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POSTNATAL ADMINISTRATION OF 2-OXOGLUTARIC ACID IMPROVES ARTICULAR AND GROWTH PLATE CARTILAGES AND BONE TISSUE MORPHOLOGY IN PIGS PRENATALLY TREATED WITH DEXAMETHASONE

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The potential effects of prenatal administration of dexamethasone (DEX) and postnatal treatment with 2-oxoglutaric acid (2-Ox) on postnatal development of connective tissue of farm animals were not examined experimentally. The aim of this study was to establish changes in morphological parameters of bone and articular and growth plate cartilages damaged by the prenatal action of DEX in piglets supplemented with 2-Ox. The 3 mg of DEX was administered by intramuscular route every second day from day 70 of pregnancy to parturition and then piglets were supplemented with 2-Ox during 35 days of postnatal life (0.4 g/kg body weight). The mechanical properties, BMD and BMC of bones, and histomorphometry of articular and growth plate cartilages were determined. Maternal treatment with DEX decreased the weight by 48%, BMD by 50% and BMC by 61% of the tibia in male piglets while such action of DEX in female piglets was not observed. DEX led to thinning of articular and growth plate cartilages and trabeculae thickness and reduced the serum GH concentration in male piglets. The administration of 2-Ox prevented the reduction of trabeculae thickness, the width of articular and growth plate cartilages in male piglets connected with higher growth hormone concentration compared with non-supplemented male piglets. The result showed that the presence of 2-Ox in the diet had a positive effect on the development of connective tissue in pigs during suckling and induced a complete recovery from bone and cartilage damage caused by prenatal DEX action.

K e y   w o r d s : *2-oxoglutaric acid, bone, cartilage, dexamethasone, glucocorticoids, growth, foetus*

INTRODUCTION

The longitudinal growth of a typical mammalian long bone composed of cartilage, cortical and trabecular bone occurs at the ends of the bone known as a growth plate (1). It is a highly organized functional unit commissioned for continuous delivery of new cartilage cells and matrix, to be succeeded by bone formation (2, 3). The structural quality of newly-formed bones is primarily determined by genes, and fluctuates with age and health under the influence of hormonal and nutritional modification during the prenatal and postnatal period in both animals and humans (4, 5). Foetal and neonatal lives are crucial periods for the growth and development of systems involved in the pathology of bone metabolism. Various experimental researches and clinical statistics confirm the hypothesis of prenatal programming of many diseases of adults including bone loss and osteoporosis (4-6). Moreover, different lesions with disturbances involving both articular and growth cartilages are a frequent and important cause of lifelong deformity, disability, and degeneration. Animals and humans suffer from osteoarthritis, osteoarthrosis, osteomyelitis, osteochondrosis, angular limb deformities, cuboidal bone malformations, leg weakness and trauma (4, 7-9).

In addition, it is known that maternal stress induces intrauterine growth retardation. Because the stress response is partially mediated by glucocorticoids (GCs), prenatal exposure to GCs may lead to retarded foetal development in both humans and animals (5).

In earlier studies authors were able to demonstrate a positive impact of diet on bone metabolism and intestinal development. Nutrition plays a major role in the early structural development of mammals and has long-term effects that are evident until later in life (10, 11). Specific compounds in food have a particular physiological function (11-13). Functional foods show a beneficial action that improve the state of health and reduce the risk of disease. Several studies have indicated that 2-oxoglutaric acid (2-Ox) known as alpha-ketoglutarate (AKG), the precursor of proline and hydroxyproline, positively influences the growth of the skeleton in fundectomized pigs that showed a lower degree of osteopenia (14). Additionally, it is a precursor of glutamine, arginine and asparagine. An earlier study showed that 2-Ox improves weight gain in piglets receiving synthetic glucocorticoid such as dexamethasone (DEX) compared with piglets under the influence of DEX alone (15). Moreover, 2-Ox administered to piglets during suckling induced a complete recovery from intestinal damage caused by prenatal DEX action (11).

The physiological skeletal changes observable prenatally are well documented histologically and morphologically. However, little is known about the protective role of 2-Ox in piglets at

weaning, after prenatal modulation of bone development with GCs, the most known modulator of prenatal maturation. The influence of prenatal overload with GCs and early neonatal diet on the development of limbs including bone and cartilage, however, has not been thoroughly studied with morphology or histomorphometry. For these reasons, the aim of this study was to establish morphological changes of bone, articular and growth plate cartilages damaged by the prenatal action of DEX in piglets supplemented with 2-Ox for 35 days of postnatal life.

MATERIAL AND METHODS

The experiment was approved by The Local Ethics Committee on Animal Experimentation of University of Life Sciences in Lublin, Poland.

Pregnant sows

The study was performed on 36 piglets born by 10 multiparous sows of Large White Polish breed. Clinically healthy sows were sired by the same boar and housed singly in separated cages under standard rearing conditions (controlled temperature, humidity and 12:12-h light-dark cycle) with free access to fresh water and fed twice a day with well balanced standard commercial diet (contained 1.6% of glutamine in its natural composition) for pregnant and lactating sows that was supplied in equal doses for all animals. The diet did not contain the additional amount of glutamate, glutamine or 2-oxoglutaric acid. The same diet described earlier (16).

To investigate the detrimental effects of prenatal GCs treatment on growth plate and articular cartilages and bone morphology, DEX was administered in sows during the last 45 days of pregnancy. Sows were randomly assigned into two groups weight- and age-matched (5 sows for each group). Sows were intramuscularly injected with DEX (0.03 mg/kg body weight/ every second day; dexamethasone, Eurovet Animal Health B.V., Bladel, The Netherlands) in the morning or with physiological saline (PhS) as a control. A total dose of DEX was about 75 mg per each pregnant DEX-treated sow during the last 45 days of pregnancy. The dose of DEX was determined from a previous study, where DEX was used in a dose of 0.03 mg/kg/48 h for 24 prenatal days and moderately significant effect on bone tissue development in neonatal piglets using this dose was found (13). Therefore in the present study it was decided to increase further the treatment period from 24 to 45 days covering the most intensive prenatal growth in pigs. The gestation length did not differ between PhS-sows and DEX-sows.

Piglets

None of the piglets born by physiological partum had congenital changes. The mean number of stillborn and live born piglets in litters from DEX-sows and PhS-sows did not differ. Females and males were treated in the same manner and used as previously described (13). At birth, unsuckled piglets were weighed and redistributed to either a control or supplemented group during the suckling period. Piglets born by sows administered with PhS belonged to the control group of male (n=6) and female (n=6). Randomly chosen and clinically healthy piglets born by DEX-sows were divided into two groups: orally supplemented with 2-Ox (the DEX/2-Ox group of female (n=6) and male (n=6) piglets) or physiological saline (the DEX group of male (n=6) and female (n=6) piglets). All 35-days-old piglets were euthanized by intravenous injection of lethal dose of pentobarbitalum natrium (Morbital, Biowet, Pulawy, Poland).

Powdered 2-Ox (2-Ox; Protista AB, Sweden) of 99% purity was mixed with distilled water to obtain a solution. The pH of this solution was buffered by the addition of NaOH to a final pH of 7.3. 2-Ox was administered orally starting on the first day of life of piglets to the end of the experiment.

During the first 35 days of postnatal life, piglets from the DEX/2-Ox group received orally the dose of 0.4 g/kg body weight of 2-Ox in a 2 ml of the solution. A dose of PhS given *per os* to piglets from control and DEX groups was identical to the volume of 2-Ox solution prepared for piglets from the DEX/2-Ox group. Experimental administration of 2-Ox was performed each morning. Because of well known general action of 2-Ox on bone development and metabolic processes, the PhS/2-Ox group in our study was intentionally omitted (17-21).

Bone analysis

After removal of soft tissues from the left tibiae, bone length and weight were measured. Each limb bone was wrapped in gauze soaked in isotonic saline and stored at –25°C for further analysis. Bone mineral density (BMD) and bone mineral content (BMC) were determined with the same method and equipment as described earlier (13). The mechanical properties of bones such as the maximum elastic (Wy) and ultimate (Wf) strengths were determined in INSTRON 3369 apparatus (Instron, Canton, MA, USA) as described previously (13, 22).

Bone, articular cartilage and growth plate histomorphometry

After removal of soft tissues, cylindrical 20 mm thick samples (cartilage and bone) were taken from the middle of the lateral condyle (the same anatomical position in the piglet joint) of tibia (proximal epiphysis and metaphysis). All of the joints were free of degenerative changes. The tissue samples were subjected to histology as described previously (13, 23). Sections were cut at 5 μm thick and were not stained. Microscopic (2D-two-dimensional) images (magnification ×100, ×200 and ×400) of autofluorescence were collected using a confocal microscope AXIOVERT 200M (Carl Zeiss, Jena, Germany) equipped with a camera AxioCam HRc (Carl Zeiss, Jena, Germany) and a fluorescent lamp (excitation wavelength 450–490 nm). The analysis of collected images was performed with the use of graphical analysis software ImageJ 1.45f (National Institute of Health USA, http://rsb.info.nih.gov/ij/index.html). The thickness of zones of reserve cartilage (I), proliferation (II), maturation (III), hypertrophy (IV), cartilage degeneration (V) and ossification (VI) was measured at four sites along the growth plate cartilage and an average was taken. Moreover, the number of chondroprogenitor cells from the generative - reserve cartilage was calculated. The four sites were selected a priori and the zones were defined as it was described by Hochberg (3) and Wikstrom *et al.* (24). Similarly, the thickness of three main zones of articular cartilage such as horizontal zone - superficial surface (I), transitional (II) and radial (III) zones was measured. Individual layers of articular cartilage were defined as it was described by Pearle *et al.* (25). Moreover, the total chondrocyte number per mm^2 of articular cartilage and the chondrocyte number per mm^2 of each zone of articular cartilage were calculated. The following parameters were analyzed in bone tissue: the thickness of trabeculae, the number of osseous lacunae per mm^2 of trabeculae and mean lacunae area, and the trabecular bone volume (BV/TV%). The non mineralized space that osteocyte occupies was defined as an osseous lacuna.

Cartilage and bone turnover markers measurement

The animal blood was collected from 35-days-old piglets using standard venipuncture of the subclavian vein, then after

clotting was centrifuged and frozen at –80°C for later analysis. None of 35-day-old piglet was fasted before blood collection. Bone metabolism was assessed by the determination of serum bone alkaline phosphatase, a highly specific and sensitive indicator of alteration in bone turnover, and osteocalcin as a bone formation marker (26). Moreover, growth hormone was used in the study to monitor the effects of prenatal administration of DEX on postnatal development.

Serum bone specific alkaline phosphatase (BAP) activity for all samples was determined in duplicate in a single BAP enzyme-linked immunosorbent assay kit (ELISA; METRA, Quidel Corporation, San Diego, USA). The minimum detection limit of the Metra BAP assay was 0.7 U/L. Osteocalcin (OC) concentration of piglets was determined using porcine osteocalcin enzyme-linked immunosorbent assay kit (Uscn Life Science Inc. Wuhan, China). The minimum detection limit of the assay was 1.6 ng/mL. Growth hormone (GH) concentrations for all samples were determined in duplicate in a single porcine growth hormone enzyme-linked immunosorbent assay kits (Uscn Life Science Inc. Wuhan, China). Minimum detectable concentration was 0.034 ng/mL.

The determination of serum hormonal concentrations was determined using Benchmark Plus microplate spectrophotometer supplied with Microplate manager Software Version 5.2.1. (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

All results are expressed as means ±S.D. (standard deviation). Differences between means were tested with the Two Way ANOVA (with treatment and gender as possible source of variation) and post hoc Tukey's test as the correction for multiple comparisons. Normal distribution of data was examined using the W. Shapiro-Wilk test and equality of variance was tested by the Brown-Forsythe test. If there was a lack of normal distribution and/or unequal variance of data, the Kruskal-Wallis ANOVA was used. A P-value of less than 0.05 was considered statistically significant. All statistical analyses were carried out by means of STATISTICA 8.0 software (StatSoft, Inc. (2008). STATISTICA (data analysis software system), version 8.0. www.statsoft.com).

RESULTS

Bone morphology and mechanical properties

Results of the weight, length and mechanical properties are presented in *Table 1*. Prenatal DEX treatment resulted in a decrease in bone weight by 48% in male piglets compared with the control group. Postnatal administration of 2-Ox resulted in an increase in bone weights in DEX-treated male (76%) and female (16%) piglets when compared with piglets receiving physiological saline. The ultimate strength was also decreased by DEX in male (about 50%) piglets when compared with the control and 2-Ox groups. This difference was significantly reversed by the administration of 2-Ox and the strength was lower only by 25% compared with the control male group. The maximum elastic strength was reduced (41%) by prenatal DEX treatment in male piglets, and the supplementation with 2-Ox enhanced the value of this parameter by 10%. While, the ultimate strength increased in 2-Ox supplemented female piglets about 20% compared with non-supplemented piglets and control female piglets. Moreover, the maximum elastic strength increased in 2-Ox supplemented female piglets by 38% and 51% when compared with non-supplemented (from the DEX group) and control female piglets, respectively.

Bone density

Results of the bone mineral density measurement is presented in *Table 1*. The control male piglets reached the highest value of BMD and BMC. The measurement of BMD and BMC of tibia showed significant decrease (more than 50%) of values obtained in prenatally DEX-treated group of male piglets whereas such action of DEX in female piglets was not observed. BMD and BMC in 2-Ox supplemented female piglets were 18% and 61% higher comparing with the control female piglets, and 8% and 46% higher compared with the DEX group. BMD and BMC in male piglets supplemented with 2-Ox increased by 71% and 34% compared with not-supplemented with 2-Ox piglets affected by DEX. Moreover, BMD and BMC in 2-Ox supplemented male piglets was lower by 15% and 48% compared with the control male piglets.

Bone, articular and growth plate cartilages morphology

The thickness of each zone of growth plate cartilage is presented in *Fig. 1A*. The thickness of superficial, transitional and radial zone of articular cartilage is presented in *Fig. 1B*. The histological sections of tibia of the control piglets showed a normal morphology of articular and growth plate cartilages. Reserve and ossification zones of growth plate were the widest, and hypertrophy and degeneration zones were the thinnest independently on the gender and treatment. Maternal administration of DEX for the last 45 days of pregnancy reduced the thickness of the zone of maturation (by 33%) in male piglets

Table 1. The effect of prenatal dexamethasone (DEX) treatment on morphology, mechanical properties, bone mineral density (BMD) and bone mineral content (BMC) of tibia in male and female piglets at the age of 35 days.

Group (n=6 in each group)	Male piglets			Female piglets		
	Control	DEX	DEX/2-Ox	Control	DEX	DEX/2-Ox
Weight (g)	33.21 ± 1.86^{ad}	17.54 ± 1.61^{b}	30.30 ± 1.80^{ad}	26.82 ± 0.93^{c}	25.37 ± 1.53^{cd}	29.18 ± 1.31^{c}
Length (cm)	8.1 ± 0.2^{a}	7.1 ± 0.7^{a}	7.8 ± 0.1^{a}	7.2 ± 0.6^{a}	7.0 ± 0.9^{a}	7.2 ± 0.5^{a}
Maximum elastic strength -Wy (N)	839 ± 48^{a}	490 ± 35^{b}	578 ± 38^{c}	548 ± 32^{c}	600 ± 45^{c}	829 ± 33^{a}
Ultimate strength -Wf (N)	1370 ± 39^{a}	687 ± 58^{b}	1017 ± 32^{c}	1130 ± 33^{c}	1107 ± 64^{c}	1354 ± 39^{a}
BMD (g/cm^2)	0.443 ± 0.005^{a}	0.221 ± 0.012^{b}	0.378 ± 0.005^{c}	0.355 ± 0.040^{c}	0.387 ± 0.007^{c}	0.418 ± 0.005^{a}
BMC (g)	6.21 ± 0.18^{a}	2.41 ± 0.75^{b}	3.24 ± 0.21^{c}	3.96 ± 0.42^{c}	4.37 ± 0.12^{c}	6.38 ± 0.20^{a}

Data are presented as mean ±S.D. Letters in superscripts indicate significant differences between the groups when P<0.05. Control group – piglets under prenatal treatment with physiological saline; DEX group – piglets being under influence of DEX for the last 45 days of prenatal life; DEX/2-Ox group – piglets after prenatal DEX administration treated with 2-oxoglutaric acid during 35 days of neonatal life. DEX – dexamethasone; 2-Ox – 2-oxoglutaric acid.

when compared with the control, and ossification in both gender (by 46% in males; 40% in females), although the total thickness of articular cartilage was not changed. 2-Ox given to male piglets after prenatal DEX administration improved the thickness of all zones of growth plate, that became wider compared with piglets from the DEX group (II – 25%; III – 22%; IV – 35%; V – 27%; VI – 70%). Similar action of 2-Ox in female piglets prenatally treated with DEX was observed, where reserve (by 58%) and ossification (62%) zones were elongate compared with the DEX group. Moreover, the reserve zone was wider by 61% in
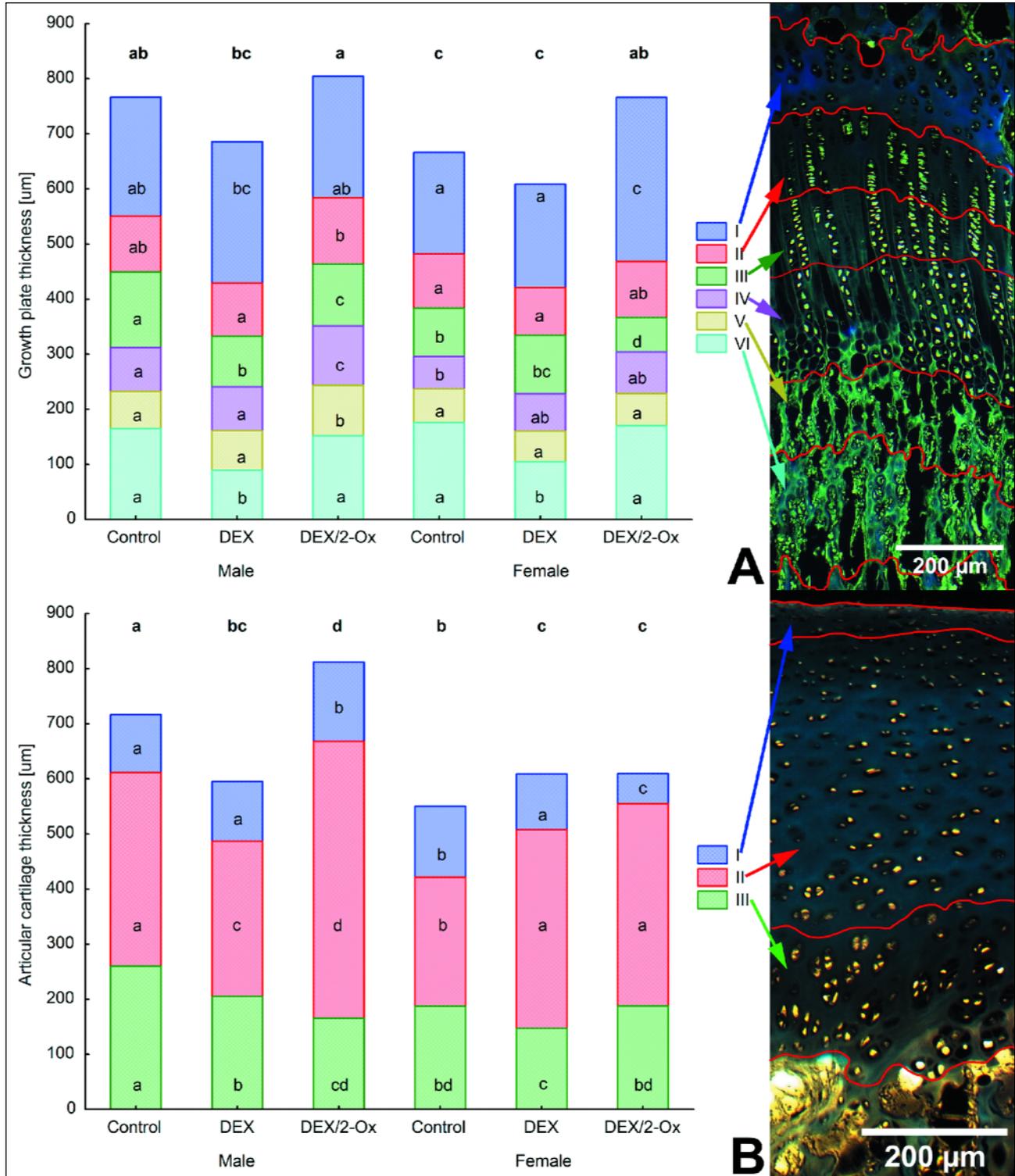

Fig. 1. Effects of postnatal administration of 2-oxoglutaric acid on the cartilage structure in newborn piglets treated with dexamethasone during prenatal time. (A) The thickness of growth plate zones (reserve cartilage – I, proliferation – II, maturation – III, hypertrophy – IV, cartilage degeneration – V, and ossification – VI) of the proximal tibial epiphysis. (B) The thickness of articular cartilage zones of proximal tibia. Different letters inside boxes of the same colour indicate differences of thickness between the groups in the appropriate zone when P<0.05. Different letters above columns indicate differences between the groups related to the total thickness when P<0.05.

Table 2. The effect of postnatal administration of 2-Ox on the chondrocyte number of articular cartilage affected by prenatal action of dexamethasone in piglets at age 35 days. Data are presented as mean ±S.D. Different letters in superscripts indicate significant differences between the groups when P<0.05. Control group – piglets under prenatal treatment with physiological saline; DEX group – piglets being under influence of DEX for the last 45 days of prenatal life; DEX/2-Ox group – piglets after prenatal DEX administration treated with 2-oxoglutaric acid during 35 days of neonatal life. DEX – dexamethasone; 2-Ox – 2-oxoglutaric acid.

Group (n=6 in each group)	Male piglets			Female piglets		
	Control	DEX	DEX/2-Ox	Control	DEX	DEX/2-Ox
Total chondrocyte number per mm² of articular cartilage	765±24ᵃ	906±58ᵇ	860±45ᵇ	956±45ᵇᶜ	1071±90ᶜ	852±45ᵃᵇ
Chondrocyte number per mm² of I zone of articular cartilage	744±40ᵃ	1596±80ᵇ	1242±112ᶜ	1683±104ᵇ	1767±184ᵇ	1312±133ᶜ
Chondrocyte number per mm² of II zone of articular cartilage	784±24ᵃ	810±56ᵃ	732±47ᵃ	861±45ᵇ	936±112ᵇ	695±47ᵃ
Chondrocyte number per mm² of III zone of articular cartilage	687±60ᵃᵇ	629±64ᵃ	825±46ᶜ	813±94ᶜᵇ	867±65ᶜ	768±60ᶜᵇ

Table 3. The effect of postnatal administration of 2-oxoglutaric acid on the morphology of bone tissue trabeculae of tibia affected by prenatal action of dexamethasone in piglets at age 35 days.

Group (n=6 in each group)	Male piglets			Female piglets		
	Control	DEX	DEX/2-Ox	Control	DEX	DEX/2-Ox
Epiphysis						
Trabecular thickness [µm]	114±22ᵃ	54±14ᵇ	109±35ᵃ	140±33ᵃ	62±32ᵇ	137±29ᵃ
Trabecular bone volume (BV/TV %)	18±3ᵃ	22±4ᵃ	21±5ᵃ	21±6ᵃ	23±3ᵃ	30±5ᵃ
Number of osseous lacunae per mm² of trabeculae	1401±214ᵃ	1035±205ᵇ	770±110ᵇ	942±175ᵇ	918±151ᵇ	853±138ᵇ
Osseous lacunae mean area [µm²]	53±16ᵃᵇ	62±18ᵇ	49±17ᵃ	60±17ᵃᵇ	53±18ᵃᵇ	50±12ᵃ
Metaphysis						
Trabecular thickness [µm]	111±28ᵃ	59±11ᵇ	159±35ᵃ	138±33ᵃ	65±15ᵇ	142±36ᵃ
Trabecular bone volume (BV/TV %)	14±2ᵃ	16±4ᵃᵇ	25±5ᵃᵇ	21±5ᵃᵇ	25±8ᵃᵇ	27±3ᵇ
Number of osseous lacunae per mm² of trabeculae	1148±227ᵇ	856±228ᵃᵇ	933±99ᵃᵇ	760±100ᵃ	777±184ᵃ	676±166ᵃ
Osseous lacunae mean area [µm²]	68±15ᵃ	58±14ᵃᵇᶜ	53±12ᵃᵇ	62±16ᵇᵃ	59±19ᵃᵇᶜ	50±13ᶜ

Data are presented as mean ±S.D. Different letters in superscripts indicate significant differences between the groups when P<0.05. Control group – piglets under prenatal treatment with physiological saline; DEX group – piglets being under influence of DEX for the last 45 days of prenatal life; DEX/2-Ox group – piglets after prenatal DEX administration treated with 2-oxoglutaric acid during 35 days of neonatal life. DEX – dexamethasone; 2-Ox – 2-oxoglutaric acid.

comparison with the control female group and about 35% compared with control and DEX/2-Ox groups. On the other hand, the zone of maturation was reduced by 40% compared with the DEX group, and 28% compared with the control female group.

The number of chondroprogenitor cells per mm² of zone cross section was similar in the control and DEX group and accounted 1023±54 and 1053±82 in male groups, and 1108±50 and 1007±129 in female groups, respectively. The number of chondroprogenitor cells per mm² of zone cross section was reduced after 2-Ox and DEX administration in male and female piglets when compared with the respective control group, and reached the value of 918±41 and 953±66, respectively.

In brief, DEX administration resulted in thinner articular cartilage in male piglets. DEX treatment significantly reduced the thickness of transitional and radial zones by 20% in male piglets, and shortened superficial and radial zones by 21% in female piglets. Prenatal administration of DEX resulted in elongated transitional zone by 57% in female piglets compared with the control group. 2-Ox given postnatally after prenatal treatment with DEX elongated the transitional zone by 43% in male piglets and by 57% in female piglets, and the superficial zone by 36% in male piglets compared with the control group. Although, the radial zone was reduced by 36% in male piglets, articular cartilage was significantly wider compared with other male groups. Moreover, the administration of 2-Ox did not improve the thickness of the superficial zone after DEX action in female piglets. This zone was reduced by 57% compared with female control piglets.

The effect of postnatal administration of 2-Ox on the chondrocyte number of articular cartilage affected by prenatal action of DEX is presented in *Table 2*. The total chondrocyte number was significantly enhanced by DEX administration in male piglets (18%) when compared with the control group that was caused by the increase of chondrocyte number in the superficial zone by 114%. Lower increase of total number of chondrocytes was observed in male piglets supplemented with 2-Ox (12%), and it was caused by the increase of the chondrocyte number in superficial (67%) and radial (20%) zones. Although, the total number of chondrocytes did not differ between female piglets, the decrease of the number of chondrocyte about 20% in superficial and transitional zones in female piglets supplemented with 2-Ox was observed when compared with the control group. Moreover, the total chondrocyte number decreased by 20% in female piglets supplemented with 2-Ox when compared with not-supplemented from the DEX group. This decrease was caused by the reduction of the chondrocyte number in transitional and superficial zones by 25%.

The effect of postnatal administration of 2-Ox on the morphology of bone tissue trabeculae of tibia affected by prenatal action of DEX is presented in *Table 3*. The analysis of trabecular bone showed that DEX treatment during prenatal time reduced the number of osseous lacunae of epiphyseal trabeculae in male piglets and the thickness of epiphyseal and metaphyseal trabeculae about 50% in both genders. The administration of 2-Ox prevented from this reduction of the trabecular thickness and reduced the area of osseous lacunae of epiphyseal trabeculae in male piglets when compared with the DEX group, and reduced the area of osseous lacunae of metaphyseal trabeculae in female piglets when compared with the control group.

Table 4. The effect of postnatal administration of 2-oxoglutaric acid on the concentration of growth hormone (GH), osteocalcin (OC) and the activity of bone alkaline phosphates (BAP) in the serum obtained from 35-day-old male and female piglets prenatally treated with dexamethasone. Data are presented as mean ± S.D. Different letters in superscripts indicate significant differences between the groups when P<0.05. Control group – piglets under prenatal treatment with physiological saline; DEX group – piglets being under influence of DEX for the last 45 days of prenatal life; DEX/2-Ox group – piglets after prenatal DEX administration treated with 2-oxoglutaric acid during 35 days of neonatal life. DEX – dexamethasone; 2-Ox – 2-oxoglutaric acid.

Group (n=6 in each group)	Male piglets			Female piglets		
	Control	DEX	DEX/2-Ox	Control	DEX	DEX/2-Ox
GH [ng/mL]	5.3±1.1[ab]	3.2±1.1[a]	5.4±1.2[ab]	4.0±1.3[ab]	4.6±3.9[ab]	8.4±0.9[b]
OC [ng/mL]	36.9±3.3[a]	35.7±1.8[a]	35.0±4.1[a]	29.0±2.3[a]	37.0±1.5[a]	37.0±1.4[a]
BAP [U/L]	120.8±29.0[ab]	149.1±28.8[ab]	149.7±3.7[ab]	106.7±29.4[a]	168.7±30.6[b]	142.0±6.7[ab]

Bone and cartilage turnover marker assay

Maternal DEX treatment for the last 45 days of pregnancy significantly reduced serum GH concentration in male piglets when compared with female piglets from the DEX/2-Ox group, where was observed the highest concentration of GH (*Table 4*).

DISCUSSION

The process of longitudinal bone growth is regulated by a complex system of endocrine signals, including growth hormone, insulin-like growth factors, GCs, thyroid hormone, estrogen, androgen, vitamin D, and leptin (27). Many of them regulate the growth plate metabolic function. Numerous human and animal skeletal disorders are caused by abnormalities in the endocrine regulation of the growth plate. Among those factors that may damage the growth plate, GCs are critical for normal prenatal development and at the same time may inhibit growth. In addition, an excess of GCs during prenatal life may play a role in the pathogenesis of low peak bone mass and then in intensified age-related bone loss. There are many ways in which GCs can exert their action on the skeleton and related tissues. Responses to GCs can occur by genes or by non-genomic mechanisms associated with their receptors (27).

Morphological analysis of the growth plate in this study revealed that the ossification zone was reduced and the tendency towards a decrease in the total thickness after prenatal DEX administration independently of gender was observed. Maternal DEX treatment also disturbed the maturation of growth plate chondrocytes in male piglets. The amount of calcified cartilage might be insufficient to form a new bone and to reach an appropriate bone mass. This was supported through the measurement of bone mineral density which showed that prenatal DEX administration reduced BMD and BMC in male piglets. In addition, analysis of trabecular bone architecture showed a loss of trabecular bone through thinning and a DEX-related increase in trabecular porosity. Maternal DEX treatment negatively influenced bone formation and led to changed trabecular architecture, mainly in male piglets. Thus, the changed trabecular architecture might explain the insufficient bone mass and low bone quality as adaptive transformations of the growing foetus in response to the excess of GCs given *via* their mothers (28). Bone architecture and bone loss leading to a drop in mineral content might be important predictors of fractures. Bone strength is derived from bone quantity, which comprises density, and quality, which consists of structure thus, mechanical bone testing revealed significantly lower values and showed that maternal DEX treatment enhances the risk of fracture in male piglets. Bone formation is carried out by chondroprogenitor cells in the growth plate through the proliferation and maturation process controlled by hormones, cytokines and growth factors. Various stages of chondroprogenitor cells with other activities occur in different zones of the growth plate. This might explain the molecular mechanism of GC induced changes in bone growth and mass. Decreased production of GH is the reason why an excess of GCs might trigger the formation of thinner trabeculae. Although maternal GCs treatment also resulted in a twofold decrease in the trabeculae thickness in DEX-treated females, an increase in the number of osseous lacunae and reduced BMD were not observed. Thus, negative effects were in male piglets. A possible explanation for the difference in DEX action was that, both local and systemic mechanisms might contribute to weaken bone and to slow growth. It has been suggested, for example, that decreased concentration of GH in male newborn piglets might play a role. Other markers of bone formation used in order to clarify the underlying pathophysiological mechanism were not affected. Additionally, the role of GCs in either local growth plate regulation or in systemic growth could also explain the observed delay. Growth plate chondrocytes having disturbed proliferation (although only a tendency towards a reduction of this zone was observed and the chondroprogenitor cells number was not changed) and maturation capacity after maternal GCs treatment were observed in male piglets. Thus, the reason for low bone mass was multifactor and could contribute to osteoporosis and the increase in fracture risk.

In vitro and *in vivo* observations demonstrate a direct inhibitory effect of GCs on multiple aspects of bone formation. It is believed that the direct inhibitory action of GCs on the osteoblast play a major role in metabolic diseases observed following a GCs excess. *In vitro* studies demonstrate that the effects of GCs are dependent on the stage of osteoblast growth and differentiation. Higher doses of GCs than a physiological range observed for example during stress, inhibit the osteoblastic function and have a negative impact on bone mass. Possibly, GCs reduce the number of bone-forming cells by decreasing their formation and inducing their apoptosis. Finally, it can damage foetal skeletal growth and development. On the other hand, a physiological concentration of GCs is needed to induce cell differentiation of the osteoblastic lineage into mature cells (29-32).

The trabeculae number does not increase during growth but remains constant and determines the amount of mineralized bone. Earlier findings show that GCs treatment results in reduced trabeculae thickness and disruption of cancellous architecture of other bones than the tibia, and the negative effect of maternal DEX treatment negatively influences bone mineral density and mechanical properties and is connected with changes in humoral factors and the free amino acid pool in male piglets (6, 16).

Genetic, hormonal and environmental factors are considered to be important triggers of metabolic bone diseases, including articular cartilage. In addition, despite the long-term intra-articular GCs use leading to accelerated cartilage degradation, little is known about the molecular mechanism underlying GCs-mediated inhibition of the chondrocyte growth (33). The primary role of articular cartilage is to provide a low-friction, wear-

resistant surface that can withstand large loads. Within the body, cartilage serves to facilitate load support and load transfer while allowing for rotation between bones. The degree of loading in a joint is dependent on its location in the body. Because of the force exerted on the hip and knee (calculated to be 3.3 and 3.5 times the bodyweight, respectively) morphological changes in articular cartilage can easily lead to joint alteration during normal functioning (27).

The presented results showed that maternal DEX treatment caused significant reduction of the articular cartilage thickness in male piglets connected with an increased chondrocyte number in the superficial zone. The decrease in the intercellular matrix might cause the alteration of cartilage properties that may become less visco-elastic and predisposed to injury later in life even under normal loading. A different effect was observed in female piglets. Despite the tendency of reduced total thickness of articular cartilage with a shortened zone of ossification, an increased of chondrocyte number was not observed.

Metabolically dynamic tissues like bone and cartilage require not only regular stimulation from weight-bearing activity but high quality nutrients for healthy growth. The stimulation of bone formation through nutrients is probably critical in establishing optimal skeletal mass and structure. Despite its obvious importance, comparatively little is known about the nutritional requirements of infants. The major food problem in a deficient diet is the scarcity of good quality protein (34). Bone mineral density, a determinant that provides 70% of the strength, is adversely affected by poor nutrition. Several earlier studies showed that 2-Ox may bring beneficial effects to human and animal existence as a functional dietary component (35, 36). Other findings proved that 2-Ox prevents bone loss associated with fundectomy, gastrectomy and GCs therapy (14, 23). Moreover, the intra-articular injection of nutritive mixture solution containing glutamine and glutamate, formulated to supply nutrients to chondrocytes, is a potent treatment that significantly delays osteoartritis progression (35, 37). Additionally, 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 (38). Beyond the physiological role of glutamate in joints, growing evidence indicates that glutamate-mediated events may contribute to the pathogenesis of human arthritic conditions. Increased levels of glutamate are seen in the knee joint of rats with early osteoarthritis and in the synovial fluid of patients with active arthropathies (38).

To our knowledge, no such effect of 2-Ox concerning the recovery of articular and growth plate cartilages after maternal GCs treatment has been previously reported in literature. The performed study showed that 2-Ox given to piglets after prenatal GC overload significantly reduced the negative action of DEX in male piglets connected with higher GH concentration compared with non-supplemented male piglets. This supplementation improved the thickness of each zone of the growth plate and two zones of articular cartilage, the first, which is more compressed than other and the second zone, which transfers and distributes the load. On the other hand 2-Ox given to male piglets prevents the decrease of intercellular space and probably improves its properties. To our knowledge, the present study showed for the first time that 2-Ox administered to piglets during suckling induced a complete recovery of growth plate damage caused by prenatal DEX action. The treatment with glutamate derivative was effective in repairing the dramatic changes of connective tissue induced by prenatal DEX action. This finding was supported by mechanical testing and morphological measurements, which revealed significant improvement of many parameters. 2-Ox also prevented the reduction of BMD and BMC in male piglets. Postnatal administration of 2-Ox to female piglets affected by the prenatal action of DEX elongated growth plate and articular cartilage compared with non-supplemented piglets. This effect of 2-Ox might prevent the risk of arthropathies because as adult cartilage still undergoes endochondral ossification, mature cartilage continues to decrease in thickness as one ages. Moreover, a tendency towards improved histomorphometrical parameters of bone tissue and other parameters including GH showed that bone became more robust. GH determines body size, including the size of the skeleton before epiphyseal closure, and regulates lean body mass and bone remodeling after epiphyseal closure.

Numerous animal models of foetal growth report differences in outcome related to foetal gender. Other studies showed a gender differences in growing animals (39). This study demonstrated interesting results in relation to the female foetus, which require further investigation. It suggested that there were significant swine foetal-gender differences in steroid metabolism (probably foetal gender-specific adverse effects on placental function) and sensitivity during pregnancy. It is also suggested that foetal and neonatal male piglets were more sensitive to the negative action of DEX, a steroid not metabolized by the placental enzyme, at a time when the sexual differentiation of the neuroendocrine system is not observed.

This study used pregnant mother conditions for a pig model, enabling investigating the effects of hormones on bone growth and joint function during the neonatal period. The result showed for the first time that diet (2-oxoglutaric acid) influenced the development of connective tissue during the neonatal period as indicated by the observed differences in the bone, growth plate and articular cartilage between the investigated groups of pigs in the neonatal period. This indicates that 2-oxoglutaric acid had a positive effect on the development of connective tissue and might protect against the prenatal action of DEX, which damages not only bone but growth plate and joint as well. A diet rich in 2-Ox might be recommended during the last trimester of gestation as a protective diet against bone and joint disease.

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