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Effect of increasing levels of glycerin on growth rate, carcass traits and liver gluconeogenesis in young bulls☆

Marcio M. Ladeira[a,*], José Rodolfo R. Carvalho[a], Mario L. Chizzotti[b], Priscilla D. Teixeira[a], Júlio César O. Dias[a], Tathyane R.S. Gionbelli[a], Aline C. Rodrigues[a], Dalton M. Oliveira[c]

[a] Department of Animal Science, Universidade Federal de Lavras, Lavras, Minas Gerais, CEP: 37.200-000, Brazil
[b] Department of Animal Science, Universidade Federal de Viçosa, Viçosa, MG, 36.570-000, Brazil
[c] Universidade Estadual de Mato Grosso do Sul, Aquidauana, MS, 79.200-000, Brazil

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ABSTRACT

This study aimed to evaluate performance, carcass traits, glycerol kinase 1 (*GK1*) and cytoplasmatic phosphoenolpyruvate carboxykinase (*PCK1*) gene expression, and glycerol kinase activity in liver of young bulls receiving different levels of crude glycerin. Forty-four crossbred young bulls (initial body weight of 368 ± 4.2 kg) were used in a completely randomized design, with four treatments and 11 replicates. The experiment period lasted 84 days, preceded by an adaptation period of 28 days. The basal diet was composed of corn silage (300 g/kg) and concentrate (700 g/kg) containing corn and soybean. The experimental treatments were as follows: without glycerin or including 60, 120 or 180 g/kg of crude glycerin in the diet. Blood samples were collected on the last day of the experiment period to evaluate biochemical parameters. After slaughter, carcass traits were measured and liver samples were collected to analyze gene expression and glycerol kinase activity. There was no effect ($P = 0.21$) of glycerin on glucose blood concentrations. However, liver glycerol kinase activity was greater ($P < 0.01$) and *GK1* and *PCK1* genes expressions were down regulate ($P < 0.01$) as glycerin levels in the diet increased. Glycerin did not affect ($P > 0.17$) performance and most of the carcass traits. However, there was a greater ($P = 0.02$) marbling score in the carcass of animals fed 120 and 180 g/kg of crude glycerin. In conclusion, the use of glycerin at a level of up to 180 g/kg is recommended in diets of feedlot beef cattle, and it increases liver glycerol kinase activity, feed efficiency and beef marbling.

Abbreviations: ADG, average daily gain; AQP 9, aquaglyceroporin; AST, aspartate aminotransferase; CCW, cold carcass weight; CK, creatine kinase; CY, carcass yield; DM, dry matter; DMI, dry matter intake; FBW, final body weight; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase gene; GGT, gamma-glutamyl aminotransferase; *GKI*, glycerol kinase 1 gene; HCW, hot carcass weight; LMA, longissimus muscle area; ME, metabolizable energy; NFC, non-fibrous carbohydrates; *PCK1*, cytoplasmatic phosphoenolpyruvate carboxykinase gene; PEPCK-C, cytosolic phosphoenolpyruvate carboxykinase enzyme; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; PPAR-gamma, peroxisome proliferator-activated receptor-γ; SFT, subcutaneous fat thickness.

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* Corresponding author.
E-mail address: mladeira@dzo.ufla.br (M.M. Ladeira).

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1. Introduction

The use of coproducts to replace commonly used ingredients such as corn and soybean could reduce feeding cost and increase profit in feedlots. Among available coproducts for use in feedlots, glycerin is emerging as an option due to the growth of biodiesel industry around the world. Some studies have shown the potential of this biodiesel industry coproduct for ruminants' feeding, being the first studies performed in dairy cows (Donkin, 2008). In beef cattle, several studies evaluated the effect of crude glycerin on performance and beef quality, but results were ambiguous and inconclusive. In other words, in some cases crude glycerin increased growth performance of the animals (Parsons et al., 2009), but in other cases, there was no positive effect (Egea et al., 2014; Van Cleef et al., 2014), while in some studies, glycerin depressed animal performance and carcass traits (Gunn et al., 2010). In addition, none analyzed performance and liver metabolism at the same time.

Chemical composition of crude glycerin still is quite variable, being important to assess whether the use of this ingredient may have toxic effects in ruminants, especially regarding methanol concentration. According to Elam et al. (2008), it has been hypothesized that glycerin supplementation may cause problems associated with hepatic lipid mobilization by increasing the liver ability to sequester and re-esterify fatty acids from tissues. Besides, glycerol may be a substrate for gluconeogenesis in the liver, increasing glycerol kinase activity and glucose turnover, which could improve beef marbling. It may occur because, according to Gilbert et al. (2003), intramuscular adipose tissue uses a high proportion of glucose for fatty acid synthesis and it is more sensitive to insulin.

Therefore, the hypothesis of this study is that glycerin will not affect negatively feedlot performance, while increases liver gene expression and activity of glycerol kinase. The objective was to evaluate performance, carcass traits, expression of glycerol kinase 1 (*GK1*) and cytoplasmatic phosphoenolpyruvate carboxykinase (*PCK1*) genes, and glycerol kinase activity in the liver of young bulls fed different levels of crude glycerin.

2. Materials and methods

Animal care and handling were approved by the Federal University of Lavras Animal Care and Use Committee. The experiment was carried out at the Animal Science Department of the Federal University of Lavras.

2.1. Animals, diet and slaughter

Fourty-four crossbred young bulls (¼ Angus, ¼ Nellore, ¼ Senepol, ¼ Caracu), with initial body weight of 368 ± 4.2 kg were used in a completely randomized design, with four treatments (0, 60, 120 and 180 g/kg of crude glycerin in the diet) and 11 replicates. Crude glycerin composition used in the experiment had the following composition: glycerol: 831 g/kg, moisture: 111 g/kg, ash: 60.6 g/kg, chlorides 36.7 g/kg, and methanol: 0.2 g/kg. Animals were housed in open-air pens with an area of 30 m^2 per animal.

This 84-day experiment was preceded by an adaptation period of 28 days, during which animals received the same experimental diet. At the beginning of the adaptation period, animals were treated for ectoparasites and endoparasites (Ivomec, Paulinia, Brazil). Aiming to measure their average daily gain (ADG) using regression analysis, animals were weighed at the beginning, every 28 days and at the end of experimental period, after 16 h of fasting.

Diets were formulated with corn silage, and four different levels of crude glycerin (Table 1) according to the National Research Council – NRC (2000) to make it isonitrogenous. Animals were fed *ad libitum* at 07:30 am and 03:30 pm. Corn gluten meal-21 was included in the diets with crude glycerin to provide similar levels of crude protein and a similar amino acid profile.

Samples of silage and concentrate were collected every 14 days. These samples supplied a composite sample that, after pre-drying in a forced-ventilation oven at 65 °C for 72 h, was ground in a mill with a 1-mm mesh sieve. Chemical analyses of the diets were performed according to AOAC (2000). The dry matter (DM) was obtained by oven drying at 100 °C for 18 h (Method 934.01). The ash content was estimated after a 2-h incineration process in a 600 °C muffle furnace (Method 942.05). The crude protein (CP) content was determined based on the N content multiplied by the 6.25 factor. The N content was obtained by the Kjeldahl procedure (Method 920.87). Evaluation of the ether extract (EE) content was performed with solvent using a Soxhlet extractor apparatus (Method 920.39). Non-fibrous carbohydrates (NFC) were determined according to Sniffen et al. (1992), and metabolizable energy (ME) was calculated according to the NRC (2001) and Mach et al. (2009).

Blood samples were collected from the coccygeal vein using Vacutainer® tubes (BD, Juiz de Fora, Brazil) at the end of the experiment after 16 h of fasting. These samples were placed in a cooler with ice and transported directly to the laboratory, where analyses of creatinine and aspartate aminotransferase (AST), gamma-glutamyl aminotransferase (GGT), and creatine kinase (CK) enzymes were performed using commercial kits (Labtest Diagnóstica, Lagoa Santa, Brazil). Blood samples for glucose analysis were collected in another Vacutainer® tube (BD, Juiz de Fora, Brazil) containing a fluoride anticoagulant.

Animals were slaughtered at a commercial abattoir by captive bolt and exsanguination, followed by hide removal and evisceration, without electrical stimulus. The carcasses were washed, divided into two equal halves and weighed to obtain the hot carcass weight (HCW) and carcass yield (CY). The subcutaneous fat thickness (SFT) and longissimus muscle area (LMA) were measured between the 12th and 13th ribs of the left half-carcass. Subsequently, they were maintained under

Table 1
Composition of ingredients and nutrients contents of the experimental diets (g/kg).

Ingredients	Glycerin levels (g/kg)			
	0	60	120	180
Corn silage	300	300	300	300
Ground corn	560	480	370	260
Soybean meal	120	120	120	120
Corn gluten meal-21	–	20	70	120
Crude glycerin[1]	–	60	120	180
Mineral mixture[2]	20	20	20	20
Nutrients				
Dry matter[3]	592	588	584	580
Crude protein[4]	131	129	131	133
NDF[4]	222	224	236	248
NFC[4]	566	566	550	533
Glycerol[4]	–	56	112	168
Ether extract[4]	34	34	33	32
ME[2] (MJ/kg MS)	11.7	11.7	11.6	11.6

[1] Guaranteed levels per kilogram of product: Glycerol: 831, Moisture: 111, Ash: 60.6, Chlorides 36.7, Methanol: 0.2.
[2] Guaranteed levels per kilogram of product: Ca: 170 g; P 31 g; Na: 155 g; Zn: 2 mg; Cu: 396 mg; Mn: 515 mg: Co: 15 mg; I: 29 mg; Se: 5.4 mg; Vit. A: 111.000 UI; Vit. D3: 22.000 UI; Vit. E: 265 UI.
[3] As fed basis.
[4] Dry matter basis.

Table 2
Target primers used in the relative quantification by q-PCR.

Gene	Primer	Sequence	Access code (GenBank)	Bp[a]	R^2	Efficiency
GK	*Glycerol kinase*	F: TCTTCTGTAGTCTGCCCTTGG R: TTTACGGCATACCTGAGAGG	BC122692.1	87	0.936	99.4
PEPCKC	*Phosphoenolpyruvate carboxykinase*	F: CCCCCAGAGATCAAGAATCA R: ATTGGAGGTGGACAGTCAGG	AY145503.1	86	0.998	98.3
β-actin	*β-actin*	F: GTCCACCTTCCAGCAGATGT R: CAGTCCGCCTAGAAGCATTT	BC142413.1	90	0.996	100.0
GAPDH	*Glyceraldehyde 3-phosphate dehydrogenase*	F: CGACTTCAACAGCGACACTC R: TTGTCGTACCAGGAAATGAGC	NM_001034034.1	96	0.998	101.4

[a] Amplicon size in base pairs.

refrigeration in a cold chamber (1 °C) for 24 h. After cooling, carcasses were reweighed to obtain the cold carcass weight (CCW). Marbling was analyzed according to Dow et al. (2011) equation, using ether-extractable fat of the longissimus muscle.

2.2. Gene expression and enzyme activity

Immediately after slaughter, liver samples were collected, frozen in liquid nitrogen and stored in −80 °C to analyze *GK1* and *PCK1* genes expression that encode glycerol kinase and cytoplasmatic phosphoenolpyruvate carboxykinase enzymes, respectively. In addition, same samples were used to measure glycerol kinase activity.

Target and reference primers were designed using sequences deposited and published in the GenBank public database, a NCBI (National Center for Biotechnology Information) platform. ORFs (Open Reading Frames) of the selected sequences were obtained by using NCBI *ORF Finder* tool, and the encoded protein sequences were obtained from the *Translate* tool of ExPASy protein database. The primer sequences were designed using the OligoPerfect™ Designer software; sequences are shown in Table 2.

Total RNA was extracted from liver samples with QIAzol reagent (QIAgen, Valencia, CA, USA) and treated with DNA-free DNase (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Total RNA was subjected to electrophoresis in 10.0 g/kg agarose gel (w/v) stained with GelRed Nucleic Acid Gel Stainand visualized in a UVITEC Fire Reader XS D-77Ls-20M for analysis of the 28 S and 18 S ribosomal RNA (rRNA) bands. Samples were quantified by spectrophotometry (Nanodrop ND-1000 Spectrophotometer) at A260 nm to determine the amount (ng/uL) and quality (260/280 and 260/230) of the RNA. The cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. After the reaction, samples were stored at −20 °C.

For quantitative gene expression analysis by RT-qPCR, ABI PRISM 7500 Real Time PCR machine (Applied Biosystems) was used with SYBR Green detection system (Applied Biosystems, Foster City, CA, USA). The reaction parameters were as follows: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 min at 95 °C, 1 min at 60 °C and 15 s at 95 °C. Data were collected and stored using 7500 Fast Software (Version 2.1, Applied Biosystems, Warrington, UK). Each reaction contained 1.0 μL of

cDNA, 0.3 μL of each primer (forward and reverse) and 5.0 μL of SYBR Green Master Mix in a final volume of 10.0 μL/sample in a MicroAmp Optical 96-well reaction plate (Applied Biosystems, Foster City, CA, USA). The RT-qPCR analyses for each gene studied were performed using cDNAs from 11 experimental units (animals), with three technical replicates for each cDNA. Results were normalized using Cycle Threshold (C_T) obtained from reference genes *β-actin* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression. The choice of these reference genes was based on a pre-study using *GAPDH*, *Actin*, *Ubiquitin* and *Cyclo Pilina*, in which *β-actin* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) had the best efficiency.

The C_T was determined from the number of cycles using the comparative C_T method. As one requisite of this method, a validation assay was performed to demonstrate that the amplification efficiencies of the target and reference genes were approximately the same (Table 2). Standard curves were generated for the studied genes with the following dilutions: 1:5, 1:25, 1:125, 1:625, and 1:3125. This procedure allows the user to define the cDNA concentrations used in each reaction (in this case, 10 ng/μL). The concentration of each primer was 1.5 μM.

The relative expression levels were calculated according to the method described by Pfaffl (2001), which is based on Ct values corrected for each primer pair amplification efficiency.

For glycerol kinase (EC 2.7.1.30) extraction, 1.5 g of frozen liver was used. The tissue was homogenized in 3 mL buffer (Tris-HCl 0.05 M and pH 8.0) using a polytron (Ultra-turrax T25 basic-IKA, Wilmington, USA). After tissue homogenization, samples were centrifuged at 15,000*g* for 20 min at 5 °C and the supernatants collected and stored at 4 °C (Bernardino et al., 2013). The enzymatic activity in the supernatants was quantified according to the method described by Kihara et al. (2009). Initially, glycerol kinase present in the samples phosphorylates glycerol to glycerol-3-phosphate and ADP, in the presence of ATP. After two sequential reactions catalyzed by glycerol-3-phosphate oxidase (EC 1.1.3.21) and peroxidase (EC 1.11.1.7), glycerol-3-phosphate is converted to quinoneimine, that can be analyzed using a colorimeter.

The kinetic assay was conducted in 96-well microplates with 880 μL of Tris-HCl buffer (50 mM, pH 8.0), 10 μL of 400 mM ATP (pH 7.0, Amresco, code: 0220-25G), 10 μL of 200 mM MgCl2, 10 μL of glycerol-3-phosphate oxidase from *Pediococcus* sp. (100 U of enzyme/500 μL of buffer; Sigma-Aldrich, code: G9637-100UN), 10 μL of horseradish peroxidase (450 U of enzyme/ml of buffer; Amresco, code: 0343-25,000U), 10 μL of 150 mM 4-aminoantipyrine (Sigma-Aldrich, code: A4382-100G), 10 μL of 150 mM phenol and 50 μL of the supernatant. To start the battery of reactions, 10 μL of 1 M glycerol were added and the plates were then incubated at 25 °C in an automatic microplate reader (Multiskan GO, Thermo Scientific, Vantaa, Finland) programmed to read the absorption at 500 nm every 20 s for a maximum period of 15 min. The blank samples were included for measuring the absorbance of the reagents used in the kinetic assay. Subsequently, data from the enzymatic assay were used to generate a linear curve.

Protein content in the samples (1 g of protein/ml of hepatic extract) was measured according to Bradford method (1976) using bovine serum albumin as a standard, to calculate specific activity of the glycerol kinase as follows:

$$\text{Specific activity (U/μg of protein)} = \text{volumetric activity/protein content in the sample.}$$

Therefore, the glycerol kinase activity was expressed in U, defined as the quantity of enzyme that generates 1 μmol of glycerol per minute of reaction in a solution with pH = 8.0 and 25 °C.

2.3. Statistical analyses

The Shapiro-Wilk test was used to analyze the data distribution. If the data were not normally distributed, transformation was performed using the PROC RANK of SAS 9.3 (SAS Inst.Inc., Cary, NC, USA). Data were analyzed as a completely randomized design using the MIXED procedure (SAS Inst. Inc., Cary, NC) with diet as fixed effect and according to the following model: $Yi,j = \mu + Ti + e$; with: Yi,j is the observation on j experimental unit receiving treatment i; μ is the overall treatment mean; Ti is the effect of having treatment level i; and e is the random error. Animals were the experimental unit, and orthogonal contrasts were used to evaluate the linear and quadratic effects of glycerin and without glycerin vs. glycerin treatment. Treatment means were computed with the LSMEANS option. Effects were considered significant at P-value of ≤ 0.05, with tendencies declared at P-values between 0.05 and 0.10.

3. Results

Blood creatinine, AST, GGT and CK were not affected by the levels of glycerin in the diet (Table 3). However, blood glucose tended ($P = 0.08$) to decrease linearly with the level of glycerin in the diets. Liver glycerol kinase activity increased linearly following glycerin inclusion (Fig. 1A). On the other hand, opposite result were detected on *GK1* and *PCK1* expressions (Fig. 1B and C). Expression of *GK1* in the liver was 1.61, 3.34 and 5.45 times lower, when the animals fed 60, 120 and 180 g/kg of crude glycerin, respectively, than in the liver of animals fed diet without glycerin. Expression of *PCK1* also presented a quadratic effect being the maximum value in the curve when glycerin content was 59.1 g/kg of DM.

Despite the lack of glycerin versus no glycerin diets effect ($P = 0.41$) on dry matter intake (DMI), linear and quadratic effects were detected (Table 4). Analyzing the means and the maximum value in the quadratic curve, 61.2 g/kg of glycerin in the diet, it is possible to conclude that the negative effect of glycerin on DMI started after this point and it was more pronounced when animals were fed 180 g/kg of glycerin in the diet. There was no effect ($P > 0.18$) of crude glycerin on animals ADG during the feedlot. However, feed efficiency increased linearly with the use of glycerin ($P = 0.04$).

Table 3
Biochemical profile of the blood of young bulls fed different levels of crude glycerin.

Variables	Glycerin levels (g/kg)				SEM	Gly[1]	L[2]	Q[3]
	0	60	120	180				
Glucose, mg/dl	83.2	73.1	85.1	71.9	5.40	0.30	0.08	0.95
Creatinine, mg/dl	1.53	1.41	1.47	1.48	0.09	0.49	0.40	0.92
AST[4], U/L	93.0	95.4	85.8	84.9	6.80	0.59	0.83	0.20
GGT[5], U/L	12.5	12.2	15.7	17.5	2.36	0.34	0.94	0.08
CK[6], U/L	346	504	376	336	60.2	0.39	0.11	0.25

[1] Gly = 0 vs. Glycerin.
[2] L = Linear.
[3] Q = Quadratic.
[4] AST = Aspartate aminotransferase.
[5] GGT = gamma-glutamyl aminotransferase.
[6] CK = creatine kinase.

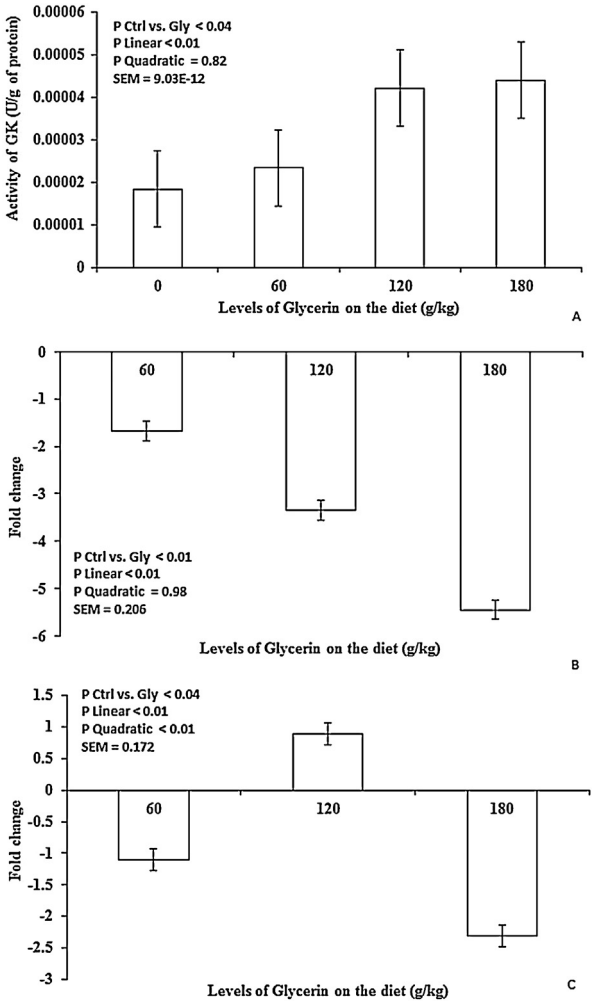

Fig. 1. Glycerol kinase activity (1A), relative expression of *GK1* (1B) and relative expression of *PCK1* (1C) in the liver of young bulls fed with different crude glycerin concentrations.

Crude glycerin did not affect ($P > 0.36$) final body weight (FBW), HCW, CCW and SFT of the animals (Table 5). Nevertheless, quadratic effects ($P < 0.05$) were observed in carcass yield, longissimus muscle area and marbling with the use crude glycerin in the diets. Maximum values in the curves for carcass yield and marbling were 212 and 244 g/kg of crude glycerin, respectively, and minimum value for longissimus muscle area was 53.9 g/kg of crude glycerin in the diet.

Table 4
Dry matter intake (DMI), average daily gain (ADG) and feed efficiency (G:F) in young bulls fed different levels of crude glycerin.

Variable	Glycerin levels (g/kg)				SEM	Gly[1]	L[2]	Q[3]
	0	60	120	180				
DMI, kg/d	11.8	11.9	11.8	11.0	0.178	0.41	<0.01	<0.01
ADG 0–28, kg/d	1.84	1.85	2.11	1.95	0.132	0.40	0.32	0.54
ADG 28–56, kg/d	1.82	2.11	2.08	1.95	0.149	0.20	0.57	0.18
ADG 56–84, kg/d	1.60	1.79	1.45	1.49	0.116	0.87	0.20	0.49
ADG 0–84, kg/d	1.75	1.92	1.88	1.80	0.007	0.32	0.80	0.21
G:F[4]	0.148	0.160	0.158	0.172	0.007	0.08	0.04	0.91

[1] Gly = 0 vs. Glycerin.
[2] L = Linear.
[3] Q = Quadratic.
[4] Gain:feed ratio.

Table 5
Final Body weight (FBW), hot carcass weight (HCW), cold carcass weight (CCW), longissimus dorsi muscle area (LMA), LMA per 100 kg cold carcass (LMA/100 kg), carcass yield (CY), subcutaneous fat thickness (SFT), and marbling score of young bulls fed different levels of crude glycerin.

Variables	Glycerin levels (g/kg)				SEM	Gly[1]	L[2]	Q[3]
	0	60	120	180				
FBW, kg	513	522	527	516	14.9	0.61	0.82	0.77
HCW, kg	284	289	299	291	9.2	0.39	0.85	0.36
CCW, kg	278	283	287	279	8.5	0.62	0.89	0.73
CY, g/kg	554	555	568	565	4.3	0.08	0.99	<0.01
LMA, cm^2	83.1	78.5	85.4	87.8	2.69	0.79	0.34	0.04
LMA, cm^2/100 kg	30.0	28.0	29.8	31.6	0.87	0.84	0.28	0.05
SFT, mm	3.18	3.27	3.18	3.18	0.433	0.95	0.89	0.91
Marbling score	336	342	540	534	62.3	0.07	0.97	<0.01

[1] Gly = 0 vs. Glycerin.
[2] L = Linear.
[3] Q = Quadratic.

4. Discussion

The concentrations of plasma creatinine are within the normal range (1–2 mg/dL) reported by Kaneko et al. (1997). This indicates that the kidney glomerular filtration rate for this metabolite was adequate, with no methanol interference of the crude glycerin. The glycerin used in the present study comprised 200 mg/kg of methanol, which is above the recommended value by United States Department of Agriculture (USDA) and Brazil Ministry of Agriculture (*Ministério da Agricultura, Pecuária e Abastecimento*) of 150 ppm.

The CK values, although not affected by the diet, were well above those recommended by Kaneko et al. (1997) of 4.8–12.1 U/L. This result can be explained by the exerted physical effort of the animals during handling for weighing; since CK is a sensitive and specific indicator of muscle injury. According to Hodgson (1994), increases of up to 500 U/L are normal in moderate physical effort situations. In joint analysis, increases in blood AST and CK may be suggestive of myonecrosis (Hodgson, 1994), what did not happen when animals fed glycerin. The GGT enzyme found in plasma is indicative of cholestasis, which is a hepatic failure in the excretion and secretion of pigments, bile acids and salts in ruminants (Santos et al., 2008). Similar to the other hepatic enzymes, there was no effect of the glycerin levels in the diet on GGT concentrations, which were also within the normal range of up to 140 U/L (Barros Filho, 1995). Therefore, glycerin added to the diets did not affect blood parameters, indicating a normal hepatic metabolism and no hepatocyte destruction.

The declining trend in blood glucose while using glycerin can indicate that tissues were more sensitive to insulin-mediated glucose uptake, since greater glycerol kinase activity occurred in the liver of these bulls, resulting in a possible greater liver gluconeogenesis. This enzyme is able to induce gluconeogenesis from the glycerol absorbed in the rumen and transported to the liver (Aschenbach et al., 2010). In this case, liver glucose production may have increased pancreas insulin secretion and then, glucose uptake by tissues as muscle. According to Majdoub et al. (2003), gluconeogenesis precursors stimulate insulin pancreatic secretion. The positive effect of the crude glycerin on marbling score also supports this theory. According to Chung et al. (2007), glucose plays a relevant role as carbon donor in *de novo* fatty acids biosynthesis.

The liver glycerol absorption occurs due to the presence of membrane proteins, specifically, aquaglyceroporin 9 (AQP 9) (Froger et al., 2001; Hibuse et al., 2005). According to Krehbiel (2008), glycerol has three fates in the rumen: escape into the post rumen (130 g/kg), fermentation in the rumen (440 g/kg) and absorption by the ruminal epithelium (430 g/kg). Therefore, due to the results in the glycerol kinase activity, a larger amount of glycerol and less propionate was probably absorbed in the rumen as glycerin levels in the diet increased. Besides, the lower expression of *PCK1*, which encodes cytosolic phosphoenolpyruvate carboxykinase enzyme (PEPCK-C), in the liver of animals fed high levels of glycerin, supports this last statement. According to Koser et al. (2008), bovine *PCK1* gene promoter is positively regulated by propionate, constituting a

feed-forward mechanism of substrate control for hepatic gluconeogenesis that is linked to the final products of rumen fermentation. Consequently, probably, ruminal propionate production increased when crude glycerin was used up to 59.1 g/kg of the diet (maximum value for *PCK1* expression curve) and then it decreased, reducing *PCK1* expression. In addition, in a study performed by Hales et al. (2015), methane production increased between 100 and 150 g/kg of glycerin, and it is well known that methane and propionate production are antagonistic, supporting the hypothesis of low propionate production when animals fed 180 g/kg of glycerin diet in the present study. Boyd et al. (2013) observed that molar proportions of butyrate were greater when animals were fed diets with glycerol inclusion and, according to these authors; ruminal butyrate would improve the growth of ruminal epithelial tissue and possibly increase nutrients absorption from the rumen, as well as glycerol.

Although glycerol kinase activity increased with the glycerin level in the diet, expression of *GK1*, the gene responsible to encode this enzyme, was inhibited with increasing levels of glycerin. Therefore, despite the positive effect of propionate in *PCK1* expression, glycerol seemed to exert a negative *feedback* in *GK1* expression. In this sense, studies evaluating which transcription factors regulate expression of genes involved in liver gluconeogenesis in ruminants are necessary. Yoon et al. (2001) showed that the Peroxisome Proliferator-Activated Receptor-γ Coactivator 1α (PGC-1α) is a key modulator of hepatic gluconeogenesis in mice, activating the *PCK* gene. In addition, Peroxisome Proliferator-Activated Receptor-γ (PPAR-gamma) activation also induces the expression of *PCK* in mice (Perry et al., 2014; Schultz et al., 2015). Probably, the important role of these transcription factors in liver of non-ruminants is a path where research in ruminants must follow.

The lower DMI when animals fed glycerin (Table 4) can be explained by a metabolic regulation and other glycerin components. In the first situation, the greater insulin secretion, as glycerin levels in the diet increased, may have affected negatively DMI. According to Plum et al. (2005) and Porte et al. (2005), insulin enter the brain and reacts with specific insulin receptors on neurons, reducing food intake. Besides the metabolic effect, according to Hales et al. (2015), high Na concentrations in cattle diet may have inhibitory effects on DMI. In the present study, glycerin had 36.7 g/kg of chlorides. Hales et al. (2015) also described that DMI reduction in cattle, as glycerin concentration is increased, seems consistent throughout literature. The linear negative effect on DMI and the lack of effect on ADG explained why feed efficiency increased linearly as glycerin increased in the diet. Therefore, animal performance observed in this study indicates that up to 180 g/kg of glycerol inclusion in DM has no negative impact, considering the type of diet used.

Similarly, Mach et al. (2009) found no effect on animals ADG when assessing up to 120 g/kg of crude glycerin inclusion in the diet. However, Parsons et al. (2009), working with diets with up to 160 g/kg of crude glycerin, observed a quadratic effect on animals ADG, and the maximum concentration recommended by the authors for glycerin was 80 g/kg on DM basis. The highest crude glycerin level analyzed for beef cattle so far was reported by Van Cleef et al. (2014), who evaluated the inclusion of up to 300 g/kg in the DM. The authors described that there were no differences among treatments for ADG and feed efficiency; however, they reported a negative effect of the treatment with 300 g/kg of glycerin on fiber digestibility. Therefore, the recommended level of crude glycerin is influenced by the glycerin quality, forage:concentrate ratio, protein source, corn processing *etc.*

One plausible explanation for the observed effect in carcass yield is a possible lower gastrointestinal content weight in the animals fed crude glycerin. Evaluating glycerol supply effect on stress, associated with long transport periods in Zebu cattle, Parker et al. (2007) observed no differences in the animals body weight after a trip of 24 h, similar to the results obtained in the present study. However, body water content increased in animals fed glycerol, possibly due to the action of glycerol in enhancing intestinal flow, and thus increasing the gastrointestinal content loss. According to Wythes et al. (1980), animal weight loss that takes place during fasting and extended transportation is a consequence of intestinal filling, urinary and defecation rates. Another explanation for the observed linear increase in carcass yield is that glycerol has positive effects on amino acids and nitrogen retention in the body, leading an economy in gluconeogenic amino acids usage. The increase in glycerol kinase activity supports this statement.

5. Conclusion

The use of glycerin increased feed efficiency and beef marbling and, therefore, at a level of up to 180 g/kg of DM is recommended for feedlot diets. Glycerol kinase activity and expression of *GK1* and *PCK1* genes were affected with the use of crude glycerin, which influence liver gluconeogenesis.

Conflict of interest

The authors declare that there are no conflicts of interest.

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