ORIGINAL ARTICLE

THE EFFECT OF DIET ENRICHED WITH PYROPHOSPHATE (E450) ON EXPRESSION OF GENES ENCODING BONE MORPHOGENETIC PROTEIN AND OSTEOCALCIN IN MOUSE EMBRYONIC MANDIBLE TISSUES

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ABSTRACT

The aim: Of our study was to measure the mRNA expression of the investigated odontogenesis factors in mandible tissue of mouse embryos (17th day of pregnancy) gestated by females, kept on a E450 rich diet since 30 days before fertilization to gestation.

Materials and methods: The effect of food supplements was studied in «Overload phosphates model». Experiments were carried out on white nonlinear outbred mice with mass 25–28g (n=40). The females from the control group were fed with standard rodent food, whereas the experimental females were fed with pyrophosphate-enriched food. The materials, used for the molecular genetic study, were the lower jaws of 17-days old mouse embryos (*E–17*).

Results: The investigated *BMP2* and osteocalcin genes are expressed at approximately the same level. Pyrophosphate-rich diet does not alter *BMP2* gene expression, but it significantly increases the expression of *osteocalcin*.

Conclusions: The present study is the first one to describe the impact of the pyrophosphate-rich diet on mRNA expression of key osteogenesis regulators – *osteocalcin* and *BMP2*.

KEY WORDS: Bone morphogenetic protein 2 (*BMP2*) expression, *osteocalcin* expression, lower jaws of mouse embryos, pyrophosphate-rich diet, food additive E450

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INTRODUCTION

An optimized and well-balanced prenatal diet is essential for the physiological fetal development and formation of oral cavity organs and tissues in embryos [1, 2]. Negative effects of food preservatives and colorings on human health, especially during pregnancy, are among greatest health concerns of our time [3, 4].

The World Health Organization (WHO) defines food additives as chemical substances and natural compounds, which are added to alimentary products for improvement in raw materials and end product quality. In Ukraine, the following definition (which does not go beyond the WHO one) is adopted: food additives are natural or artificial substances and their compounds, which are added to alimentary products at the production stage to give them specific properties and (or) preserve their quality.

Food additives are substances added to alimentary products due to the processing reasons, namely for preservation of organoleptical properties and extension of shelf life.

The food additives consumption by the population has significantly increased in recent years. This is due primarily to the expansion of the list of substances authorized for addition to food. A big deal of daily use products contains some amount of food additives (cereal and meat products, cheeses, oils, mayonnaise, NA beverages, confectionary goods). The world production of food additives is continuously increasing, both quantitatively and qualitatively: by 10-15% in Asia, by 4,4% in the USA, and only by 2% in Europe [5].

We focused our attention on one of the stabilizers, encoded E450, which is a pyrophosphate $H_4P_2O_7$ and it is authorized as a stabilizing agent, though in some countries it is classified as hazardous to health [6]. It can be found in meat products, sausages, bacons, semi-finished products, jam, condensed milk, chocolate and processed cheese spreads, lemonade, sugary foods, etc [4]. Despite this, E450 is classified as a food additive, causing gastrointestinal and kidney diseases and calcium-phosphorus imbalance with further development of osteoporosis. It should be noted, however, that pyrophosphates are safe if consumed moderately [4].

Excessive intake of phosphates impairs calcium absorption [5], what can be crucial at the tooth bud mineralization stage [7]. The impact of maternal nutrition on the fetal odontogenesis has been well studied, but there are no studies dealing with teeth germination disorders, caused

by excessive pyrophosphate (food additive E450) intake.

Of special interest are the studies, focusing on expression of the genes, the protein products of which play a crucial role in the mentioned processes at all stages of odontogenesis. Considerable attention is being paid to the bone morphogenetic protein (encoded by *BMP2* gene) and the *osteocalcin* (*Bglap* gene) because of their dominant role in the calcification of a tooth bud [8, 9, 10].

It was investigated, that the *BMP2*, which is a growth factor for the tooth bud cells [11], promotes the differentiation of follicular cells into cementoblasts / -osteoblasts [12]. Such *BMP2* action is caused by the expression of a number of genes (type I collagen, osteonectin, dentin sialophosphoprotein, nestin) in cells of dental pulp [13]. The expression of *BMP2* gene, in turn, is controlled by the growth hormone (somatotropin) and insulin-like growth factor-1, that both can increase the *BMP2* expression in dental pulp fibroblasts *in vitro* in 4–5 times [14]. Moreover, the differentiation of enamel organ cells is also regulated by growth factors, in particular by transforming growth factor alpha (TGF- α) and epidermal growth factor (EGF) [15]. The *BMP2* protein is able to activate the receptors of osteoblasts located on the tooth germ surface and stimulate the proliferation of dental pulp mesenchymal cells with following differentiation in odontoblasts, thus providing the formation of osteodentine and tubular dentine [14]. *BMP2* is predominantly expressed in epithelial cells at the early stage (up to 13th day of a mouse embryo development) of tooth development. However, at the later stages it begins to be expressed mainly in the mesenchymal cells of dental papilla, initiating the intensification of dentinogenesis. Thus, *BMP2* is responsible for dental mesenchyme cells' fate during the tooth formation [16].

Odontoblasts also synthesize calcium-binding proteins - osteocalcin and osteonectin, which are expressed in dentine as well as in bone tissue [15]. Osteocalcin belongs to the proteins containing three residues of γ-carboxyglutamic acid that binds free calcium and prevents the apatite formation. Osteocalcin is a vitamin K-dependent matrix protein, which is able to bind to hydroxyapatites. It is synthesized by the dentine odontoblasts and it is a key factor in dental connective tissue mineralization [17].

We failed to find any published data regarding the effect of excessive pyrophosphates intake by a mouse female during pregnancy on mRNA expression of *BMP2* and *osteocalcin*.

THE AIM

The aim of this study was to measure mRNA expression of these odontogenesis factors in mandible tissue of embryos (17th day of pregnancy) gestated by females, kept on a E450 rich diet since 30 days before fertilization to gestation.

MATERIALS AND METHODS

In calculating the diet for the experimental mice group we have considered such data as: the actual nutrition of pregnant women, which was evaluated by questionnaire-polling method [1], recommended daily set of products for a pregnant woman[1], the content of E450 in foodstuffs, M.A.C.[2] of E450 in some foodstuffs[3]. Approximate daily set of products for a pregnant woman: 200g of meat or fish, 1 liter of milk in any form, 100-150g of curd, 20-30g of cheese, 1 egg, 600g of vegetables, 200-300g of fruits.

The effect of this food additive was studied in «phosphate overload model» (we add 2 g of E450 (sodium pyrophosphate, Israel) per 100g of standard rodent food). We did not apply for any modification to a basic model [18].

Experiments were carried out on white nonlinear outbred mice with mass 25–28g (n=40). All mice were separated into 2 groups: control group and experimental group. The females from the control group were fed with standard rodent food, whereas the experimental females were fed with pyrophosphate-enriched food (2% of pyrophosphate, Israel). Females in proestrus or estrus phase were kept with the males in proportion 4:1 30 days later. The presence of spermatozoa in the vaginal smear was considered as an indicator of fertilization and first day of pregnancy. Animals were fed with standard food (control group) or pyrophosphate rich food (experimental group). Pregnant mice (n=6 per group) were sacrificed by the carbon dioxide expose on the 17th day of pregnancy (E–17). Mousekins were sacrificed on day 2 (*D–2*) or day 28 (D–28) after the birth. Experiments were performed in accordance with the European Community Standards.

The material of the molecular-genetic study were the lower jaws of 17-days old mouse embryos (*E–17*), since *bone morphogenetic protein 2* (*BMP–2*) and *osteocalcin* (encoded by *Bglap*) [8, 9, 10] are crucial for the tooth bud calcification at all stages of odontogenesis, especially at the bell stage (period from 16.5 to 18.5 days of pregnancy) [19].

RNA samples were extracted from mandible tissue with phenol/chloroform using *Sigma-Aldrich* (USA) reagents. RNA concentration was measured by NanoDrop 1000 spectrophotometer by *Thermo Scientific* (USA). Reverse transcription was performed on 200-300 μg of total RNA using *First Strand cDNA Synthesis Kit* (*Fermentas,* Lithuania) and $(Oligo(dT)_{18}$ Primer. The resulting cDNA product was used for PCR amplification. Quantification of BMP2 and *bone gamma carboxyglutamate protein* (*Bglap, osteocalcin*) expression was performed by real-time PCR using primers shown below:

BMP2 Up: 5`-GTGGAGGAACTTCCAGAGATGA-3`;
BMP2 Dw: 5`-CTGCAGATGTGAGAAACTCGTC-3`;
Osteocalcin Up: 5`-CAGGAGGGCAATAAGGTAGTGA-3`;
Osteocalcin Dw: 5`-CAGGGTTAAGCTCACACTGCTC-3`.

[1]Maximum allowable concentration.
[2]Maximum allowable concentration of the food additive E450 in some foodstuffs: baked goods - 10 000 mg / kg, desserts - 3000 mg / kg, ice cream - 1 000 mg / kg, flour - 2 500 mg / kg, raw eggs - 10 000 mg / kg, sauces - 5000 mg / kg, processed cheese - 9000 mg / kg, meat and fish products - from 100 to 5000 mg / kg (0.3% of the total weight of the forcemeat).
[3]The maximum acceptable daily consumption of E450 is 70 mg/kg body weight.

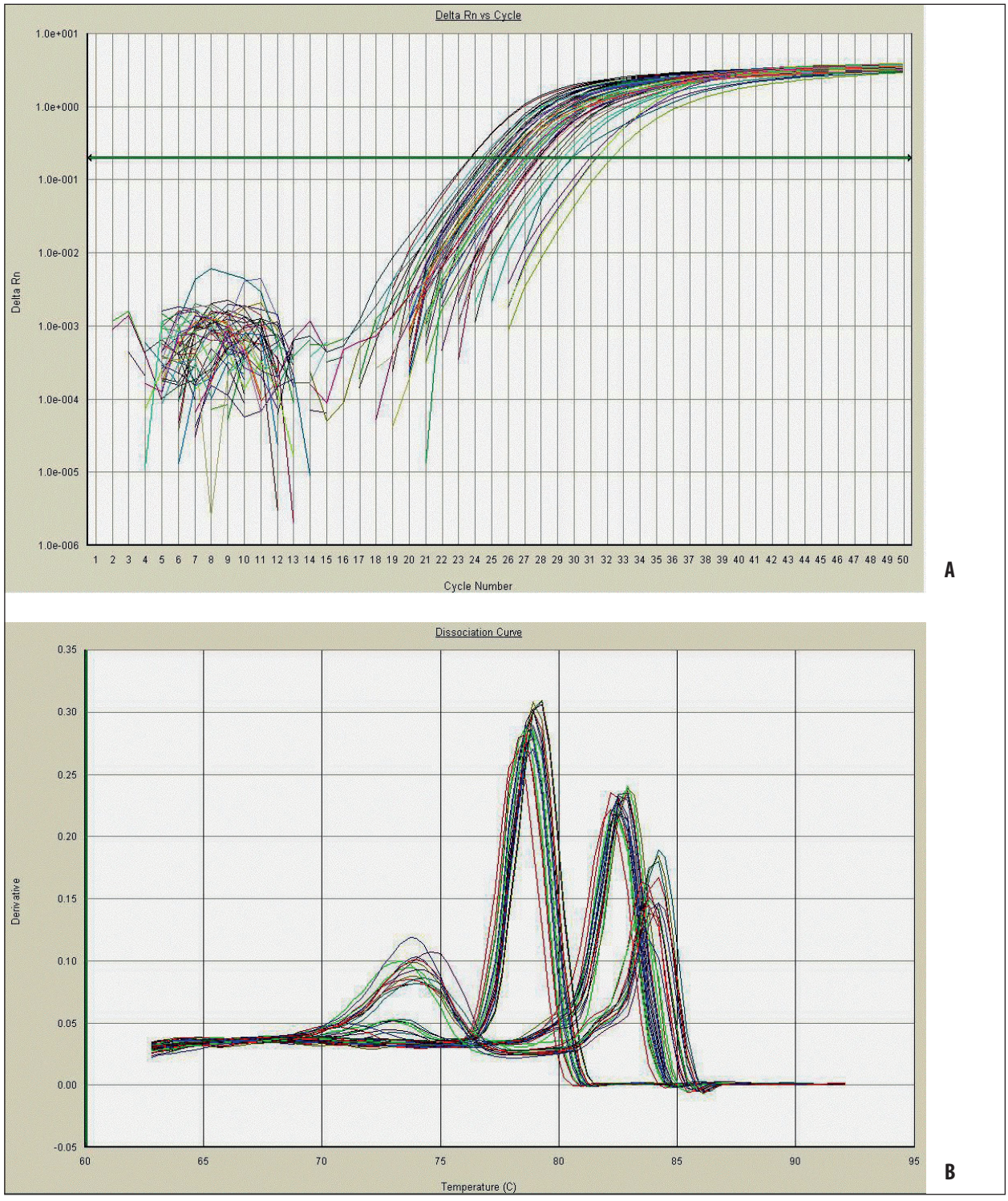

Fig. 1. Actin, BMP2 and osteocalcin amplification products concentration relative to amplification cycle number curve (A) and amplification products dissociation curve (B).

PCR amplification reactions were performed in 20 μL of SYBR Green *PCR Master Mix* containing 25 pM of each primer. Thermal cycling conditions comprised an initial denaturation and AmpliTaq Gold® DNA polymerase activation step at 95°C for 10 min, followed by 50 cycles of denaturation for 15 s at 95°C and 60°C (or 61°C, in case of *osteocalcin* gene) for 1 min. In addition, melting curve analysis was performed to control the specificity of PCR product fluorescence: the sample was *gradually* heated from 60(61) to 94° C while simultaneously detecting decrease of DNA-*SYBR Green* complex fluorescence intensity.

Amplification products concentration vs cycle curve and amplification products dissociation curve are shown in fig. 1.

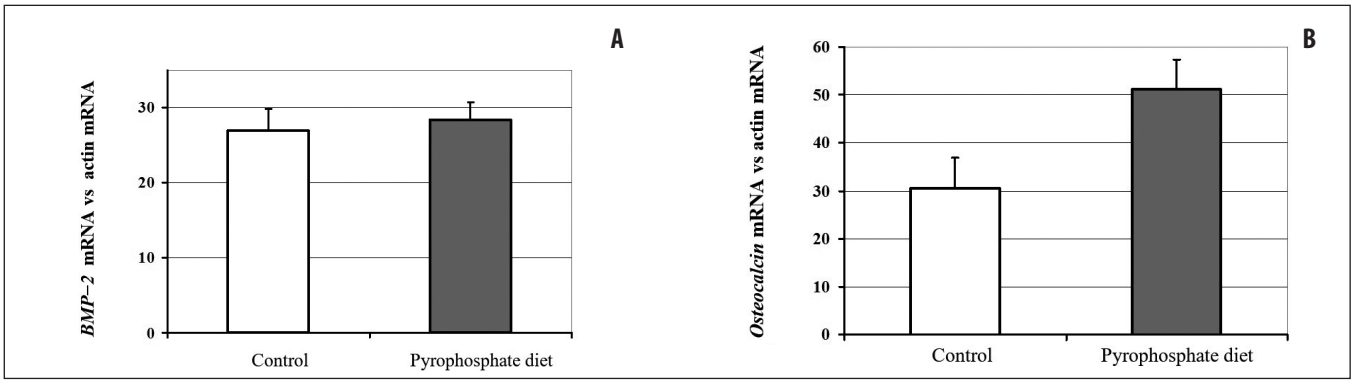

Fig. 2. BMP2 (A) and osteocalcin (B) mRNA expression relative to actin in lower jaw tissue of mouse embryos from control and experimental (high pyrophosphate diet) groups.

The analysis of the data received was performed using 7500 Fast Real-time PCR Software.

The extrapolation of the obtained results to humans is theoretically possible due to the great described genes sequence similarity between humans and mice [20, 21].

Statistical analysis. Statistical analysis of the digital data was performed using Excel 2000 and Origin 7.0. Probability distribution of mean ($P<0.05$) was calculated using Student's t-test.

RESULTS AND DISCUSSION

The expression of *BMP2* in jaws was the same in animals from control and experimental groups ($p=0,71$; $p>0,05$). Nevertheless, pyrophosphate-enriched diet caused 1,8-fold increase of *osteocalcin* expression ($p=0,047$; $p<0,05$) to 51,2±6,20 (fig. 2).

The analysis of the data shows that a diet with high content of sodium pyrophosphate does not change the expression of *BMP2* gene. Given that *BMP2* is a key factor of odontoblasts differentiation [22], we can hypothesize that excessive pyrophosphate in maternal diet would not influence the odontogenesis in the embryo. However, sodium pyrophosphate rich diet is likely to increase the expression of osteocalcin. On the one hand, raised osteocalcin expression seems to be a positive sign, since osteocalcin ensures the mineralization of tooth bud tissues, and that means intensification of apatite formation in animals with gained expression of osteocalcin. But on the other hand, the hyperexpression of osteocalcin could cause the premature tooth mineralization that can disrupt the processes of teeth formation and odontogenesis all in all.

Our results do not fully agree with those in the study [23], where the authors researched the effect of inorganic phosphate/pyrophosphate on expression of a wide range of genes (using real-time PCR) in mouse cementoblasts. It was investigated that pyrophosphate at the concentration of 5mM caused increase of ogosteopontin and dentin matrix protein-1 expression and decreased mRNA expression of bone sialoprotein (Bsp), osteocalcin and type I collagen. Differences in the results could be explained by the differences in selected methodology (experiments were conducted in vitro on isolated cells) and by briefness of pyrophosphate exposure (to 48 hours). Our study shows the effect of 50 days-long pyrophosphate exposure of the whole organism. We evaluated the gene expression in lower jaw tissues instead of using cultivated cells for this purpose. Nevertheless, the most important fact is that pyrophosphate can alter the expression of some genes significant for odontogenesis.

CONCLUSIONS

Thus, we first described the impact of pyrophosphate-enriched diet on mRNA expression of *osteocalcin* and *BMP2* which are crucial regulators of osteogenesis. Further observations on histopathological changes in mouse tooth bud would give a possibility to match the genetic changes with the pathomorphological changes and clarify the functional role of the alterations in expression of the investigated genes.

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The Authors declare no conflict of interest.

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