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The effect of dietary administration of 2-oxoglutaric acid on the cartilage and bone of growing rats

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Abstract

2-Oxoglutaric acid (2-Ox), a precursor to hydroxyproline – the most abundant amino acid in bone collagen, exerts protective effects on bone development during different stages of organism development; however, little is known about the action of 2-Ox on cartilage. The aim of the present study was to elucidate the influence of dietary 2-Ox supplementation on the growth plate, articular cartilage and bone of growing rats. A total of twelve male Sprague–Dawley rats were used in the study. Half of the rats received 2-oxoglutarate at a dose of 0·75 g/kg body weight per d in their drinking-water. Body and organ weights were measured. Histomorphometric analyses of the cartilage and bone tissue of the femora and tibiae were conducted, as well as bone densitometry and peripheral quantitative computed tomography (pQCT). Rats receiving 2-Ox had an increased body mass ($P<0·001$) and absolute liver weight ($P=0·031$). Femoral length ($P=0·045$) and bone mineral density ($P=0·014$), overall thickness of growth plate (femur $P=0·036$ and tibia $P=0·026$) and the thickness of femoral articular cartilage ($P<0·001$) were also increased. 2-Ox administration had no effect on the mechanical properties or on any of the measured pQCT parameters for both bones analysed. There were also no significant differences in histomorphometric parameters of tibial articular cartilage and autofluorescence of femoral and tibial growth plate cartilage. Dietary supplementation with 2-Ox to growing rats exerts its effects mainly on cartilage tissue, having only a slight influence on bone. The effect of 2-Ox administration was selective, depending on the particular bone and type of cartilage analysed.

Key words: 2-Oxoglutaric acid: Bone: Growth plate: Articular cartilage: Growing rats

Nutrition plays a key role in every stage of life, from prenatal development to the ageing of an organism. Nutrition is also one of the most important factors in the maintenance of good health during an organism's lifetime[1–3]. Various nutritional supplements are available, which improve the overall health status of an organism and reduce the risk of disease by improving the functioning of the gut, bone and other organs[4–9].

Several studies have shown that 2-oxoglutaric acid (2-Ox), the precursor to proline, hydroxyproline, glutamate, arginine and asparagine, has an effect on the growth of both human and animal bones. Dietary supplementation with 2-Ox also counteracted the bone loss observed in gastrectomised, fundectomised and ovariectomised animals, as well as in post-menopausal women[5,6,8–14]. 2-Ox affects the synthesis of amino acids and proteins, as well as improving their absorption from the gut[15]. It seems that dietary 2-Ox may be an important factor for the improvement of protein anabolism, especially collagen anabolism. 2-Ox, together with Fe^{2+}, have been proposed to be active participants in the conversion of proline to hydroxyproline, the main amino acid of bone collagen[9]. Moreover, 2-Ox acts as a cofactor for Fe^{2+} absorption from the intestine[16]. This may be one of the most important routes in the overall relation between nutrition, the digestive tract and skeletal condition.

Abbreviations: 2-Ox, 2-oxoglutaric acid; A, administration; aBMD, areal bone mineral density; BMC, bone mineral content; BV, bone volume; BV/TV%, relative bone volume; C, control; pQCT, peripheral quantitative computed tomography; TV, tissue volume.

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British Journal of Nutrition

The influence of 2-Ox supplementation on skeletal development observed prenatally and in the early neonatal stages of life, as well as in mature and ageing animals' is well documented[9,17]. However, little is known about the influence of 2-Ox administration on animals or human subjects during the growth period, especially regarding the influence of 2-Ox on the morphological and histological structure of cartilage. This influence could be verified in an animal model like the rat, with continued growth (active growth plates) after skeletal maturity[18]. Oral supplementation with 2-Ox may improve bone growth, as well as the quality of bone, through its influence on the growth plate and articular cartilage.

The aim of the present study was to establish the effect (if any) of 2-Ox administration in rats during intensive growth periods, on the histological and morphological properties of cartilage tissue (articular and growth plate cartilage in particular) and on the histological, mineral and mechanical parameters of bone.

Experimental methods

Experimental design and laboratory animals

A total of twelve, 7-week-old, male Sprague–Dawley rats (120–150 g) were used in the present study. Rats were clinically healthy and housed individually in Macrolon® cages (EHRET GmbH & Co. KG), under standard laboratory conditions (controlled temperature, humidity and 12 h light–12 h dark cycle), with free access to water and solid food. Rats were fed a diet of standard feed pellets (Lactamin). Feed composition was based on the nutrient requirements of laboratory animals[19]. The feed was weighed weekly and feed consumption was calculated (results showed no differences in feed consumption throughout the duration of the experimental period – data not shown). Rats were randomly divided into two groups, with six rats in each group. Rats in the administration (A) group received the 2-oxoglutarate sodium salt (Rexim S.A.S.), administered in the drinking-water, at a dose of 0·75 g/kg body weight per d (pH 7·3). Rats in the control (C) group received only the vehicle composed of tap water and NaCl (1·17 g/l). Vehicle was titrated with 0·01 M-NaOH to achieve a pH of 7·3. A measure of 30 g/l of glucose and 15 g/l of sucrose were added to both solutions to improve the taste. Water consumption was measured daily and the mean daily consumption between the two groups for each week was compared. No differences were observed in water consumption between the groups throughout the duration of the experimental period (data not shown).

Rat activity was observed twice daily: in the morning (for half an hour before changing feed and water (10.00 hours) and half an hour after) and in the afternoon (for half an hour starting at 18.00 hours). There were no changes in behaviour and activity of rats between the groups (data not shown).

Rats were weighed at the start of the experiment and then weekly during the experimental period. The dose of 2-Ox was adjusted according to body weight gain. The study lasted for 7 weeks. At the end of the experiment, rats were euthanised, one by one, with CO_2 and by dislocation of the spine.

The experiment was approved by the local Animal Welfare Committee, Lund, Sweden.

Tissue collection and analysis

Tissue collection. At autopsy, the internal organs were evaluated in terms of location, colour, volume and the accuracy of the anatomy, as well as adipose tissue distribution. Tissue samples were collected from each rat. Stomach, liver, pancreas and left kidney were weighed.

Right femora and tibiae were isolated and cleaned of remaining soft tissues. Bone length and weight were measured and bone samples were wrapped in gauze, soaked in isotonic saline and stored at −25°C until further analyses. Left femora and tibiae were dissected out intact from each rat, cleaned of soft tissue and fixed in 4% buffered formaldehyde (pH 7·0).

Bone mineral density and bone mineral content. Areal bone mineral density (aBMD) and areal bone mineral content (BMC) were determined for each right femora and tibiae using the dual-energy X-ray absorptiometry method and a Norland Excell plus densitometer (Norland), with a resolution of $0{\cdot}5 \times 0{\cdot}5\,mm^2$ and speed of 30 mm/s, using Small Subject Scan software version 3.9.6. (Visual MED, Inc. and Norland a CooperSurgical Company). The machine was calibrated daily with a phantom provided by the manufacturer.

Peripheral quantitative computed tomography. Right femora and tibiae epiphysis, 5 mm from distal and proximal end, respectively, were scanned with a peripheral quantitative computed tomography (pQCT) XCT Research SA Plus system, using software version 5.5 D (Stratec Medizintechnik GmbH), as described previously[20]. Upon completion of scanning, the following parameters were determined: densitometric – the volumetric BMD and content (volumetric BMC); the trabecular (trabecular BMD) and cortical (cortical BMD) bone density; the trabecular (trabecular BMC) and cortical (cortical BMC) BMC; geometric – the total bone area; the trabecular area and cortical area; the mean cortical thickness; and the periosteal circumference and the endosteal circumference. Results showed no statistically significant differences between the groups in all the aforementioned pQCT parameters (data not shown).

Mechanical properties of bone. Mechanical properties of right femora and tibiae were examined on a Zwick Z020/TN2S universal testing machine (Zwick GmbH & Company KG), equipped with a measuring head KAP-Z (Zwick GmbH & Company KG) of operation range up till 100 N, linked to a computer with testXpert 7.1 software (Zwick GmbH & Company KG), as described previously[21]. The ultimate strength, work to fracture and Young's modulus were determined. There were no significant differences between the groups regarding mechanical properties of the bones (data not shown).

Histomorphometry. The left femora and tibiae were subjected to histology as described previously, except that 5 μm-thick sections were cut and were not stained[5]. Sagittal sections through the middle of the lateral condyle of the

femur and tibia were cut. Microscopic (two-dimensional) images (magnification ×200) of autofluorescence were collected using a confocal microscope AXIOVERT 200M (Carl Zeiss) equipped with a camera AxioCam HRc (Carl Zeiss) and a fluorescent lamp (excitation wavelength 450–490 nm). Structure of articular cartilage and growth plate, as well as cortical and trabecular bone of the femur (distal end) and tibia (proximal end) (both epiphysis and metaphysis) were examined by microscopic observation. Analysis of collected images was performed with the use of graphical analysis software ImageJ 1.46i (National Institutes of Health USA, http://rsb.info.nih.gov/ij/index.html).

Measurements of growth plate width (the resting, proliferative, hypertrophy and calcification zone width) were taken as described previously[22,23].

The parameters examined for articular cartilage were as follows: the number of isogenous groups/mm^2 of hyaline cartilage (two or more lacuna per group means isogenous group), the number of chondrocytes/mm^2 of hyaline cartilage and the width of articular cartilage. Three zones of articular cartilage were defined (the superficial, the intermediate and the deep zone) as described previously and their width was measured[24–26]. Tibial articular cartilage parameters did not differ significantly (data not shown).

The bone volume (BV), tissue volume (TV) and relative BV (BV/TV%) were measured in photographs of bone tissue sections using the pixel count, as described previously[5]. The parameters examined for trabecular bone (epiphysis and metaphysis) were as follows: the BV/TV%, the trabecular thickness, the number of osteocyte/mm^2 of trabecula and the trabecular separation defined as the distance between edges of adjacent trabeculae (measured directly).

The parameters examined for cortical bone were as follows: the number of osteocytes/mm^2 of cortical bone, the number of Haversian and Volkman canals/mm^2 of cortical bone tissue and the cortical bone thickness. Results showed no significant differences between the groups regarding cortical bone histomorphometry parameters (data not shown)

Since autofluorescence is related to the hydroxyproline content, which in turn reflects the collagen content, as described previously by Hagiwara *et al.*[27], the mean intensity of intercellular matrix autofluorescence (measured in 8 bit grey value which showed proportional correlation – the higher the result, the stronger the tissue autofluorescence) and the integrated density of intercellular matrix autofluorescence were measured for: growth plate, articular cartilage, trabecular bone (epiphysis and metaphysis) and cortical bone.

Statistical analysis

All results are expressed as means and standard deviations. Differences between means in body mass, as well as feed and water consumption, were analysed using a repeated measures ANOVA. Differences between all other means were analysed using the Student's *t* test. Normal distribution of data was examined using the Shapiro–Wilk test and equality of variance tested by the Brown–Forsythe tests. When data were not normally distributed and/or there was unequal variance of data, we had to make use of the Mann–Whitney *U* test to analyse differences between means. A *P* value of less than 0·05 was considered statistically significant. All statistical analyses were carried out using STATISTICA 8.0 software (StatSoft, Inc. (2008), STATISTICA (data analysis software system), version 8.0. www.statsoft.com).

Results

Body mass, bone and selected organ weights and bone length

The body mass gain is presented in Fig. 1. 2-Ox accelerated the body mass gain, which was significantly higher already following the 5th week of the study ($P=0·017$). At the end of the experimental period, rats receiving 2-Ox were 24% heavier than control rats ($P<0·001$) (Table 1).

The absolute and relative weights of the bones and organs are presented in Table 1. 2-Ox increased absolute liver weight ($P=0·031$) and decreased the relative weight of the stomach ($P=0·003$) and both bones that were analysed ($P<0·001$).

Analysis revealed no differences between the groups regarding the anatomy of the internal organs and adipose tissue distribution. However, 2-Ox significantly increased the length of the femur (38·07 (SD 0·67) mm) compared with the control rats (35·83 (SD 3·82) mm) ($P=0·045$). However, the length of the tibia was not significantly affected (C – 42·85 (SD 0·86) mm, A – 42·68 (SD 0·67) mm).

Bone mineral density and bone mineral content

2-Ox administered to rats significantly increased the femoral aBMD (0·112 (SD 0·002) g/cm^2) compared with the C group (0·107 (SD 0·004) g/cm^2) ($P=0·014$), whereas femoral areal BMC and tibial aBMD and areal BMC were not affected (data not shown).

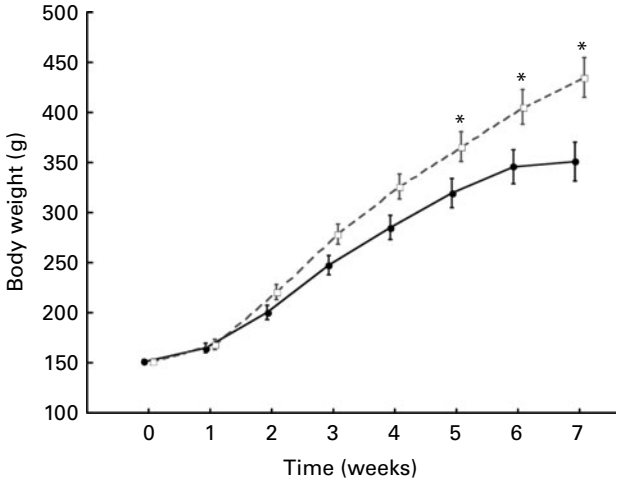

Fig. 1. Effect of 2-oxoglutarate (2-Ox) on body weight gain of rats. ▯, Animals receiving 2-Ox; ●, animals not receiving 2-Ox sodium salt (0·76 g/kg body weight per d), administered in drinking-water. Values are means, with standard deviations represented by vertical bars. * Mean value was significantly different ($P<0·05$).

Table 1. Effect of 2-oxoglutarate (2-Ox†) on bone and organ weights
(Mean values and standard deviations)

	Absolute weight (g)				Relative weight (g/kg body weight)			
	C		A		C		A	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Femur	1·20	0·06	1·17	0·05	3·42	0·20	2·69*	0·12
Tibia	0·88	0·08	0·86	0·04	2·50	0·17	1·99*	0·08
Stomach	2·12	0·24	2·14	0·29	6·02	0·28	4·92*	0·63
Liver	12·58	1·33	15·05*	2·02	35·81	2·17	34·56	3·69
Pancreas	1·02	0·07	1·13	0·31	2·91	0·35	2·61	0·68
Kidney	1·21	0·16	1·43	0·20	3·45	0·23	3·28	0·39
Body weight	351	26·9	435*	27·3				

C, rats not receiving 2-Ox; A, rats receiving 2-Ox sodium salt (0·76 g/kg body weight per d).
* Mean value was significantly different between the groups ($P<0·05$).
† 2-Ox was administered in drinking-water.

Growth plate and articular cartilage histomorphometry

Growth plate and the articular cartilage thickness are presented in Figs. 2–4. Rats receiving 2-Ox had significantly thicker femoral ($P=0·036$) and tibial ($P=0·026$) growth plates. Moreover, resting ($P=0·008$) and calcification ($P=0·016$) zones of the femoral growth plate and calcification zone ($P<0·001$) of the tibial growth plate were significantly thicker in the A group (Figs. 2 and 3).

The articular cartilage of the femur of rats in the A group was much thicker than in the C group ($P<0·001$). Moreover, all zones of cartilage were also significantly thicker in the A group compared with the C group (superficial $P=0·006$; intermediate $P<0·001$; deep $P<0·001$) (Fig. 4). Furthermore, the integrated density of intercellular matrix autofluorescence was higher in the A group ($12·0 \times 10^6$ (SD $5·1 \times 10^6$)) compared with the C group ($9·6 \times 10^6$ (SD $3·6 \times 10^6$)) ($P=0·033$). However, the mean intensity of intercellular matrix autofluorescence was lower in the A group (112 (SD 19)) than in the C group (124 (SD 20)) ($P=0·017$). Results showed no significant differences in the number of isogenous groups/mm^2 (C – 41 (SD 21), A – 49 (SD 32)) ($P=0·27$) and in the number of chondrocytes/mm^2 of articular cartilage between the groups (C – 1627 (SD 390), A – 1558 (SD 376)) ($P=0·47$).

There were no statistically significant differences in the mean intensity and integrated density of intercellular matrix autofluorescence of growth plate cartilage in both analysed bones (data not shown).

Trabecular and cortical bone histomorphometry

Data from trabecular histomorphometry are presented in Table 2. Treatment with 2-Ox significantly increased the mean intensity ($P=0·014$) and integrated density ($P=0·006$) of trabecular bone autofluorescence of the femoral epiphysis; however, the mean intensity of autofluorescence of the femoral metaphysis ($P=0·035$) as well as the integrated density of autofluorescene of the tibial epiphysis ($P=0·002$) were reduced by 2-Ox administration. Rats in the A group had wider trabecular spacing of tibial metaphysis than those in the C group ($P=0·004$). However, trabecular spacing of the femoral epiphysis was lowered by 2-Ox administration ($P=0·002$). The trabecular thickness of the tibia epiphysis ($P=0·030$) and metaphysis ($P=0·036$), as well as the BV/TV% of tibia epiphysis ($P=0·004$), were significantly reduced by supplementation with 2-Ox (A group) compared with the C group.

Discussion

2-Ox, a metabolite of the Krebs cycle and a precursor to the main amino acid of collagen, hydroxyproline, is a substance with great protective potential, especially with regard to metabolism and the condition of the skeleton and the whole organism in general[9]. Several previous studies have shown that dietary 2-Ox could prevent systemic diseases, like osteoporosis or increases in cholesterol, as well as improve kidney and muscle function[5,13,28–30]. Other studies have demonstrated the anabolic properties of 2-Ox regarding amino acid and protein utilisation, energy expenditure, the maintenance of body mass, as well as acceleration of body mass gain. However, these effects were dependent on sex, age or health status[6,7,9,11,31,32]. In the present study, body mass

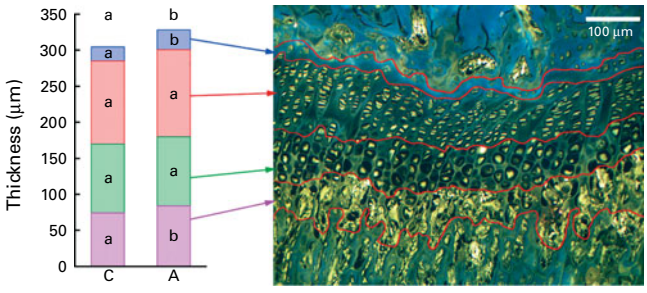

Fig. 2. Effect of 2-oxoglutarate (2-Ox) on femoral growth plate thickness. C – animals not receiving 2-Ox; A – animals receiving 2-Ox sodium salt (0·76 g/kg body weight per d), administered in drinking-water. Appropriate zones of growth plate are indicated by different colours, and coloured arrows point to the corresponding zones marked on the sample photograph. Letters inside the boxes of the same colour indicate differences of thickness between the groups in the appropriate zone. Letters above the columns indicate differences between the groups related to total thickness. [a,b] Values with unlike letters in columns were significantly different between the groups ($P<0·05$). ■, Resting; ■, proliferative; ■, hypertrophy; ■, calcification.

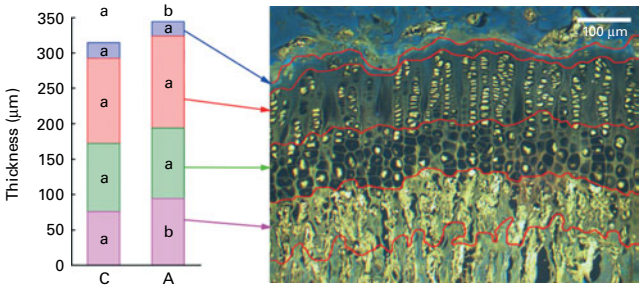

Fig. 3. Effect of 2-oxoglutarate (2-Ox) on tibial growth plate thickness. C – animals not receiving 2-Ox; A – animals receiving 2-Ox sodium salt (0·76 g/kg body weight per d), administered in drinking-water. Appropriate zones of growth plate are indicated by different colours, and coloured arrows point to the corresponding zones marked on the sample photograph. Letters inside the boxes of the same colour indicate differences of thickness between the groups in the appropriate zone. Letters above the columns indicate differences between the groups related to total thickness. [a,b] Values with unlike letters in columns were significantly different between the groups ($P<0·05$). ■, Resting; ■, proliferative; ■, hypertrophy; ■, calcification.

gain was accelerated by 2-Ox administration (Fig. 1); however, no differences were observed in the absolute weight of the bones examined or in the weight of any of the other organs (except the liver) when compared with the C group. The increase in absolute liver weight after 2-Ox administration may be a reflection of the increase in cell volume, intercellular space, average size of the nuclei, as well as the number and total area of nuclei of liver cells, which was observed in our previous study (Śliwa *et al.*[33]). Such changes in liver histology, leading to an increase in the weight and volume of this organ, according to Haussinger *et al.*[34], are related to glutamine metabolism.

No differences were found in food and water consumption between the groups. It must be emphasised that the relative weights of the femur, tibia and stomach were in fact decreased in the A group, although the absolute bone mass was not altered (Table 1), which means that the relative decrease in bone mass must have been caused by accelerated muscle or adipose tissue development. Observations showed no differences in the activity of rats throughout the experimental period or in the adipose tissue deposition in the abdominal cavity observed during sections. 2-Ox is a N scavenger and a source and precursor of glutamine, synthesised in human skeletal muscles, which improves and stimulates protein synthesis and inhibits protein degradation in skeletal muscles[9]. Thus, 2-Ox supplementation serves to enhance muscle development, as reviewed by Brüggemann *et al.*[30]. However, several studies reviewed by Harrison & Pierzynowski[9] showed that 2-Ox administered orally stimulates collagen synthesis and bone formation. In the present study, dietary supplementation with 2-Ox to growing rats selectively affected the hyaline cartilage rather than the bone tissue. Moreover, 2-Ox showed selective action regarding the type of bone, with preference towards the femur, regardless of the type of tissue. To our knowledge, little is known about the influence of 2-Ox on cartilage, especially in growing animals or human subjects. In rats, the growth rate increases between 1 and 5 weeks of age, then declines until skeletal maturity is reached, which is achieved by 11·5–13 weeks of age, after which the bones continue to grow, at a reduced rate, until 26 weeks of age, where growth virtually ceases[18]. Our experimental period ended after the rats were 14 weeks of age; hence, we examined the active and not ossified growth plate, and all morphological features of the growth plate for growing animals were maintained as described by Roach *et al.*[18]. In the present study, 2-Ox caused a significant increase in cartilage thickness in both the articular and growth plate sections. Furthermore, the effect of 2-Ox was more pronounced in the articular cartilage compared with that of the growth plate, since all of the zones examined within the articular cartilage had an increased thickness compared with those of the control. Although the stronger effect observed in articular cartilage might have been related to the loading of the joints by the higher body mass of the rats treated with 2-Ox, data obtained by Eckstein *et al.*[35] and Koszyca *et al.*[36] demonstrated that the thickness of articular cartilage is related to functional demand, and since cartilage plays an important role in the ability of a joint to deal with loading, the thicker the cartilage, the better it is able to deform with compression. The question that arises is whether 2-Ox is the cause of the cartilage thickening, in particular the articular cartilage, or whether the bone loading (by a heavier body mass) is the key factor stimulating better nutrient utilisation facilitated by 2-Ox abundance? Within the femur articular cartilage, which is the most loaded point of the knee joint, thickening was the predominant reaction to additional 2-Ox. Several earlier studies showed that 2-Ox may bring about beneficial effects within human subjects and animals as a functional dietary component[9,37]. Moreover, the intra-articular injection of a nutritive mixture solution containing glutamine and glutamate, formulated to supply nutrients to chondrocytes, is a potent treatment that significantly delays osteoarthritis progression[37,38]. On the other hand, the glutamate functional signal in peripheral tissues (neuronal and non-neuronal) may act as an autocrine and/or paracrine mediator to influence many cellular activities[39]. Additionally,

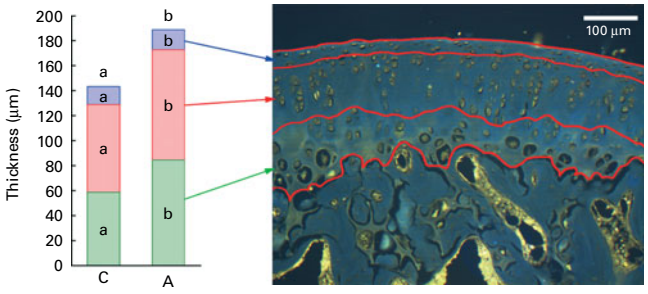

Fig. 4. Effect of 2-oxoglutarate (2-Ox) on femoral articular cartilage thickness. C – animals not receiving 2-Ox; A – animals receiving 2-Ox sodium salt (0·76 g/kg body weight per d), administered in drinking-water. Appropriate zones of articular cartilage are indicated by different colours, and coloured arrows point to the corresponding zones marked on the sample photograph. Letters inside the boxes of the same colour indicate differences of thickness between the groups in the appropriate zone. Letters above the columns indicate differences between the groups related to total thickness. [a,b] Values with unlike letters in columns were significantly different between the groups ($P<0·05$). ■, Superficial; ■, intermediate; ■, deep.

British Journal of Nutrition

Table 2. Effect of 2-oxoglutarate (2-Ox†) on trabecular (Tb.) bone morphology
(Mean values and standard deviations)

	C		A	
	Mean	SD	Mean	SD
Femur epiphysis				
BV/TV%	32·1	10·0	35·5	6·3
Mean intensity of autofluorescence Tb. (8 bit grey scale)	64·3	8·5	70·0*	9·1
Integrated density of autofluorescence	$3{\cdot}0 \times 10^7$	$8{\cdot}2 \times 10^6$	$3{\cdot}6 \times 10^{7}$*	$5{\cdot}0 \times 10^6$
Tb.Th (μm)	139·1	69·2	129·0	52·4
N.Ot/mm^{2}Tb	950·2	355·4	817·3	208·3
Tb.Sp (μm)	294·2	136·2	252·1*	102·0
Femur metaphysic				
BV/TV%	35·3	5·6	39·7	4·8
Mean intensity of autofluorescence Tb. (8 bit grey scale)	72·0	7·0	67·5*	4·5
Integrated density of autofluorescence	$3{\cdot}8 \times 10^7$	$6{\cdot}0 \times 10^6$	$4{\cdot}2 \times 10^7$	$7{\cdot}0 \times 10^6$
Tb.Th (μm)	108·4	46·9	107·8	42·9
N.Ot/mm^{2}Tb	918·5	232·8	1039·0	362·9
Tb.Sp (μm)	200·0	93·4	171·7	77·8
Tibia epiphysis				
BV/TV%	44·5	3·1	34·7*	9·8
Mean intensity of autofluorescence Tb. (8 bit grey value)	60·7	6·1	64·9	6·7
Integrated density of autofluorescence	$4{\cdot}1 \times 10^7$	$3{\cdot}4 \times 10^6$	$3{\cdot}3 \times 10^{7}$*	$7{\cdot}3 \times 10^6$
Tb.Th (μm)	128·1	48·4	115·1*	48·5
N.Ot/mm^{2}Tb	870·6	331·2	814·5	189·6
Tb.Sp (μm)	257·0	117·3	251·2	117·6
Tibia metaphysic				
BV/TV%	33·3	7·9	28·3	7·3
Mean intensity of autofluorescence Tb. (8 bit grey scale)	76·6	7·5	75·4	13·5
Integrated density of autofluorescence	$3{\cdot}7 \times 10^7$	$9{\cdot}3 \times 10^6$	$3{\cdot}0 \times 10^7$	$5{\cdot}5 \times 10^6$
Tb.Th (μm)	90·7	47·3	78·9*	48·2
N.Ot/mm^{2}Tb	1207·4	482·8	1147·2	448·5
Tb.Sp (μm)	157·2	100·2	195·5*	87·8

C, rats not receiving 2-Ox; A, rats receiving 2-Ox sodium salt (0·76 g/kg body weight per d); BV/TV%, relative bone volume; Tb.Th, trabecular thickness; N.Ot/mm^{2}Tb, number of osteocyte/mm^2 of trabecula; Tb.Sp, trabecular separation.
* Mean value was significantly different between the groups ($P<0{\cdot}05$).
† 2-Ox was administered in drinking-water.

unlike articular cartilage, growth plate cartilage is a tissue which is sensitive to load and overload and may, to some extent, slow down the growth, which is mostly related to the hypertrophy zone of this particular tissue[18,40]. And yet we observed an increase in growth plate thickness, in the present study, in the rats treated with 2-Ox. Furthermore, considering the antiproliferative properties of 2-Ox, the most probable answer is that exogenous factors are in control of the use of additional 2-Ox depending on the organism's demand[9]. Thus, at this stage, considering articular and growth plate cartilage thickness after 2-Ox supplementation, we do not have a definite answer regarding whether the 2-Ox or the bone load is the key factor. Additional studies are required to address this issue.

Surprisingly, no changes were found in bone mechanical properties and no convincing changes in bone mineralisation (except for the increased femoral aBMD) or cortical bone histomorphometry, although the administration of 2-Ox caused an increased femur length. Moreover, the histomorphometry analysis of trabecular bone showed unclear differences caused by 2-Ox, pronounced mostly in autofluorescence, which reflects the content of proline and hydroxyproline[22]. The present results from the bone tissue analysis are somewhat in contrast to previous findings, which showed stimulation of bone formation, mineralisation and mechanical properties[5,6,8–11,13]. However, data presented in those studies refer to simulated skeletal or gastrointestinal tract injuries, which were to be the consequence of natural damaging factors or diseases. Moreover, the present study focuses on a particular stage of development, adolescence, which was not covered by previous findings. On the other hand, administration of 2-Ox must have led to an improvement in bone properties, as the femur was significantly longer, had a higher aBMD and intensity and integrated density of autofluorescence (higher bone mineral and collagen density).

In conclusion, the present study showed that dietary supplementation with 2-Ox of growing rats exerts its effects predominantly on cartilage tissue, with a very small influence on bone. The most interesting aspect is the selective effect of 2-Ox on a particular bone and type of cartilage. Different effects of 2-Ox exerted on injured or healthy organisms may be evidence of a regulatory mechanism of nutrient utilisation. 2-Ox may be a key substance in this mechanism of controlling the use of proteins and amino acids, in which there is a regulatory substance or substances (activated by 2-Ox) that enable the appropriate use of nutrients, depending on the stage of development in which the organism is. Nevertheless, this assumption needs further study to elucidate this hypothetical mechanism.

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