be explained by the superoxide anion dismutation to hydrogen peroxide caused by the overproduction of the superoxide anion linked to oxidative stress. Depression of the protective capability against oxidative stress by SOD may lead to greater tissue damage and initiate a vicious cycle by increasing free radical production, thereby exceeding the antioxidant liver capacity and resulting in further oxidative damage (Deger *et al*. 2008; Nazari *et al*. 2019). Glucose is the chief source for the existence and generation of ROS and endocytosis for neutrophils.

Table 4 Concentration of nitric oxide and antioxidant enzymes in blood of fattened lambs by adding glutamine and different protein levels

Items	Experimental treatments				SEM	P-value			
	C	G	P	GP		Treat	Protein	Glutamine	Protein × glutamine
NO, µmol/L									
Day 15	7.77^{ab}	6.39^{b}	8.40^{a}	7.52^{ab}	0.170	0.0185	0.0328	0.0105	0.4778
Day 30	10.63	8.17	10.28	8.21	0.396	0.1112	0.8466	0.0211	0.8087
Day 45	9.79^{ab}	8.49^{bc}	10.89a	6.97^{c}	0.230	0.0017	0.6705	0.0005	0.0216
SOD, u/gHb									
Day 15	1111.70^{b}	1315.00^{ab}	900.00^{b}	1555.00^{a}	47.791	0.0070	0.8858	0.0020	0.0458
Day 30	1408.30	1418.30	1078.30	1265.00	47.346	0.1071	0.0341	0.3294	0.3782
Day 45	935.00^{b}	1281.70^{ab}	1003.30^{b}	1625.00^{a}	57.043	0.0101	0.1089	0.0028	0.2626
GPX, u/gHb									
Day 15	42.67^{b}	59.33^{a}	44.00^{b}	55.33^{a}	0.957	0.0006	0.5060	< 0.0001	0.2012
Day 30	62.67^{b}	62.67^{b}	65.00^{b}	80.67^{a}	1.010	0.0006	0.0010	0.0047	0.0047
Day 45	54.33^{c}	77.00^{a}	60.33^{bc}	65.67^{b}	1.108	0.0005	0.2635	0.0002	0.0045
TAS, mmol/L									
Day 15	0.19^{b}	0.43^{a}	0.37^{a}	0.36^{a}	0.012	0.0006	0.0519	0.0012	0.0010
Day 30	0.25	0.32	0.24	0.27	0.011	0.2187	0.3557	0.0856	0.4252
Day 45	0.20^{b}	0.23^{ab}	0.24^{ab}	0.27^{a}	0.005	0.0172	0.0105	0.0254	0.6742

C: basal diet; G: basal diet supplemented with glutamine at the rate of 0.2 g/kg of body weight; CP: basal diet with 10% higher protein and GP: basal diet with 10% higher protein and glutamine.
NO: nitric oxide; SOD: superoxide dismutase; GPx: glutathione peroxidase and TAS: total antioxidant status.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

However, as an energy metabolite, glucose is not the only source of energy for these cells. Interestingly, neutrophils utilize more glutamine than other leukocytes such as lymphocytes and macrophages (Pithon-Curi *et al.* 2004). In neutrophils, most of the glutamine is converted into aspartate, glutamate, and lactate through the Krebs cycle. Glutamine and glutamate are essential for the proper function of leukocytes and the production of vital compounds such as glutathione (GSH) and its metabolism under optimal conditions. Neutrophils use structural proteins which have chromatin (uncondensed) and antimicrobial factors known as neutrophilic extracellular traps (NETs). NETs need the formation of ROS, enzyme production (myeloperoxidase and elastase) and other compounds capable of killing extracellular bacteria and overriding the virulence factors (Branzk *et al*. 2014). The mechanism requires ROS to turn on the initiation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 complex. Due to the role of glutamine for the synthesis of malate, the malic enzyme is used for the production of a higher quantity of NADPH; meanwhile, NADPH is essential for the synthesis of superoxide anion (O2−), which represents action against the microbes. Likewise, glutamine is also used by the macrophages for arginine and consequently, the synthesis of nitric oxide via the activity of inducible NO synthase enzyme (iNOS) through utilizing NADPH as a source of energy. In the neutrophil, glutamine enhances the production of superoxide by the activity of NADPH oxidase. Numerous research studies have shown that the metabolism of glutamine plays an important role in lymphocyte activation.

To accomplish the quick activity of proliferation below a certain amount, lymphocytes change from oxidative phosphorylation to glycolysis (aerobic) and glutaminolysis, and thus, noticeably upsurge glutamine and glucose consumption. Different metabolites of the krebs cycle, which are produced during the metabolism of glutamine and glucose, such as citrate, fumarate, and succinate, take part in inflammation control and immunity in both adaptive and innate immunity (Mills *et al*. 2017).

Metabolic changes associated with oxidative stress, inflammation, and infection can alter the metabolism of amino acids and proteins, thereby altering the pattern of need for them. Studies have shown that in such conditions, glutamine is one of the most important amino acids (Tanha *et al.* 2011). It has been shown that sulfhydryl (SH) groups due to the amino acid cysteine and proteins made in the liver, especially albumin, have a very important role in the body's antioxidant defense (Tanha *et al*. 2011). It seems that increasing access to glutamine in the intestine by passing it to prevent fermentation in the rumen can have a protective effect on the oxidation of methionine and phenylalanine in the liver. It is clear that increasing the availability of methionine can increase physiological levels of cysteine and increase the level of sulfhydryl or SH groups in plasma. Glutamine also plays a key role in the structure of glutathione peroxidase, which is an important component of the antioxidant system. Glutathione is mainly made de novo in the liver from glutamate, glycine and cysteine. The liver has a unique ability to convert methionine to cysteine, and reducing its function can disrupt glutathione production.

Glutamine as a precursor of glutamate and by preventing the oxidation of methionine can have positive effects on increasing cysteine. The general conclusion from the above statements can be that increasing the availability of glutamine to the animal can increase glutathione production in various ways. Due to the role of glutamine in the production of glutathione, increasing its consumption can increase plasma glutathione peroxidase activity and increase total plasma antioxidant capacity and reduce oxidative stress due to increased plasma antioxidant capacity.

Non-esterified fatty acids, AST and ALT

Glutamine increased NEFA concentration and decreased AST concentrations without significant effect on ALT concentration. Increased protein levels at the beginning of fattening did not have a significant effect on the concentration of NEFA, AST and ALT, although after that those decreased significantly. The interaction effect of glutamine-protein levels decreased NEFA and ALT concentrations but it did not significant effect on AST concentrations. The highest concentration of NEFA was determined at group G (basal protein with glutamine) (Table 5). The NEFA concentrations were significantly lower in groups C and P (glutamine-free) and group GP (higher protein with glutamine), respectively. The enzymes determined were within the normal range.

The role of metabolic regulators in assessing the physiological response to heat stress through various enzymes involved in metabolic reactions in the blood is very important. Decreased levels of NEFA in heat stress in livestock have led to an increase in glucose fuel as a possible solution to reduce the production of metabolic heat in the body of animals.

There is a direct relationship between serum NEFA and lipolysis severity, as well as serum glucose and gluconeogenesis in ruminants. Stress hormones such as epinephrine and cortisol naturally stimulate lipolysis and increase the concentration of NEFA. But in heat stress, despite significant increases in circulating of cortisol, norepinephrine, and epinephrine levels, catabolic signals that normally stimulate lipolysis and adipose mobilization, and a significant reduction in feed intake, lack of an increase in NEFA is especially surprising, and blood lipid depletion is potentially due to a decrease in adipose tissue mobilization. Because heat stress has been shown to decrease the concentration of NEFA plasma in ruminants; dairy cows sheep and pigs. In a study, the treatments with the high level of metabolizable energy and protein were able significantly to avoid to reduce energy carriers (glucose, cholesterol, triglycerides) and also reduce the level of ketone bodies and NEFA in blood metabolites (Moradi *et al*. 2018).

Thyroid hormones are associated with energy and heat production and are known to stimulate lipolysis and the use of NEFA (Mullur *et al*. 2014). Therefore, reducing thyroid hormones in heat stress is effective in reducing lipolysis. Increased in ALT and AST in hot period is in agreement with the findings of Banerjee *et al*. (2015) and Rathwa *et al*. (2017). The increase in ALT and AST may be due to increase in gluconeogenesis. In addition, in an experiment on cross-dairy cows, Alameen and Abdelatif (2012) showed an increase in AST levels and a decrease in ALT levels during the summer compared to winter. Therefore, alteration in the levels of both AST and ALT are correlated to adaptive potential of livestock to environmental challenges.

Table 5 Biochemical indicators in the blood of fattened lambs by adding glutamine and different protein levels

Items	Experimental treatments				SEM	P-value			
	C	G	P	GP		Treat	Protein	Glutamine	Protein × glutamine
NEFA, mmol/L									
Day 15	0.16	0.15	0.13	0.14	0.008	0.6754	0.3310	0.7849	0.5287
Day 30	0.18^{b}	0.29^{a}	0.17^{b}	0.21^{ab}	0.010	0.0130	0.0695	0.0060	0.1457
Day 45	0.14^{b}	0.22^{a}	0.14^{b}	0.15^{b}	0.005	0.0039	0.0265	0.0048	0.0171
AST, U/L									
Day 15	88.00	79.33	86.66	81.00	1.114	0.0656	0.9423	0.0124	0.5201
Day 30	106. 33^{a}	105.00a	97.66^{ab}	87.33^{b}	1.155	0.0014	0.0005	0.0355	0.0872
Day 45	101.33	102.00	99.00	95.66	3.022	0.8779	0.4949	0.8314	0.7498
ALT, U/L									
Day 15	12.66	15.00	16.00	13.33	0.449	0.1026	0.3803	0.8573	0.0237
Day 30	19.00^{ab}	21.33^{a}	18.66^{ab}	15.00^{b}	0.471	0.0096	0.0077	0.4996	0.0130
Day 45	20.00^{a}	23.00^{a}	14.00^{b}	13.00^{b}	0.456	0.0001	<.0001	0.3052	0.0598

C: basal diet; G: basal diet supplemented with glutamine at the rate of 0.2 g/kg of body weight; CP: basal diet with 10% higher protein and GP: basal diet with 10% higher protein and glutamine.
NEFA: non-esterified fatty acids; AST: aspartate transaminase and ALT: alanine transaminase.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Nemati *et al*. (2018) demonstrated that glutamine supplementation decreased the NEFA and AST concentrations. In other study, glutamine treatment had no effect on plasma glucose and NEFA concentrations but did tend to increase plasma urea N concentration (Cruzat *et al*. 2018). In the present study, NEFA levels increased in glutamine supplementation treatment. It seems that the increase in NEFA levels in this study is related to the increase in thyroid hormones in the treatment glutamine. Thyroid hormones have been reported to be associated with energy and heat production and have been shown to stimulate lipolysis and the use of NEFA (Mullur *et al*. 2014). Therefore, an increase in thyroid hormones in glutamine treatment is effective in increasing lipolysis. In addition, serum AST concentration reduced significantly with glutamine supplementation that can confirm the better status of liver cells.

CONCLUSION

The results showed that higher protein levels not have a significant effect on cortisol, T_3 and T_4 hormones but it decreased NEFA, AST and ALT and increased TAS concentrations. Glutamine decreased levels of cortisol and NO but it increased SOD, GPx, TAS, NEFA, T_3 and T_4 concentrations. Glutamine also decreased AST concentrations without significant effect on ALT concentration. The interaction of glutamine and higher protein levels on AST, TAS, NEFA, T_3 and T_4 was not significant but they increased GPx and decreased cortisol and ALT concentrations. From the results of the present study, despite to economic aspect, it could be concluded that feeding protected glutamine from ruminal fermentation under heat stress, at a concentration of 0.2 g/kg body weight of fattening lambs, via increasing thyroid hormones, antioxidant enzymes, and lowering cortisol levels improved health status.

ACKNOWLEDGEMENT

The authors thank all the teams who worked on the experiments and provided technical assistance in the laboratory during this study.

REFERENCES

Adams F. and Ohene-Yankyera K. (2014). Socio-economic characteristics of subsistent small ruminant farmers in three regions of Northern Ghana. *Asian J. Appl. Sci. Eng*. **3,** 93-106.

Afsal A., Sejian V., Bagath M., Krishnan G., Devaraj C. and Bhatta R. (2018). Heat stress and livestock adaptation: Neuro-endocrine regulation. *Int. J. Vet. Anim. Med*. **1(2),** 108-119.

Aizawa S., Sakai T. and Sakata I. (2012). Glutamine and glutamic acid enhance thyroid-stimulating hormone *b* subunit mRNA expression in the rat pars tuberalis. *J. Endocrinol*. **212,** 383-394

Alameen A.O. and Abdelatif A.M. (2012). Endocrine responses of crossbred dairy cows in relation to pregnancy and season under tropical conditions. *American Eurasian J. Agric. Environ. Sci.* **12,** 1065-1074.

Banerjee D., Upadhyay R.C., Chaudhary U.B., Kumar R., Singh S., Das A.T.K. and De S. (2015). Seasonal variations in physio-biochemical profiles of Indian goats in the paradigm of hot and cold climate. *Biol. Rhythm Res.* **46,** 221-236.

Branzk N., Lubojemska A., Hardison S.E., Wang Q., Gutierrez M.G., Brown G.D. and Papayannopoulos V. (2014). Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat. Immunol*. **15,** 1017-1025.

Chaudhary S.S., Singh V.K., Upadhyay R.C., Puri G., Odedara A.B. and Patel P.A. (2015). Evaluation of physiological and biochemical responses in different seasons in Surti buffaloes. *Vet. Worl.* **8(6),** 727-731.

Cichosz G. and Czeczot H. (2013). Controversions around diet proteins. *Polish Med. J*. **35(210),** 397-401

Cong X., Zhang Q., Li H., Jiang Z., Cao R., Gao S. and Tian W. (2017). Puerarin ameliorates heat stress–induced oxidative damage and apoptosis in bovine Sertoli cells by suppressing ROS production and upregulating Hsp72 expression. *J. Theriogenol*. **88,** 215-227.

Cortas N. and Wakid N. (1990). Determination of inorganic nitrate in serum and urine by a kinetic cadmium-reduction method. *Clin. Chem*. **36(8),** 1440-1443.

Cruzat V., Macedo Rogero M., Noel Keane K., Curi R. and Newsholme P. (2018). Glutamine: Metabolism and immune function, supplementation and clinical translation. *Nutrients*. **10,** 1564.

Deger Y., Ertekin A., Deger S. and Mert H. (2008). Lipid peroxidation and antioxidant potential of sheep liver infected naturally with distomatosis. *Turkiye Parazitol. Derg.* **32(1),** 23-26.

Fekete C. and Lechan R.M. (2013). Central regulation of hypothalamic-pituitary-thyroid axis under physiological and pathophysiological conditions. *Endocr Rev*. **35,** 159-194.

Gonzales R., Auclair C., Voisin E., Gautero H., Dhermy D. and Boivin P. (1984). Superoxide dismutase, catalase and glutathione peroxydase in red blood cells from patients with malignant diseases. *Cancer Res.* **44,** 4137-4139.

Joy A., Pragna P., Archana P.R., Sejian P.R. and Bagath M. (2016). Significance of metabolic response in livestock for adapting to heat stress challenges. *Asian J. Anim. Sci.* **10,** 224-234.

Karami S., Kashef M. and Mehri Alvar Y. (2014). Protective effect of glutamine by the expression of HSP70 and reduction of cortisol on exercise induced stress. *J. Arak Univ. Med. Sci.* **17(91),** 65-73.

McManus C.M., Paludo G.R., Louvandini H., Gugel R., Sasaki L.C.B. and Paiva S.R. (2009). Heat tolerance in Brazilian sheep: physiological and blood parameters. *Trop. Anim. Health Prod*. **41,** 95-101.

Mills E.L., Kelly B. and O'Neill L.A. (2017). Mitochondria are

the powerhouses of immunity. *Nat. Immunol.* **18,** 488-498.

Moradi M., Chashnidel Y., Teimouri Yansari A. and Dirandeh E. (2018). Effects of increasing level of metabolizable energy and protein on feed intake, nutrient digestibility, performance and blood metabolites of Zell pregnant ewes at late gestation. *Res. Anim. Prod.* **9(22),** 60-71.

Mullur R., Liu Y.Y. and Brent G.A. (2014). Thyroid hormone regulation of metabolism. *Physiol. Rev.* **94,** 355-382.

Nazari A., Dirandeh E., Ansari-Pirsaraei Z. and Deldar H. (2019). Antioxidant levels, copper and zinc concentrations were associated with postpartum luteal activity, pregnancy loss and pregnancy status in Holstein dairy cows. *Theriogenology*. **15,** 133:97-103.

Nemati M., Menatian S., Joz Ghasemi S., Hooshmandfar R., Taheri M. and Saifi T. (2018). Effect of protected-glutamine supplementation on performance, milk composition and some blood metabolites in fresh Holstein cows. *Iranian J. Vet. Res.* **19(3),** 225-228.

Nisar A., Sultana M. and Ashraf H. (2013). Oxidative stress-threat to animal health and production. *Int. J. Livest. Res*. **3,** 76-83.

Niyas P.A.A., Chaidanya K., Shaji S., Sejian V. and Bhatta R. (2015). Adaptation of livestock to environmental challenges. *J. Vet. Sci. Med. Diagn*. **4,** 3-13.

Okoruwa M.I. (2014). Effect of heat stress on thermoregulatory, live bodyweight and physiological responses of dwarf goats in southern Nigeria. *European Sci. J.* **10,** 255-264.

Ou C.Y., He Y.H., Sun Y., Yang L., Shi W.X. and Li S. (2019). Effects of sub-acute manganese exposure on thyroid hormone and glutamine (Gln)/glutamate (Glu)-γaminobutyric acid (GABA) cycle in serum of rats. *Int. J. Environ. Res. Public Health*. **16(12),** 2157-2168.

Pithon-Curi T.C., De Melo M.P. and Curi R. (2004). Glucose and glutamine utilization by rat lymphocytes, monocytes and neutrophils in culture: A comparative study. *Cell Biochem*. Funct. **22,** 321-326.

Rathwa S.D., Vasava A.A., Pathan M.M., Madhira S.P., Patel Y.G. and Pande A.M. (2017). Effect of season on physiological, biochemical, hormonal, and oxidative stress parameters of indigenous sheep. *Vet. World*. **10(6),** 650-654.

SAS Institute. (2004). SAS®/STAT Software, Release 9.4. SAS Institute, Inc., Cary, NC. USA.

Sejian V., Maurya V.P. and Naqvi S.M. (2010). Adaptive capability as indicated by endocrine and biochemical responses of Malpura ewes subjected to combined stresses (thermal and nutritional) in a semi-arid tropical environment. *Int. J. Biometeorol*. **54(6),** 653-661.

Shaji S., Sejian V., Bagath M., Manjunathareddy G.B., Kurien E.K., Varma G. and Bhatta R. (2017). Summer season related heat and nutritional stresses on the adaptive capability of goats based on blood biochemical response and hepatic *HSP70* gene expression. *Biol. Rhythm Res*. **48,** 65-83.

Stachowicz M. and Lebiedzinska A. (2016). The effect of diet components on the level of cortisol. *European Food Res. Technol*. **242,** 2001-2009.

Tanha T., Amanlou H., Chamani M., Ebrahimnezhad Y., Salamatdost R., Maheri N. and Fathi M. (2011). Effect of glutamine enhancement on oxidative stress and reproduction in Holstein dairy cows during transition period. *J. Anim. Vet. Adv.* **10(21),** 2838-2845.