

Dietary 2-oxoglutarate prevents bone loss caused by neonatal treatment with maximal dexamethasone dose

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Impact statement

The present study has showed, for the first time, that dietary 2-oxoglutarate (2-Ox) administered postnatally has a potential to improve/maintain bone structure of animals simultaneously treated with maximal therapeutic doses of dexamethasone (Dex). It may open the new direction in searching and developing combined treatment for children treated with glucocorticoids (GCs) since growing group of children is exposed to synthetic GCs and adverse effects such as glucocorticoid-induced osteoporosis and growth retardation are recognized. Currently proposed combined therapies have numerous side effects. Thus, this study proposed a new direction in combined therapies utilizing dietary supplementation with glutamine derivative. Impairment caused by Dex in presented long bones animal model was prevented by dietary supplementation with 2-Ox and vast majority of assessed bone parameters were restored almost to the control level. These results support previous thesis on the regulatory mechanism of nutrient utilization regulated by glutamine derivatives and enrich the nutritional science.

Abstract

Synthetic glucocorticoids (GCs) are widely used in the variety of dosages for treatment of premature infants with chronic lung disease, respiratory distress syndrome, allergies, asthma, and other inflammatory and autoimmune conditions. Yet, adverse effects such as glucocorticoid-induced osteoporosis and growth retardation are recognized. Conversely, 2-oxoglutarate (2-Ox), a precursor of glutamine, glutamate, and collagen amino acids, exerts protective effects on bone development. Our aim was to elucidate the effect of dietary administered 2-Ox on bone loss caused by neonatal treatment with clinically relevant maximal therapeutic dexamethasone (Dex) dose. Long bones of neonatal female piglets receiving Dex, Dex+2-Ox, or untreated were examined through measurements of mechanical properties, density, mineralization, geometry, histomorphometry, and histology. Selected hormones, bone turnover, and growth markers were also analyzed. Neonatal administration of clinically relevant maximal dose of Dex alone led to over 30% decrease in bone mass and the ultimate strength ($P < 0.001$ for all). The length (13 and 7% for femur and humerus, respectively) and other geometrical parameters (13–45%) decreased compared to the control ($P < 0.001$ for all). Dex impaired bone growth and caused hormonal imbalance. Dietary 2-Ox prevented Dex influence and vast majority of assessed bone parameters were restored almost to the control level. Piglets receiving 2-Ox had heavier, denser, and stronger bones; higher levels of growth hormone and osteocalcin concentration; and preserved microarchitecture of trabecular bone compared to the Dex group. 2-Ox administered postnatally had a potential to maintain bone structure of animals

simultaneously treated with maximal therapeutic doses of Dex, which, in our opinion, may open up a new opportunity in developing combined treatment for children treated with GCs.

Keywords: Glutamine derivative, glucocorticoids, newborn, osteoporosis, supplementation

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Introduction

Glucocorticoids (GCs) are widely used in pediatric practice.^{1–6} GCs administration is an established therapy to lessen chronic lung disease, in the treatment of asthma

and croup, and to reduce mortality in premature infants with respiratory distress syndrome.^{4,7–10} A growing group of children is chronically exposed to steroids through the increasing incidence and prevalence of childhood atopy,

related to environmental pollution and processed food, and it is estimated that 10% of children and adolescents have or require some form of GCs treatment.^{3,6}

Despite this variety of beneficial applications of GCs, often lifesaving, there is a growing body of evidence for their adverse effects, i.e. suppression of the hypothalamic-pituitary-adrenal axis, decrease in weight gain, bone loss and growth retardation, and many more.^{3-7,11-13} Furthermore, glucocorticoid-induced osteoporosis (GIO) is the most common iatrogenic cause of secondary osteoporosis as a long-term side effect of clinical use of GCs.⁴ Children treated with GCs have lower spine bone mineral density (BMD) and higher rates of morphometric fractures compared to healthy ones.² Nevertheless, several possible mechanisms of GIO are proposed in this regard. On the one hand, inhibition of bone formation by bone cells imbalance caused by increased apoptosis and lower activity of osteoblasts and osteocytes along with additional effect of GCs on regulatory proteins ratio. On the other hand, a rapid bone loss related to diminished synthesis of proteins such as collagen or osteocalcin (OC) as well as disturbance in cell signaling leading to malfunction of repair mechanisms and bone fragility following microdamage accumulation.^{1,2,4,9} However, evidence-based management guidelines for children who require a long-term GC therapy are not well established. Since clinical trials involving children on GCs are sparse, treatment options for GIO in children are greatly limited.^{1,2} Recent studies show limited information on bisphosphonates, vitamin K₂, D, calcitriol, and calcium supplementation in this regard.^{1,3,4,9} On the other hand, studies in animals and humans revealed protective effects of 2-oxoglutarate (2-Ox) (alpha-ketoglutarate) on bone loss caused by gastrectomy, ovariectomy, menopause, and GCs prenatal administration.^{14,15}

2-Ox is a crucial intermediate in the tricarboxylic acid cycle, taking part in the cellular energy metabolism and in a variety of metabolic pathways as an important nitrogen transporter. It bridges cellular energy metabolism with the metabolism of amino acids, does not show toxic properties, and is highly stable in water solutions. However, since it is impossible to utilize 2-Ox from the tricarboxylic acid cycle in the synthesis of amino acids, it has to be provided as a pure dietary supplement in order to do so. Further, such enteric supplementation of 2-Ox can increase plasma levels of insulin, growth hormone (GH), and insulin-like growth factor-1 (IGF-1). 2-Ox is converted into proline, leucine, and other amino acids in the enterocytes. In addition, it has been demonstrated that 2-Ox is involved in collagen metabolism through a variety of mechanisms and that it counteracts bone loss.^{14,16}

In our previous studies we showed bone loss caused by dexamethasone (Dex), a synthetic GC, administered pre- and postnatally in animal models. We also showed that dietary supplementation with 2-Ox has beneficial effects on growth and performance and that it can prevent bone loss after prenatal Dex treatment.^{15,17,18} Based on the above knowledge about GCs' adverse effects in children and on our previous studies, we hypothesize that 2-Ox may have a potential to prevent or at least diminish bone loss after neonatal GCs treatment. Hence, the aim of this study was to

elucidate the effect of dietary administered 2-Ox on bone loss caused by neonatal treatment with maximal Dex dose.

Materials and methods

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

Animal breeding and experimental design

Ten clinically healthy multiparous sows (Large Polish White) were mated naturally with the same boar of the same breed and their litters were used in this experiment. All sows showed similar care about their previous offspring. The time of parturition was calculated from the second day of the mating, which lasted three days. Sows were singly housed in separate cages under standard rearing conditions (constant temperature, humidity, and 12/12h day night cycle) with free access to fresh water and fed twice a day with standard commercial diets for pregnant and lactating sows (2.3 kg/day). All piglets were born naturally, had no congenital infections, and litters contained eight or more piglets. On the day of birth, litters were weighed and sexed before suckling. Female piglets were used in the subsequent study to account for sex differences. Piglets were not separated from mother after farrowing and were held in the same cage, thus were not transferred from one sow to another. During suckling piglets did not receive feed, iron, or other medication. Piglets were randomly divided into three experimental, weight-matched groups (N = 12 in each): the Dex group, in which piglets received intramuscular injections of Dex at the dose of 0.5 mg/kg b.w. daily (Dex pro inj. 0.2%, Eurovet Animal Health B.V., Bladel, The Netherlands); the Dex plus 2-oxoglutaric acid group (Dex+2-Ox), in which piglets received Dex at the same dose, manner, and time, and additionally a solution of 2-oxoglutaric acid at the dose of 0.4 g/kg b.w./day administered orally and the control group, in which piglets received injections with physiological saline at the volume and manner corresponding to Dex-treated groups. Powdered 2-oxoglutaric acid of 99% purity (Protista Biotechnology AB, Lund, Sweden) was mixed with distilled water and buffered by NaOH to a final pH of 7.3. Animals from Dex and control groups also received orally a solution of physiological saline to balance the amount of Na ions in all three groups. The duration of all described treatments lasted from one to 35 day of piglets life and animals were weighed every second day to calculate Dex and 2-Ox doses. At the end of the experiment all piglets were weighed and euthanized by intravenous injections of a lethal dose of pentobarbitalum natrium (Morbital, Biowet, Puławy, Poland).

Bone analysis

Left femora, humeri, and four ribs (sixth and seventh taken from each side) were isolated from all piglets. Once cleaned of remaining soft tissues, the bones were weighed and measured. Each limb bone and ribs was wrapped

in gauze, soaked in isotonic saline, and stored at -25°C for further analysis.

BMD and bone mineral content (BMC) were determined by dual energy X-ray absorptiometry using a Discovery W Hologic X-ray densitometer (Bedford, MA, USA) and APEX 3.0.1 software with Small Animals Studies option for investigation of bones from various types of animals. Densitometer self-calibration was performed before the measurements using Hologic Automatic Internal Reference System. An optional scout scan was available to assist the operator in defining the scan region. The analysis was performed on the scan data using an operator-defined region of interest and numeric results were calculated and displayed. The measurements of BMC and BMD were performed using the following parameters: additional calibration with a phantom provided by the manufacturer, the resolution 0.5 line pairs/mm. The region of interest after the scout scan was defined manually. Measurements were performed for the whole bone samples and for proximal part of the limb bone including both the trabecular and cortical bone compartments, and for the neck of ribs (i.e. the flattened portion which extends laterally from the head, about 2 cm in length).

Volumetric bone density of the femora and humeri was measured by quantitative computed tomography using a GE LightSpeed VCT 64 Slice CT apparatus (GE Healthcare, Pollards Wood, United Kingdom) supplied with LightSpeed VCT Select with 64-channel upgrade 12HW14.6 software (GE Healthcare, Little Chalfont, United Kingdom) in terms of both trabecular and cortical bone by means of 2 mm thick, cross-sectional, metaphyseal and diaphyseal scans. Trabecular bone density (Td) of femur was measured in the distal metaphysis with the scan positioned just below the distal growth plate, proximal to the distal growth plate. Td of humerus was measured in the proximal metaphysis with the scan positioned just below the proximal growth plate. Cortical bone density (Cd) was assessed at 50% of the length of both bones.¹⁹

Mechanical properties of bones were determined after thawing at room temperature using a three-point bending test in INSTRON 4302 apparatus (Instron, Canton, MA, USA). The distance between supports was set at 40% of total bone length and bone samples were loaded with a constant speed of 10 mm/min. The maximum elastic strength (Wy) and the ultimate strength (Wf) of the bones were determined as described previously.¹⁷

Geometry of bones was estimated on the basis of horizontal and vertical diameter measurements of the mid-diaphyseal cross-section of bone. The cross-section area (A), the mean relative wall thickness (MRWT), cortical index (CI), and the second moment of inertia (Ix) were determined.^{17,20}

Histomorphometry

The fragments of femora, humeri, and ribs were subjected to histology as previously described,²¹ except sections were cut 4 μm thick. Samples of rectangular shape, 2 mm thick, from the surface of epiphysis to 5 mm below the growth plate of metaphysis, were cut out from each femur

(from the central part of the lateral condyle, sagittal section) and each humerus (from the central part of the head, transverse section). Fragments of rib bone from the head of the rib through the neck to a distance of 5 mm past the tubercles were also taken. Eighteen sections from each bone (at 40 μm intervals after each series of three sections) were cut from each individual piglet with a microtome (Microm HM 360, Microm, Walldorf, Germany). Total thickness of section plane was 272 μm for each bone. For histological analysis, the sections were stained with hematoxylin and eosin. Microscopic (two-dimensional) images of fluorescence (10 images magnification $\times 50$ and 25 images magnification $\times 630$) of each slice were taken using a confocal microscope (AXIOVERT 200 M, Carl Zeiss, Jena, Germany) equipped with a color digital camera (AxioCam HRc, Carl Zeiss, Jena, Germany) and a fluorescent lamp (excitation wavelength 450–490 nm).²² The analysis of collected images was performed using graphic analysis software ImageJ 1.50 a (National Institute of Health USA, <http://rsb.info.nih.gov/ij/index.html>) equipped with BoneJ, a series of plugins dedicated for bone analysis. The following parameters were analyzed for epiphysis and metaphysis of each bone: the fractal dimension of the trabecular bone, the trabecular bone volume (BV/TV%), mean and maximum trabecular thickness (Tb.Th), mean and maximum trabecular separation (Tb.Sp). Additionally, for the trabecular bone, the number of osseous lacunae per square millimeter of trabeculae, the total area of osseous lacunae per square millimeter of trabeculae, and the mean cross-sectional area of osseous lacunae were assessed as previously described.^{18,21,23}

Blood sample collection and analysis

Blood samples collection and analysis were carried out as described previously.^{15,24} Briefly, blood of not fasted piglets was collected from subclavian vein. Serum and plasma samples, following blood centrifugation (clotted or total blood, respectively) (20 min at 3000 rpm; Versatile Sigma 2-16P centrifuge, Sigma Laborzentrifugen GmbH, Harz, Germany), were partitioned and stored at -80°C until further analysis.

Selected hormones serum concentrations were determined using commercially available Enzyme-Linked Immunosorbent kits according to manufacturer's protocols: GH, OC, and leptin (Diagnostic System Laboratories, Inc., Webster, USA), bone-specific alkaline phosphatase (BAP) (METRA, Quidel Corporation, San Diego, USA), IGF-1 (Uscn Life Science Inc., Wuhan, P. R. China), and cortisol (MyBioSource, Inc., San Diego, USA). The determination of serum hormonal concentrations was performed using Benchmark Plus microplate spectrophotometer supplied with Microplate manager Software Version 5.2.1. (Bio-Rad Laboratories, Inc., Hercules, CA, USA).^{15,17,24}

The plasma concentrations of glycine, glutamine, glutamate, and proline were analyzed using ion-exchange chromatography on an INGOS AAA 400 automatic analyzer (Ingos, Praha, Czech Republic).²⁵ Serum concentration of calcium, phosphorus, and sodium was determined using energy-dispersive X-ray spectrometry (ED 2000 Spectrometer, Oxford Instruments, Buckinghamshire, UK)

in dry matter of the lyophilized samples as described previously.²⁵ Urea, uric acid, creatinine, albumin, and total protein were determined in the blood serum using kits from Cormay (Warsaw, Poland).²⁶

Statistical analysis

All results are expressed as mean \pm standard deviation. Differences between means were tested with the use of one-way analysis of variance (ANOVA) and a *post hoc* Tukey's honest significant difference test as a 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. When there was a lack of a normal distribution and/or an unequal variance of data, the Kruskal-Wallis test was used to analyze the differences between means and *post hoc* tests for mean range for every pair of groups. The two-sided significance level (*P* value) of less than 0.05 was considered statistically significant. All statistical analyses were carried out by means of STATISTICA (data analysis software system), version 12. StatSoft, Inc. (2014). STATISTICA Power Analysis tool was used to calculate the size of the sample and the statistical power as a function of the error and the type and size of the effect for the one-way ANOVA. High power of statistical tests (86–99%) was reached for assessed parameters. The effect size was calculated and interpreted according to Cohen *f* according to Kotrlik *et al.*²⁷ All presented statistically significant differences had large effect sizes (over 0.56), except for the cross-sectional area of osseous lacunae where medium effect size (0.38) was noticed.

Results

Dex treatment resulted in significant weight loss in both groups receiving Dex (Dex 3300 ± 246 g, Dex + 2-Ox 4612 ± 285 g) compared to the control group 5045 ± 482 g ($P < 0.001$ and $P = 0.013$, respectively). However, weight loss was considerably lower in piglets supplemented with 2-Ox compared to the Dex group ($P < 0.001$).

The weight; length; mechanical and geometrical properties; as well as BMD and content of femora, humeri, and ribs are presented in Table 1. The majority of assessed parameters in the Dex group declined significantly compared to the control group, except MRWT and proximal BMD of humeri, and length of ribs. On the other hand, supplementation with 2-Ox prevented most of such declines or at least reduced influence of Dex (Table 1). All examined bones had lower weight in the Dex group compared to the control, starting from 34% for humerus, through 35% for femur, to 45% for rib ($P < 0.001$ for all). The bone weight of piglets receiving Dex+2-Ox was similar to the control group except for ribs which were significantly lighter (30%), but still 28% heavier than in the Dex group. Very similar effect was observed in the length of femur and humerus. Piglets from the Dex group had considerably shorter long bones compared to the control (13% for femur and 7% for humerus, $P < 0.001$ for both bones) and supplementation with 2-Ox abolished this effect, thus there was no difference between the Dex+2-Ox and the control group. There was no significant difference in the length of ribs among all groups

(Table 1). The ultimate strength of femur and humerus in the Dex group was 30% lower than in the control animals and ribs were even weaker (42%) ($P < 0.001$ for all). Supplementation with 2-Ox significantly reversed this effect in humerus, which was 40% more resistant to breakage than in the Dex group. The effect of 2-Ox on Wf of femora and ribs was not as substantial, but still these bones of piglets from the Dex+2-Ox group were stronger (13 and 23%, respectively) than in the Dex group, although not significantly. Similarly, the elastic strength of femora, humeri, and ribs declined in the Dex group, compared to the control by 20, 15, and 40% ($P = 0.004$, $P = 0.014$, $P < 0.001$), respectively. In the case of Wy, supplementation with 2-Ox preserved the flexibility of assessed bones, although to a lesser extent in femur, and there were no significant differences between the Dex+2-Ox and the control group (Table 1). Dex administered alone reduced all geometric parameters (A, MRWT, CI, and Ix) in femur by 29 ($P < 0.001$), 19 ($P = 0.013$), 13 ($P = 0.012$), and 45% ($P < 0.001$), respectively; in rib by 15, 49, 37, and 9% ($P < 0.001$ in all parameters), respectively; and in humerus by 20 ($P = 0.012$), 15 ($P = 0.096$), 13 ($P = 0.033$), and 34% ($P = 0.003$), respectively, as compared to the control group. In other words, bone shafts of piglets from the Dex group had thinner walls than in the control. On the other hand, the impact of Dex on bone geometry was almost completely abolished in the Dex+2-Ox group, since there were no substantial differences in geometry of femora, ribs, and partly in humeri compared to the control. MRWT of humeri did not differ significantly among all groups (Table 1). Total BMD and BMC of femora, humeri, and ribs were reduced in the Dex group compared to the control by an average of 21 and 33%, respectively ($P < 0.001$ for femur and humerus, and $P = 0.004$ for rib). Although 2-oxoglutarate improved total BMD and BMC in femora of animals from the Dex+2-Ox group compared to the Dex group (11%, $P = 0.016$ and 53%, $P < 0.001$, respectively), the mineralization was still significantly lower than in the control (11% $P = 0.002$ for BMD, 13% $P = 0.009$ for BMC). Total BMD in humerus was also improved by supplementation with 2-Ox compared to the Dex group (14%, $P < 0.001$), although it was still beneath the control level (11%, $P = 0.016$). Total BMC was lower in humeri of both Dex and Dex+2-Ox groups than in the control (25% $P = 0.015$, 33% $P = 0.003$, respectively). Similarly, total BMD and BMC of ribs were significantly lower in both Dex and Dex+2-Ox groups than in the control (on average 25%, $P < 0.001$ for all). Dex decreased BMD in proximal femur regardless of the group (17%, $P = 0.016$ for the Dex group and 14%, $P = 0.041$ for the Dex+2-Ox), as well as proximal BMC of humerus (29%, $P = 0.020$ for the Dex group and 27%, $P = 0.030$ for the Dex+2-Ox). On the other hand, although BMC of proximal part of femur was significantly decreased by Dex (39%, $P < 0.001$) compared to the control, supplementation with 2-Ox prevented such dramatic decrease in the Dex+2-Ox group where proximal BMC was 28% higher than in the Dex group ($P = 0.010$), however still 22% lower than in the control group ($P = 0.001$). Dex had no substantial influence on proximal humerus BMD (6% decrease, $P = 0.195$), nevertheless piglets from the Dex+2-Ox group had this

Table 1 The mechanical, geometrical, and densitometry properties of selected bones of 35-day-old female piglets treated neonatally with dexamethasone (Dex) or Dex and 2-oxoglutaric acid (2-Ox)

Group (n = 12/group)	Femur						Humerus						Rib					
	Control			Dex			Control			Dex			Control			Dex		
	mean	SD		mean	SD		mean	SD		mean	SD		mean	SD		mean	SD	
Weight (g)	27.1	2.7a		17.7	2.4b		24.0	3.6a		15.9	2.2b		23.6	1.8a		2.7	0.3a	
Length (mm)	81.1	1.8a		70.6	3.9b		72.1	2.2a		67.2	4.0b		74.2	3.1a		95.5	7.3a	
Wf (N)	1122	58a		782	99b		904	94a		635	96b		886	112a		76	11a	
Wy (N)	705	91a		564	60b		565	84a		479	21b		538	28a		42	13a	
A (mm ²)	46.6	6.5a		33.1	3.6b		48.4	7.2a		38.7	3.5b		43.4	8.3ab		7.8	0.4a	
MRWT	0.57	0.09a		0.46	0.03b		0.55	0.05a		0.47	0.04a		0.49	0.11a		0.68	0.16a	
CI	36.3	3.8a		31.5	1.5b		36.7	3.5a		32.1	1.8b		32.5	4.8ab		38.6	5.7a	
Ix (mm ⁴)	487	96a		267	73b		626	146a		415	51b		567	137a		6.7	0.1a	
Total BMD (g/cm ³)	0.35	0.03a		0.28	0.02b		0.36	0.01a		0.28	0.02b		0.32	0.02c		0.09	0.01a	
Prox. BMD (g/cm ²)	0.35	0.04a		0.29	0.02b		0.31	0.02ab		0.29	0.02a		0.33	0.04b		-	-	
Total BMC (g)	2.62	0.30a		1.50	0.16b		2.51	0.46a		1.87	0.16b		1.69	0.15b		0.12	0.01a	
Prox. BMC (g)	1.89	0.26a		1.15	0.12b		1.56	0.35a		1.11	0.09b		1.14	0.10b		-	-	
Td (g/cm ³)	1.31	0.02a		1.28	0.01b		1.30	0.02a		1.24	0.06b		1.31	0.02a		-	-	
Cd (g/cm ³)	2.06	0.13a		1.81	0.08b		1.98	0.11a		1.81	0.08b		2.09	0.09a		-	-	

A: cross-sectional area; BMC: bone mineral content; BMD: bone mineral density; Cd: cortical volumetric bone mineral density; CI: cortical index; Ix: second moment of inertia; MRWT: mean relative wall thickness; prox.: proximal; Td: trabecular volumetric bone mineral density; Wf: ultimate strength; Wy: maximal elastic strength.
Data are given as mean and SD. Dex (0.5 mg/kg b.w. daily), 2-Ox (0.4 g/kg b.w. daily). Differences between letters mean significant differences with $P < 0.05$. Comparisons are made within particular bones.

parameter elevated by 14% ($P=0.027$) relative to the Dex group (Table 1). Trabecular and cortical volumetric BMD of femora and humeri was decreased in the Dex group compared to the control (Td 3% $P=0.045$ and Cd 12%, $P<0.001$ for femur, Td 5% $P=0.038$ and Cd 9%, $P=0.012$ for humerus) and supplementation with 2-Ox abolished this effect, since piglets from the Dex+2-Ox group had higher Td (by 2% $P=0.043$ and 15% $P=0.011$, respectively) and Cd (by 18%, $P<0.001$ and 15%, $P=0.013$, respectively) than these from the Dex group (Table 1).

Results from histomorphometry measurements are presented in Table 2, individually for epiphysis and metaphysis of assessed bones; in addition, the structure of bones trabeculae is presented in Figures 1 and 2. Dex administered separately to piglets significantly increased the fractal dimension of trabeculae in the epiphysis of femora, humeri, and ribs ($P=0.015$, $P=0.003$, $P=0.003$, respectively), as well as in femora metaphysis ($P<0.001$), however given with 2-Ox did not cause any significant changes of this parameter in epiphysis. 2-oxoglutarate exerted little less protective influence on metaphysis of femora, since piglets from Dex+2-Ox group had higher fractal dimension than in the control ($P=0.010$), however still much lower than in the Dex group ($P<0.001$). Even though Dex had no influence on the fractal dimension of metaphysis of humeri and ribs ($P=0.289$ and $P=0.082$, respectively) compared to the control, animals from the Dex + 2-Ox group had this parameter significantly lower ($P=0.008$, $P=0.033$, respectively) compared to the Dex group (Table 2). The greater the fractal dimension, the more complex surface structure of trabeculae, more cracks and crevices (Figures 1 and 2). The trabecular bone volume was decreased by Dex treatment in the epiphysis of femora and humeri (38 and 34%, respectively, both $P<0.001$) as well as in the metaphysis of femora, humeri, and ribs (16%, $P=0.033$; 41%, $P<0.001$; and 22%, $P=0.018$, respectively). Supplementation with 2-Ox abolished this effect of Dex completely in metaphysis of femora and ribs (28%, $P=0.002$ and 29%, $P=0.014$, respectively; higher BV/TV than in the Dex group). Moreover, piglets from the Dex+2-Ox group had substantially greater trabecular bone volume in the epiphysis of femora and humeri (17%, $P=0.048$, and 20%, $P=0.018$, respectively) as well as in metaphysis of humeri (33%, $P<0.001$) comparing to the Dex group, nevertheless still smaller than in the control (27, 20, 22%, $P<0.001$ for all). There were no significant differences in BV/TV of ribs epiphysis (Table 2, Figure 1). Dex reduced the mean trabecular thickness in both epiphysis (30 and 24% in femur and humerus $P<0.001$, 16% in rib $P=0.006$) and metaphysis (31, 32, 28%, respectively, $P<0.001$) of all assessed bones comparing to the control. Conversely, the mean thickness of epiphyseal and metaphyseal trabeculae of ribs in the Dex+2-Ox group was at the level of the control. Although supplementation with 2-Ox was less preventive in femora and humeri (14% $P=0.014$, 15% $P<0.001$, respectively, for epiphysis and 11 and 14%, respectively, both $P<0.001$ in metaphysis) comparing to the control, yet still mean trabecular thickness was significantly higher than in the Dex group—both in epiphysis (23% for femora and 13% for humeri, both $P=0.004$) and metaphysis (29 and 26%,

respectively, both $P<0.001$) (Table 2). In contrast, maximal trabecular thickness was not affected significantly by Dex in any evaluated point. Nevertheless, thick trabeculae in the Dex+2-Ox group were significantly thicker in epiphyses of femora and humeri (24% $P=0.023$ and 25% $P=0.020$, respectively) as well as in metaphysis of ribs (31% $P<0.001$) compared to the Dex group (Table 2). The mean trabecular separation was reduced in the Dex group, in femora and humeri epiphysis (19% $P=0.018$ and 19% $P=0.010$, respectively) as well as in metaphysis (32% $P<0.001$ and 17% $P=0.002$, respectively) compared to the control. Supplementation with 2-Ox prevented aforementioned changes of trabecular separation in femora epiphysis and metaphysis (35% $P<0.001$ and 34% $P<0.001$, respectively) as well as in humeri metaphysis (36% $P<0.001$) compared to the Dex group. There were no statistically significant changes in ribs mean trabecular separation. Moreover, maximal trabecular separation was not changed in any assessed bone and any evaluated point (Table 2). Generally, some features of cancellous bone, like space occupied by trabeculae and their thickness and related differences among groups were clearly visible by the microscopic histological assessment (Figures 1 and 2). Moreover, observation in higher magnification revealed more osteoid structures in trabeculae of animals treated with Dex only (Figure 2). There were also other alterations regarding the Dex impact, like number of osseous lacunae per square millimeter of trabeculae which was significantly elevated in the Dex group (246 ± 30) compared to the control (137 ± 25) and the Dex+2-Ox (151 ± 29) (both $P<0.001$). Similar differences were observed in the area of osseous lacunae (μm^2) per square millimeter of trabeculae which was the greatest in the Dex group (6886 ± 2168) compared to the control (4814 ± 1904 , $P=0.006$) and to the Dex+2-Ox group (4496 ± 990 , $P<0.001$), as well as in the cross-sectional area of osseous lacunae (μm^2) which was also bigger in the Dex group (32 ± 1) compared to the control (29 ± 2 , $P=0.015$) and to the Dex+2-Ox group (30 ± 3 , $P=0.023$).

Serum levels of bone markers, selected hormones, nitrogen metabolites, and proteins are presented in Table 3. Dex administered alone caused significant decrease in GH, IGF-1, OC, cortisol, and urea concentration (48% $P<0.001$, 35% $P=0.048$, 32% $P<0.001$, 55% $P<0.001$, and 10% $P=0.0027$, respectively) comparing to the control. Simultaneously, leptin and albumin levels were higher in the Dex group relating to the control (138 and 37%, $P<0.001$). BAP, uric acid, creatinine, and total protein levels were not affected significantly by Dex alone. In contrast, animals supplemented with 2-Ox had substantially higher OC, GH, urea, uric acid, and creatinine concentrations than these from the Dex group (39% $P<0.001$, 362% $P<0.001$, 15% $P=0.002$, 150% $P=0.002$, and 46% $P<0.001$, respectively). Moreover, GH, uric acid, and creatinine levels were even higher than in the control animals (139% $P<0.001$, 67% $P=0.046$, and 42% $P<0.001$, respectively). Albumin serum concentration was preserved by supplementation with 2-Ox compared to the Dex group (21% $P=0.004$). However, 2-oxoglutarate had no influence on IGF-1, cortisol, and leptin. There were no significant

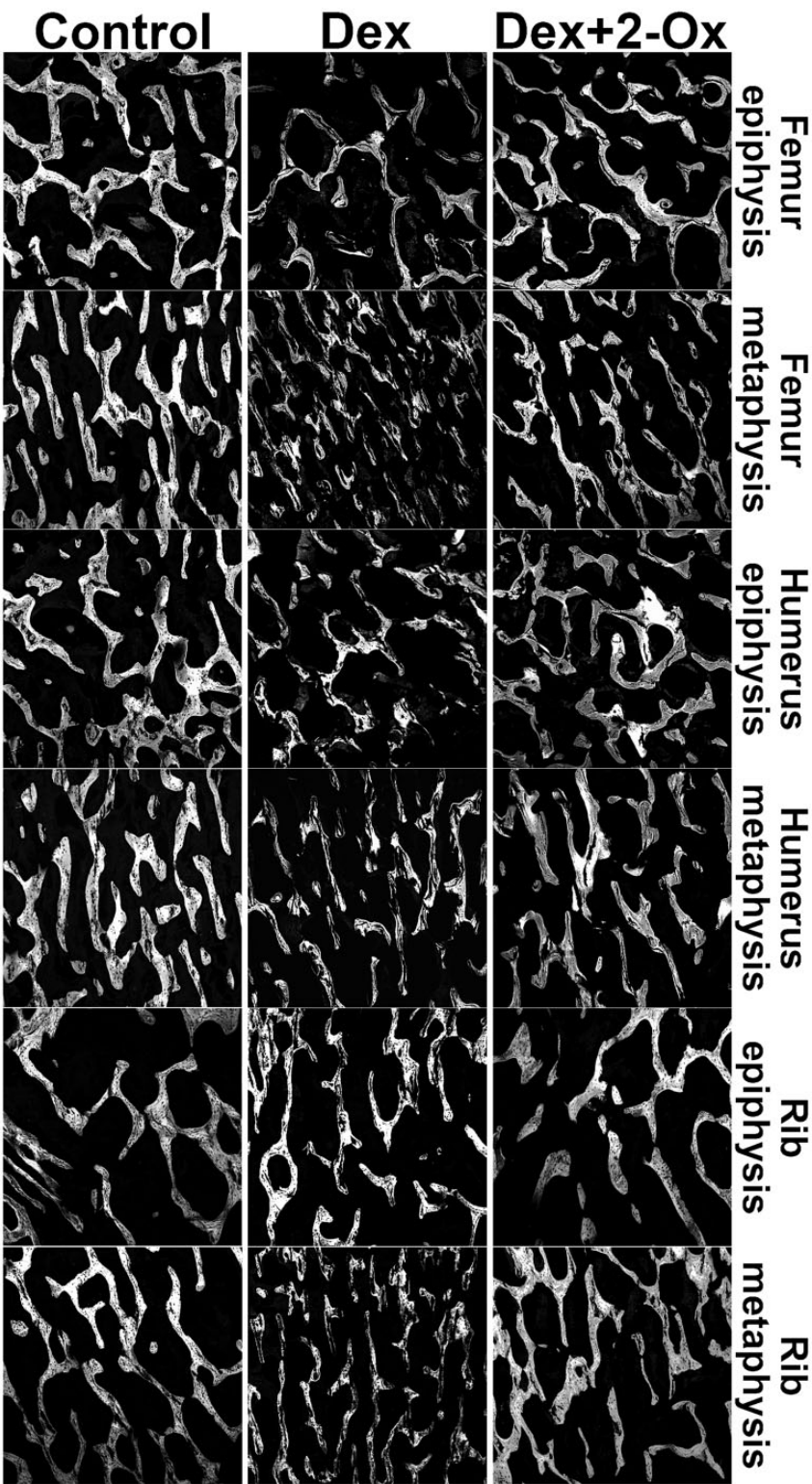


Figure 1 The effect of Dex or Dex with 2-Ox administered neonatally on cancellous bone of female piglets. Gray color—bone trabeculae, black color—bone marrow space. Magnification in all microphotographs is $\times 50$. Dex: dexamethasone; 2-Ox: 2-oxoglutarate

changes in BAP and total protein concentrations among all groups (Table 3).
The plasma concentration of glycine, glutamine, and glutamate was significantly elevated in the Dex group (709 ± 24 , 299 ± 28 , $180 \pm 15 \mu\text{mol/L}$, respectively)

compared to the control (498 ± 38 , 171 ± 33 , $91 \pm 33 \mu\text{mol/L}$, respectively) $P < 0.001$. Conversely, proline level decreased after exclusive Dex treatment ($540 \pm 41 \mu\text{mol/L}$) compared to the control ($614 \pm 10 \mu\text{mol/L}$) $P = 0.001$. Supplementation with 2-Ox had, to some extent, a

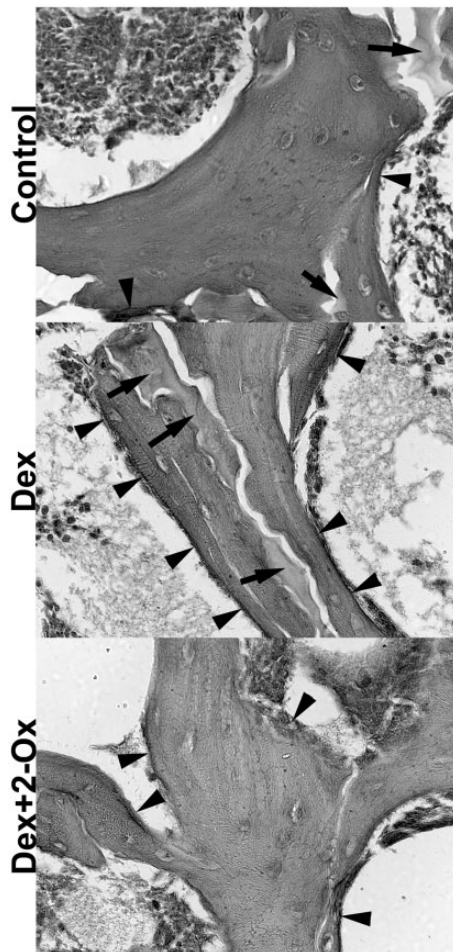


Figure 2 The effect of Dex and Dex with 2-Ox administered neonatally on amount and area of unstructured and acellular bone material as well as structural disturbances observed in trabecular bone. Black arrows indicate areas with unstructured and acellular bone, arrowheads indicate osteoid areas. Magnification in all microphotographs is $\times 630$. Dex: dexamethasone; 2-Ox: 2-oxoglutarate

protective effect, since glycine, glutamine, and glutamate concentrations decreased (632 ± 38 , 248 ± 28 , and $133 \pm 31 \mu\text{mol/L}$, respectively) and proline increased ($624 \pm 36 \mu\text{mol/L}$) relating to the Dex group ($P = 0.001$, $P < 0.001$, $P = 0.013$, and $P < 0.001$, respectively).

The serum concentration of calcium, phosphorus, and sodium did not differ between control, Dex, and Dex + 2-Ox groups (Ca: 1234 ± 130 , 1245 ± 86 , and $1222 \pm 152 \text{ ppm}$; P: 2897 ± 243 , 2737 ± 263 , and $2566 \pm 298 \text{ ppm}$; Na: 4.6 ± 0.4 , 4.0 ± 0.6 , and $4.3 \pm 0.9\%$, respectively).

Discussion

In the present study neonatal administration of Dex alone led to osteoporosis that was demonstrated in geometry, durability, density, mineralization, histology, and histomorphometry of bones examined (Tables 1 and 2 and Figure 1). These findings were in accordance with variety of earlier studies on rats, mice, and piglets treated pre-, neo-, or postnatally with GCs.^{15,17,24,25,28-31} The clinically relevant maximal dose of Dex used impaired bone growth, which was evident in shorter long bones in the Dex group. Such growth impairment was also found in previous studies on animals and reviewed in children's clinical trials.^{4,31-33} For example, mice and rats treated with Dex had femur growth reduced by 47 and 31%, respectively, so much more than in the present experiment. Four and two times higher doses were however used (for mice and rats, respectively) in these referred studies.^{31,32} Nevertheless, other studies show that GC-induced bone quality aggravation is partly reversible.³ Indeed, impairment caused by Dex in piglets' long bones was prevented by 2-Ox dietary supplementation and vast majority of assessed bone parameters were restored almost to the control level, as presented in Tables 1 and 2. Also, according to the best of our knowledge, this is the first study utilizing 2-Ox to prevent osteopenia induced by neonatal administration of GCs. Moreover, dietary 2-Ox

Table 3 The serum levels of bone markers, selected hormones, nitrogen metabolites, and proteins of 35-day-old female piglets treated neonatally with dexamethasone (Dex) or Dex and 2-oxoglutaric acid (2-Ox)

Group (n = 12/group)	Control		Dex		Dex+2-Ox	
	Mean	SD	Mean	SD	Mean	SD
BAP (U/L)	82.8	6.7a	80.4	8.4a	83.5	22.5a
OC ($\mu\text{g/L}$)	37.8	1.7a	25.8	1.7b	35.9	2.5a
GH ($\mu\text{g/L}$)	3.47	1.43a	1.80	0.11b	8.31	1.93c
IGF-1 ($\mu\text{g/L}$)	75.5	7.7a	49.3	13.0b	46.3	23.7b
Leptin ($\mu\text{g/L}$)	0.56	0.20a	1.33	0.09b	1.15	0.12b
Cortisol ($\mu\text{g/L}$)	66.2	9.6a	29.8	4.6b	25.2	3.9b
Urea (mmol/L)	3.76	0.31a	3.40	0.19b	3.91	0.07a
Uric acid (mmol/L)	0.03	0.01a	0.02	0.00a	0.05	0.01b
Creatinine ($\mu\text{mol/L}$)	112	8a	108	10a	158	1b
Albumin (g/L)	30.1	0.6a	41.2	6.3b	32.5	1.8a
Total protein (g/L)	74.1	3.4a	74.1	5.1a	70.2	5.4a

BAP: bone alkaline phosphatase; GH: growth hormone; IGF-1: insulin like growth factor 1; OC: osteocalcin; 2-Ox: 2-oxoglutarate. Data are given as mean and SD. Dex (0.5 mg/kg b.w. daily), 2-Ox (0.4 g/kg b.w. daily). Differences between letters mean significant differences with $P < 0.05$.

appears to be even more effective in preserving bone structure and mineralization in GCs induced rather than in gastrectomy-induced osteopenia, since there was no improvement in femoral and tibial BMD and BMC of gastrectomized rats receiving even higher dose of 2-Ox in our previous study.²¹

The ability to withstand the mechanical forces acting upon a bone and to resist fractures is related to its geometry, mineralization, and quality of structural components such as shape, microarchitecture of trabecular bone, the turnover, collagen and cells regularly spaced throughout the mineralized matrix.^{19,28} There are several possible mechanisms of GIO presented in literature: inhibition of bone formation by suppression of genesis and activity of osteoblasts with accelerated osteocyte and osteoblasts apoptosis as well as acceleration of bone resorption by extension of osteoclasts life span. Furthermore, GCs interrupt regulatory proteins ratio by stimulating osteoclastogenesis-inducing RANKL and inhibiting antiresorptive osteoprotegerin.^{1,4,9,28,30,34} Such cellular imbalance contributes to disruption of the osteocyte signaling network, which is responsible for damage detection and repair initiation, leading to microdamage accumulation and further increased bone fragility. This leads to higher frequency of fractures and at higher BMD values than in patients with osteoporosis not induced by GCs.^{4,28}

A rapid bone loss (mostly trabecular mass), loss of BMD, and bone strength/quality after the initiation of GCs therapy is likely to be associated with the loss of structural integrity as well as loss of osteoblast-generated proteins such as collagen or OC referred to as microarchitectural deterioration.^{1,3,4} Although OC level was decreased in the Dex group, our results do not differentiate between cell types and cannot directly confirm cellular proportion/imbalance. However, we have noticed microstructural and architectural bone deterioration reflecting in significant enlargement of trabecular fractal dimension in the Dex group which implies impaired, irregular surface structure and the complexity/integrity of trabecular bone. Thus, not only did we show the reduced trabecular bone volume, thinner trabeculae, less mineralized bone, but also cracked trabeculae and impaired trabecular microstructure. Furthermore, GCs can modify osteocyte microenvironment by causing changes in bone material, including an enlargement of their lacunar space and generation of areas with hypomineralized bone.³⁵ Suchlike expansion of osseous lacunae and its total area as well as more osteoid were also observed in the present study in piglets from the Dex group.

On the other hand, animals supplemented with 2-Ox had preserved microarchitecture of trabecular bone, since trabecular thickness, amount of osteoid, lacunar cross-sectional area, and trabecular fractal dimension were very similar to the control. This observed effect of glutamine precursor presumably involves mechanisms related to stimulation of bone anabolism, since 2-Ox acts as an energy donor and as a substrate for the synthesis of amino acids—principally proline—leading to increased collagen synthesis and formation in the cell and organism. Several mechanisms by which 2-Ox is involved in collagen metabolism were distinguished beginning from being a cofactor of prolyl-4-hydroxylase which catalyze 4-

hydroxyproline, crucial for the formation of collagen triple helix, through facilitate collagen synthesis by increasing the pool of proline residues via glutamate, and finally by being also a precursor of pyrroline-5-carboxylate an intermediate in the interconversion of proline.^{14,16} Moreover, enteral 2-Ox administration may cause an anabolic effect on bone tissue metabolism via glutamate innervation as well as significantly increased plasma level of GH and IGF-1 as reviewed previously.^{14,16,19} In addition, 2-Ox affects bone tissue probably also by its effect on the endocrine system by conversion to glutamine and glutamate followed by transformation into ornithine and then arginine. Both ornithine and arginine stimulate secretion of GH and IGF-1, thus well-described functional axis GH-IGF-1 with strong osteotropic effect.¹⁴ Such stimulation of growth was observed in our study by highest GH level in the Dex+2-Ox group, which might have a direct effect on osteoblasts reflected in higher OC concentration than in the Dex group. This observation highlights the effect of 2-oxoglutarate, especially since OC is known to be negatively regulated by GCs.³⁰ Conversely, Dex alone decreased GH secretion which was consistent with previous review.⁶ And again, although our results do not distinguish cell types, the elevated OC level in the Dex+2-Ox group may suggest at least increase in osteoblast metabolism, if not increase in osteoblast number. Conversely, while IGF-1 was reported to be negatively correlated with bone resorption, treatment with 2-Ox in first 14 days of postnatal life had no significant effect on plasma IGF-1 concentration in growing lambs.¹⁹ Our results confirm these findings since we showed no effect of 2-Ox on IGF-1 concentration, lowered by Dex. It seems that even if enteral 2-Ox treatment early in postnatal development can have a long lasting effect on bone structure, these changes are probably independent of the plasma concentration of IGF-1, and not only in growing lambs.^{14,19} Moreover, since all animals receiving Dex had decreased plasma IGF-1, a potent inhibitor of programmed cell death, we assumed that GC-induced structural changes might have been related to the increased apoptosis as observed previously.³²

GCs accelerate bone resorption by extension of osteoclasts life span and stimulation of osteoclastogenesis.⁴ Further, osteoclast activation can be promoted by hypoxia-inducible factor 1 (HIF-1 α) stabilization followed by increased bone resorption and decreased bone mass. Whereas, 2-Ox is known inhibitor of HIF-1 α , and by such properties and inhibitors of HIF-1 α can protect from osteoclast activation and bone loss. Hence there is another possible mechanism of positive effects of 2-Ox on bone tissue maintenance.^{14,36,37}

Changes in bone remodeling, e.g. increased trabecular surface remodeling are responsible for altered trabecular bone volume and architecture, which may further influence fracture risk and further confirm, however indirectly, prevalence of cellular regulation by GCs.³⁵ Structural changes caused by GCs in the trabecular fractal dimension were completely different from these observed in gastrectomy-evoked osteopenia in our previous study.²¹ Gastrectomized rats had lower trabecular fractal dimension than control animals, suggesting greater reduction in the

amount of extracellular matrix rather than in its quality, since BV/TV and BMD and BMC decreased as well.²¹ Although piglets receiving Dex alone also had reduced bone volume and mineralization, the mechanism behind this present observation is most probably different from the lack of gastric factor promoting trabecular bone formation and calcium absorption and its transport from blood to bone, as postulated for gastrectomy.^{21,22} Nevertheless, supplementation with 2-Ox in both these cases revealed stimulating effect on bone structure. In this regard, dietary 2-Ox may maintain bone structure regardless of mechanism of action of damaging agent, probably by being involved in regulatory mechanism of nutrients utilization. 2-Ox may act by improving protein anabolism and absorption from the gut, namely by stimulating the gut mucosa to synthesize proline, the main amino acid required for bone collagen synthesis.^{14,16} Moreover, proline concentration in the Dex+2-Ox group was indeed significantly higher than in animals receiving only Dex. On the other hand, the excess of GCs showed catabolic effect revealed by elevated glutamine, glutamate, and glycine levels in animals receiving Dex. This might have been related to increased proteolysis in skeletal muscle as assumed previously.^{3,29} Nevertheless, what is substantial is that this effect was also largely abolished by supplementation with 2-Ox.

Chronic GCs therapy is related also to reduced intestinal calcium absorption and promotion of renal calcium excretion, resulting further in an increase in PTH secretion, which may promote bone resorption.^{1,34} However, no differences in serum calcium or phosphorus levels were noticed in the present study which leads us to the strengthening of previously presented mechanism related rather to the GC-induced bone remodeling by alterations in bone cells viability.³⁵ Moreover, particular life period and length of Dex treatment may also be important factors, and there were several studies regarding long period of GCs treatment and its outcomes.^{1,2,4,6,8,11,13} The duration of the presented experiment, 35 days, was imposed by the moment of natural weaning of piglets, a point until when continuous and uninterrupted growth occurs. We have chosen this particular time in order to avoid the changes in digestive system caused by weaning which affects both nutrient absorption and skeleton.

The main problem associated with the GCs treatment is that they act simultaneously on various organs and systems, e.g. immune system, metabolic organs, and bone. Concomitantly, GCs affect the levels of other hormones by negative-feedback loops influencing the hypothalamic-pituitary-adrenal axis.³⁸ In the present study, the Dex treatment, regardless of 2-Ox supplementation, resulted in decreased level of cortisol and increased leptin, similarly as described previously.^{5,25,30,38} Therefore, there are two main ways to prevent GCs' side effect: the use of weaker GCs and lowering the most effective dose of systemic steroids, or the implementation of combined therapies to ameliorate GC-induced adverse effects.⁷ However, although weaker GCs are less harmful, they are also less effective in promoting beneficial effects.⁷ The second approach, regarding combined therapies, involve vitamins C, E, K, and D as well as bisphosphonates.^{1,3,4,7,34,39} Nonetheless,

bisphosphonates themselves have many adverse effects and raise safety concerns related to reproduction and gastrointestinal side effects. Moreover, although vitamin D derivatives as well as vitamin K can be considered suitable treatment options, it still lacks Food and Drug Administration approval, since hypercalcemia and hypercalciuria are well-known unwanted effects of vitamin D derivatives.¹ Nevertheless, there are also glutamine derivatives widely described in the context of bone health which have beneficial influence on the whole organism, e.g. 2-oxoglutaric acid.^{14,16,18,19,21,22} Moreover, recently described antioxidative action of 2-Ox strengthens the possible use of this natural glutamine derivative in ameliorating of the GC-induced side effects in the skeleton.^{7,14,40}

In conclusion, the present study has showed, for the first time, that dietary 2-Ox administered postnatally has a potential to improve/maintain bone structure of animals simultaneously treated with maximal therapeutic doses of Dex which, in our opinion, may open a new direction in developing combined treatment for children treated with GCs. Moreover, present findings also support our previous thesis on the regulatory mechanism of nutrient utilization regulated by glutamine derivatives.^{18,22} Presumably, 2-Ox may take part in the specific mechanism of metabolic regulation activated in a state of health problems by utilization of proteins and amino acids depending on the stage of development in which the organism is to facilitate better use of nutrients. Also the mechanism of action of 2-Ox on the functional axis GH-IGF-1 and its osteotropic effect needs further studies.

Author's contributions: All authors participated in acquisition, analysis, and interpretation of the data; final approval of the article; provision of study materials; administrative, technical, or logistic support; PD—conception and design, collection and assembly of data, statistical expertise, drafting of the article; PD, ET, SGP—critical revision of the article for important intellectual content.

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DECLARATION OF CONFLICTING INTERESTS

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