Effect of Late Gestational Betaine Supplementation on Intermediate Metabolites, Homocysteine and Lipid Peroxidation in Pregnant Ewes and Their Offspring

H.R. Sahraei¹, A. Kiani¹*, A. Azarfar¹ and H. Khamisabadi²

ABSTRACT

Betaine (trimethylglycine) is a methyl group donor involved in important physiological processes including homocysteine synthesis, alleviating the oxidative stress, and reducing the lipid peroxidation. In the present study, the effect of dietary betaine supplementation on blood concentrations of beta-hydroxybutyrate (BHB), homocysteine, glutathione peroxidase (GPx), catalase (CAT), and malondialdehyde (MDA) and serum concentrations of glucose, urea, total protein in pregnant ewes were evaluated. Furthermore, the influence of prepartum supplementation of ewes with betaine on serum concentrations of glucose, urea, and insulin in their lambs was investigated. During the last month of gestation, 20 multiparous pregnant Sanjabi ewes were fed either a basal diet (control: 71.2±3.6 kg BW) or a basal diet supplemented with 5 g betaine hydrochloride per day per head (betaine: 71.6±3.8 kg BW). Blood samples were taken from ewes at parturition, and from lambs at birth, 14 and 28 days of age. Betaine supplemented ewes had lower BHB (0.55±0.18 vs. 1.88±0.37 mmol/L) and MDA (8.1±0.51 vs. 9.1±0.61 µmol/L) than the control ewes, while blood concentrations of homocysteine, antioxidant enzymes, glucose, urea, and total protein remained unchanged. Lambs of betaine ewes tended to be heavier at birth compared to those born from control ewes (4.41±0.18 vs. 3.95±0.18 kg; P=0.06). However, all lambs had similar growth performance until day 60 of age. Late gestational betaine supplementation did not affect circulating glucose, insulin, and urea in lambs. In conclusion, betaine supplementation reduced circulating BHB most likely via suppressing lipid oxidation in pregnant ewes leading to greater birth weight of lambs.

KEY WORDS betaine, blood metabolites, homocysteine, sheep.

INTRODUCTION

Betaine or trimethylglycine is a methyl donor compound which is involved in many physiological processes and plays important roles in glycine and glutathione synthesis (Ueland et al. 2005). Betaine is involved in the reaction of homocysteine to methionine conversion (Ueland et al. 2005). Betaine as a methyl donor provides the labile methyl groups for the synthesis of several metabolically active substances including glutathione which provides a protection mechanism against the oxidative stress (Lu, 2009). During the pregnancy, high quantities of reactive oxygen species generate which have detrimental effects on both mother and fetus which may retard the fetal growth (Mutinati et al. 2013). Betaine as an antioxidant (Zhang et al. 2015) could prevent lipid peroxidation by sustaining the glutathione level in the body (Haddad, 2002). Betaine through its relation with choline might affect the growth and development...

Betaine originates either from choline oxidation or from dietary sources (Eklund et al. 2005). As a product of choline oxidation, betaine is involved in transmethylation reactions in the body. Dietary supplementation with betaine may decrease the requirements for the other methyl donors such as methionine and choline leading to an elevation in circulating methionine (Eklund et al. 2005). There is also some suggestion for enhanced methionine availability after dietary supplementation of betaine in small ruminants (Puchala et al. 1995).

Alterations in the distribution pattern of protein and fat in the body have been reported following the betaine supplementation in farm animals (Eklund et al. 2005). The potential effects of betaine supplementation on animal performance have been studied (Puchala et al. 1995; Eklund et al. 2005). In a study, dietary supplementation with betaine reduced the thickness of subcutaneous fat in growing lambs (Fernández et al. 1998).

In the present study, we hypothesized that dietary supplementation of pregnant ewes with betaine in late pregnancy can improve antioxidant defense, reduce lipid peroxidation, and subsequently might affect pre- and postnatal growth of their offspring.

We also hypothesized that betaine supplementation of ewes in late pregnancy changes the circulating homocysteine in them.

The objective of the present study was to investigate betaine effects on blood concentrations of beta-hydroxybutyrate (BHB), homocysteine, antioxidant enzymes, malondialdehyde, and the body measurements of their lambs at birth.

MATERIALS AND METHODS

The current study was carried out at the experimental farm of at Kermanshah Agricultural and Natural Resources Research and Training Center (Kermanshah, Iran), and all procedures were approved by the Committee of Animal Ethics and Rights in Lorestan University (ET-no: 4013).

Animals, managements, and treatments

Twenty multiparous Sanjabi ewes that were singleton pregnant ewes were used in the present study. In mating time (September 2016), the ewes were estrus synchronized using 20 mg intravaginal fluorogestone acetate sponges (Pharmpect, Australia) for 14 days with an intramuscular injection of 400 IU of pregnant mare's serum gonadotropin right after removing the sponges. The ewes were naturally mated with rams. The pregnancy was diagnosed with an ultrasonography device (Medison Sonovet 600, USA). The ewes were kept individually in boxes (1.5×1.5) during the last four weeks of pre-partum. Half of the ewes were fed 5 g betaine hydrochloride per day per head (Betaine; n=10, BW: 71.6±3.8 kg), and the rest were fed without betaine (control; n=10, BW: 71.2±3.6 kg). The ingredient and chemical composition of the basal diet is presented in Table 1. The amount of 5 g betaine was chosen according to other experiments which have used betaine in ruminant nutrition (Fernández et al. 2004; Peterson et al. 2012; DiGiacomo et al. 2016; Hall et al. 2016). Betaine used in this experiment were as betaine hydrochloride (CAS: 590-46-5, Weifang Sunwin Chemicals Co., Ltd. Shandong, China). Ewes were fed in two equal meals at 08:30 and 16:00 h, first, the concentrate and then the forage portion of the daily ration was offered.

Table 1. Ingredients and chemical composition of the basal diet

<table>
<thead>
<tr>
<th>Ingredients (g/kg as-fed)</th>
<th>Alfalfa hay</th>
<th>620</th>
<th>Corn silage</th>
<th>343</th>
<th>Barley grain</th>
<th>27</th>
<th>Common Salt</th>
<th>7.5</th>
<th>Mineral salt</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical composition</td>
<td>DE* (Mcal/kg DM)</td>
<td>2.34</td>
<td>ME* (Mcal/kg DM)</td>
<td>1.88</td>
<td>Crude protein (%)</td>
<td>10.6</td>
<td>ADFom (%)</td>
<td>38.0</td>
<td>aNDF (%)</td>
<td>54.4</td>
</tr>
</tbody>
</table>

* Values are calculated according to NRC equations (NRC, 2007).
DE: digestible energy; ME: metabolizable energy; ADFom: acid detergent fiber and aNDF: neutral detergent fiber.
Betaine was mixed with 30 g of milled barley grain and then added to the concentrate portion of the daily ration. Feed intake and orts (if any) were recorded daily. There was no leftover of concentrate portion of the diet. The ewes had free access to the drinking water and minerals supplements. The body condition score of ewes was determined at parturition.

**Chemical analyses**

Feed ingredients were dried at 55 °C for 72 h, grounded (1 mm screen using Cyclotech Mill, Tecator, Sweden) and chemically analyzed using the standard methods of AOAC (1990) for DM (100 °C in air-forced oven for 24 h; method 967.03), crude ash (550 °C in ashing furnace for 6 h; method 942.05), CP (Kjeldahl procedure; method 976.06), EE (method 920.29) and Acid detergent fiber (ADFom, method 973.18). Neutral detergent fiber (aNDFom) was determined, according to Van Soest et al. (1991), with the addition of heat-stable α-amylase and sodium sulfite and the results were calculated without residual ash. The digestible energy and metabolizable energy of the diet were estimated after calculating these values for each ingredient using the NRC (2007).

**Lambing and growing period**

After lambing, lambs were placed next to their mothers. Birth weight, wither height, body length (from crown to rump), and rectal temperature of lambs was recorded at the birth. Thereafter, bodyweight of lambs was recorded weekly until the age of eight weeks.

**Blood sampling and assays**

Blood samples were taken from the jugular vein at the day of parturition. The blood was collected in tubes with (EDTA) and without an anticoagulant agent. The tube without anticoagulant agent remained at room temperature (25 °C) until complete coagulation. Thereafter, the tube was centrifuged at 1800 g for 10 minutes and resulted serum was stored at -20 °C pending analyses. Serum concentration of glucose (Cork, Ireland), urea (Cork, Ireland), total protein (Cork, Ireland), insulin (Saluggia, Italy), beta-hydroxybutyrate (East, Biofarm, Hangzhou, China), and homocysteine (Diazyme, Hanover, Germany) were determined by commercial kits in accordance with the manufacturer guidelines.

The EDTA tubes containing whole blood were cooled immediately on ice and used for determination of glutathione peroxidase (GPx), catalase (CAT), and malondialdehyde (MDA) levels. Lipid peroxidation products were quantified by the thiobarbituric acid (TBA) method (Placer et al. 1966). GSHPx activity was measured according to the method of Paglia and Valentine (1967) using commercial kit (Cat. NO, RS 505, Ransel, Randox, UK). Catalase activity was determined as explained previously in detail by Alirezaei et al. (2011).

Lambs were also blood sampled at 1, 14 and 28 days of age. The blood was collected in tubes containing anticoagulant (EDTA). The tubes were cooled on ice and centrifuged at 1800 g for 15 minutes. The resulted plasma was analyzed for concentration of glucose (Cork, Ireland), urea (Cork, Ireland), and insulin (Saluggia, Italy).

**Statistical analysis**

Normality of residuals was tested using the Shapiro-Wilks test. Not-repeated data were analyzed using a one-way ANOVA test and repeated measurements of serum metabolites in lambs were analyzed using MIXED procedure of SAS (2004). The model included the effects of treatment, blood sampling time as the fixed effects, and animal as the random effect. The interaction effect between treatment and sampling time included in the model if significant (P<0.05), otherwise it was excluded from the model. Based on likelihood ratio test, the covariance structure of the repeated measurements was modeled as compound symmetry (Littell et al. 2000). Relative standard division (RSD) is presented unless otherwise mentioned.

**RESULTS AND DISCUSSION**

Body weight of ewes at parturition did not differ between Betaine (73.6±3.6 kg BW) and control ewes (72.5±3.9 kg BW). Body condition score of ewes on the day of parturition was 3.68 and 3.63 for betaine and control ewes respectively. Ewes in Betaine had lower (P<0.05) circulating BHB and MDA than those in control group (Table 2). Betaine supplementation had no effect on homocysteine, glutathione peroxidase and catalase concentrations in ewes (Table 2).

In total twenty lambs were born (betaine: 6 male and 4 female; control: 6 male and 4 female). Lambs in betaine tended to be heavier at the birth (4.41 vs. 3.95 kg, P=0.06). Lambs in Betaine group tended to have greater shoulder height than control lambs (P=0.06) (Table 3). The average daily gain of lambs from birth until 56 days of age was 221 ±18 and 224 ±23 g for control and betaine respectively. Body weight of lambs was significantly different at 56 days of age (Figure 1). However, from birth until 49 days of age, the daily growth rates of betaine-fed lambs were similar to those of control.

In the present study, due to the low number of lambs, the gender effect was not significant. Therefore, pooled data from both sexes were used for statistical analysis (Tables 3 and 4). Betaine supplementation had no significant effect on the measured blood metabolites in lambs (Table 4).
Table 2: Effect of dietary betaine supplementation on concentration of β-hydroxybutyrate (BHB), homocysteine, and antioxidant enzymes in ewes at parturition (Means±Standard deviation)

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>Betaine</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>3.3±0.26</td>
<td>3.0±0.29</td>
<td>0.44</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>67±2.1</td>
<td>73±2.4</td>
<td>0.09</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>9.3±1.13</td>
<td>7.4±1.24</td>
<td>0.28</td>
</tr>
<tr>
<td>BHB (mmol/L)</td>
<td>1.9±0.37</td>
<td>0.55±0.18</td>
<td>0.02</td>
</tr>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>7.88±0.14</td>
<td>7.95±0.17</td>
<td>0.75</td>
</tr>
<tr>
<td>Malondialdehyde (µmol/L)</td>
<td>9.1±0.61</td>
<td>8.1±0.51</td>
<td>0.03</td>
</tr>
<tr>
<td>Glutathione peroxidase (U/mL)</td>
<td>1.86±0.26</td>
<td>1.74±0.22</td>
<td>0.74</td>
</tr>
<tr>
<td>Catalase (U/mL)</td>
<td>514±69</td>
<td>574±59</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Table 3: Effect of late gestational betaine supplementation in ewes on birth weight and morphologic traits of new-born lambs

<table>
<thead>
<tr>
<th>Treatment (TRT)</th>
<th>Gender</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Item</td>
<td>Control</td>
<td>Betaine</td>
</tr>
<tr>
<td>Number of lambs</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>3.9±0.2</td>
<td>4.4±0.2</td>
</tr>
<tr>
<td>Shoulder height (cm)</td>
<td>39.1±0.6</td>
<td>40.6±0.5</td>
</tr>
<tr>
<td>Body length (cm)</td>
<td>38.7±0.8</td>
<td>40.1±0.7</td>
</tr>
<tr>
<td>Rectal temperature (˚C)</td>
<td>39.2±0.1</td>
<td>39.3±0.1</td>
</tr>
</tbody>
</table>

SEM: standard error of the means.

Figure 1: Body weight (kg) of suckling lamb born to dams that were fed either without (control: ■) or with 5 g betaine per day per head (betaine: □) during late gestation

Table 4: Effect of late gestational betaine supplementation on circulating concentrations of glucose, insulin, and urea in suckling lambs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose (mmol/L)</th>
<th>Insulin (µmol/L)</th>
<th>Urea (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.71±0.31</td>
<td>0.37±0.08</td>
<td>14.8±0.59</td>
</tr>
<tr>
<td>Betaine</td>
<td>6.43±0.25</td>
<td>0.42±0.05</td>
<td>14.0±0.76</td>
</tr>
</tbody>
</table>

Time of blood sampling

<table>
<thead>
<tr>
<th>Day</th>
<th>Glucose (mmol/L)</th>
<th>Insulin (µmol/L)</th>
<th>Urea (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.95±0.38</td>
<td>0.64±0.1</td>
<td>16.2±0.99</td>
</tr>
<tr>
<td>14</td>
<td>7.31±0.33</td>
<td>0.41±0.08</td>
<td>13.1±0.86</td>
</tr>
<tr>
<td>28</td>
<td>5.82±0.36</td>
<td>0.12±0.06</td>
<td>13.8±0.96</td>
</tr>
</tbody>
</table>

P-value

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose</th>
<th>Insulin</th>
<th>Urea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.51</td>
<td>0.62</td>
<td>0.43</td>
</tr>
<tr>
<td>Time</td>
<td>0.03</td>
<td>&lt;0.01</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Glucose concentration on day 14 of age was higher than on days 1 and 28 days of age. The serum concentration of insulin decreased significantly from birth (0.64±0.1 ng/ml) to 28 days of age (0.12±0.06 ng/mL; Table 4).

In the present study, betaine supplementation reduced the circulating BHB in pregnant ewes. Beta-hydroxybutyrate is a product of incomplete oxidation of non-esterified fatty acids (NEFA) via ketogenesis process in the liver (Drackley et al. 1991).

Circulating BHB in ruminant animals may also originate from the conversion of butyrate to BHB in the ruminal epithelium and liver upon the absorption (Emmanuel, 1980). The liver extracts NEFA from the blood in proportion to the hepatic supply (Alison, 1986), and it is directly related to rate of NEFA mobilization from adipose tissue depots.

Increasing plasma concentration of NEFA is associated with a proportional increase in hepatic NEFA uptake as well as increased hepatic formation of ketone bodies (Drackley et al. 1991; Andersen et al. 2002). In pregnant ewes, plasma BHB is expected to be high due to mobilization of body fat reservoirs. It is known that betaine plays a role in the metabolism of fats by affecting the expression of genes associated with the absorption and transporting fatty acids into cells (Li et al. 2017). Betaine supplementation increases fatty acid uptake and oxidation in muscles in pigs (Li et al. 2017). Less circulating NEFA and less BHB in betaine supplemented ewes in our study might indicate that betaine may prevent ketogenesis and alleviate pregnancy toxemia in pregnant ewes.

In the present study, late gestational betaine supplementation reduced MDA. Malondialdehyde is an end-product of lipid peroxidation (Halliwell and Chirico, 1993). In general, during pregnancy, both mothers and fetuses are exposed to oxidative stress (Garrel et al. 2010) which increases the level of free radicals in them (Mutinati et al. 2013). Therefore, the activity of many antioxidant enzymes especially glutathione peroxidase increases during the course of pregnancy (Aurousseau et al. 2006). Free oxygen-induced radicals include superoxide, hydrogen peroxide, and hydroxyl that is produced during the oxidative metabolism during pregnancy (Mutinati et al. 2013). Glutathione peroxidase, superoxide dismutase, and catalase are part of the antioxidant defense system which protects tissues against oxidative stress (Sies, 1997). The proper antioxidant level during pre- and post-partum is essential for reducing fetal death and improving the survival of the newborns (Mutinati et al. 2013). Lipid peroxidation is a highly destructive phenomenon which occurs when reactive oxygen species (ROS) and free radicals attack the double bonds of polyunsaturated fatty acids in biological membranes (Halliwell and Chirico, 1993).

The protective effects of betaine against the detrimental effects of free radicals may be attributed to its scavenging actions (Zhang et al. 2015). It has been reported that betaine increased the hepatic S-adenosine methionine (SAM) level and lowered the accumulation of hepatic triglycerides (Barak et al. 1996). Betaine reduced fat accumulation in rat liver (Deminice et al. 2015a), and caused changes in fat metabolism in the single stomach animals (Eklund et al. 2005). Contrary to our expectation, betaine had no effect on blood concentration of homocysteine in the pregnant ewes. It has shown that the betaine supplementation in rat increased SAM production and an increase in the level of SAM, and decreases the activity of the betaine homocysteine methyltransferase (Deminice et al. 2015b). Betaine-homocysteine S-methyl transferase (BHMT) converts homocysteine into methionine using betaine (Obied, 2013).

Increasing the amount of SAM decreases the activity of BHMT. However, BHMT is expressed in the early stages embryonic growth (Zhang et al. 2015) and in the fetal livers (Feng et al. 2011). This may justify why betaine supplementation of ewes in the late gestation had no effect on blood concentration of homocysteine in the present study. Late gestational betaine supplementation tended to increase the blood total protein in pregnant ewes. It has been reported that betaine supplementation may decrease the requirements for methionine and choline leading to an elevation in circulating methionine (Eklund et al. 2005). Alterations in the distribution pattern of protein in the body have been also reported following the betaine supplementation in farm animals (Eklund et al. 2005).

Lambs born from Betaine-fed ewes were heavier than those born from control ewes. Alirezaei et al. (2011) showed that adding betaine to the pregnant rat diet increased fetus length (Alirezaei et al. 2011). Recently, a positive association has been reported between placenta concentration of betaine and embryo weight (King et al. 2017), which supports the role of betaine in promoting fetal growth (Zhang et al. 2015). However, the reason behind the relation between betaine and greater birth weight of lambs in betaine-fed ewes remained unclear. It might be due to enhanced transfer of betaine from the placenta to the fetus or / and due to the fact that betaine is a source of nitrogen. Betaine may also reduce osmotic stress (Lever and Slow, 2010), which could have contributed to the observed improvements in placental efficiency. In betaine-fed ewes, oxidation of betaine into dimethylglycine might have improved the embryo growth. Betaine involves in production of glycine for glutathione synthesis and indirectly for embryonic development (Mutinati et al. 2013). Inhibition of lipids peroxidation as reflected by a lower MDA in betaine-fed ewes might also have helped the fetus growth.
CONCLUSION

In conclusion, the betaine supplementation reduced circulating BHB most likely via suppressing lipid oxidation in pregnant ewes leading to greater birth weight of lambs.

ACKNOWLEDGEMENT

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