

Effect of HMB and 2-Ox administered during pregnancy on bone properties in primiparous and multiparous minks (*Neivison vison*)

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Abstract

The aim of the study was to determine the mechanical and geometric properties as well as bone tissue density of long bones in primiparous and multiparous dams of minks supplemented with β -hydroxy β -methylbutyrate (HMB) and/or 2-oxoketoglutarate (2-Ox) during gestation. Powdered 2-Ox was given at the daily dosage of 0.4 g/kg b.w. separately or simultaneously with HMB, which was administered at the daily dosage of 0.02 g/kg b.w. The study demonstrates for the first time that administration of 2-Ox and/or HMB to dams markedly influences bone tissue density and the mechanical and geometrical properties of mother's bones in minks. Moreover, it was demonstrated that the supplementation was more effective in the thoracic limb, which was comprehensively used in contrast to the pelvic limb. The mechanical parameters and bone tissue density significantly increased in the humerus in multiparous minks. Only such diet may provide satisfactory production results in the animals. Nutritional deficiencies occurring during pregnancies may trigger body's own reserves to cover the bone mass increase in developing foetuses and support milk production. This can prevent regeneration of dams' organisms, which negatively affects their reproductive performance. 2-Ox or HMB may be regarded as a protective metabolite when administered orally to minks, counteracting the negative influences of pregnancy and lactation periods on bones condition. Both simultaneous treatment with 2-Ox and HMB and their separate administration were equally effective.

Keywords: mink, bone, pregnancy, 2-oxoglutaric acid, β -hydroxy- β -methylbutyrate.

Introduction

Biochemical data suggest that human and animal gestations are accompanied by alterations in the uptake

and release of calcium and other minerals from the maternal skeleton. Gestation is also accompanied by changes in weight and advances in age, which influence the bone tissue density independently. Moreover, bone

metabolism in a postpartum skeleton is additionally affected by lactation and nursing (17). Lactation is associated with changes in numerous hormones and other factors that regulate maternal calcium and bone metabolism. During lactation, skeletal resorption is the main mechanism by which calcium is supplied to the milk, supported by elevated prolactin and decreased oestradiol concentrations, which normalise as lactation finishes (17). Maternal adaptation to pregnancy and lactation evolve differently over time. All these events lead to bone loss. Thus, during pregnancy, additional supplementation is needed, especially during its late period (14).

Currently, there is a growing number of information about the role of the most preferable derivative of glutamate, 2-oxoglutaric acid (α -ketoglutaric acid), i.e. a signalling molecule playing an important role in nutrition. Studies conducted in recent years have shown that 2-Ox supplementation improves body weight gain. It decreases protein catabolism and increases protein synthesis in the skeletal muscles (31).

Other studies have proved that mammalian gastrointestinal tract acts as an important system influencing bone development and mineralisation. 2-Ox is the main source of energy for the cells of the gastrointestinal tract, induces proliferation of intestinal cells, and is an important factor for microbial flora present in the healthy gut (23). Enteral administration of 2-Ox leads also to its conversion into glutamine, proline, and hydroxyproline (main amino acids of collagen), as well as arginine and asparagine (22, 23). 2-Ox administered *per os* has positive effects on prenatal or postnatal skeletal development and growth progress in animals, e.g. post-hatching turkeys (4, 11, 16, 27-30). 2-Ox also exerts a protective effect against the negative action of dexamethasone on bone and cartilage in intrauterine restricted animals (30). Moreover, long-term oral administration of 2-Ox improved bone mineralisation and mechanical endurance of long bones in fundectomised animals compared with non-supplemented individuals (26). 2-Ox has shown beneficial effects through nitrogen balance in clinical studies on septic, trauma, or surgical patients (2, 33). In addition, 2-Ox is the main donor of carbon units required for the synthesis of alanine and glutamine during the gestation period (14).

The positive impact of specific compounds in food is evident. Functional food improves the health state and can reduce the risk of various diseases. Some elements can influence bone metabolism and intestinal development as well (29). Among these specific compounds, there is also the bioactive metabolite of leucine – β -hydroxy- β -methylbutyrate (HMB). Approximately 5% of leucine metabolism leads to endogenous synthesis of HMB, which serves as a key carbon source for *de novo* cholesterol synthesis in tissues, which is necessary to maintain maximal cell function (21). Earlier studies have shown that dietary supplementation with HMB results in enhanced wound

collagen deposition in rats. Similar results were obtained in another study on humans, where HMB administration enhanced the hydroxyproline content, thereby increasing wound repair processes (32). Moreover, maternal administration of HMB has positive long-term effects on the skeletal system in offspring, improving bone mineral density as well as the geometrical and mechanical properties (28).

There is no knowledge about the effects of 2-Ox or HMB (given during the whole pregnancy) on bone properties in minks, especially in dams that gave birth and lactated.

The aim of the study was to determine the mechanical and geometric properties as well as bone density of long bones in primiparous and multiparous female minks supplemented with HMB and/or 2-Ox during their pregnancy.

Material and Methods

The study was carried out on a mink-breeding farm located in south-eastern Poland.

Animal breeding and experimental design. Sixty clinically healthy multiparous (after two or three pregnancies; the standard farm procedure is to keep minks that had and reared the largest number of kits during three consecutive years) and 60 primiparous (at the age of 10 months; never gave birth) minks (*Neovison vison*) of the standard dark brown type were used. After mating, they were housed singly in separate cages under standard breeding/farming conditions and the natural photoperiod with free access to fresh water. The animals were fed a well-balanced standard ranch diet once a day throughout the study. The mating was performed in March. Female minks were mated only twice to obtain an accurate measure of the length of the gestation period. Parturitions occurred at the end of April and the beginning of May. Routine farm procedures were employed in the feeding, care, and breeding of the animals. The minks were checked twice a day; the day on which a litter was found was considered the day of parturition and the supplementation was ended. Mink kits were kept as family groups with their dams until approximately two months of age, and then they were placed into the same gender pairs.

The basal diet (45.65% of moisture, 35% of protein, 24.15% of fat, 9.43% of carbohydrates, 2.79% of crude fibre, 9.8% of ash, 2.03% of calcium, and 1.78% of phosphorus) comprised commercial mink cereal, beef liver, raw poultry by-products, and fish.

After the mating, the minks were randomly assigned into four groups: control group (n = 15; not supplemented), group supplemented with 2-Ox (n = 15; 2-Ox), group supplemented with HMB (n = 15; HMB), and group supplemented with 2-Ox+HMB (n = 15; 2-Ox+HMB). HMB and/or 2-Ox were administered to the minks (with the basal diet) since day one after the mating until the end of the gestation (about 46 d).

In accordance with the farm procedures and Polish national legislation, all the multiparous and primiparous dams were euthanized by carbon monoxide inhalation at pelt harvesting and skinned carcasses were delivered to the laboratory (1, 6).

Supplementation. Powdered 2-Ox (Olimp L-Glutamina Mega Caps, Olimp Laboratories, Poland) was given at a daily dosage of 0.4 g/kg of body weight. Powdered HMB (DR Seidla HMB, Laboratorium DermaPharm, Poland) was given at a daily dosage of 0.02 g/kg b.w.

Bone analysis. The bone length and weight were measured after removal of soft tissues from the femora and humeri. Each bone was wrapped in gauze, soaked in isotonic saline, and stored at -25°C for further analysis.

The mechanical properties of bones were determined for all the groups after three-hour thawing at room temperature using the three-point bending test. The mechanical properties were examined on a Zwick Z010 universal testing machine (Zwick GmbH & Company KG, Germany), equipped with a measuring head (Zwick GmbH & Company KG, Germany) of an operation range up to 10 kN, linked to a computer with test TestXpert II 3.1 software (Zwick GmbH & Company KG, Germany). The distance between supports was set at 40% of total bone length. The measuring head loaded bone samples with a constant speed of 10 mm/min. The maximum elastic strength (Wy) and the ultimate strength (Wf) were determined as described previously (7, 26). On the basis of measurements of the horizontal and vertical diameters of the mid-diaphyseal cross-section of the bone, the cross-sectional area (A) and the mean relative wall thickness (MRWT) were calculated (7). Moreover, the

cortical index (CI) of the bone was estimated as described previously (26).

Bone mineral measurement. The measurement of bone tissue density (BTD) for the whole bone was performed with a helium gas pycnometer (AccuPyc 1330; Micromeritics, USA) equipped with a 10 cm³ metal measuring cylinder. Prior to the analysis, the samples were heated in an oven at 105°C for 24 h to remove the bound water, and then cooled to room temperature in vacuum desiccators (5, 20). The dehydrated masses of the samples were measured to the nearest 0.0001 g, and then the volume was measured to the 0.0001 cm³ and specified in the analysis mode before the measurement. The parameters of the measurement cycle were adjusted to the number of purges – 4, purge fill pressure – 18.000 psig, the number of cycles per sample – 4, cycle fill pressure – 2 bar, equilibration rate – 0.005 bar/min.

Statistical analysis. All results are expressed as means $\pm$ SD (standard deviation). The differences between the means were tested with a two-way ANOVA (with supplementation and female groups as factors) and Dunnet's 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. The normality and equality of variance assumptions of BTD and length in the femur and humerus as well as of MRWT and A in the humerus were not fulfilled, hence Kruskal-Wallis one-way ANOVA with correction for female groups was used for these parameters. $P < 0.05$ was considered statistically significant. All statistical analyses were performed by means of Statistica 12 software (StatSoft, Inc., USA; <http://www.statsoft.com>).

Table 1. The effect of 2-Ox and/or HMB given during pregnancy on femur and humerus properties in primiparous and multiparous minks (n = 15 in each group) at pelts obtaining

Group		Control	2-Ox	HMB	2-Ox+HMB	Control	2-Ox	HMB	2-Ox+HMB
Bone	Properties	Primiparous				Multiparous			
Femur	Weight (g)	1.65 $\pm$ 0.35	2.01 $\pm$ 0.22	1.93 $\pm$ 0.44	1.56 $\pm$ 0.14	1.62 $\pm$ 0.24	1.98* $\pm$ 0.04	1.76 $\pm$ 0.09	1.82 $\pm$ 0.23
	Length (mm)	48.48 $\pm$ 3.19	48.67 $\pm$ 1.55	50.19 $\pm$ 2.87	47.32 $\pm$ 0.85	47.12 $\pm$ 2.61	51.29* $\pm$ 0.54	48.67 $\pm$ 1.55	49.13 $\pm$ 1.59
	Wf (N)	196.5 $\pm$ 43.7	211.5 $\pm$ 24.1	231.3 $\pm$ 89.8	213.0 $\pm$ 35.5	183.7 $\pm$ 20.9	238.2* $\pm$ 19.3	258.0* $\pm$ 32.9	232.9 $\pm$ 50.5
	Wy (N)	159.2 $\pm$ 41.5	171.2 $\pm$ 31.4	178.3 $\pm$ 61.1	174.2 $\pm$ 29.1	141.6 $\pm$ 9.8	165.0 $\pm$ 12.6	215.0* $\pm$ 15.1	168.7 $\pm$ 38.4
	A (mm ²)	10.91 $\pm$ 1.82	12.03 $\pm$ 1.32	11.41 $\pm$ 1.91	9.80 $\pm$ 0.95	9.10 $\pm$ 0.33	10.53 $\pm$ 0.91	10.52 $\pm$ 1.19	10.53 $\pm$ 1.57
	MRWT	2.11 $\pm$ 0.55	1.86 $\pm$ 0.39	1.77 $\pm$ 0.20	1.93 $\pm$ 0.55	1.34 $\pm$ 0.22	1.23 $\pm$ 0.53	1.42 $\pm$ 0.36	1.18 $\pm$ 0.17
	CI	66.38 $\pm$ 6.35	64.31 $\pm$ 4.66	63.65 $\pm$ 2.63	64.44 $\pm$ 7.58	56.75 $\pm$ 3.98	52.82 $\pm$ 10.41	57.76 $\pm$ 5.59	51.37 $\pm$ 4.14
	BTD (g/cm ³)	2.06 $\pm$ 0.06	2.10 $\pm$ 0.09	2.11 $\pm$ 0.09	2.12 $\pm$ 0.04	2.09 $\pm$ 0.04	2.10 $\pm$ 0.07	2.18 $\pm$ 0.06	2.19 $\pm$ 0.05
Humerus	Weight (g)	1.38 $\pm$ 0.30	1.76 $\pm$ 0.12	1.61 $\pm$ 0.34	1.34 $\pm$ 0.19	1.31 $\pm$ 0.15	1.64* $\pm$ 0.04	1.46 $\pm$ 0.07	1.48 $\pm$ 0.17
	Length (mm)	44.67 $\pm$ 2.32	48.54* $\pm$ 1.14	47.67 $\pm$ 2.59	45.59 $\pm$ 1.76	44.34 $\pm$ 2.17	48.16* $\pm$ 0.58	49.13 $\pm$ 3.59	46.35 $\pm$ 1.28
	Wf (N)	162.8 $\pm$ 42.4	246.2* $\pm$ 36.62	294.2* $\pm$ 93.5	262.8* $\pm$ 40.5	183.0 $\pm$ 20.5	304.2* $\pm$ 33.2	187.0 $\pm$ 47.4	220.5 $\pm$ 71.3
	Wy (N)	129.2 $\pm$ 34.4	182.5 $\pm$ 74.3	197.0 $\pm$ 42.6	191.7 $\pm$ 38.6	141.6 $\pm$ 9.8	213.0* $\pm$ 17.8	132.5 $\pm$ 28.6	161.2 $\pm$ 38.4
	A (mm ²)	9.86 $\pm$ 0.37	11.53 $\pm$ 1.51	11.97 $\pm$ 2.23	9.94 $\pm$ 1.34	9.03 $\pm$ 1.22	10.73* $\pm$ 0.61	10.89 $\pm$ 1.18	10.97 $\pm$ 1.62
	MRWT	2.68 $\pm$ 0.96	2.35 $\pm$ 0.59	2.39 $\pm$ 1.36	1.65 $\pm$ 0.29	1.89 $\pm$ 0.39	1.18 $\pm$ 0.06	3.17 $\pm$ 1.93	2.22 $\pm$ 0.75
	CI	70.66 $\pm$ 6.69	68.27 $\pm$ 5.54	65.89 $\pm$ 10.54	61.05 $\pm$ 4.40	64.50 $\pm$ 5.02	51.54* $\pm$ 1.37	70.50 $\pm$ 11.05	67.14 $\pm$ 5.05
	BTD (g/cm ³)	2.13 $\pm$ 0.10	2.15 $\pm$ 0.09	2.15 $\pm$ 0.10	2.16 $\pm$ 0.08	2.15 $\pm$ 0.06	2.22 $\pm$ 0.08	2.12 $\pm$ 0.08	2.21* $\pm$ 0.07

Data are presented as means $\pm$ SD, * indicates significant differences between the control and other group where $P < 0.05$; Wy - maximum elastic strength; Wf - ultimate strength; A - bone cross-sectional area; MRWT - the mean relative wall thickness; CI - the cortical index; BTD - bone tissue density

Results

There was no difference in the number of stillborn or born offspring among all the dams.

Body weight. The final mean body weight of the primiparous minks did not differ among the groups. Similarly, the mean body weight of the multiparous minks did not differ among the groups at obtaining pelts.

Bone density, morphology, geometry, and mechanical properties. The supplementation of 2-Ox during pregnancy in primiparous minks led to an increase in the length and both strengths of their humeri. The value of both strengths of the humerus increased after the HMB supplementation, and after simultaneous supplementation with 2-Ox and HMB (Table 1). Although there were no significant differences in geometric parameters (except CI of the humerus in the 2-Ox group of multiparous dams) among primiparous or multiparous minks, the influence of the number of pregnancies on the geometry of the femur was evident, because the cross-sectional area and wall thickness of the midshaft were smaller in multiparous minks.

The 2-Ox supplementation to multiparous minks resulted in longer and heavier bones. An increase in the mechanical and geometric parameters was observed. HMB given to the pregnant multiparous minks increased the mechanical parameters in the femur (Table 1). The influence of the number of pregnancies and lactations was observed, since higher values of mechanical parameters were found in multiparous minks.

Discussion

One of the most important criteria that are used to estimate the breeding benefits is the animals' reproductive performance measured by fertility and fecundity. Both features determine the economic results of reproduction (9). Young minks reach sexual maturity at the age of about 10 months and exhibit high reproductive performance until the age of two or three years. The number of reared animals is an important indicator in the assessment of the usability of breeding minks. The percentage of young animals kept until the slaughter period is considered to be the number of reared minks, which is counted in relation to the number of born kits (9). Moreover, this number depends on the condition of their mothers. The adaptation to pregnancy includes anatomical, physiological, and metabolic changes in the mother (12, 14). Pregnancy is a physiological state when calcium homeostasis is changed in order to build the foetal skeleton and to maintain normal range of calcium concentrations in blood. The metabolic processes in bone tissue of the pregnant dam and foetus and later of the offspring and lactating mothers are temporarily

subordinated to each other and remain in close dependence on the physiological regulatory mechanisms of the foetus and mother in both humans and animals (18). During gestation, hormones such as parathyroid hormone and 1,25 dihydroxyvitamin D, the active form of vitamin D, are two important regulators of calcium homeostasis. They maintain calcium level within a narrow range *via* the following mechanisms: stimulating renal calcium reabsorption, increasing intestinal calcium absorption, and mobilising calcium from the bones. There is a significant transfer of calcium into the foetus to provide enough calcium for foetal bone and tooth development, and a decrease in maternal bone density during pregnancy and lactation. The primary mechanism by which calcium is supplied for the support of milk production is the reduction of maternal bone mass. Bone density remains lower during continued lactation, which is independent of parathormone and vitamin D contents (8, 13, 15, 18, 19).

Our study showed that a few pregnancies and lactations did not influence significantly the bone development and density in dams. However, it was evident that the values of the particular parameters were different (geometric and mechanical parameters), *i.e.* the number of pregnancies (with multiple foetuses) and lactation affected bone development in dams (even though it was assessed at pelt harvesting). The requirements for calcium in the lactation period depend on the amount of produced milk (determined by the number of kits) and the quantity of calcium deposited in the milk.

At the time the kits start to intake solid feed, the dams increase feed intake because they lose weight during the lactation period, which is on average 15% of the body weight at the parturition (10). Further changes in the calcium storage occur in dams after weaning when lactation is ended. There is an increase in the absorption of calcium by the intestine. This intestinal calcium absorption is the main mechanism by which the amount of calcium is reconstituted in mother's bone after weaning (18). Furthermore, a long pause between the end of lactation and the next pregnancy constitutes the period of increased demand for minerals in mothers.

Our study also showed that the administration of 2-Ox during the whole time of the gestation, which was interrupted at the parturition, resulted in a bigger humerus as well as its better endurance in primiparous or multiparous minks. The results obtained not only document the changes in the skeletal system after gestation and lactation, but also precisely show how oral administration of 2-Ox (during pregnancy only) improves essential mechanical and geometrical parameters connected with higher bone mineralisation. Essentially, 2-Ox may increase absorption of those metabolites which influence the processes of growth and mineralisation of the skeletal system. Importantly, this effect was not observed in other groups.

It should be noted that the metabolic pathway of 2-Ox is associated with the synthesis of proline and hydroxyproline, amino acids important qualitatively and quantitatively in type I collagen (2). Moreover, the increase in endochondral growth in early pregnancy is accompanied by an increase in periosteal apposition and an increase in periosteal diameter (3). This is an example of modelling-dependent bone gain and, since it occurs on the periosteal surface, it could have a greater impact on bone biomechanics than when added onto the endocortical surfaces. It has been suggested that these early changes in skeletal tissues are adaptive mechanisms that serve to prepare the maternal skeleton for the ensuing calcium demands of foetal skeletal mineralisation and lactation (3).

The data presented here also support the studies that show improved morphological and mechanical parameters as well as bone density after HMB supplementation. In our study, HMB administered during the gestation period affected the endurance of the humerus in the primiparous minks and the mechanics of the femur in the multiparous minks. However, when 2-Ox and HMB were given simultaneously to the dams during the whole gestation, they did not affect mother's skeleton as significantly as expected. This phenomenon was observed for the first time and it should be further investigated. Moreover, this study supported the observation that the thoracic limb in minks is comprehensively used, as evident in the supplemented group, in which the mechanical parameters and density significantly increased in the humerus in contrast to the pelvic limb.

This study is novel in that the results indicate for the first time that maternal 2-Ox and/or HMB administration also influences mechanical and geometrical properties of mother's bones in minks. Dietary supplements given to pregnant dams can program postnatal development of their offspring (28). Currently, 2-Ox or HMB may be treated as a protective metabolite when administered orally in minks, counteracting negative influences of pregnancy and lactation periods on bones. Bone mass and density are related to body weight and its components like muscles (24). Moreover, it is obvious that the small body size is associated with reduced muscle mass and strength, which depend not only on genetic factors but also on postnatal musculoskeletal development (25). Leg deformities and bone weakness and fractures can disturb animal welfare and cause serious economic losses.

When individual treatment of animal is not possible, nutrition should meet the highest quality standards. Balancing doses at the level of basic nutrients is not enough. Feed additives that improve nutrition are necessary. Only a diet containing appropriate supplements can provide satisfactory production results in animals, because nutritional deficiencies might trigger body's own reserves to cover

the increased needs connected with the bone mass of developing fetuses and ensuing milk production.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Animal Rights Statement: The experimental procedures used throughout this study were approved by the II Local Ethics Committee on Animal Experimentation of University of Life Sciences in Lublin, Poland.

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