Ciência Rural, Santa Maria, v.50:8, e20190948, 2020
http://doi.org/10.1590/0103-8478cr20190948
ISSNe 1678-4596
ANIMAL PRODUCTION

Guanidinoacetic acid supplementation on growth performance and molecular mechanisms of lean mass gain in nursery pigs

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ABSTRACT: *The objective with the present study was to evaluate the effect of guanidinoacetic acid (GAA) on the growth performance of nursery piglets as well as a possible molecular mechanism of action on lean mass gain. Seventy-two pigs, weaned at 21 d, weighing 6.80 ± 1.2 kg were distributed in a completely randomized design into one of three dietary treatments (control, control + 1.2 g/kg GAA or control + 2.4 g/kg GAA) and 8 replicates per treatment. The control diet was an animal protein-free diet based on corn and soybean meal. Body weight, average daily weight gain, average daily feed intake and feed efficiency were evaluated at 35, 49, and 56 days. At the end of the experiment, one animal per pen was slaughtered and samples of the vastus lateralis muscle were collected for RT-qPCR and protein abundance analysis. Overall (from 21 to 56 d), GAA supplementation improved feed efficiency (P < 0.03). Skeletal muscle of pigs fed with GAA diet had greater mRNA expression of Akt (P < 0.04) and RPS6KB2 (P<0.01). In conclusion, supplementation with 2.4 g/kg GAA to nursery piglets improves feed efficiency and activates molecular mechanisms important to lean mass gain.*
Key words: *gene expression, growth performance, guanidinoacetic acid, piglet, protein synthesis.*

Suplementação de ácido guanidinoacético no desempenho e mecanismo moleculares de ganho de massa magra em leitões

RESUMO: *O objetivo do presente estudo foi avaliar o efeito do ácido guanidinoacético (GAA) no desempenho de leitões, bem como um possível mecanismo de ação molecular no ganho de massa magra. Setenta e dois leitões, desmamados aos 21 dias, pesando 6,80 ± 1,2 kg, foram distribuídos em um delineamento inteiramente casualizado com três tratamentos dietéticos (controle, controle + 1,2 g / kg ou controle + 2,4 g / kg GAA) e 8 repetições por tratamento. A dieta controle não continha proteína animal e foi formulada a base de milho e farelo de soja. O desempenho dos animais foi avaliado aos 35, 49 e 56 dias. Ao final do experimento, um animal por unidade experimental foi abatido e amostras do músculo Vastus lateralis foram coletadas para análise de RT-qPCR e abundância de proteínas. A suplementação com GAA melhorou a eficiência alimentar (P<0,03) aos 56 dias. O músculo dos leitões suplementados apresentou maior expressão de mRNA de Akt (P<0,04) e RPS6KB2 (P <0,01). Em conclusão, a suplementação de 2,4 g / kg de GAA em leitões (21 a 56 d) melhora a eficiência alimentar e ativa mecanismos moleculares importantes para o ganho de massa magra.*
Palavras-chave: *ácido guanidinoacético, desempenho, expressão gênica, leitões, síntese proteica.*

INTRODUCTION

Interest in guanidinoacetic acid (GAA), as a nutritional supplement in muscle energy metabolism, has been discussed in recent years (MICHIELS et al., 2012; RAHIMNEJAD et al., 2017; HE et al., 2018). GAA is a natural creatine precursor, synthesized mainly in the kidneys from the amino acids glycine and arginine, and is subsequently methylated into creatine within the liver, by de novo synthesis. It enhances creatine availability (RINGEL et al., 2007; HE et al., 2018), a key component in energy metabolism, transferring energy from ADP to ATP in the muscle cell.

Corn and soybean-based diets, as generally used in pig production, have low creatine content. Therefore, the capacity of de novo synthesis might be a limiting factor and supplementation via feed can be beneficial. Unlike creatine, GAA is more stable during feed processing, less expensive and highly bioavailable (BAKER, 2009). Hence, GAA supplementation could substitute dietary creatine, improve its synthesis, and save amino acids for other functions such as protein synthesis and components to cope with oxidative stress (nitric oxide) (MICHIELS et al., 2012).

Received 12.01.19 Approved 04.14.20 Returned by the author 05.22.20
CR-2019-0948.R1

Supplementation with GAA has been determined to increase animal performance and carcass characteristics in growing-finishing pigs (WANG et al., 2012; HE et al., 2018). Therefore, in weaned piglets, dietary GAA could also improve protein deposition by restoring creatine levels in muscle and enhancing genes expression related to muscle hypertrophy, as the demand for creatine is proportionately higher in growing animals than in adults, due to their rapidly growing tissues ratio (BROSNAN et al., 2009). It has been suggested that GAA plays a key role in protein synthesis by stimulating myogenic differentiation (e.g., growth factors – *MyoD*, *MyoG*), or regulating components in the anabolic protein synthesis signaling pathways (e.g., miRNA miR-133a-3-p- and miR-1a-3p) (WANG et al., 2018).

Although, there are extensive reports of the effects of GAA and creatine on performance in humans and pigs, there is limited information regarding the effects of dietary supplementation with GAA on the expression of genes involved in the mammalian target of rapamycin (mTOR), which is the primary biological pathway controlling protein synthesis. Therefore, we hypothesized that GAA supplementation would improve growth performance and activate molecular mechanisms important in lean mass gain. The objective of the present study was to evaluate the effect of GAA on the growth performance of nursery pigs as well as a possible molecular mechanism of action on lean mass gain.

MATERIALS AND METHODS

Animals and housing

Seventy-two pigs (AGPIC 415×Camborough [Agroceres PIC, Patos de Minas, MG, Brazil]), females and castrated males, weaned at 21 d, weighing 6.80 ± 1.2 kg were randomly allocated to 1.65 × 1.05 m suspended pens with plastic floors at the swine research facility of Universidade Federal de Viçosa, Viçosa, MG, Brazil. Each pen housed 3 pigs (0.58 m²/pig) and had a dry feeder and a nipple drinker. Pigs had free access to feed and water throughout the 35-d feeding trial.

Experimental design and diets

Pens were allotted in a completely randomized design with three dietary treatments: control, control + 1.2 g/kg GAA, or control + 2.4 g/kg GAA (purity>96%; CreAMINO®). The analyzed levels of GAA in the diets were: undetectable, 1.1 g/kg, and 2.3 g/kg, respectively.

A pen with 3 pigs was considered as the experimental unit, males and females were evenly distributed between treatments. There were 8 replicates per treatment. The control diet was an animal protein-free diet based on corn and soybean meal and it was formulated according to the ideal amino acid profile recommended by the Brazilian Tables for Poultry and Swine (ROSTAGNO et al., 2011; Table 1). The GAA was added on top over the control diet.

Performance and sample collection

Throughout the trial, feed was weighed before feeding and feed wastage was collected and weighed daily to determine average daily feed intake. At 35, 49 and 56 d (end of the experiment), pigs were individually weighed to determine body weight (BW), average daily gain, and feed efficiency.

At 56 d, after a 12 h fasting, one pig per pen, with BW closest to pen average, was slaughtered. The pig was rendered unconscious using head-only electrical stunning (240V, 1.3A). Right after exsanguinated, biopsy samples were taken from the *vastus lateralis* and were immediately stored in RNA later (Life Technologies, Carlsbad, CA, USA) and kept at -20 °C until RNA and protein extraction analysis (only control and control + 2.4 g/kg of GAA treatments were analyzed for practical reasons).

RNA extraction, cDNA synthesis and RT-qPCR

Total RNA extraction was performed from 50 mg of the *vastus lateralis* using TRIzol® (Invitrogen™) according to the manufacturer's instructions. Briefly, 1 mL of TRizol® was added to a tube containing 50 mg of tissue and homogenized for 30 seconds using tissue disrupter TX10 (Ika, Königswinter, Germany) for 30 seconds. After homogenization, 0.2 mL of chloroform was added and the tubes were shaken vigorously. The homogenate was incubated for 5 min at room temperature and then centrifuged at 12,000 x g and 4°C for 10 minutes. After centrifugation, the supernatant was transferred to a tube containing 0.5 mL of isopropyl alcohol, incubated for 5 min at room temperature, and then centrifuged at 12,000 x g and 4°C for 10 min. The pellet was washed twice by adding 75% (v/v) ethanol the centrifuged at 12,000 x g and 4 °C for 8 minutes. The final precipitate was rehydrated with 30 µL of UltraPure® DNase/RNase-Free water. The RNA concentration was estimated using a NanoVue Plus TM spectrophotometer (GE Healthcare, Munich, Germany), with A260/A280 ratios between 1.8 and 2.0 serving as purity control. The RNA was then

Table 1 - Ingredients and calculated nutritional composition of the control diets fed from 21 to 35, 35 to 49 and 49 to 56 days old.

Ingredients[1], g/kg	----------Days of age----------		
	21-35	35-49	49-56
Corn	449.6	507.3	561.2
Soybean meal	148.7	175.0	261.5
Soybean micronized	180.0	180.0	100.0
Dried whey	130.0	100.0	50.0
Milk whole, dried	50.0	0.0	0.0
Dicalcium phosphate	15.9	15.5	13.4
Limestone	5.7	6.9	7.6
Salt	3.7	3.4	3.6
Zinc Oxide	3.0	2.0	0.0
Mineral-vitamin premix[2]	2.0	2.0	2.0
L-lysine, 78%	4.9	3.9	0.5
DL-methionine	2.5	1.8	0.2
L-threonine	2.3	1.6	0.0
L-valine	1.2	0.4	0.0
L-tryptophan	0.5	0.2	0.0
----------Calculated nutritional composition----------			
Metabolized energy, kcal/kg	3400	3375	3230
Crude protein, %	21.00	21.00	21.00
SID lysine[3], %	1.45	1.33	1.07
SID threonine, %	0.91	0.83	0.70
SID Met + Cys	0.81	0.74	0.60
SID tryptophan	0.26	0.23	0.22
SID valine	1.00	0.91	0.88
Calcium, %	0.85	0.82	0.77
Available phosphorus, %	0.50	0.45	0.38
Sodium, %	0.28	0.23	0.20

[1]Experimental diets were obtained by adding CreAMINO® (purity > 96%, Evonik Degussa Gmbh, Essen, Germany) at 1.2 and 2.4 g/kg into the control diet on top.
[2]Vitamin-mineral premix provided the following per kilogram of complete diet: vitamin 12,000 IU; vitamin D3 - 2,250 IU; vitamin E - 65 IU; vitamin K – 3 mg; thiamine - 2.25; riboflavin - 6mg; pyridoxine - 2.25 mg; vitamin B12 - 27mcg; folic acid - 400mcg; biotin – 150 mcg; pantothenic acid - 22.5mg; niacin - 45mg; copper – 10 mg; iodine - 1.5mg; iron – 100 mg; manganese - 40mg; selenium 0,3 mg; zinc – 100 mg;
[3]SID = standardized ileal digestible.

treated with RNase-free DNase I, (Thermo Scientific, Massachusetts, USA) following the manufacturer's instructions. The quality and integrity of the extracted RNA were verified using a 1% agarose gel. Subsequently, the samples were reverse transcribed into cDNA using the GoScript Reverse Transcription (RT) Kit (Promega, Madison, WI), following the manufacturer's recommendations. The cDNA samples were stored at -20°C until use in real-time quantitative polymerase chain reaction (RT-qPCR).

Primers for amplification of the target (translation initiation factor 4E binding protein 1, 4E-BP1; serine/threonine kinase 1, Akt; ribosomal protein S6 kinase B1, RPS6KB1; ribosomal protein S6 kinase B2, RPS6KB2) and endogenous gene fragments were designed using PrimerQuest software provided by Integrated DNA Technologies, Inc. (Coralville, IA) using nucleotide sequences obtained from the GenBank database (http: // www. ncbi.nlm. nih.gov) (Table 2).

The β-actin, HPRT1 (hypoxanthine-guanine phosphoribosyltransferase 1), and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) genes were tested as reference genes to be used in standardization analyses to minimize possible variations in the amount of initial mRNA and the efficiency of reverse transcription. Selection of the reference gene was based on the amplification

Table 2 - List of primers sequences for gene expression analysis by RT-qPCR.

Item[1]	GeneBank no.	Primer sequence	Size (bp)
4E-BP1	NM_001244225.1	F: CAGCCAGGCCTTATGAAAG	134
		R: AGGCACATGGAGGTATCTG	
Akt	NM_001159776.1	F: TCAGGGCTGCTCAAGAA	112
		R: CATACACATCCTGCCACAC	
RPS6KB1	XM_021067293.1	F: CAGTGAAAGTGCCAACCA	109
		R: TCGAGGTGACCGGATTT	
RPS6KB2	XM_021082757.1	F: GCAATGCCAAGGACACA	127
		R: CACTCGAGGATGAGGTAGAG	

Abbreviation: 4E-BP1, Translation initiation factor 4E binding protein 1; Akt, Serine/threonine kinase 1; RPS6KB1, Ribosomal protein S6 kinase B1; RPS6KB2, Ribosomal protein S6 kinase B2; F: Forward; R: Reverse; Annealing temperature: 60 °C.

efficiency of candidate genes from the efficiency calculation for each primer pair (at 100, 200 and 400 mM concentrations) using the formula E= 10(-1/slope of the line)-1, where E is the reaction efficiency (PFAFFL, 2001). In addition, the profile of the amplification and dissociation curves and the amplification stability of these genes between treatments were used. Due to the lack of difference in mRNA expression between treatments (P<0.10), β-actin was chosen as reference gene.

The RT-qPCR analyses were performed in duplicate in an ABI Prism 7300 Sequence Detection Systems thermocycler (Applied Biosystems - Foster City, CA, USA) using the Relative Quantification method and applying the SYBR® Green system (Applied Biosystems - Foster City, CA, USA) and the GoTaq® qPCR Master Mix kit (Promega Corporation, Madison, USA). The PCR reactions were subjected to 95 ºC for 3 minutes, 40 cycles of 95 ºC for 15 seconds, and 60ºC for 1 minute. The target gene values of the threshold cycle (Ct) obtained were later normalized (ΔCt) based on the Ct values obtained for the endogenous control gene (β-actin). Calculation of the relative gene expression levels was performed according to the $2^{-\Delta Ct}$ method (LIVAK & SCHMITTGEN, 2001).

Protein abundance quantification by Western-blot analysis

Total protein of *vastus lateralis* muscle was extracted from 0.1 g of macerated tissue in 1 mL of lysis buffer (10 mM Tris HCl, 100 mM of NaCl, 0.5 mM of DDT [dithiothreitol], 2.5 mM of 0.5% MgCl2, and triton X-100) and 1% protease inhibitor cocktail (Sigma-Aldrich®). Protein content was measured by the Bradford protein assay (Bio-Rad, Hercules, CA), and samples stored at -80 °C.

Proteins were separated by SDS-PAGE, with 10% gels loaded with 60 µg of protein per sample. Proteins were transferred to nitrocellulose membranes and blocked with blocking solution (3% BSA [bovine serum albumin, Sigma-Aldrich®]) in TBS1x (tris-buffered saline) for 1 h with gentle agitation at room temperature. Membranes were then incubated with primary antibodies against p-AMPK and p-mTOR (no. 2535 and no. 2971, respectively, [Cell Signaling, Danvers, MA, USA]). Only the abundance of primary proteins was assessed because both proteins are highly produced by any tape of cell and we had no hypothesis for changing in non-phosphorylated proteins as consequence of dietary treatments. Primary antibodies were incubated at 1:1000 diluted in 10 mL blocking solution for 12 h at 4 °C with gentle agitation. After incubation with primary antibodies, membranes were washed 3 times, for 5 minutes each, at 4 ºC with Tris-Buffered Saline and 0.1% Tween® (TBSt) and incubated with the secondary antibody (anti-rabbit IgG- Cell signaling®) at 1:5000 dilution, for 1h at 4 ºC with gentle agitation. Subsequently, membranes were washed 3 times (5 min each) with TBSt, revealed by ECL ClarityTM substrate (Bio-Rad, Hercules, CA), the images were generated by c-Digit® Blot scanner (Licor Biosciences, Nebraska, USA), and bands quantified by densitometry using the software Image Studio Digits Lite version 5.2 (Licor Biosciences). Each SDS-PAGE gel contained protein extracted from all the treatments, as well as an internal loading control sample used for signal normalization. For the internal control, we used two reference samples from the same tissue and experiment loaded on each gel. The internal control which presented greater band intensity (expressed by optic densitometry units) was

used to normalize the remaining samples as described by CRUZEN et al. (2014).

Statistical analysis

For gene expression and protein analysis samples from one pig per pen were collected. This data was analyzed following the model: $y_{ij}=m+T_j+e_{ij}$, where, y_{ij} is the observed variable of i^{th} animal in treatment j; m is the mean; T_j is the j^{th} treatment (control or control + 2.4 g/kg GAA); and e_{ij} is the random residual.

Performance data were analyzed using the same model including initial BW as co-variable; however, if initial BW covariate was not significant (P > 0.05) it was removed from the model. .

The residuals from all analyses were submitted to Shapiro-Wilk normality test. Only the residuals of 4EBP1 gene expression data did not follow normal distribution and it was transformed using: $\log(2^{-\Delta Ct}+1)$.

The normality test and ANOVA analyses were performed using the functions shapiro.test and anova of R software (https://cran.r-project.org/). The least-squares means of treatments were computed using the lsmeans package (https://cran.r-project.org/web/packages/lsmeans/lsmeans.pdf) of R software. Results were deemed significant when $P < 0.05$; trends were determined as $0.10 > P > 0.05$. When necessary, performance average values were compared using the Tukey test.

RESULTS

The supplementation of GAA did not influence (P>0.10) piglet growth performance from 21 to 35 d and from 21 to 49 d (Table 3). However, from 21 to 56 d, pigs fed 2.4 g/kg GAA had improved feed efficiency (P<0.03) compared to the control group with no difference from 1.2 g/kg GAA group, while differences in final BW, average daily gain and feed intake were not significant (P>0.10).

The mRNA expression of target genes and abundance of target proteins in skeletal muscle tissue were assessed only in pigs fed the control and the 2.4 g/kg GAA diets due to the lack of significance in pig performance with 1.2 g/kg GAA supplementation. Skeletal muscle of pigs fed 2.4 g/kg GAA had greater mRNA expression of *Akt* (P<0.04) and *RPS6KB2* (P<0.01; Figure 1). The mRNA expression of 4E-BP1 (P = 0.06) tended to be greater in skeletal muscle

Table 3 - Performance of nursery pigs supplemented with 0.0, 1.2 or 2.4 g/kg of guanidinoacetic acid[1].

Item[2]	----------Guanidinoacetic acid, g/kg----------			SEM[3]	*P-value*
	0.0	1.2	2.4		
21-35 d					
Initial BW, kg	7.2	6.6	6.9	-	-
BW 35 d, kg	10.9	10.9	11.0	0.38	0.96
ADG 35 d, g	288	283	294	27.4	0.96
ADFI 35 d, g	384	380	370	31.1	0.94
G:F 35 d	744	743	785	21.7	0.31
21-49 d					
BW 49 d, kg	20.4	19.9	20.4	0.66	0.84
ADG 49 d, g	481	464	481	23.7	0.84
ADFI 49 d, g	697	664	664	35.8	0.75
G:F 49 d	693	702	727	11.8	0.13
21-56 d					
BW 56 d, kg	24.3	23.9	24.7	0.75	0.71
ADG 56 d, g	495	482	507	21.6	0.71
ADFI 56 d, g	791	761	768	33.2	0.79
G:F 56 d	627a	634ab	661b	9.4	0.03

[1]Values are the means of eight replicates per treatment with 3 pigs per pen.
[2]BW, Body weight; ADG, average daily weight gain; ADFI, average daily feed intake; G:F, feed efficiency.
[3]Standard error of the mean.
[a-b]Means within a row with different letters differ (P < 0.05) using the Tukey test.

of pigs fed 2.4 g/kg GAA compared to pigs fed the control diet. The mRNA expression of *RPS6KB1* did not differ (P>0.10).

In the assessment of protein abundances as energy metabolism markers in the skeletal muscle, there was a tendency in the abundance of p-AMPK (P = 0.08) and p-mTOR (P = 0.06) in skeletal muscle of pigs fed 2.4 g/kg GAA compared to those fed the control diet (Figure 2).

DISCUSSION

It is estimated that approximately 2/3−3/4 of the daily creatine requirement is synthesized *de novo*, while the remainder must be supplied by the feed (RINGEL et al., 2007). However, in growing livestock, *de novo* synthesis may not be sufficient to sustain the growth potential. We hypothesized that dietary GAA, the single immediate creatine precursor in animal tissue, could improve creatine storage in the skeletal muscles of pigs and accordingly improve growth performance. Although, 1.2 g/kg is the practical level used in commercial production systems, 2.4 g/kg GAA was chosen to evaluate how pigs would alter their energy metabolism when higher levels of GAA are supplemented.

There was no effect of 1.2 g/kg GAA supplementation over performance compared to the control group, consistent with TEIXEIRA et al. (2017) using similar level of GAA. Supplementation of 2.4 g/kg GAA did not influence performance from 21 to 35 d or 21 to 49 d, and it significantly improved feed efficiency from 21 to 56 d (overall phase) compared to the control group. This suggests that to improve feed efficiency, creatine storage has to build up to be effective, which takes time to achieve. Moreover, because creatine provides ATP for muscle contraction and metabolism, it likely resulted in better feed efficiency in the pigs supplemented with 2.4 g/kg GAA. Increased feed efficiency with dietary supplementation of GAA in growing-finishing pigs was observed by HE et al. (2018), who also reported that in general, the longer the experiment lasted, the more sufficiently the effects of a feed additive can be evaluated.

In the current study we observed that 2.4 g/kg GAA supplementation increased the mRNA expression of *RPS6KB2* and tended to increase *4E-BP1* in skeletal muscle of pigs. These genes encode proteins that are downstream-regulated by mTOR and among many roles, control ribosome biogenesis,

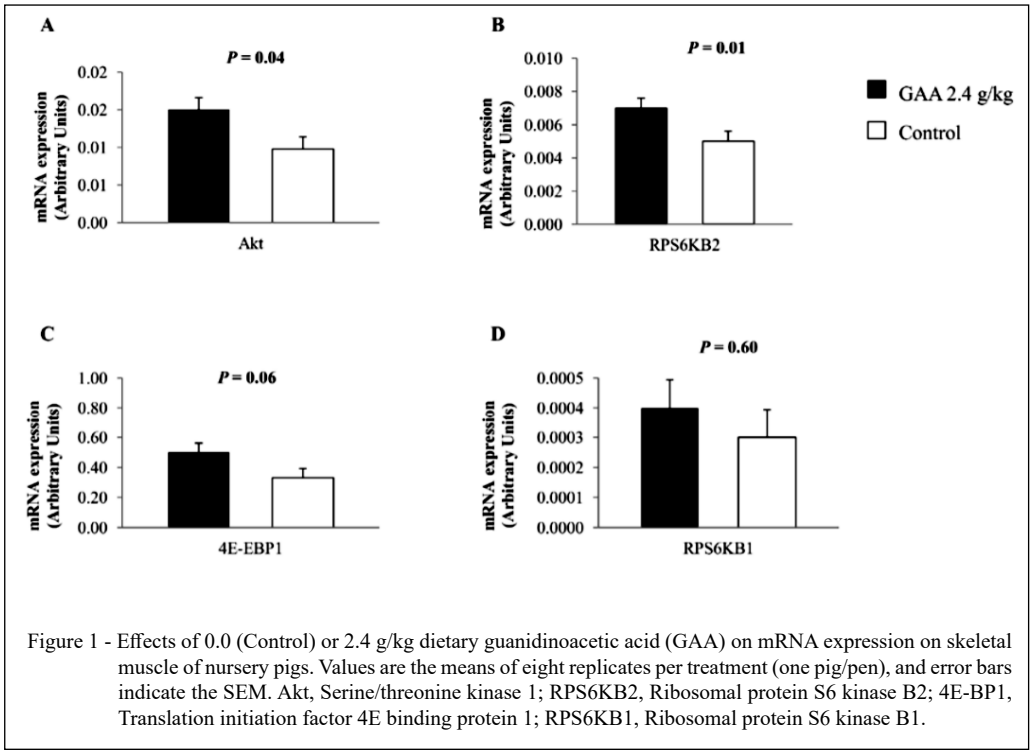

Figure 1 - Effects of 0.0 (Control) or 2.4 g/kg dietary guanidinoacetic acid (GAA) on mRNA expression on skeletal muscle of nursery pigs. Values are the means of eight replicates per treatment (one pig/pen), and error bars indicate the SEM. Akt, Serine/threonine kinase 1; RPS6KB2, Ribosomal protein S6 kinase B2; 4E-BP1, Translation initiation factor 4E binding protein 1; RPS6KB1, Ribosomal protein S6 kinase B1.

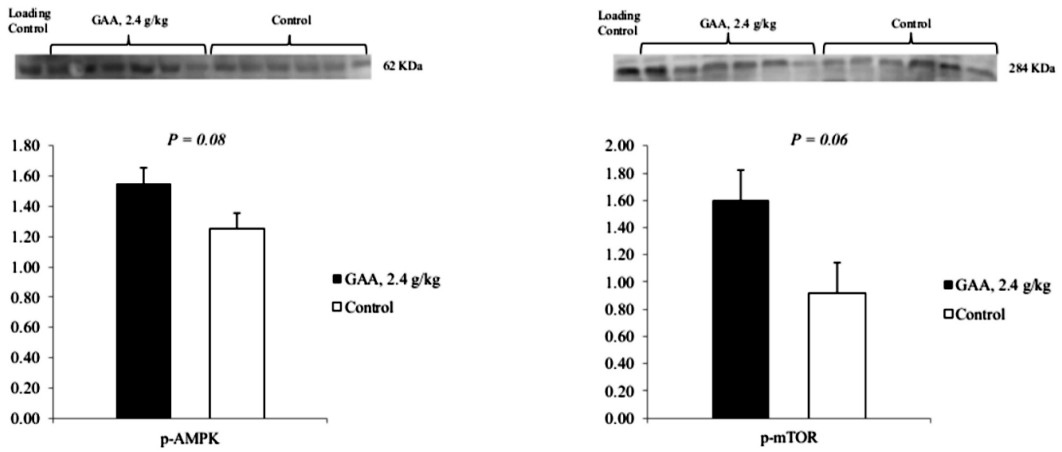

Figure 2 - Representative images of western-blot analysis and protein abundance for p-AMPK (A) and p-mTOR (B). The protein abundance was measured in skeletal muscle of nursery pigs supplemented with 0.0 (Control) and 2.4 g/kg guanidinoacetic acid (GAA). Values are the means of eight replicates per treatment (one pig/pen), and error bars indicate the SEM.

stimulate mRNA translation, and inhibit apoptosis (LAPLANTE & SABATINI, 2009), which leads to increase in cell size. These events have been shown to result from an action of mTOR on 4E-BP1 and ribosomal S6 kinases (LAPLANTE & SABATINI, 2009; MA & BLENIS, 2009). Moreover, our results also show that the mRNA expression of *Akt* was greater in pigs supplemented with 2.4 g/kg GAA compared to the control group. This may reinforce the action of the supplement in muscle hypertrophy through the mTOR pathway, as the activation of Akt/mTOR pathway is a requisite for muscle hypertrophy *in vivo* (BODINE et al., 2001).

Previous studies have associated the effects of creatine supplementation and increased lean deposition with the mTOR signaling pathway (FARNAZ et al., 2017; FERRETTI et al., 2018). The mTOR is a serine/threonine kinase that plays an essential role in skeletal muscle deposition. As such, the mTOR signaling pathway is sensitive to several environmental and intracellular changes such as nutrient availability and energy status (YOON, 2017), and coordinates diverse cellular processes including cell growth, differentiation, autophagy, survival, and metabolism (LAPLANTE & SABATINI, 2012). In the current study, in addition to changes in the mRNA expression of genes that encode key proteins in mTOR pathway, we also observed that animals supplemented with 2.4 g/kg GAA tended to have a greater abundance of p-mTOR compared to animals from the control group. Altogether, our data suggest that that mTOR pathway is somehow changed as a consequence of GAA supplementation and may contribute to changes in overall feed efficiency of nursery pigs.

Another notable result of the current study was a tendency for greater p-AMPK on skeletal muscle of pigs supplemented with 2.4 g/kg GAA, compared to the control group. It has been shown that during energy stress AMPK is activated when ATP breakdown to ADP accelerates (HARDIE, 2018). Moreover, AMPK's response to the decrease in the ATP:AMP ratio is crucial for the maintenance of appropriate ATP levels because it causes ATP-generating catabolic process (THOMSON, 2018). One of the main catabolic process led by activation of AMPK is fatty acid oxidation (O'NEILL et al., 2013). Therefore, the tendency of greater p-AMPK in skeletal muscle of GAA-supplemented pigs may have occurred as a consequence of increased muscle mass and/or mitochondrial biogenesis, as both events are caused by increased mTOR activation. Altogether, our data are consistent with the hypothesis that creatine would be increased by providing dietary GAA (RINGEL et al., 2007; HE et al., 2018), which ultimately would increase the skeletal muscle requirement for ATP; and consequently, cause changes in cellular energy pathways such as AMPK and mTOR.

CONCLUSION

In conclusion, supplementation of 2.4 g/kg of GAA to nursery pigs (21 to 56 d) improves feed efficiency and actives molecular mechanisms important to lean mass gain.

ACKNOWLEDGMENT

We gratefully thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, code 001), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Instituto Nacional de Ciência Animal (INCT-CA).

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The experimental protocol has followed the ethical principles in animal welfare of the Brazilian National Council for Animal Experimentation Control (CONCEA, 2016) and was previously approved by the Ethical Committee on Animal Use of Universidade Federal de Viçosa (UFV) [protocol no. 01/2018].

DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript.

REFERENCES

BAKER, D. H. Advances in protein–amino acid nutrition of poultry. **Amino Acids**, v.37, n.1, p.29-41. 2009. Available from: <https://doi.org/10.1007/s00726-008-0198-3>. Accessed: Mar. 18, 2018. doi: 10.1007/s00726-008-0198-3.

BODINE, S. C., et al. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. **Nature Cell Biology**, v.3, p.1014. 2001. Available from: <https://doi.org/10.1038/ncb1101-1014>. Accessed: Feb. 01, 2019. doi: 10.1038/ncb1101-1014.

FARNAZ, F., et al. Creatine supplementation and skeletal muscle metabolism for building muscle mass-review of the potential mechanisms of action. **Current Protein & Peptide Science**, v.18, n.12, p.1273-1287. 2017. Available from: <http://www.eurekaselect.com/node/152946/article>. Accessed: Jun. 30, 2018. doi: http://dx.doi.org/10.2174/1389203718666170606105108.

FERRETTI, R., et al. High-fat diet suppresses the positive effect of creatine supplementation on skeletal muscle function by reducing protein expression of IGF-PI3K-AKT-mTOR pathway. **Plos One**, v.13, n.10, p.e0199728. 2018. Available from: <https://doi.org/10.1371/journal.pone.0199728>. Accessed Jul. 30, 2019. doi: http://dx.doi.org/10.1371/journal.pone.0199728.

HARDIE, D. G. Keeping the home fires burning: AMP-activated protein kinase. **J R Soc Interface**, v.15, n.138. 2018. Available from: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=29343628>. Acesso em. doi: rsif.2017.0774 [pii] 10.1098/rsif.2017.0774.

HE, D. T., et al. Effects of guanidinoacetic acid on growth performance, creatine and energy metabolism, and carcass characteristics in growing-finishing pigs1. **Journal of Animal Science**, v.96, n.8, p.3264-3273. 2018. Available from: <http://dx.doi.org/10.1093/jas/sky186>. Accessed Jul. 15, 2019. doi:10.1093/jas/sky186.

LAPLANTE, M.; D. M. SABATINI. mTOR signaling at a glance. **Journal of Cell Science**, v.122, n.20, p.3589. 2009. Available from: <http://jcs.biologists.org/content/122/20/3589.abstract>. Accessed: Jan. 01 2018. doi: 10.1242/jcs.051011.

LAPLANTE, M.; DAVID M. SABATINI. mTOR Signaling in Growth Control and Disease. **Cell**, v.149, n.2, p.274-293. 2012. Available from: <http://www.sciencedirect.com/science/article/pii/S0092867412003510>. Accessed. Oct. 12, 2013. doi: https://doi.org/10.1016/j.cell.2012.03.017.

LIVAK, K. J.; T. D. SCHMITTGEN. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. **Methods**, v.25, n.4, p.402-408. 2001. Available from: <http://www.sciencedirect.com/science/article/pii/S1046202301912629>. Accessed: Oct. 12, 2013. doi: http://dx.doi.org/10.1006/meth.2001.1262.

MA, X. M.; J. BLENIS. Molecular mechanisms of mTOR-mediated translational control. **Nature Reviews Molecular Cell Biology**, v.10, p.307. 2009. Available from: <https://doi.org/10.1038/nrm2672>. Accessed: Jan. 22, 2019 doi: 10.1038/nrm2672.

O'NEILL, H. M., et al. AMPK regulation of fatty acid metabolism and mitochondrial biogenesis: Implications for obesity. **Molecular and Cellular Endocrinology**, v.366, n.2, p.135-151. 2013. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0303720712003334>. Acesso em. doi: https://doi.org/10.1016/j.mce.2012.06.019.

PFAFFL M. W. A new mathematical model for relative quantification in real-time RT-PCR. **Nucleic Acids Research**, v.29, n.9, p.e45-e45, 2001. Available from: <https://academic.oup.com/nar/article/29/9/e45/2384081>. Accessed: Jan. 11, 2018. doi: https://doi.org/10.1093/nar/29.9.e45.

RINGEL, J., et al. Effects of graded levels of creatine and guanidino acetic acid in vegetable-based diets on performance and biochemical parameters in muscle tissue. **Proceedings of the 16th European Symposium on Poultry Nutrition**, 2007. p.387-390.

ROSTAGNO, H. S., et al. **Brazilian tables for poultry and swine. Composition of feedstuffs and nutritional requirements**. Viçosa: Suprema Gráfica. 2011.

THOMSON, D. M. The Role of AMPK in the Regulation of Skeletal Muscle Size, Hypertrophy, and Regeneration. **Int J Mol Sci**, v.19, n.10. 2018. Disponível em: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=30314396>. Acesso em. doi: ijms19103125 [pii] 10.3390/ijms19103125.

YOON, M.-S. mTOR as a Key Regulator in Maintaining Skeletal Muscle Mass. **Frontiers in physiology**, v.8, p.788-788. 2017. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/29089899>. Accessed: Jan. 24, 2019. doi: 10.3389/fphys.2017.00788.