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Insulin-Like Growth Factor I and II mRNA Levels in Rumen Wall of Calves Fed with Different Physical Forms of Diets

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ABSTRACT

This study was designed to investigate the effects of physical forms and hay contents of diets on gene expression of insulin-like growth factor *(IGF) I* and *II* in rumen epithelium of Holstein calves. Twelve male calves were assigned to 4 treatments: ground (GR), texturized (TX), pellet (PL), and ground+10% forage (GF). Calves were weaned on day 50 of age and then slaughtered on day 70 after birth. Rumen epithelial tissue samples were immediately collected for quantification of mRNA abundance. Results indicated that only *IGF I* expression was influenced by the dietary treatments. A significant ($p<0.05$) correlation between *IGF I* expression and each of histological parameters denoted as length of rumen villi and diameter of keratinocyte layer was observed. No significant correlation between *IGF II* expression and rumen histological parameters was found ($p>0.05$). Regarding the results, higher *IGF I* expression in PL and TX treatments despite the low growth rate might be due to the challenging condition of developing rumen in calves. In fact, the rumen tissue attempted to maintain rumen pH at least by induction of a higher *IGF I* expression.

Keywords: *IGF I*; *IGF II*; Keratinocyte layer; Rumen villi, Holstein calves.

INTRODUCTION

Newborn dairy calves do not have any effective rumen function at early ages. Feeding with solid foods can increase relative weight of rumen compared to that of whole body weight (BW). Fermentation of the starter diets and feedstuff in rumen enhances the amounts of volatile fatty acids (VFA), especially propionic and butyric acids (Dai *et al.*, 2010). These critical materials stimulate development of the ruminal mucosa in calves.

The main goal of using starter diets is to accelerate the growth of rumen papillae and finally gastrointestinal tract (GIT) and increase the ability of calves to be weaned sooner and consume milk replacers using other sources of foods as a ruminant (Malmuthuge *et al.*, 2013). The GIT grows rapidly and changes notably its function during the initial postnatal period. Moreover, it is a major factor and affected by the somatotropic axis in endocrine, autocrine, and paracrine processes (Ontsouka *et al.*, 2004b).

Apart from the significant effect of serum IGFs and *IGF* expression in the liver, studies have shown that local expression of *IGF* mRNAs occurs in ovine and bovine rumen and intestine (Georgieva *et al.*, 2003a, b ; Flaga *et al.*, 2011; Cheng *et al.*, 2012). Higher levels of *IGF I* mRNA in intestine, but not in liver, of pre-term pregnancy than in full-term pregnancy calves at birth suggests autocrine-paracrine roles of IGF I for regulation of intestine growth and indicates that IGF I is an essential factor in

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pre-term intestinal activities. The IGF I mediates growth and differentiation of tissues during postnatal period (Hammon and Blum, 2002). It influences enterocyte proliferation and maturation and GIT morphology and functions in calves (Georgiev *et al.*, 2003a; Roffler *et al.*, 2003). In turn, *IGF II* expression, has been found to be involved in the mechanisms affecting the intestinal epithelium differentiation in calves, and is mostly important during prenatal period and for fetal growth and development (Georgieva *et al.*, 2003a; Ontsouka *et al.*, 2004b). Besides, it has been shown that age-dependent decrease in *IGF*s transcripts is related to the reduction in rumen papillae length and tunica muscularis thickness in calves (Flaga *et al.*, 2011).

During immediate postnatal period, GIT changes morphologically and functionally in calves (Ontsouka *et al.*, 2004b). Investigation of the factors involved in functional development of GIT improves rate of compatibility during postnatal period, and it would be possible to help reduce digestive defects and increase the efficiency of digestive system, health status, and growth performance (Georgiev *et al.*, 2003a, b). Maturation of GIT and its associated organs in calves are influenced by dietary strategies and weaning-feeding managements in early life (Khan *et al*., 2011). Although there have been some studies considering effect of physical forms of dietary treatments on feed intakes and histological parameters, it is still questioned if different physical forms of diets can affect gene expression of growth factors in rumen tissue. Therefore, this study was designed to investigate the expression level of *IGF I* and *IGF II* genes in rumen epithelium of Holstein calves (*Bos taurus*) fed with different physical forms of starter diets and hay contents to achieve more information about GIT changes. In addition, the relation between *IGF*s gene expression and histological parameters of the rumen was evaluated.

MATERIALS AND METHODS

Research was performed in compliance with the Federation of Animal Science Societies in the Guide for the Care and Use of Agricultural Animals in Research and Teaching. Also, the killing procedure was according to Humane Methods of Slaughter Act (Federation of Animal Science Societies, 2010).

Animals, Feeding, and Experimental Design

This study was managed at FKA (Agro-Animal Husbandry Corporation, Isfahan, Iran) between February and May 2015. For comparison of gene expression profile in different treatments, 12 Holstein calves (*Bos taurus*) (41.3 ± 0.4 kg BW) were selected and randomly assigned to 4 treatments. Each treatment consisted of 3 male calves in a completely randomized design, according to previous researches. (Connor *et al.*, 2008; Connor *et al*., 2014). All neonatal calves were housed in individual pens (1.2 m × 2.5 m) while separated from their dams at birth. The bed was filled with fresh sawdust, which was renewed daily. All calves received colostrum for 3 days (4 L/day). They were then fed by (4 L/day) milk in steel buckets twice daily from days 3 to 45. Feeding by milk reduced to half (i.e. 2 L/day and by morning feeding) from days 46 to 49 of age. All calves were weaned on day 50. From day 7 onward, all calves received the experimental diets. They had *ad libitum* access to starter diet and water. The total study duration was 70 d.

Dietary treatments were: 1) ground (GR), 2) texturized (steam flake corn and steam flake barley), (TX), 3) pellet (commercial pelleted starter mixture) (PL), and 4) ground + 10% forage (GF). The GF treatment was a mixture of GR starter feed supplemented by 10% alfalfa hay. Diets were formulated to be iso-energetic and iso-nitrogenous and with similar nutrient compositions, but the treatments were different in particle size

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(Table 1). Geometric mean particle size of starter diets was calculated using the method described by the American Society of Agricultural Engineers (ASAE, 1983). Starter diets were separately analyzed for dry matter, ash, crude protein, crude fat, Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) and metabolizable energy using standard procedures (AOAC, 2000) (Table 2).

Tissue Sampling and Processing

On day 70 of age, the calves were slaughtered at the neck area by captive bolt stunning and cutting of the veins. The gastrointestinal tract was removed immediately and washed. The tissue samples were collected from cranio-ventral sac of rumen. Muscular tissues were separated from the lamina propria layer and washed by phosphate-buffered saline solution. Then, epithelial layer was inserted into 1.5 mL tubes containing DEPC (Sigma Aldrich Company, Germany) water and finally frozen in liquid nitrogen, and stored at −80 °C until analysis for *IGF I* and *IGF II* mRNA expression.

A previous data of villi length, villi width, and thickness of keratinocyte layer, epithelium, circular muscle, longitudinal muscle, total muscle, and rumen wall for the same animals (Pazoki *et al.*, 2017) were used to calculate the correlations between rumen histological parameters and expression levels of *IGF I* and *II* mRNAs.

RNA Extraction, RT-PCR and Real-Time-PCR

Frozen tissue samples were grounded in liquid nitrogen in a mortar. Total RNA was extracted using TRiZol® Reagent (Thermo Fisher Scientific Inc. Invitrogen, USA). Quantity and quality of extracted RNA was verified using electrophoresis and NanoDrop spectrophotometer (Thermo Scientific, USA). An amount of 2000 ng RNA was

Table 1. Particle size of different diets.

Dietary Treatments [a]	Particle Size [b] (mm)	Standard Error
GR	0.72	3.34
TX	3.61	2.07
PL	4.53	1.58
GF	1.02	3.04

[a] Dietary treatments were 1: GR (ground), 2: TX (texturized: steam flake corn and steam flake barley), 3: PL (commercial pelleted starter mixture) and 4: GF (ground + 10% forage). [b] Geometric mean particle size was calculated as described by ASAE, 1983.

Table 2. Chemical composition of different diets.

Chemical Composition (% of DM of total food) [b]	Dietary Treatments [a]			
	GR	TX	PL	GF
DM	98.30	98.02	98.50	98.05
CP	23.88	21.09	23.53	20.29
Crude fat	2.94	2.97	2.98	2.93
NDF	15.2	15.4	14.6	21.10
ADF	8.85	8.58	7.57	13.1
Calcium	0.82	0.82	0.82	0.85
Phosphorus	0.68	0.68	0.68	0.64
Ash	6.9	6.4	6.7	7.01
ME (Mcal/kg) [c]	2.75	2.75	2.75	2.69

[a] Dietary treatments symbols are defined under Table 1. [b] Chemical composition was explained in % dry matter. [c] ME was calculated from NRC (2001) and was shown in Mcal/kg.

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used to construct cDNA by Fermentas RevertAid First Strand cDNA Synthesis kit (Hanover, Maryland, USA). The temperature condition of the thermal cycler (B960, Nison Instrument Limited, Shanghai, China) was practiced as following: 25 °C for 10 min at first, 42 °C for 60 min, and finally 90 °C for 10 min. All cDNA samples were stored at −20 °C until next analyses.

Primers used for the amplification of *IGF I*, *IGF II* and *β-actin* genes were selected from previous studies (Armstrong *et al.*, 2000; Kasimanickam and Kasimanickam, 2011; Nasiri *et al.*, 2012). Nonetheless, in order to have the best choice, bioinformatic analysis was performed by considering melting temperature (Tm), GC %, ΔG, dimer and hairpin formation using Oligo (7.0) software and Primer-BLAST order of NCBI database. The sequences and length of utilized primers, Tm, GC %, and length of PCR products are shown in Table 3. The PCR reaction components were 4.5 µL PCR-master-mix (Viragene Akam Company, Tehran, Iran) (2.5 mM), 7.5 µL ddH2O, 2 µL cDNA, and 0.5 µL of each forward and reverse primers (10 pmol/µL) for a 15 µL final reaction solution. PCR program included a preliminary denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58, 53, and 63 °C for *β-actin*, *IGF I*, and *IGF II*, respectively for 30 sec, extension at 72 °C for 30 sec, and 5 min at 72 °C as final extension. Polymerase chain reaction was carried out to amplify 200, 196, and 200 bp fragments for *β-actin*, *IGF I* (parts of exons 2 and 3), and *IGF II* (parts of exons 2 and 3) genes, respectively. A negative control (water instead of cDNA) was considered in each run to check the accuracy of the reaction. PCR products were visualized in 1% agarose-TBE gel, after electrophoresis and staining.

In order to determine the changes in *IGF* gene expression in response to different treatments, Real-Time PCR was performed including a negative control, and cDNA of 3 calves of each treatment for each gene. *β-actin* was used as an internal control to normalize the expression of *IGF* mRNAs, as it was used in previous studies (Dervishi *et al.*, 2010; Muscher *et al.*, 2010; Tao *et al.*, 2014) and it is considered as an stable reference gene in rumen samples in different rations (Die *et al.*, 2017). Reaction components for Real-Time-PCR consisted of 5 µL SYBR Green kit (2X), 3 µL ddH2O, 1 µL cDNA, and 0.5 µL of each forward and reverse primers (5 pmol/µL) for a 10 µL final reaction solution. The Real-Time PCR (Applied Biosystems StepOne Plus) program was as explained before. Also, in order to ensure the absence of primer-dimer formation and unspecific amplification, a melting curve program (60-95 °C) was included.

Statistical Analyses

The abundance of *IGF I* and *II* mRNAs were calculated using relative quantification by $2^{-\Delta\Delta CT}$ formula. The effect of different feeding types on *IGF*s expression was evaluated using the GLM procedure of SAS

Table 3. Characteristics of specific primers.

Fragment Length	Tm	Primer [a] Length	%GC	Sequence (5`==> 3`)	Gene
200	61.68	18	66.7	F [b]: TCG CCC GAG TCC ACA CAG	β-actin
	61.9	19	63.16	R [c]: ACC TCA ACC CGC TCC CAA G	
196	66.77	21	66.67	F: CCT CTG CGG GGC TGA GTT GGT	IGF I
	68.09	22	68.18	R: CGA CTT GGC GGG CTT GAG AGG	
200	61.42	20	60	F: CTG GTG GAC ACC CTC CAG TT	IGF II
	60.67	18	61.11	R: TCC GGA AGC ACG GTC GTA	

[a] Primers of *β-catin*, *IGF I*, and *IGF II* genes suggested by Nasiri *et al.* 2012; Kasimanickam and Kasimanickam, 2011; and Armstrong 2000, respectively. [b] forward, [c] reverse.

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software version 9.2 (SAS Institute, Cary, NC, USA), as a completely randomized design, using the following model (1):

$$Y_{jkl} = \mu + T_j + IBW_k + e_{jkl} \quad (1)$$

Where, Y_{jkl} was the gene expression ratio, μ was the overall mean, T_j was the fixed effect of the treatments (dietary groups), IBW_k was the covariate effect of the initial body weight of calf, and e_{jkl} was the random error.

Tukey-Kramer test was used for comparison of the treatment means. Pearson method was used to clarify the correlations of rumen histological parameters with expression levels of *IGF I* and *II* mRNAs (Georgiev *et al.*, 2003a, b; Kravchenko *et al*., 2008). The significance level was declared at $p \leq 0.05$ for all the tests.

RESULTS

RNA electrophoresis and NanoDrop spectrophotometer verified the extracted RNA integrity and purity. The optical density ratio of 260 and 280 nm was between 1.8 and 2 for all samples. PCR results showed the amplification of 196, 200 and 200 fragments for *IGF I*, *IGF II*, and *β-actin*, respectively (Figure 1).

Quantitative PCR showed that abundance of *IGF I* mRNA differed significantly ($p < 0.001$) among different dietary treatments, but there was no significant difference in *IGF II* mRNA levels ($p > 0.05$). The average expression levels of *IGF I* and *II* mRNAs are shown in Table 4. The results showed that the physical form of starter diet can significantly affect the expression of *IGF I*, which increased in PL and TX ($p < 0.05$) (order of treatments: PL and TX > GR > GF).

Investigated traits and their correlation coefficients are presented in Table 5. Results showed that correlation between *IGF I* and *IGF II* was not significant. The correlation between *IGF I* gene expression and 2 of the histological parameters of the rumen was significant [-0.72 and 0.73] for length of villi and diameter of keratinocyte layer, respectively. The correlation between the expression of *IGF II* and histological parameters of rumen was not statistically significant.

DISCUSSION

Several reports have revealed that consumption of different foods and their

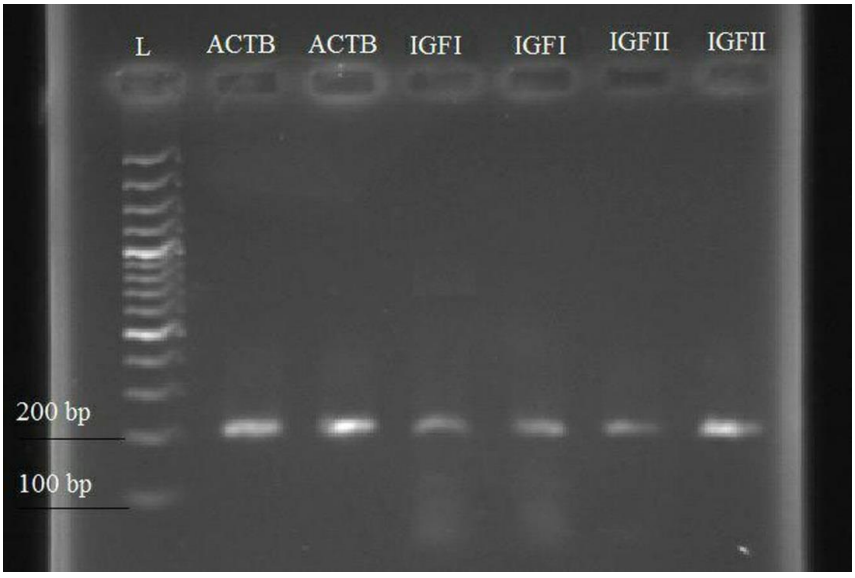

Figure 1. PCR amplification results of *IGF I*, *IGF II* and *β-actin* (ACTB) in 1% agarose-TBE gel, L: 100 bp DNA ladder.

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Table 4. The expression levels of *IGF I* and *II* mRNAs normalized to *β-actin*, in rumen wall in different dietary treatments.

	Dietary Treatments [a]				
mRNA	GR	TX	PL	GF	Standard Error
IGF I	2.91[b]	4.24[a]	4.99[a]	2.56[b]	1.23
IGF II	1.39	2.49	1.98	1.37	1.19

[a] Dietary treatments symbols are defined under Table 1. [a-b] Values with different superscript letters are significantly different ($p < 0.05$).

Table 5. The coefficients of correlation between the expression of IGF I and II mRNAs and histological parameters of Holstein calves rumen.

	Histological Parameters Coefficients Correlation (Significant level)							
mRNA	Villi length	Villi width	Keratinocyte layer thickness	Epithelium thickness	Circular muscle thickness	Longitudinal muscle thickness	Total muscle thickness	Wall thickness
IGF I	-0.727* (0.03)	0.261 (0.50)	0.729* (0.03)	0.111 (0.78)	-0.66 (0.11)	-0.50 (0.32)	-0.59 (0.22)	-0.63 (0.18)
IGF II	-0.526 (0.15)	0.087 (0.83)	0.362 (0.34)	0.022 (0.95)	-0.153 (0.75)	0.625 (0.19)	0.451 (0.37)	0.41 (0.42)

* $P < 0.05$.

physical form can increase the mRNA expression of *IGF*s and then modulate the amplification of epithelial cells and development of muscles of digestive tract (Shen, 2005; Flaga *et al.*, 2009; Flaga *et al*., 2011). Ontsouka *et al*. (2004b) showed that adding dexamethasone (DEXA; 30 µg/kg BW per day) into an isoenergetic milk-based formula increased mRNA levels of *IGF I* in esophagus, rumen, jejunum, and colon, while it reduced *IGF I* levels in esophagus, fundus, duodenum, and ileum in colostrum-fed calves. They concluded that different feedings affected gene expression of the components of somatotropic axis in GIT of neonatal calves.

Moeini *et al*. (2016) studied the effects of physical forms of diet and hay content on blood metabolites, liver composition and intestinal morphology. They showed that feed efficiency in the texturized treatment was better and the ground treatment had the least efficiency among the treatments. As physical forms of PL and TX diets in starter mixture in the current study caused the highest expression level of *IGF I* mRNA among the experimental treatments, it seems that more nutrients in these treatments got the chance (through processing) to have an effect on expression of growth factor genes. Available nutrients in PL and TX treatments (through processing) may stimulate the expression of *IGF I* at rumen wall. However, it is notable that the expression of *IGF I* is affected by various stimuli controlled by different factors such as hormonal and nutritional status, specific characteristics of each tissue, and the mechanical pressure (Dai *et al*., 2010).

Calves in PL treatment showed the lowest ADG, DMI and feed efficiency (Moeini *et al*., 2016). Interestingly, the expression level of *IGF I* was the highest in PL treatment and then the order was followed by TX (numerally), GR and GF (statistically) treatments, respectively. The PL treatment, which had shown a weak performance in the study of Moeini *et al.* (2016), had the highest expression level of *IGF I* mRNA in the current study.

It has been demonstrated that using forage in starter diets stimulated the physical abrasion of feed on rumen papilla (Beiranvand *et al*., 2014; Omidi-Mirzaei *et al.*, 2015) and improved the rumen wall (Suarez *et al*., 2007). Furthermore, GF

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treatment, which was reported to have the highest ruminal pH, ADG and DMI among the treatments (Moeini *et al.*, 2016), showed the lowest *IGF I* expression level in the present study. Therefore, we concluded that there might be a relation between ruminal pH, ADG, DMI and the expression of *IGF I*.

Yang *et al.* (1996) introduced IGF IEc, an isoform of *IGF I*, as a factor related to mechanical stimulation, and named it as mechano growth factor (MGF). It has been further demonstrated that acidic status and increased temperatures enhance the expression of MGF isoform in mice myoblasts (Kravchenko *et al.*, 2008). It has been reported that the rumen pH was reduced in treatments lacking forage, because of decrease in rumination time span, amount of salivary secretion, and the ability of rumen to absorb VFA (Moeini *et al*., 2016). Hence, we concluded that the acidic status of the rumen injured the rumen wall and then, in response to this challenging situation, the rumen increased the expression of *IGF I* in PL and TX treatments compared with GF treatment. The higher expression of *IGF I* mRNA may be due to the damage resulting from a reduction in the rumen pH which makes a challenging condition for rumen at these treatments. However, the increased expression could not affect the tissue growth. It has been previously reported that diets consisting of more concentrate showed lower-developed rumen mucosa than those including concentrate and 10% hay (Beiranvand *et al.*, 2014). Another assumption is that IGF I receptors might be damaged due to the acidic condition of rumen, or their expression might be hypothetically down-regulated as a result of low pH and, therefore, no cellular response would happen after *IGF I* expression. Consequently, the cells might have tried to express more *IGF I* transcripts to overcome the deficiency.

This study indicated a considerable, but not significant, difference in *IGF II* mRNA levels and, numerically, the order of treatments was as TX > PL > GR > GF. Flaga *et al.* (2011) studied the age-dependent changes (days 5, 12, 19 and 26 of calves' life) in *IGF I* and *IGF II* mRNA levels in relation to GIT development in newborn calves. They detected that both *IGF I* and *IGF II* mRNAs were at the highest level on 5th day of life, and significantly decreased on days 12 to 19 in most of analyzed GIT parts, and started to increase slightly thereafter. They concluded that mRNA expression level depended on age and feed type.

Georgieva *et al.* (2003a, b) demonstrated that the expression of *IGF II* mRNA in liver and intestine of preterm calves (born after 277 days of pregnancy) was higher in comparison to full-term calves (born after 290 days of pregnancy), but not significant between preterm calves on 8th day of life after being fed for 7 day and full-term calves . These results confirm that *IGF II* is more functional during prenatal life and its function decreases in postnatal period. It was reported that *IGF II* expression level was affected by feeding type and dexamethasone treatment. Abundance of *IGF II* mRNA in rumen and jejunum was higher ($p < 0.05$) in formula-fed than in colostrum-fed calves and in pylorus was higher ($p < 0.05$) in DEXA-treated than in non-treated calves (calves on 5th day of life) (Ontsouka *et al.*, 2004b). Considering previous studies, this is the first time that the expression level of *IGF I* and *IGF II* mRNAs is investigated in different physical forms of starter mixture. Hence, more studies are needed in this field to get a comprehensive knowledge.

The effect of dietary energy–dependent alterations has been studied on the expression of growth factor genes and a positive correlation has been reported between *IGF*s expression and rumen papilla length (Shen *et al*., 2004; Shen, 2005; Flaga *et al.*, 2011). However, our study showed a positive correlation between *IGF I* expression and the diameter of keratinocyte layer and a negative correlation between the expression of *IGF I* and villi length ($p < 0.05$). In a previous study, it was demonstrated that the diameter of keratinocyte layer in dairy calves increased

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in concentrate treatments compared with concentrate + 10% forage (Beiranvand *et al.*, 2014). Feeding calves with concentrate rich with fast fermentable carbohydrates can quickly reduce ruminal pH (Beharka *et al*., 1998), and cause overgrowth and keratinization of ruminal papillae (Bull *et al.*, 1965). Beharka *et al*. (1998) have shown that calves fed with ground starter diets had shorter villi and lower villi surface area in the rumen compared with calves fed with unground diets, which clearly demonstrates that physical forms of starter diets affect papillary development of rumen. According to the present study, the most *IGF I* expression was in PL and TX treatments. Hence, there would be an assumption that body in the high-*IGF I* expression group confronts a challenging acidic rumen environment and attempts to alleviate this problem by increasing *IGF I* expression. In fact, GF treatment which has been shown in different reports to have the highest growth rate (Franklin *et al*., 2003; Coverdale *et al.*, 2004; Moeini *et al.*, 2016), had some expression of *IGF I* gene, but it was not as high as PL treatment.

Reports indicate a negative correlation between the expression of *IGF I* and II mRNAs and villi length in small intestine (Ontsouka *et al.*, 2004a). It has been shown that intestinal villi width was independent of *IGF I* and *IGF II* (Flaga *et al.*, 2011). In an investigation upon 24 calves on 8th day of life, no correlation was detected between histological parameters and the expression levels of *IGF I* mRNA expression in intestine (Velayudhan *et al.*, 2008). High expression of *IGF I* mRNA in intestine on 5th day of life was accompanied by decrease in villi length. The reduction in *IGF*s mRNA levels after 5th day of life in the duodenum (*IGF I* and *IGF II*) and jejunum (*IGF I*) was associated with reduction in villi length (duodenum and jejunum). In general, it may be concluded that *IGF I* has a fluctuating role in different parts of digestive system and is affected by diet (Flaga *et al.*, 2009). However, more studies considering more repetitions per treatment may be needed to understand the exact role of growth factors in changes of rumen histological parameters.

A comprehensive conclusion is listed in the following:

IGF I and not *IGF II* expression was affected by diet and ruminal environmental status.

High level of *IGF I* expression is not always an indicator of the rumen development and this may happen due to the cellular-molecular compatibility of the rumen to the environmental status (e.g. acidic environment of rumen).

Given the lack of significant correlation between *IGF I* and *IGF II*, the expression of these genes may be regulated independently and *IGF II* may have a less important role compared to *IGF I* in the rumen development at birth time.

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سطوح mRNA فاکتورهای رشد شبه انسولین ۱ و ۲ در دیواره‌ی شکمبه‌ی گوساله-های تغذیه شده با شکل‌های فیزیکی متفاوت جیره

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چکیده

مطالعه‌ی حاضر به منظور بررسی اثرات شکل فیزیکی و مقدار علوفه‌ی جیره بر بیان ژن های *IGF I* و *IGF II* در اپیتلیوم شکمبه‌ی گوساله‌های هلشتاین طراحی شد. دوازده گوساله‌ی نر به چهار گروه تیماری اختصاص داده شد: آردی (GR)، آجیلی (TX)، پلت (PL) و آردی با ده درصد علوفه (GF). گوساله‌ها در سن ۵۰ روزگی از شیر گرفته شدند و سپس در روز ۷۰ بعد از تولد کشتار شدند. نمونه‌های بافت اپیتلیوم شکمبه بلافاصله برای اندازه گیری فراوانی mRNA جمع‌آوری شدند. نتایج نشان داد که تنها بیان *IGF I* تحت تأثیر تیمارها قرار گرفت. همبستگی معنی داری بین بیان *IGF I* و هر کدام از فراسنجه‌هی بافتی شکمبه که به نام طول پرزهای شکمبه و قطر لایه‌ی کراتینوسایت، مشاهده شد ($p < 0.05$). همبستگی معنی‌داری بین بیان *IGF II* و فراسنجه‌های بافتی شکمبه دیده نشد. با توجه به نتایج، بیان بالاتر *IGF I* در تیمارهای پلیت و آجیلی با وجود نرخ کم رشد، ممکن است به علت شرایط چالش برانگیز شکمبه‌ی در حال توسعه در گوساله‌ها باشد. در واقع، بافت شکمبه جهت حفظ pH شکمبه در حداقل توسط افزایش بیان *IGF I* تلاش می‌کند.

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