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Influence of the forms of α-ketoglutarate as feed additive on some blood indices and performance of growing rats

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Summary

Alpha-ketoglutarate (AKG), a derivative of glutaminic acid and glutamate, was shown to increase muscle protein synthesis as well as to have a positive effect on the quality of bone strength. The aim of this study was to investigate the effects of AKG supplemented either as a salt (Ca + AKG) of pH 5 – AKG 5, or in the pure form of pH 2 (AKG 2) on rats' growth, performance, feed utilization, some blood parameters and histology of the small intestine. Sixty four rats were divided into 4 treatments and stayed on trial for 9 (n = 6) or 18 days (n = 10). The AKG 2 treated rats were observed to generally have the lowest average daily gain (ADG) with a high average daily food intake (ADFI). The only significant difference found was a reduced ($P < 0.03$) feed efficacy on day 9 of the AKG 2 treatment from that of the control group. All dietary treatments showed higher Hb levels than the controls on day 9, with those of dextrose and AKG 2 being significant ($P < 0.03$ and $P < 0.005$, respectively). The enterocyte crypt depth in the proximal small intestine of the AKG 2 treated rats was significantly enlarged in comparison to that of the dextrose group. From day 9 to day 18, the control as well as the dextrose and the AKG 2 treatments showed an increase in the free Gln levels, while the AKG 5 group showed a decrease in free Gln levels over time. In the AKG 2 group, the level of peptide bound (PB) Gln + Glu was higher than in controls.

Keywords: α-ketoglutaric acid, rats

The information about the possible effect of orally administered of AKG on growth and performance is limited. Pharmacokinetics studies showed that large amounts of AKG are metabolized in first-pass by the splanchnic tissues (80% intake), however, nonoxidative pathways predominate over complete metabolism to CO_2 (2, 6, 18, 19). Some AKG present in the diet is metabolized to proline, which justifies its possible application in treatment of bone metabolic diseases e.g. osteoporosis (5, 12, 16, 17, 24, 25). AKG increased plasma proline levels together with enhancing both mineralization and mechanical properties of bone tissue in turkeys (21, 22) and piglets (11).

Human studies suggested the potential usefulness of α-ketoglutarate combined with calcium in preventing bone loss in postmenopausal women. AKG--Calcium induced beneficial changes in serum levels of the bone resorption marker – CTX, which was consistent with the preservation of bone mass in the lumbar spine (7). However in above and other studies (10), the enteral AKG was provided to animals in different forms e.g. calcium salt or acid form, therefore the exact effect of acidity (pH) of administered AKG is still not known.

From the nutritional point of view, chemical form of a nutrient is important due to possible interfering of the complementary ions (e.g. Ca) with both release and absorption. Furthermore, acid or alkaline reaction can independently influence the metabolic effects of feed supplementation, e.g. low acidity in the stomach can inhibit intestinal Fe absorption and lead to sideropenic anemia. Therefore, the comparison of all of above mentioned clinical and experimental studies with AKG is difficult, because AKG was provided as pure acid form (10, 12), or as a Calcium (3, 7, 9, 15) and ornithine (6, 8, 13, 23) salts. Bearring in mind the biological role of AKG, except bone metabolism, growth, performance, maturation of the alimentary tract together with the levels of amino acids and hemoglobin seem to be important when analyzing the metabolic features of AKG supplementation (3, 8, 9, 15, 20).

The aim of this study was to investigate the effects of AKG supplemented either as a salt (Ca + AKG) of pH 5 – AKG 5, or in pure form of pH 2 (AKG 2) on

growing rat performance, feed utilization, some blood parameters and histology of the small intestine.

Material and methods

Animals and experimental design. Sixty four young (22 days old) male Sprague Dawley® outbred laboratory rats (M&B A/S, 8680 Rye, Denmark), were randomly divided into 4 experimental groups: 1 – control, 2 – dextrose, 3 – AKG 2, 4 – AKG 5, differing according to dietary treatment (tab. 1). The crude protein levels in all the test diets were 50% that of the standard rat feed (see below) to simulate poor nutritional status. Dextrose was tested as a positive control group, since AKG to some extent, has an energy supplementing effect. The effect of different chemical forms was tested using of α-ketoglutarate – one, as a pure AKG having a low reaction pH 2 (AKG 2) and one having an almost neutral reaction pH 5 (AKG 5). Rats were caged individually and had *ad libitum* access to feed and water. During a 6-d adaptation period, the rats were fed a BeeKay Standard Rat and Mouse Diet No 2. (BK002ES) (B&K Universal AB, Sollentuna, Sweden). They were maintained at 60% humidity with 12 h of light per 24 h at the special animal quarters of the Dept. of Animal Physiology, Lund University, and finally put in trial at 4 weeks of age. The body weight of the rats at trial start was 93 g ± 1 g, and groups were equalized with regard to body weight. Body weight, feed and water intake was measured every 3rd day. Treatments and experimental conditions were conducted according to the recommendations of the Federation of the European Laboratory Animal Science Associations (FELASA) concerning the protection of experimental animals, and the Lund University Ethical Committee gave approval. The trial consisted of two periods. Period I ran from day 0 to day 9, and on day 9, 6 rats from each group were selected and euthanized after collecting blood samples for the analyses. Selection of the 24 rats was performed by picking up at random one rat from each group. The remaining forty rats (10 in each group) stayed in trial till day 18, when they were euthanized with collecting blood samples. In addition, samples for GI-tract investigation were taken for histological analyses.

Blood sampling and amino acid analysis. Rats were euthanized with CO_2. From each rat, 2.0 mL of blood was collected by heart puncture, and immediately analyzed for the content of haemoglobin (Hb) (HemoCue B-Haemoglobin, HemoCue AB, Helsingborg, Sweden). The blood was then frozen and analyzed for the content of free amino acids (FAA) and total amino acids (TAA). Amino acid analyses from whole blood samples (TAA and FAA) were performed in triplicate by the Waters HPLC system and Waters AccQ Tag analysis kit (Waters Corporate, Milford, Mass. USA). Free amino acids were analyzed in samples that had been deproteinized by adding 6 mL of a 0.2 mol/L sulfosalicylic acid solution to 2.0 mL whole blood. Total amino acids were analyzed from the deproteinized samples hydrolyzed with 6.0 M HCl for 20 h. In order to obtain data with a minimal error, the AA analyses were performed in duplicate using two different chromatographic systems (Waters, 2000a, Waters, 2000b).

Histological analyses. One cm long whole thickness segments of the small intestine (proximal and distal) were excised and collected for histological analyses. The intestinal samples were fixed in Bouin's solution for 4 d, and stored in 70% ethanol for preparation. Afterwards, specimens were dehydrated in 96% and 99.8% ethanol, put in xylene and embedded in paraffin. Then, the serial, histological sections of 5 µm thickness were stained with hematoxylin and eosin for light microscopy analysis. The depth of crypts, length and width of villi, and thickness of the tunica mucosa of each sample were measured. On each slide, twenty well-oriented villi and crypts lying outside the area with Payer's patches were measured at small magnification with a Nikon optical binocular microscope coupled via the Nikon Camera to a PC computer with MultiScan v 6.08 software (4). The villi length was taken as the distance from the crypt opening to the tip of the villi, while the depth of crypt was measured from the base of the crypt to the level of the crypt opening.

Tab. 1. Composition of experimental diet [ab]

Components	Control	Dextrose	AKG 2	AKG 5
Ingredient, %				
Wheat	44.00	44.00	44.00	44.00
Corn starch (N-free)	40.63	40.13	40.13	40.13
Fish meal (70% CP)	6.08	6.08	6.08	6.08
Soya Oil	4.36	4.36	4.36	4.36
Monocalcium phosphate	1.27	1.27	1.27	1.27
Cellulose, 100%	1.27	1.27	1.27	1.27
Limestone	0.48	0.48	0.48	0.35
Vitamin premix	1.00	1.00	1.00	1.00
Salt	0.59	0.59	0.59	0.59
Lysine HCL, 100%	0.21	0.21	0.21	0.21
Threonine, 100%	0.11	0.11	0.11	0.11
AKG	–	–	0.50	–
Ca - AKG	–	–	–	0.629
Dextrose	–	0.50	–	–
Crude Protein, %	10.80	10.50	10.40	10.20

Explanations: a – average energy content, MJ, ME/kg = 14.74; b – average diet composition (crude protein): lysine = 5.43%, threonine = 3.53%, methionine + cysteine = 2.61%, calcium = 6.50%, phosphate = 4.88%; c – provided the following per 1000 g of diet: vitamin A, 5000 I.U; vitamin D, 500 I.U; vitamin E, 40 mg; vitamin K, 2 mg; vitamin B1, 2 mg; vitamin B2, 4 mg; pantothenic acid, 10 mg; niacin, 20 mg; vitamin B12, 20 µg; biotin, 0,2 mg

Statistical analysis. The concentration of peptide bound amino acids (PBAA) was calculated according to the following equation; PBAA = TAA – FAA where TAA (total amino acids) = concentration of amino acids after hydrolysis of deproteinized whole blood, and FAA (free amino acids) = concentration of deproteinized free amino acids in whole blood. During hydrolysis, the content of whole blood glutamine was converted to whole blood glutamate, in which case the total level of peptide-bound glutamine plus glutamate was calculated as: PB Gln + Glu = T Glu – (F Gln + F Glu).

Data on rat performance are expressed as mean values with standard error of the mean (means ± SEM). Statistical significance is considered to be $p < 0.05$. Student's t-test

and one-way ANOVA followed by the Tukey-Kramer multiple comparison test, have been used to compare the control and treated rats (InStat for 147 Macintosh v. 2.03, Graph Pad Software, Ca. USA).

Results and discussion

The analysis of AKG content in the feeds showed that it ranged between 0.41-0.43% (active ingredients), which was some 14-18% below the expected AKG level of 0.5% (active ingredients). All animals remained healthy during the study.

Performance. No single treatment proved to be significantly different from the control group with respect to growth performance (tab. 2). The AKG 2 treated rats were observed to have generally the lowest average daily gains (ADG) with a high average daily feed intake (ADFI). The only significant difference found, was a worse ($p < 0.03$) feed conversion ratio (FCR), on day 9, of the AKG 2 treatment in comparison with that of the control group. The rats in the AKG 5 treatment showed very similar performance results to those of the control group, but had better performance results than did those in the AKG 2 treatment, although the only significant difference was found a significantly lower ($p < 0.04$) feed efficacy, on day 18, for the AKG 2 treated rats.

Haemoglobin. All dietary treatments showed higher Hb levels than the controls on day 9, with those of dextrose and AKG 2 being significant ($p < 0.03$ and $p < 0.005$, respectively) (tab. 3). On day 18, there were no significant differences in haemoglobin levels between the control group and the treatments.

Histology. In general, the morphometric parameters (crypt depth, villi size and tunica mucosa thickness) did not show any significant differences in samples of the proximal and distal small intestine obtained from the different treatments, as compared to those found in the control group (data not shown). Only the enterocyte crypt depth in the proximal small intestine of the AKG 2 treated rats was significantly enlarged ($p < 0.05$) in comparison to that of the dextrose treated animals (fig. 1).

Amino acids and peptides. The results containing blood levels of amino acids are presented in tab. 4. The levels of blood TAA in the dietary treatments were generally lower on day 9 then on day 18. TAA levels increased over time in both control and treatment groups. The free glutamine (Gln) levels on day 9 were generally higher in the experimental groups than those of the control, however on 18 d of trial, all treatments showed lower free Gln levels than the control, with the lowest values being seen in the AKG 5 group. From day 9 to d 18, only the control treatment showed an increase in the free Gln level, while the experimental groups showed a decrease in free Gln levels over time.

On day 9, the free Glu levels in the AKG and dextrose supplemented rats were generally lower comparing to controls, with the lowest values observed in dextrose group. On 18 d of trial, the free Glu levels in the control group had decreased, in comparison to the levels observed on day 9. The Dextrose and AKG 5 experimental groups showed an increase in the free Glu levels, with the greatest increase found for the AKG 5 treatment. With the exception of AKG 2 group, all treatments showed a lower level of peptide bound (PB) Gln + Glu than did the controls on day 9

Tab. 2. Growth performance of rats (means ± SEM) according to dietary treatment

Days	Parameters	Control	Dextrose	AKG 2	AKG 5
0-9 (n = 16)	Average daily gain, g/d	7.2 ± 0.2	6.9 ± 0.2	6.7 ± 0.2	7.0 ± 0.3
	Average daily feed intake, g/d	21.3 ± 0.3	21.4 ± 0.1	21.7 ± 0.2	21.1 ± 0.3
	Feed conversion ratio kg/kg	0.33 ± 0.007	0.32 ± 0.007	0.30 ± 0.01*	0.33 ± 0.012
0-18 (n = 10)	Average daily gain, g/d	7.1 ± 0.3	6.8 ± 0.2	6.7 ± 0.2	7.1 ± 0.3
	Average daily feed intake, g/d	24.5 ± 0.5	24.6 ± 0.4	25.2 ± 0.9	24.4 ± 0.6
	Feed conversion ratio kg/kg#	0.30 ± 0.009	0. 28 ± 0.007	0.27 ± 0.007	0.3 ± 0.007

Explanations: * $p \leq 0.03$; #AKG 2 vs. AKG 5 ($p < 0.04$)

Tab. 3. The haemoglobin level (g/L) (means ± SEM) of rats in d 9 and d 18 of trial, respectively, according to dietary treatment

Days	Control	Dextrose	AKG 2	AKG 5
0-9 (n = 6)	92.3 ± 6	113.2 ± 6*	120.8 ± 5**	106.2 ± 8
0-18 (n = 10)	121.9 ± 4	120.4 ± 2	122.2 ± 2	122.4 ± 2

Explanations: * $p \leq 0.03$; ** $p \leq 0.005$

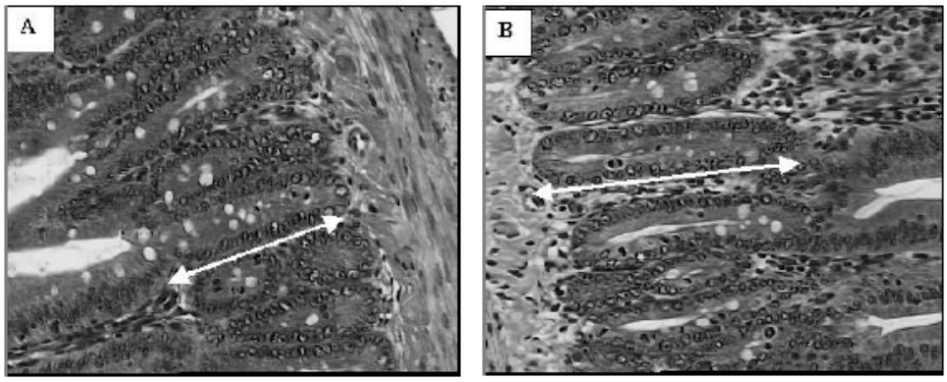

Fig. 1. The crypt depth of enterocytes from the proximal small intestine of dextrose (A) and AKG 2 (B) supplemented rats (A and B × 100). The enterocyte crypt depth in AKG 2 treated rats is significantly larger (P < 0.05) than those of the dextrose treated rats (arrows)

Tab. 4. The concentration of glutamine (gln), glutamate (glu) and arginine (arg), in the whole blood after dietary AKG supplementation as a calcium salt (AKG 5), or in pure form (AKG 2)

Parameters	Control		Dextrose		AKG 2		AKG 5	
	I (µmol/L)	II (µmol/L)	I (µmol/L)	II (µmol/L)	I (µmol/L)	II (µmol/L)	I (µmol/L)	II (µmol/L)
Total Glu + Gln	1670	2102	1439	1961	1389	2106	1530	n.d.
Free Gln + Glu	919	896	844	891	855	874	916	950
• Free Gln	489	527	503	505	509	515	516	478
• Free Glu	430	369	341	386	347	359	399	473
PB Gln + Glu	751	1205	596	1070	534	1231	614	n.d.
Total Arg	330	465	350	375	320	389	310	n.d.
Free Arg	193	272	235	242	218	246	210	303
PB Arg	137	193	115	133	102	143	100	n.d.

Explanations: I: day 9 (n = 6), II: day 18 (n = 10); values from the treated groups are presented as percentage of the control group; n.d. = no data; PB – peptide bound

and day 18 (tab. 4). All treatments had an increase in the levels of these PBAA from day 9 to day 18, with AKG 2 showing the greatest increase. The total Arg levels, showed a decrease over time for all treatments in comparison to that of the control group. Only the dextrose treatment had a level higher than that of the control on day 9. All treatments had higher free Arg levels than the control group at day 9; however, the increase with time was very small. The levels of PB Arg in the various treatments were all considerably lower than those of the controls both after day 9 and day 18. The levels of this PBAA increased from day 9 to day 18 for all treatments as well as the control.

The growth rates observed in this trial of male Sprague Dawley ® outbred laboratory rats were equivalent to the growth rates found in the lower deviation range of the standard growth curve, as given by the supplier (M&B 2001). The AKG pH 2 treated animals had the lowest growth rates during the entire trial, which could be explained by the low pH level of their diets interfering with other metabolic effects of feed supplementation.

According to intestinal morphology, a decrease in VH (villi height – indicator of enterocyte destruction), as well as an increase in LPD (the intestinal *lamina propria* depth – an indicator of crypt depth and increased numbers of less mature cells) are both considered as morphological symptoms of malabsorbtion in piglets (26). It was shown in piglets, that glutamine added to diet can enhance maturation of the intestinal crypt cell in the jejunum (26). Irrespectively of the fact that both glutamine and dextrose are metabolic fuels for cellular differentiation and development, glutamine was found to have a preventive potential of increasing the jejunal LPD (14). In present study in the AKG pH 2 treated rats, the enterocyte crypt depth of the proximal jejunum showed a significant increase, but only when compared to the dextrose treatment. This could mean that the AKG 2 treatment had some influence on the turnover of the intestinal crypt cells (enterocytes), and, therefore, on nutritional absorption. The actual difference in absorption appeared to be limited, since there was no difference in villi height between the two treatments nor was the growth rate better.

After 9 days, a significant difference in Hb levels was noted between controls and dextrose group, as well as between controls and AKG 2 group; however, after 18 d the Hb levels equalized in all treatments groups. This could possibly be explained by the fact that at the beginning of the study, low acidity or calcium addition inhibited the Fe absorption. Although in these groups a positive relationship between Hb levels and ADG was observed, it can not be concluded that it is an impact of the pH itself.

According to the expectations (1), an increase in free Glu with time was generally accompanied by the reduction in the level of free Gln, which is an evidence of metabolic adaptation. In the AKG 2 treatment, the largest increase in the PB Gln + Glu levels over time was noted. Surprisingly, in the AKG group, an increase in the PB Gln + Glu levels over time was observed, together with the lowest level of performance with respect to ADG and feed efficacy ratio.

Conclusions. The objective of this trial aimed to investigate the effects of AKG supplemented either as a salt (Ca + AKG), or in pure form on growing rat performance, feed utilization, some blood parameters and histology of the small intestine. The study showed no difference in growth performance between treatment groups and control, but the results of the study indicate that chemical form of orally administered AKG has some influence on growth performance. The AKG 2 treated rats had the lowest average daily gains (ADG) and largest increases in the peptide bound glutamine and glutamate levels (PB Gln + Glu), which was linked to the highest average daily feed intake (ADFI). Acid form of AKG exerted positive effect on blood Hb, but this effect was seen only during the first 3 weeks of treatment. We found no evidence showing that in growing rats AKG has any impact on crypt depth or the turnover of the intestinal crypt cells in proximal jejunum, independently of the chemical form administered.

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