RELATION BETWEEN GROWTH AND BONE COLLAGEN CONTENT IN YOUNG PIGS; EFFECTS OF DIETARY α-KETOGLUTARATE SUPPLEMENTATION

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Abstract

The aim of this study was to determine the effect of dietary supplementation with α-ketoglutarate (AKG) sodium salt on growth rate in relation to bone collagen formation during the first 70 d of postnatal life in piglets. The results show that dietary AKG supplementation increased body weight of the experimental piglets in comparison to the controls, especially between 21th and 56th d of life (P≤0.01). Moreover, the area of collagen trabeculae slightly increased in experimental age sub-groups and reached the highest differences between 14th (P≤0.01) and 70th d of piglets life (P≤0.001). In contrast, the highest values for the number of collagen trabeculae were observed in piglets at 2nd d of age, regardless of treatment group. The positive effect of AKG supplementation on the number of collagen trabeculae was found between 3rd and 35th d of life, with statistical confirmation at days 14, 35, and 56 (P≤0.01). The data-lines of the bone strain showed similar course during the whole experimental period, except 56th d of life, when the experimental piglets reached statistically significant, higher values in comparison to the controls (P≤0.05). Similarly, the blood plasma osteocalcin reached the highest concentration in experimental sub-groups from 21st d of life in comparison to the controls, with statistical significance at the age of 56 (P≤0.05). These data indicate that dietary AKG supplementation effectively stimulated collagen synthesis in young growing piglets, both before and after weaning.

Key words: piglets, bone, collagen, α-ketoglutarate.

Bone physiology of newborns is clearly different from that of adults (19, 26). One of the most important differences is the rate of bone tissue metabolism, recognised in newborns and reported as bone modelling (17-19, 23). It is generally known that bone is a complex tissue of which the principal function is to resist mechanical forces and fractures. Bone properties depends not only on the quantity of bone tissue but also on the quality, which is characterised by the geometry and the shape of bones, microarchitecture of the trabecular bones, turnover, mineral content, and finally the collagen (6). In fact, the process of collagen maturation is one of the two main factors, which are responsible for further – adult bone resistance to mechanical loads. The second major limiting factor is mineralisation of bone, comprising accumulation of the calcium and other bone mineral constituents. Therefore, in bone tissue, collagen fibrils are stiffened by integration of the mineral phase (20). The abovementioned factors remain tightly coupled since the beginning of the bone formation, because any disturbances between these processes can easily lead to various pathological states of the adult bones. In general, bone is composed of two basic types, namely cortical and trabecular. Cortical bone constitutes approximately 80% of the total skeletal mass, whereas trabecular bone only 20%. However, in fact, the trabecular bone is much more rapidly remodelled than cortical bone, with approximately 25% renewal each year compared with only 3% of cortical bone (30). The proper bone
homeostasis consisted of processes of bone formation and resorption, also called bone turnover, is an essential part of bone health (30). This process of bone tissue modelling requires the synthesis of collagen, which forms the main structure of bone, where later the minerals are deposited alongside the collagen network. Approximately 85%-90% of the total bone protein consists of collagen (21). An essential part of the amino acids from foetal bone collagen can be used for the re-synthesis of the postnatal bone collagen. However, there are two limiting amino acids, proline and hydroxyproline. Hydroxyproline in new bone collagen must be synthesised from proline, one of the most abundant amino acids in sow milk (28). However, in the second part of lactation, sow milk covers not more than 40% of the piglets’ requirements for growth. Thus, bone collagen synthesis will also be impaired because of the lack of proline, although the enterocytes are the site of proline synthesis from glutamate (13). However, dietary glutamate is 70%-90% oxidised in the first pass of metabolism. Another potential source of glutamate can be gluatamine, but this amino acid is also oxidised in 95% during the first pass (22). Dietary α-ketoglutarate (AKG) is oxidised only by 30% during the first pass (8). Thus, AKG can be a source of glutamine and proline. Indeed, 25% of the dietary AKG is converted to proline in the enterocytes (12). Bearing in mind that AKG can also be converted in the intestine and the indispensability of proline for bone collagen synthesis, the main aim of the study was to determine the influence of dietary AKG on bone collagen synthesis in the early stages of skeleton development in piglets. Attention was especially paid to the time intervals when the natural dietary sources of proline could be too low.

Material and Methods

Experimental design and sampling procedure. The study was approved by the Ethical Review Committee for Animal Experiments of the University of Life Sciences in Lublin, and conducted according to the European Community regulations concerning the protection of experimental animals. The experiment was performed on 120 piglets of the Large Polish White breed derived from 10 litters, mixed males and females, obtained from the Research Farm of the University of Life Sciences in Lublin. Pigs were housed in a piggery with flat deck pens (2 x 2.5 m). Each pen had wire-mesh sides that allowed contact between the animals. Pens contained a sleeping area, a feeding trough and a nipple drinker. Temperature was controlled thermostatically at 22 ± 2°C. Before weaning, from the 7th d of life and after weaning at the 28th d of age, the piglets were fed twice a day a standard commercial diet assigned for respective groups of age. At birth, the piglets were randomly divided within litters into two main groups: the experimental and the control, with sixty animals in each group.

The experimental and control groups were tested at 6 age periods, namely at 3, 14, 21, 35, 56, and 70 d of piglet life, respectively. The experimental procedure was conducted from the 1st to the 70th d of postnatal life. The experimental piglets were treated per os with an aqueous solution of sodium salt of AKG, buffered with NaOH to pH 7.3, in the dosage of 2.0 ml/kg b.w./d, which contained 0.4 g of AKG. At the same time, the piglets from the control group were treated per os with physiological saline (PhS) in an equal capacity of 2.0 ml/kg b.w./d. The AKG and PhS supplementations started from the first day and were continued up to 70 d of piglet life. The animals remained healthy throughout the experiment, and pathological signs were not detected in any of the piglets. After the set up periods, the piglets within litters were weighed and treated with a single intramuscular injection of 4 mg/kg b.w. of azaperonum (Stresnil; Janssen Pharmaceutical Ltd, Belgium) to induce a sedation and then euthanised with an intravenous lethal dose of pentobarbital natrium (Morbital, Biowet, Poland). After euthanasia, their femora were isolated and prepared for further analysis.

Histomorphometry. The right femora were subjected to histology. The epiphysis was collected from the distal part of each femur and fixed in buffered formaldehyde, decalcified in 7% nitric acid for 10 d, dehydrated in graded ethanol solutions, and embedded in paraffin. Longitudinal sections (6 μm thick) were cut in a microtome (Microm HM 360, Microm GmbH, Germany). Twenty such sections were cut (at 20 μm interval after each series of five sections) from the femur of each piglet. The sections were stained with haematoxylin and eosin (HE). All analyses were carried out at a magnification of 50×. Microscopic images were collected using an AXIOVERT 200M-light microscope, supplied with laser recording head LSM Pascal 5 (Carl Zeiss, Germany) and digital camera. Excitation of eosin was obtained with the argonic laser at the excitation wavelength 514 nm. The microscopic images of the epiphyseal distal parts of the femur were subjected to computerised analysis using the EsiVision 3.0 programme. The total area and number of collagen trabeculae were determined and then the obtained results were recalculated to mean values per one microscopic field 3.39 mm².

Bone elasticity. All femora were mechanically tested to failure using the three-point bending test according to the method of Ferretti et al. (3, 4). These tests were conducted using a material testing machine INSTRON 4302 (Instron Corp., USA) linked to a computer, registering the relationship between the force acting perpendicularly to the length of the bone, and resulting in displacement. In this apparatus, the bones were placed on two supports at a distance of 40% of the total length of the investigated bone. The bones were loaded by the head of the apparatus at the middle of the bone length at a displacement rate of 1.0 mm/s until failure. The results of this mechanical analysis were presented graphically and the test curves were analysed to determine the standard mechanical properties of the bones. The bone stiffness or resistance to deflection, depending largely on the elastic quality of the bone substance was calculated by the subtraction of the values
of the elastic strength causing bone deflection, from the values of the ultimate strength causing bone fracture. The range of the bone susceptibility to deformation between the initial state (bone deflection) and the final state (bone failure) was defined as bone strain.

**Blood sampling.** Blood samples were collected directly from the jugular vein using a 10 ml syringe. Then the blood was transferred into tubes containing ethylenediaminetetraacetic acid (EDTA), centrifuged at 1,500 g for 20 min within 30 min from sampling, and plasma was harvested. The plasma was stored at −25°C until further analyses.

**Osteocalcin.** The plasma concentration of osteocalcin (OC) was measured using a commercial radioimmunoassay DSL-6900 (DSLabs Inc., Webster, USA) and γ-radiance detector PACKARD-CANNBERA. Before all analyses, the plasma samples were allowed to reach room temperature (−18°C).

**Statistical analysis.** All data are presented as a mean ± standard error (± S.E.). Differences between the groups were analysed by two way analysis of variance (ANOVA) and the Fisher test (correction for multiple comparisons). P<0.01 was considered statistically significant.

### Results

**Body weight of the piglets.** During entire experimental period, the body weights of both groups of piglets increased in parallel (Fig. 1A). From day 21, the AKG-treated piglets reached a statistically higher body weight in comparison to that of the controls (P≤0.01). The significant differences were observed at the age of 21, 35, and 56 d of life (Fig. 1A).

**Area of the collagen trabeculae.** The mean values of the area of the collagen trabeculae of the epiphysis part of the femora increased and reached a plateau at the 14th d of the experiment. They were significantly higher during the entire experimental period, starting at day 14 in piglets, which received AKG as compared to that of the controls. (Fig. 1B). In the control group, the collagen area decreased continuously by day 21 and showed a slight increased by the last day of the experiment (Fig. 1B).

**Number of the collagen trabeculae.** The number of collagen trabeculae was the highest in both experimental and control piglets on day 3, and then the number gradually decreased. At the first four check points (days 3, 14, 21, and 35 of life), the mean values of this parameter were higher in the group, which received AKG in comparison to the controls, with statistical significance at the age of 14, 21, and 35 d of age (P≤0.01), respectively (Fig. 2 AB). At the last two test days, 56 and 70 d of age, the number of the collagen trabeculae was the same in both groups (Fig. 1C and Fig. 2CD).

**Bone elasticity.** The AKG-treated and control groups of piglets showed a very similar course of bone elasticity values during the first 35 d of age. Thereafter, from 35 d of age to the end of the experimental period, the piglets which received AKG (P≤0.01) showed higher values of bone elasticity (Fig. 1D).

**Osteocalcin.** The analysis of the osteocalcin level showed that oral AKG administration increased the concentration of this hormone in blood plasma from the 21st d of life, with statistical difference being found between the experimental group and controls on the 56th d (P≤0.01) (Fig. 1E).

### Discussion

The presented study aimed to highlight the importance of skeleton development - by determining the relationship between collagen synthesis in the femur and growth of pigs supplemented with dietary AKG. It has been shown previously that dietary AKG strongly improved the synthesis of femur collagen and increased not only the bone elasticity, but also other physical parameters, e.g., the maximum elastic strength of the bones (10). This observation permitted us to assume that the growth of the entire skeleton would be improved by supplementation with AKG in the diet. The growth was also improved in the piglets, which were given AKG. Thus, we conclude that skeletal growth in the early stages of development is a limiting factor for piglet’s growth overall.

The most probable explanation for the observed events is as follows. Dietary AKG via glutamate synthesis in the gut epithelium improves proline synthesis in enterocytes (14). Proline, as one of the conditionally essential amino acids in the early stages of development is the limiting amino acid for bone collagen synthesis (1, 5). Proline and hydroxyproline account for about 70% of amino acids in bone collagen structure (27). Exogenous proline, provided intravenously, definitively improves the synthesis of bone collagen and indicates the fact that the turnover time of bone collagen is underestimated (1). The amount of proline in sow milk is rather high, but the amount of milk is low, especially during the late phase of lactation (31). In the post weaning period, the dietary proline levels are also low and do not match piglets’ requirements (9). Thus, under such conditions, any additional source of proline or precursors for proline synthesis can be of importance for skeleton growth. Moreover, Jaksic et al. (7) documented previously that proline concentration in blood plasma drops significantly when it is absent from the diet.

It was shown in the presented study that piglets receiving AKG supplementation had a significantly higher amount of collagen than the control group and do not show any changes in bone collagen structure during the experimental period and exhibited the best growth. The control piglets showed a lower content of collagen in the bone, poorer growth, and a correlated fluctuation of the bone collagen level with dietary changes. Moreover, the quality of the bone in pigs receiving AKG was essentially improved by the end of experiment, with respect to the bone elasticity of the femur characterised by the same amount of collagen trabeculae.
Figs 1 A-E. The effect of AKG dietary supplementation on body weight and bone parameters of young piglets during the first 70 d of postnatal life. A – mean body weight of piglets (kg); B – mean area of collagen trabeculae per one microscopic field (3.39 µm²); C – mean number of collagen trabeculae per one microscopic field; D – mean values of bone strain (N); E – mean concentration of osteocalcin in blood plasma (ng/mL). The broken line represents the control group, and the solid line represents the AKG treated group. Different letters given with results for a specific treatment describe statistical significance where \( P \leq 0.01 \). Stars given with results describe statistical differences \( ** P \leq 0.01, *** P \leq 0.001 \) between treatment and control group at a given time point. The x axes present following experimental days of piglet life (from 3rd to 70th d).

The importance of trabecular microarchitecture of bones, as a factor determining bone elastic properties, was confirmed previously (16, 25, 29, 32). The effect of AKG supplementation on bone collagen synthesis was also shown indirectly in these studies by elevated plasma OC levels in the piglets receiving AKG. Osteocalcin is well-known as one of the most important markers and components of bone formation (2, 15). Moreover, the concentration of OC in the blood plasma correlated with the bone mineral density (BMD), which is universally recognised as an important determinant of bone fracture risk (24). Measurements of the BMD of the femora in piglets receiving AKG indicated that AKG significantly increased the bone mineral content, which can be useful in practice to protect the bones against local damage, as demonstrated in previous studies (10, 11). Results of the present study confirmed that the bone network of collagen fibers in young growing piglets, composes the scaffolding for further bone mineralisation, and enables bones to grow.

In summary, the growth of the piglets is correlated with bone collagen synthesis and bone collagen synthesis can be essentially improved by dietary AKG supplementation, since it is the precursor of proline, the main amino acid of bone collagen.
Figs 2 A-D. Photomicrographs of representative cross-sections from distal epiphysis of piglets’ femora stained with HE and viewed under laser light. The collagen trabeculae are visible as white structures on the black background. The presented specimens are from piglets of 14 d of age from experimental – AKG treated (A) and control (B) groups and from piglets 56 d of age from experimental (C) and control (D) groups. The area shown is 1.842×1.842 mm at 50x.

References