ORIGINAL ARTICLE

THE EFFECT OF DIET ENRICHED WITH PYROPHOSPHATE (E450) ON MORPHOLOGICAL CHANGES OF TOOTH GERMS OF MOUSE EMBRYOS

DOI: 10.36740/WLek202202115

Inessa I. Yakubova[1], Victor Dosenko[2], Lesya Tumanovska[2], Volodymyr Ostrianko[3], Sergii Tsypan[1], Yurii Skrypnyk[1]
[1]PRIVATE HIGHER EDUCATIONAL ESTABLISHMENT «KYIV MEDICAL UNIVERSITY», KYIV, UKRAINE
[2]BOGOMOLETS NATIONAL MEDICAL UNIVERSITY, KYIV, UKRAINE
[3]SHUPYK NATIONAL HEALTHCARE UNIVERSITY OF UKRAINE, KYIV, UKRAINE

ABSTRACT

The aim: To reveal the effect of pyrophosphates on the tooth germ structure in the mandible of embryos (17th day of pregnancy) gestated by females, kept on a pyrophosphate-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 material for the morphological study were the mandible of 17-day-old mouse embryos (E-17), which were examined under a microscope with subsequent photofixation.

Results: The examination of the mandible of 17-day-old mouse embryos, gestated by females on a pyrophosphate-rich diet, showed morphological changes in tooth germs at the dental follicle development stage.

Conclusions: The experimentation revealed that the pyrophosphate excessive intake during dental follicle development leads to early dentinogenesis and oppression of ectodermal structures of tooth germs.

KEY WORDS: pyrophosphate diet, odontoblasts, enamel, dentin, mouse embryo mandible, food supplement E-450 (pyrophosphate)

Wiad Lek. 2022;75(2):410-415

INTRODUCTION

Diet is an important determinant of offspring health, starting from the conception [1]. Nutritional needs increase during pregnancy to maintain maternal metabolism and tissue accretion while supporting foetal growth and development [2]. Poor dietary intakes or deficiencies in key macronutrients and micronutrients can therefore have a substantial impact on pregnancy outcomes and neonatal health. Increasing evidence suggests that the effects of foetal nutrition may persist well into adulthood, with possible intergenerational effects [2].

One of the current health concerns, particularly relevant to pregnant women, is the addition of preservatives and dyes to alimentary products [3,4].

Among the stabilizing agents our attention was drawn to a food supplement encoded E-450 (salts and esters of pyrophosphoric acid - pyrophosphates).

There are 8 types of pyrophosphates [5,6]:

(i) Disodium diphosphate;
(ii) Trisodium diphosphate;
(iii) Tetrasodium diphosphate;
(iv) Dipotassium diphosphate;
(v) Tetrapotassium diphosphate;
(vi) Dicalcium diphosphate;
(vii) Calcium dihydrogen diphosphate;
(viii) Dimagnesium diphosphate.

E-450 is widely used in food industry as a preservative, stabilizer and raising agent in the production of meat products, semi-finished products (sausages, wursts, dumplings, carbonade, deli meats, dried meat), minced meat, canned food; cheeses, processed cheeses and some dairy products (sour cream, condensed milk); canned seafood, jams, lemonades, sweets, baking soda [5,6]:

The World Health Organization (WHO) classifies the inorganic pyrophosphate as a nontoxic physiological metabolite with a maximum tolerable daily intake value (MTDI) of 70mg/kg [7].

The Food and Drug Administration (FDA) of the United States classifies the inorganic pyrophosphate as Generally Recognized Safe (GRAS), while in Europe it is designated as food supplement E-450 [8].

Dietary inorganic pyrophosphate is readily absorbed in humans [9] and its level of oral absorption, observed with human volunteers, is comparable to absorption from water [10].

Excess of phosphates impairs the absorption of calcium in the body, causing calcium-phosphorus imbalance [11, 12], which can be crucial at the tooth bud mineralization stage.

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 supplement E-450) intake.

We failed to find any published data regarding the morphological changes of tooth germs caused by excessive pyrophosphate intake by a mouse female during pregnancy, therefore we initiated a study in pursuit of the following:

examining of the impact of excessive E-450 intake on the structure of the tooth germs in the mandible of mouse embryos (17th day of pregnancy) gestated by females, kept on a pyrophosphate rich diet since 30 days before fertilization to gestation.

THE AIM

The aim of this study was to reveal the effect of pyrophosphates on the tooth germ structure in the mandible of embryos (17th day of pregnancy) gestated by females, kept on a pyrophosphate-rich diet since 30 days before fertilization to gestation.

MATERIALS AND METHODS

The *in vivo* study of the E-450 (pyrophosphate) effect was carried out on «phosphate overload model». We did not apply any modification to a basic model [12].

The experiments were performed in compliance with the «Rules and Regulations for Carrying Out Animal Research Work».

Experiments were carried out on white nonlinear outbred mice (total 40 animals, weighing 25-28g), housed in stainless steel cages under controlled conditions (50–60% relative humidity, artificial 12-h light-dark cycle). The mice were kept at 23±1°C with a 12-h light–dark cycle, light at 7 a.m., and were allowed ad libitum access to tap water and food.

The room was located in the vivarium of the Bogomoletz Institute of Physiology NAS of Ukraine.

All mice were separated into 2 groups: control group and experimental group.

Experimental hyperphosphatemia (2%) was simulated by adding to the diet food supplement E-450 (sodium pyrophosphate, chemical formula $Na_4P_2O_7$) for 60 days.

Mice of the experimental group received a diet of vivarium with the addition of 2g. sodium pyrophosphate (made in Israel) per 100 gr. stern.

Mice in the control group received a diet of vivarium (24% protein, 11% fat, 48% carbohydrates, 5.5% fiber, 6% vitamin 5.5% ash).

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.

Pregnant females were kept in cages and 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).

The object of the morphological investigation were the mandibles of 17-day-old mouse embryos (E-17), when odontogenesis underwent the stage of the bell (period from 16.5 to 18.5 days of pregnancy) [13].

Experiments were performed in accordance with the European Community Standards.

The mandibles were fixed by 2% glutaraldehyde in cacodylate buffer followed by decalcification, postfixation in 1 % osmium oxide and embedding in epoxy resin. Semithin sections were stained with methylene blue and fuchsine that allowed identification of mesenchymal and epithelial tissues. Obtained morphological sections had 1,0-1,5 μm in thickness. The images were obtained using *Nikon Eclipse E200 (Fryer Co., Huntley, IL,* USA*)* microscope in combination with *Nikon DS-F11* camera.

To describe micrographs with magnification x10, x20, x40 we used a schematic representation of successive stages of differentiation of ameloblast cells (Fig. 1).

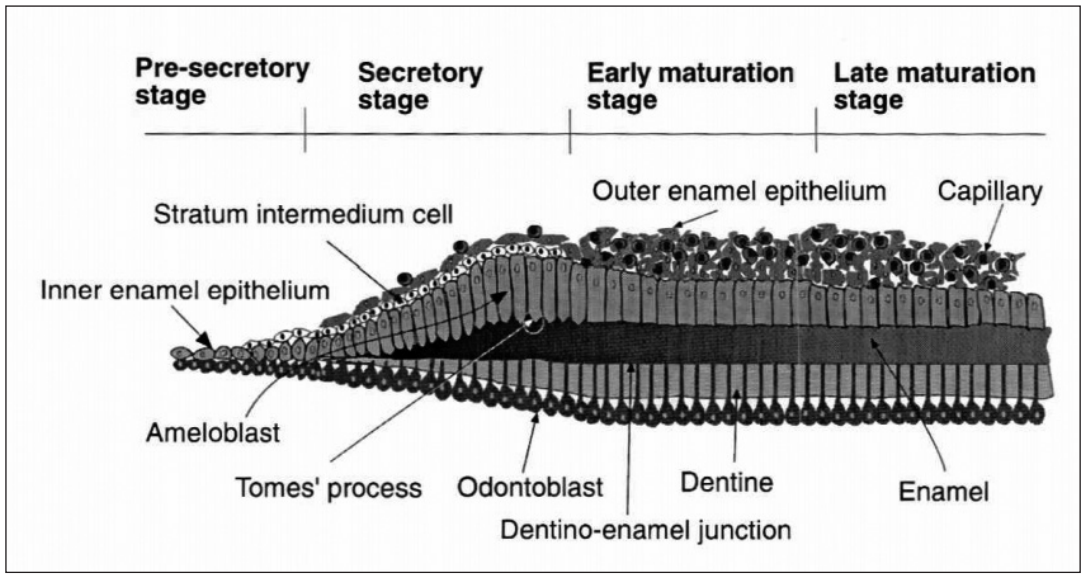

Fig. 1. Schematic representation of the sequence of stages of ameloblast cells differentiation [14]

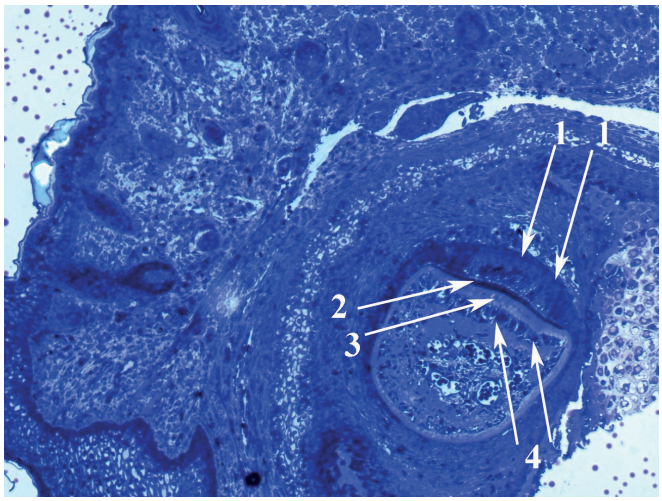

Fig. 2. Morphological study of the tooth germs of a 17-day-old embryo of control mice, x10, staining with methylene blue: 1 - ameloblasts, 2 - enamel, 3 - dentin, 4 - odontoblasts.

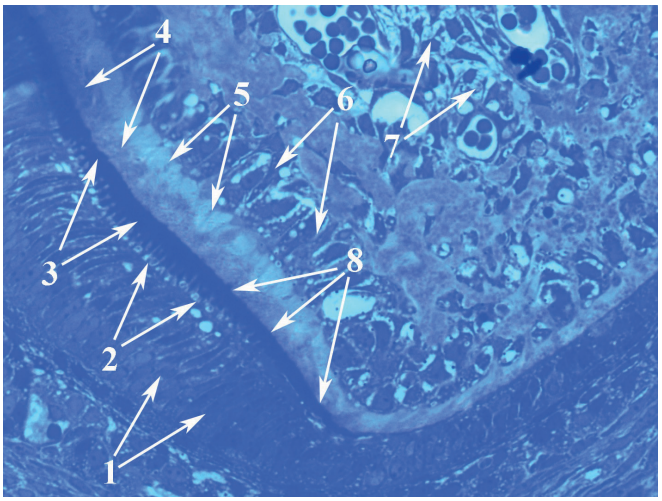

Fig. 3. Morphological study of the tooth germs of a 17-day-old embryo of control mice, x40, staining with methylene blue: 1 - ameloblasts, 2 - Thomes' processes, 3 - enamel, 4 - dentin, 5 - predentin, 6 - odontoblasts, 7 - pulp, 8 - enamel-dentin connection.

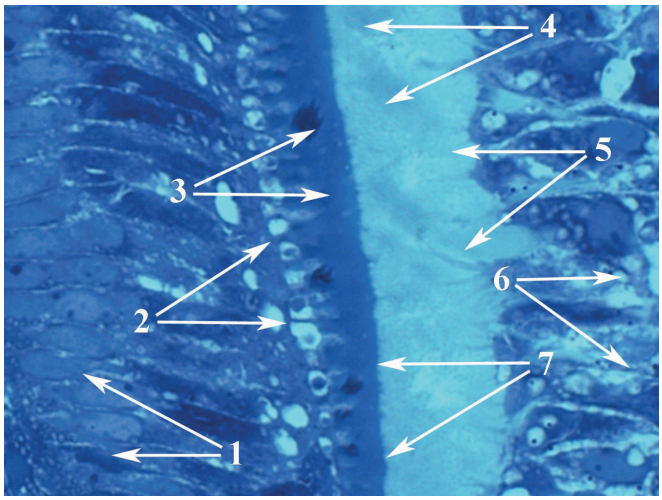

Fig. 4. Morphological study of the tooth germs of a 17-day-old embryo of control mice, x100, staining with methylene blue: 1 - ameloblasts, 2 - Thomes' processes, 3 - enamel, 4 - dentin, 5 – predentin, 6 - odontoblasts, 7 - enamel-dentin connection.

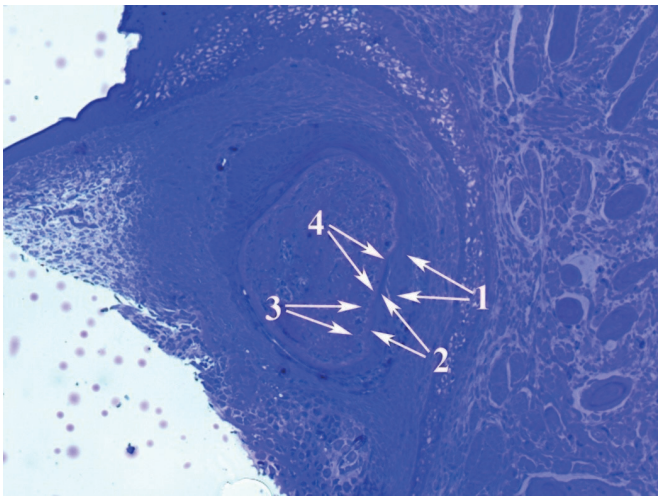

Fig. 5. Micrograph of the dental germ structure of a 17-day-old embryo of experimental mice (pyrophosphate (E450) rich diet). Magnification: x10; staining: methylene blue. 1 - ameloblasts, 2, 4 - enamel, 3 – odontoblasts.

STATISTICAL ANALYSIS

Statistical analysis of obtained 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

Our morphological research of dental germs from embryos gestated by mice kept on a pyrophosphate-enriched diet before and during pregnancy showed tooth morphogenesis disturbances.

The obtained micrographs of the control group showed the following.

At an increase of x10 in the control group, ameloblasts formed a uniform layer with clear polarization, on the apical part of cylindrical cells there were narrowed areas - Tomes' processes, key structures responsible for the secretion of the enamel matrix, the presence of which indicates high differentiation of ameloblasts and corresponds to secretory stage development of these cells. The functional activity of the ameloblast layer was reflected by the presence of a pronounced dark-dark band between the dentin and ameloblasts, which morphologically corresponds to tooth enamel (Fig. 2).

With increasing x40 in the control group (Fig.3), observed was the formation of a uniform layer of odontoblasts, which took a cylindrical shape, were located in parallel and formed processes in the predentin layer. Dentin and predentin had the form of two tightly connected but evenly spaced bands, differing in color by about one tone - predentin lighter, dentin darker. When the predentin layer reaches a thickness of 40-80 μm, it is pushed to the periphery by the newly formed layers of predentin, in which the fibers have a different direction - they are located parallel to

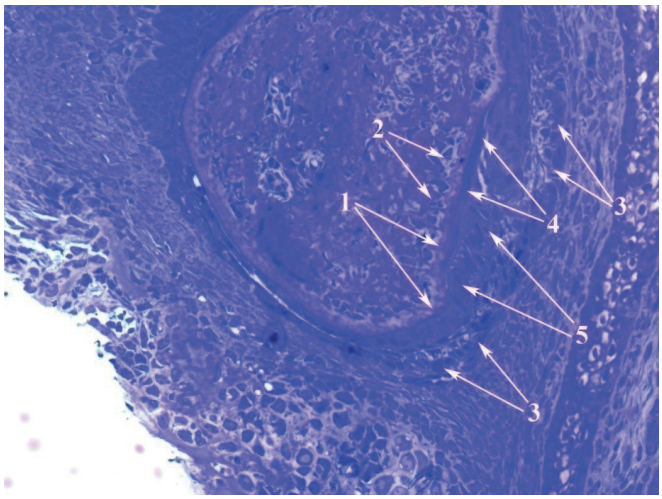

Fig. 6. Micrograph of the dental germ structure of a 17-day-old embryo of experimental mice (pyrophosphate (E450) rich diet). Magnification: x20; staining: methylene blue. 1 – dentin, 2 – odontoblasts, 3- outer enamel epithelium, 4 – enamel, 5 – ameloblasts.

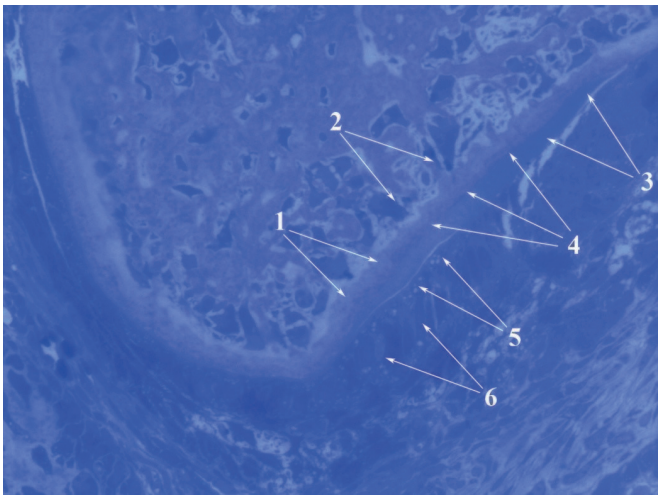

Fig. 7. Micrograph of the dental germ structure of a 17-day-old embryo of experimental mice (pyrophosphate (E450) rich diet). Magnification: x40; staining: methylene blue. 1 – dentin, 2 – odontoblasts, 3 – enamel, 4 – enamel-dentin connection, 5 – Tomes' process, 6 – ameloblasts.

the surface of the papilla. Subsequently, these inner layers of dentin, rich in tangential fibers, form pulp dentin in the formed tooth, and the radial fibers lying in the outer layers of dentin, which was formed first, - mantle dentin.

With increasing x100 in the control group (Fig. 4), the uniform distribution of dentin and predentin bands on lighter and darker was observed. There is a higher differentiation of cells of both odontoblasts and ameloblasts. Ameloblasts have a cylindrical shape with pronounced thinning in the apical part, the clear location and uniform distribution of cell layers allow us to conclude about the high differentiation and sequence of function of all cells.

The following differences are observed in the obtained micrographs of the experimental group.

With an increase in x10 in the experimental group (Fig. 5), the enamel was expressed in form of uneven barely noticeable line in a limited area. It was probably resulted from significant structural alterations in ameloblast layer, to be more precise – in formation of unevenly ordered layer composed from unpolarized cells with feebly pronounced cylindrical shape.

With increasing x20 in the experimental group (Fig. 6) the outer enamel epithelium was expressed in form of uneven layers of chaotically distributed cells distinguishable by their polarization and intensity of staining.

With increasing x40 in the experimental group (Fig. 7) the line of hard tissues formation contained nonuniform bands in teeth from experimental group, a phenomenon that was observed in none of the control samples. The thinnings on the apical part of ameloblasts were observable only on the very limited area, determined thinning - *Tomes' process* (Fig 7).

The hypertrophic nucleuses of ameloblasts occupied almost the entire cytoplasmic space, that indicates decreased cell differentiation and only the beginning of cell transition from *pre-secretory* into *secretory* phase of differentiation (Fig. 7).

Unevenly located ameloblasts differed from each other by phase of their maturation (most of them were on the *early maturation stage).* The unpolarized chaotically distributed cell aggregations were observed, in front of which the enamel was not disclosed (Fig 5, 6).

Some slight disorientation and disorganization of odontoblasts, as like as absolute their absence in some areas of the organ, was observed at x40. Dentin has the appearance of a light irregular strip without distinctive division between the predentin and dentin. Since predentin and dentin could not be distinguished in the preparation, it is clear that the specimens from the experimental group underwent some alterations caused by uneven and even sometimes chaotic arrangement of dentine collagen fibers and disbalance of process of dentine layer satiation with organic and inorganic components. The thin layer of dentin is one more indicator of its reduction; the disorganization of the odontoblasts layer was also observed (Fig. 7).

DISCUSSION

The experiment focused on the features of morphogenesis of tooth germs of 17-day-old mouse embryos. In mice of the experimental group the study revealed changes in the morphological structure of teeth, caused by the sodium pyrophosphate (food additive E450) rich diet, including significant structural changes in the layer of ameloblasts, uneven layers of chaotically arranged cells of the outer enamel, nonuniform bands in the line of hard tissues formation. Tomes' processes were determined in a very limited area of apical parts of ameloblasts, hypertrophied nuclei of ameloblasts covered almost the entire cytoplasmic space, unevenly spaced ameloblasts were mainly at the stage of early maturation, disorientation and disorganization of individual odontoblasts. In the experimental group there were changes caused by uneven and sometimes chaotic

arrangement of dentin collagen fibers, imbalance in the saturation of the dentin layer with organic and inorganic components.

Thus, in all samples of the tooth germs of the experimental group there were significant differences as opposed to the control ones. This is due to the fact that the most pronounced effect of food additive E450 (pyrophosphate) occurs during the follicular development of teeth, which leads to early dentinogenesis and inhibition of ectodermal structures of tooth germs.

The researchers [15] conducted the study of the effect of excessive amounts of pyrophosphates in the diet of pregnant mouse females on the expression of BMP2 mRNA and osteocalcin. It was found that a diet, enriched with sodium pyrophosphate, does not alter BMP2 gene expression [15]. Given that BMP2 is a key factor of odontoblasts differentiation [16], 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 [15]. On the one hand, raised osteocalcin expression seems to be a positive sign, since osteocalcin ensures the mineralization of the 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.

Studies of pathohistological changes in the tooth germs of the mandible of 17-day-old mouse embryos allowed to compare genetic changes [16] with pathomorphological and to establish the functional significance of changes in the expression of the studied genes.

Under clinical conditions, this creates a favorable basis for the development of systemic hypoplasia of the enamel, and in the long run - focal demineralization of hard tissues and carious process [17].

CONCLUSIONS

Experiments on the mandible of 17-day-old mouse embryos, influenced by food supplement E-450 (pyrophosphate), showed the availability of morphological changes in teeth buds. In all samples of the experimental group there were significant structural differences in the formation of tooth germs as opposed to the samples of the control group. The study has revealed that food supplement E-450 (pyrophosphate) can cause early dentinogenesis and oppression of ectoderm derived structures of teeth at the stage of their follicular development. In clinical conditions, it may induce the development of system enamel hypoplasia, focal demineralization of hard tissues and hereafter may cause caries.

REFERENCES

1. Marangoni F., Cetin I., Verduci E. et al. Maternal Diet and Nutrient Requirements in Pregnancy and Breastfeeding. An Italian Consensus Document. Nutrients. 2016;8(10):629. doi:10.3390/nu8100629.
2. Mousa A., Naqash A., Lim S. Macronutrient and Micronutrient Intake during Pregnancy: An Overview of Recent Evidence. Nutrients. 2019;11(2):443. doi:10.3390/nu11020443.
3. Trasande L., Shaffer R.M., Sathyanarayana S. Food Additives and Child Health. Pediatrics. 2018;142(2):e20181410. doi:10.1542/peds.2018-1410.
4. Potera C. DIET AND NUTRITION: The Artificial Food Dye Blues. Environ Health Perspect. 2010;118(10). doi:10.1289/ehp.118-a428.
5. Younes M., Aquilina G., Castle L. et al. Re–evaluation of phosphoric acid–phosphates – di–, tri– and polyphosphates (E 338–341, E 343, E 450–452) as food additives and the safety of proposed extension of use. EFSA J. 2019;17(6). doi:10.2903/j.efsa.2019.5674.
6. Cooke A. Dietary Food-Additive Phosphate and Human Health Outcomes. Compr Rev Food Sci Food Saf. 2017;16(5):906-1021. doi:10.1111/1541-4337.12275.
7. Summary of Evaluations Performed by the Joint FAO/WHO Expert Committee on Food Additives. http://www.inchem.org/documents/jecfa/jeceval/jec_2259.htm [date access 20.01.2021]
8. Commission Regulation (EU) No 1129/2011 of 11 November 2011 Amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by Establishing a Union List of Food Additives. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:02011R1129-20131121 [date access 20.01.2021]
9. Pomozi V., Julian C.B., Zoll J. et al. Dietary Pyrophosphate Modulates Calcification in a Mouse Model of Pseudoxanthoma Elasticum: Implication for Treatment of Patients. J Invest Dermatol. 2019;139(5):1082-1088. doi:10.1016/j.jid.2018.10.040.
10. Dedinszki D., Szeri F., Kozák E. et al. Oral administration of pyrophosphate inhibits connective tissue calcification. EMBO Mol Med. 2017;9(11):1463-1470. doi:10.15252/emmm.201707532.
11. Moe S.M. Disorders Involving Calcium, Phosphorus, and Magnesium. Prim Care Clin Off Pract. 2008;35(2):215-237. doi:10.1016/j.pop.2008.01.007.
12. Karkishchenko N.N. Guidance on Laboratory Animals and Alternative Models in Biomedical Technology. (S.V. G, ed.). Profile; 2010, 54p.
13. Åberg T., Wozney J., Thesleff I. Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. Dev Dyn. 1997;210(4):383-396.
14. Lee S.K., Krebsbach P.H., Matsuki Y. et al. Ameloblastin expression in rat incisors and human tooth germs. Int J Dev Biol. 1996;40(6):1141-1150.
15. Yakubova I.I., Dosenko V.E., Tumanovska L.V., Ostrianko V.I. The effect of diet enriched with pyrophosphate (E450) on expression of genes encoding bone morphogenetic protein and osteocalcin in mouse embryonic mandible tissues. Wiadomości Lek. 2021;74(1):43-47. doi:10.36740/WLek202101108.
16. Popowics T., Foster B.L., Swanson E.C. et al. Defining the Roots of Cementum Formation. Cells Tissues Organs. 2005;181(3-4):248-257. doi:10.1159/000091386.
17. Yakubova I.I. A Compendium on Oral Health of Children around the World: Early Childhood Caries. Nova Scien. ; 2017. https://novapublishers.com/shop/a-compendium-on-oral-health-of-children-around-the-world-early-childhood-caries/ [date access 20.01.2021]

ORCID and contributionship:

Inessa I. Yakubova: 0000-0003-2780-2460 [A-D,F]
Victor Dosenko: 0000-0002-6919-7724[A,E,F]
Lesya Tumanovska: 0000-0002-7984-5270[A,E,F]
Volodymyr Ostrianko: 0000-0001-6525-7526[B-E]
Sergii Tsypan: 0000-0003-4451-9990[E]
Yurii Skrypnyk: 0000-0003-1196-8204[E]

Conflict of interest:
The Authors declare no conflict of interest.

CORRESPONDING AUTHOR
Inessa I. Yakubova
Kyiv Medical University
2 Boryspilska St., 02099 Kyiv, Ukraine
tel: +380677132097
e-mail: yakubova.inessa@gmail.com

Received: 04.03.2021
Accepted: 28.08.2021

A - Work concept and design, **B** – Data collection and analysis, **C** – Responsibility for statistical analysis, **D** – Writing the article, **E** – Critical review, **F** – Final approval of the article