Increasing metabolisable energy and protein supplementation to stimulate the subsequent milk production during late gestation by increasing proliferation and reducing apoptosis in goat mammary gland prepartum

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Abstract. In total, 32 pregnant goats were assigned randomly to four diets fed from Day 100 of pregnancy to Day 30 after parturition, to determine the effects of metabolisable energy (ME) and metabolisable protein (MP) supplementation levels on feed intake, subsequent colostrum and milk production and expression of genes regulating mammary-cell proliferation and apoptosis. Diets were as follows: (1) diet with ME and MP provided according to NRC recommendations (control), (2) diet with extra 10% ME, (3) diet with extra 10% MP, and (4) diet 1 with 10% extra of both ME and MP. Mammary biopsies were obtained from each udder half 24 h after parturition. Feed intake (g/day), and colostrum (kg/day) and milk (kg/month) production increased when the extra ME and MP were provided together prepartum and in early lactation ($P<0.05$). Relative mRNA expressions significantly increased in the mammary gland of insulin-like growth factor 1 (IGF-1, 4.3-fold), IGF-1 receptor (IGF-1R, 3.6-fold) and B-cell lymphoma 2 (Bcl-2, 4.6-fold), whereas insulin-like growth factor binding protein 3 (IGFBP-3, 3.2-fold), Bcl-2-associated X protein (Bax, 16.7-fold) and the ratio of Bax : Bcl-2 expressions significantly decreased (69.8-fold) with increased ME and MP levels fed in late gestation. In conclusion, colostrum production and milk yield in the early lactation period are sensitive to nutrient supply during gestation, where increased dietary ME as well as MP supplementation levels during late gestation will favour mammary development, by increasing expression of genes stimulating cellular proliferation (IGF-1, IGF-1R, Bcl-2) and reduced those stimulating apoptosis (IGFBP-3, Bax).

Additional keywords: gene expression, lactation, periparturient nutrition, ruminant.

Introduction

Studies conducted over the past decades have shown that milk-production efficiency and milk composition in dairy animals are related to the number and activity of secretory cells in the mammary gland, and the formation of new secretory cells before parturition as well (Safayi et al. 2010). In small ruminants, ~80% of fetal growth occurs during the last 2 months of pregnancy, and this can result in a significant increase in energy and protein requirements of the dam (Bell 1995). There is also a large increase in the requirement of net protein for udder development and colostrum production in the last 2 weeks of gestation (Mellor and Murray 1985). Mellor and Murray (1985) reported that inadequate feed intake during late gestation leads to a reduction in mammary development and milk production in the subsequent lactation. Therefore, there might be a potential for improving energy and protein utilisation in goats by changing their energy requirements and recommended protein intake to better account for the differences in requirements during late gestation, as well as during the lactation period.

While mammogenesis is initiated during embryonic life, the majority of mammary development occurs postnatally, particularly during late gestation when the gland is prepared for lactation (McManaman and Neville 2003). The mammary gland undergoes cyclical periods of growth, differentiation, lactation and regression that are coordinated to provide nutrients for offspring or are driven by strategies to coordinate the events of reproduction and milk production of mammals (Capuco and Ellis 2013). Although the rate of milk composition is influenced by many factors, such as the nutrition of the animal, systemic hormones and internal factors within the gland, ultimately, it is the number and activity of secretory cells that are present in the gland during different time points of lactation that determine the milk yield (Safayi et al. 2010). Thus, formation of secretory tissue during pre-partum is critical for subsequent lactation performance (Knight and Peaker 1984; Capuco et al. 2001; Boutinaud et al. 2004; Rahmani et al. 2017). In adult sheep, mammary development is accomplished essentially through parturition and there is
only limited mammary growth during early lactation (Neville et al. 2002). Such coordinated regulation of developmental and functional events is likely to be mediated by changes in gene expression related to proliferation and apoptosis (Hennighausen and Robinson 2001, 2005; Paten et al. 2015). Understanding the regulation of these two crucial processes in mammary remodelling during the transition from pregnancy to lactation is essential to be able to evaluate to what extent we can intervene in mammary development, e.g. through nutrition. Therefore, the objective of the present study was to address the following questions: (1) can increased provision of dietary metabolisable energy (ME) and metabolisable protein (MP) influence the expression of genes related to proliferation and apoptosis during late gestation to favour mammary gland development in goats; and (2) does dietary-induced alterations in mammary cell remodelling have implications for colostrum and milk production in the subsequent lactation?.

Materials and methods

The experimental procedures followed the Iran Ministry of Agriculture Protocol on Animal Protection and were approved by the ministerial Committee of Animal Experiments (experimental permission no. 1021).

The present study was conducted in 2016, on the herd kept in Sari Agricultural Sciences and Natural Resources University (SANRU) located in the north of Iran (36.33°N, 53.06°E). During the study, the ambient temperature and the relative humidity of the region were ~11–27°C and 52–64% respectively.

Animals and treatments

Sistani multiparous pregnant goats (n = 32) that had the same parity number, were aged 3–4 years old, and had completed the lactation, were used. Two months before the mean expected date of delivery, goats were assigned randomly to four treatment groups balanced on the basis of their initial bodyweight (25.5 ± 1.6 kg; mean ± s.e.m.) and body condition score (2.6 ± 0.50 points). Each of the four groups received their experimental diet from 100 days prepartum to 30 days after parturition, as follows: Group 1 received ME and MP according to NRC recommendations (control), Group 2 received extra 10% of ME, Group 3 received extra 10% of MP and Group 4 received extra 10% of both ME and MP (Table 1). Animals had free access to fresh water and a vitamin-mineral supplement. They were milked manually at 0900 hours and 1530 hours and were fed twice a day at 0730 hours and 1430 hours, half the ration being given at each feeding. The kids were separated from dams after parturition. Colostrum and milk production were recorded for 1 day and 1 month respectively. Milk yield was recorded daily at each milking (morning and afternoon) and then was averaged.

Goats were housed in individual stalls (1.2 m × 0.7 m) in an open shed with a metal roof and natural light, which protected them against wind and rain. Two weeks before parturition, the animals were moved to individual large straw-bedded pens (720 × 440 cm) so as to allow animals to be adapted to the presence of an observer.

Table 1. Ingredients and composition of the rations fed to dairy goats in late gestation and early lactation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ME</th>
<th>MP</th>
<th>MEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolisable energy (Mcal/day)</td>
<td>2.1</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Dry matter (%)</td>
<td>77</td>
<td>78</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Crude protein (%)</td>
<td>9.8</td>
<td>9.8</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>Neutral detergent fibre (%)</td>
<td>42.4</td>
<td>40.4</td>
<td>41.3</td>
<td></td>
</tr>
<tr>
<td>Ash (%)</td>
<td>6.1</td>
<td>6.7</td>
<td>5.6</td>
<td>5.9</td>
</tr>
<tr>
<td>Ether extract (%)</td>
<td>2.4</td>
<td>2.03</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Non-fibre carbohydrate (%)</td>
<td>39.2</td>
<td>42.0</td>
<td>40.7</td>
<td></td>
</tr>
</tbody>
</table>

Materials

Two fine-needle biopsy samples were obtained from both mammary glands of each goat exactly 24 h after parturition, and they were snap-frozen immediately in liquid nitrogen and then stored at −80°C until RNA extraction. Biopsies were taken according to the procedure described in the study by Safayi et al. (2010). Briefly, mammary biopsies were collected from each udder half immediately after the morning milking, so as to minimise the amount of milk in the udder.

After washing with a surgical iodine scrub, the caudal surface of the udder halves were shaved and were disinfected with an iodine–70% ethanol solution. The biopsy site was anaesthetised by injecting ~3 mL of lidocaine 2% (20 mg/mL) to multiple sites subcutaneously and surrounding the biopsy site. Biopsies were obtained from the skin with a semi-automatic 14G biopsy needle.
gun (TSK Laboratory, Tochigi-Ken, Japan). Each sample was contained of ~15–20 mg of tissue. After biopsy sampling, the udder half was milked by hand to check for the presence of blood in milk and to remove any blood clots from mammary ducts. After three or four milkings (~1.5 days after sampling), there was no visible evidence of blood in milk.

Real-time polymerase chain reaction (PCR)

Gene expression was assessed by quantitative real-time PCR. Approximately 10 mg of mammary tissue was homogenised in 350 μL of RNeasy lysis buffer (Qiagen, Albertslund, Denmark) and diluted (1:1) with 70% ethanol. The RNA was purified using the RNeasy mini kit (Qiagen) and reverse-transcribed with oligo-dT and Superscript II RNase H reverse transcription kit (Invitrogen, Taastrup, Denmark), according to the manufacturer’s protocol as described by Diranesh et al. (2016). Electrophoresis through a 2% agarose gel was used to confirm the quality of the RNA. Total RNA (1 μg) was first treated with 1 U DNase (Canada) to digest any contaminating DNA. Real-time PCR was conducted in a Corbett Rotor-GeneTM 3000 quantitative PCR system (Corbett Life Sciences, Sydney, NSW, Australia) with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA) and the specific primers that were used for amplifying IGF-1, IGF-1R, IGFBP-3, IGFBP-5, Bcl-2, Bax and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) cDNA were previously published by Safayi et al. (2010) and Amiri et al. (2016).

Common thermal-cycling parameters (3 min at 95°C and 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C) were used to amplify each transcript. Samples were run in duplicate, and were expressed relative to YWHAZ as a housekeeping gene, which was stable under the conditions used. Data were normalised to a calibrator sample by using the ΔΔCt method with correction for amplification efficiency.

Statistical analyses

Homogeneity of variance was tested with O’Brien and Brown–Forsythe tests. The analysis of variance was performed with JMP software (SAS Institute, Cary, NC, USA), with treatment as the main effect and udder half as the random variable in the F-test. The Tukey–Kramer honestly significant difference test was used for differences among means for multiple comparisons. Significance was declared at $P < 0.05$.

Results

Neither daily feed intake of milk (g/day; kg/day) nor milk yield was influenced by treatments and both were increased significantly by providing the extra ME and MP in combination compared with control groups ($P < 0.05$; Table 2 and Fig. 1). Increasing the ME and MP supplementation levels did not influence milk (Fig. 1) and colostrum production ($P > 0.05$; Table 2). Milk yield increased over time ($P < 0.05$). Milk protein content was similar among treatments ($P > 0.05$) but milk fat percentage was lowest in the MEP group ($P < 0.05$; Table 2).

Providing the extra ME and MP together increased the relative mRNA expression of proliferation-related genes (IGF-1 mRNA ($P = 0.004$, 4.31-fold) and IGF-1R mRNA ($P = 0.01$, 3.61-fold) significantly Fig. 2), compared with the controls, whereas, IGFBP-3 mRNA was decreased ($P = 0.01$, 3.16-fold).

Expression of IGFBP-5 ($P = 0.28$) was not affected (Fig. 2). Expression of apoptosis-related genes (Bcl-2 ($P = 0.003$), Bax ($P = 0.001$) and Bcl-2 : Bax ratio ($P = 0.007$)) was significantly affected by diets. Compared with the control, increasing the ME and MP either alone or in combination increased the relative gene expression of Bcl-2 ($P = 0.007$) and decreased the Bcl-2 : Bax mRNA ratio ($P = 0.003$, 3.16-fold).

Discussion

The present study investigated the effect of maternal nutrition and regulating the amount of milk production, which involves changes in the activity of mammary secreting cells, and also changes in the activities of the gene regulating cell proliferation and death. During declining phase of lactation in goats, the balance between cell proliferation and apoptosis is in favour of net cellular loss. While the structural changes, and roles of hormones, are well known in events from late pregnancy to lactation (Hovey et al. 2002; Paten et al. 2015; Barfurouosii et al. 2018), there is some evidence of the underlying molecular mechanisms that regulate differentiation of the mammary epithelium to a secretory phenotype (Paten et al. 2015).

Results of the present study showed that the increase in energy and protein levels together, but not individually, resulted in a greater milk and colostrum production than for other groups. In agreement with Sahlu et al. (1995) who found that, as gestation progressed, dry matter intake (DMI) was increased by increasing the dietary protein concentration, which was associated with the changes in the fractional passage rate of digesta from the rumen. The DMI also may be

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>Control</th>
<th>MP</th>
<th>ME</th>
<th>MEP</th>
<th>s.e.m.</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily feed intake (g/day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>893.2b</td>
<td>967.5b</td>
<td>996.4b</td>
<td>1282.2a</td>
<td>27.50</td>
<td>0.01</td>
</tr>
<tr>
<td>Colostrum production (kg/day)</td>
<td></td>
<td>0.3b</td>
<td>0.3b</td>
<td>0.4b</td>
<td>0.5a</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>Milk fat (%)</td>
<td></td>
<td>5.4b</td>
<td>5.4b</td>
<td>5.5b</td>
<td>6.2a</td>
<td>0.12</td>
<td>0.01</td>
</tr>
<tr>
<td>Milk protein (%)</td>
<td></td>
<td>2.4a</td>
<td>2.4a</td>
<td>2.5a</td>
<td>2.5a</td>
<td>0.08</td>
<td>0.07</td>
</tr>
</tbody>
</table>
influenced by the dietary protein if it enhances the ruminal retention time. With greater DMI and milk yield, a smaller proportion of dietary nutrients is used for maintaining functions, which, in turn, makes animals experience a lower degree of fat mobilisation and negative energy balance, and also improves health status and productive and reproductive efficiency (de Ondarza and Tricario 2017). Feed typically accounts for 50–60% of the operating expenses on a dairy farm, making it a logical focal point when it comes to increasing the efficiency (Knoblauch et al. 2012). The objective of the farm is to maximise net economic returns generally, while converting a greater percentage of feed nutrients into milk with little wastage feed.

It is known that there is a positive correlation between cell proliferation and death balance and milk production, although there have not been many studies demonstrating this correlation (Sorensen et al. 1998; Capuco et al. 2001); however, more recent studies have shown (Andersen et al. 2005) that when cell renewal is disturbed by continued milking throughout the dry period and in late pregnancy, it results in a negative effect on milk production of dairy cows. Although goats appear to be much less susceptible to this negative effect (Safayi et al. 2010), the studies on cows have shown the importance regarding the formation of new cells in late gestation, in determining milk-production potential in early lactation. Throughout pregnancy, the growth of fetus is controlled by the maternal diet, both directly, which is through the supply of essential nutrients, and indirectly, by altering the expression of key hormones and growth factors that regulate the uptake and utilisation of nutrients by the fetus (Jenkinson 2003). Low provision of maternal nutrition (maintenance × 1.0) throughout pregnancy is detrimental to the development of the fetal mammary gland. Fetal mammary growth has been shown to be two times greater in sheep treated with high provision of nutrition (maintenance × 1.5) than in those retained at a maintenance level (Jenkinson 2003). Moreover, in most cases, the gland was able to initiate compensatory growth by receiving a high provision of nutrition at any time during pregnancy. The mediator role of fetal growth axis (GH-IGF-1) in the nutritional response has been supported by the results showing an increase in plasma growth hormone (GH) and a decrease in plasma IGF-1 concentrations for both the ewe and its fetus under maternal nutrition during late gestation (Bauer et al. 1995). The increase in fetal cortisol concentration leads to an increase in IGF-1, which, in turn, mediates the nutritional response (Li et al. 1993). IGF-1 receptors are downregulated in response to a nutritionally mediated decline in glucose concentrations (Jenkinson 2003).

In the present study, relative expression of IGF-1 and IGF-1R mRNA was increased in goats fed with greater levels of combined ME and MP compared with the control. It could be speculated that the effects of increasing dietary energy level and protein concentration are mediated by IGF-1 and the IGF-1-R. It has been reported that plasma IGF-1, as a local factor secreted by the stroma and adipocytes, participates in epithelial cell proliferation in heifer mammary tissue after activation by GH (Connor et al. 2007) and is elevated significantly during mammary development or increased metabolic activity (Nielsen et al. 2001). Norgaard et al. (2008) reported that dry, pregnant sheep that were exposed to energy restriction (50% of requirements) during the last 6 weeks of gestation had significantly lower plasma IGF-1 concentrations than did ewes that were nourished adequately. These last weeks of gestation coincided with formation of 90% of mammary epithelial cells (Hennighausen and Robinson 2001), and the energy restriction was associated with a decrease in colostrum production postpartum (Norgaard et al. 2008). Also Allan et al. (2004) have reported that IGF-1 plays an important role as a survival factor for mammary development and remodelling during involution. Prosser et al. (1990) found that milk yield and mammary blood flow were stimulated in goats when close-arterial infusion of IGF-1 was administered to one mammary gland. As it can be seen, IGF-1 is the factor that is responsible for cell-proliferation regulation and the regulation of cell turnover by IGF-1 can be influenced by dietary levels of energy and protein.

Results obtained in the present study showed that the relative expression of apoptosis genes is influenced by a change in both energy level and protein concentration. Members of the bcl-2 gene family mediate apoptosis by forming homo- and heterodimers (Sedlak et al. 1995) and the relative concentration of each protein in an individual cell may determine whether a cell will undergo apoptosis (Oltvai and Korsmeyer 1994). Haughn et al. (2003) reported Bcl-2 (B-cell leukemia/lymphoma) as an anti-apoptotic factor. A strong immunohistochemistry expression of Bax protein was seen in dying cells of goat mammary tissue, which confirmed a correlation between the presence of Bax and apoptosis (Heermeier et al. 1996). Increased amounts of Bax protein are found in apoptotic alveolar cells, which are being shed from collapsing and remodelled alveoli. These results strongly indicate that Bax plays an important role in the apoptosis of mammary alveolar cells (Heermeier et al. 1996).

The results of Bcl-2 and Bax gene expression showed that we changed the expression of factors that are known to depict...
Fig. 2. Relative mRNA expression of insulin-like growth factor 1 (IGF-1), insulin-like growth factor 1 receptor (IGF-1R), insulin-like growth factor binding protein 3 (IGFBP-3), insulin-like growth factor binding protein 5 (IGFBP-5), Bcl-2-associated X protein (Bax) and B-cell lymphoma (2Bcl-2) in mammary tissue. Diets were as follows: (1) diet providing metabolisable energy (ME) and metabolisable protein (MP) according to NRC recommendations (C), (2) diet with extra 10% ME (E), (3) diet with extra 10% MP (P), and (4) diet 1 with 10% extra of both ME and MP (EP). Data are presented as the mean ± s.e. Means without a common superscript differ significantly (at \( P = 0.05 \)).
proliferating or apoptotic cells. Changes in cell turnover result in changes in the numbers of active cells synthesising milk and, hence, mammary synthetic capacity at parturition (Capuco and Byatt 1997; Capuco et al. 2001; Paten et al. 2015). Such changes, promoting formation of new cells and inhibiting apoptosis, will expand mammary synthetic capacity at the time of parturition. Other studies have shown that conditions in which there is an impairment in the formation of new cells and a decrease in IGF-1 during late gestation can be associated with a decrease in subsequent colostrum and milk production of the following lactation in cows and sheep (Capuco et al. 2001; Paten et al. 2015).

In conclusion, we have found that expression of factors involved in cell proliferation and apoptosis is sensitive to energy level and protein concentration supplied in the diet; changes in the expression of these factors are related to simultaneous changes in expressions of factors involved in IGF-1 signalling and in paracrine synthesis of IGF-1 itself as well. IGF-1 signalling in the mammary gland is important for this process and, hence, it is sensitive towards energy and protein. So as to find out that whether it is favourable for animal health, we need to know the adequate duration needed for increasing the IGF-1 concentration, according to the timing of cellular events in the mammary gland.

Acknowledgements
This research was supported by Sari Agricultural Sciences and Natural Resources University (SANRU). The authors thank Professor Mette Olaf Nielsen (University of Copenhagen, Denmark) for critical review of the manuscript. The authors acknowledge Miss Roghayeh Rahmani, Miss Mansourore Ghobaraliania and Dr Ali Rezaei Roodbari for their assistance during the study.

References


Handling editor: David Yanez-Ruiz