

ORIGINAL ARTICLE

INFLUENCE OF CHOLESTEROL ENRICHED DIET ON GENES EXPRESSION ENCODING BONE MORPHOGENETIC PROTEIN-2 AND OSTEOCALCIN IN MOUSE MANDIBLE

DOI: 10.36740/WLek202307114

Inessa I. Yakubova¹, Volodymyr Ostriancko², Victor Dosenko³, Liliia Bielova¹, Yurii Skrypnyk¹, Ganna Viun¹¹PRIVATE HIGHER EDUCATIONAL ESTABLISHMENT «KYIV MEDICAL UNIVERSITY», KYIV, UKRAINE²SHUPYK NATIONAL HEALTHCARE UNIVERSITY OF UKRAINE, KYIV, UKRAINE³THE BOGOMOLETZ INSTITUTE OF PHYSIOLOGY NAS OF UKRAINE, KYIV, UKRAINE

ABSTRACT

The aim: To evaluate the mRNA expression of the key regulators of osteogenesis – osteocalcin and BMP-2 in the mouse embryos mandible (17th day of pregnancy) which were borne by females on high-cholesterol diet for 30 days before fertilization and throughout pregnancy.

Materials and methods: Experimental hypercholesterolemia (2%) was simulated by adding Cholesterol to the diet for 60 days. In experiment were used 40 mature female white mice that were randomly divided to control and experimental groups. The control group were fed with standard chow diet, the experimental group with diet with cholesterol enriched diet (with addition of 2 grams of Cholesterol per 100 grams of standard chow). The mandibles of mouse embryos (E–17) were examined by using molecular genetic methods.

Results: In control group the relative level of BMP-2 mRNA / actin mRNA was 27.0 ± 2.82 , the relative level of and osteocalcin mRNA / actin mRNA was 30.5 ± 6.28 . In the jaws of animals in the experimental group with cholesterol enriched diet, the expression relative level of BMP-2 was 30.9 ± 5.81 that is by 14,4% higher than in control group. Therefore, the expression level of osteocalcin, on the contrary, decreased by 22.3% and was 23.7 ± 5.31 .

Conclusions: Our study report influence of the cholesterol enriched diet (2%) on mRNA expression of BMP-2 and osteocalcin encoding genes. The embryos from mouse on cholesterol enriched diet (2%) had increased level of BMP-2 gene expression, however significantly decreased level of osteocalcin gene expression.

KEY WORDS: bone morphogenetic protein-2, osteocalcin, mouse embryos mandible, hypercholesterolemic diet, cholesterol

Wiad Lek. 2023;76(7):1608-1614

INTRODUCTION

Adequate and balanced nutrition of the pregnant woman ensures the normal fetal development and is the basis for the formation of organs and tissues of the oral cavity in the baby. Nutrition during pregnancy play an important role in tooth development, that basically include influence on primary tooth formation and the start of mineralization. More over in future may account for dental caries susceptibility in children [1].

In all trimesters, more than a half of the sample group reported fat intakes as percentages of energy that exceeded the acceptable range of 20–35% [2]. These results were similar to those [3] in which a third of the 1533 studied pregnant women had total fat intakes as a percentage of energy exceeded the Acceptable Micronutrient Distribution Range. Furthermore, a meta-analysis [4] of the studies also found that the average fat intakes in pregnant women also above recommendations (as percentages of energy intake) and were 35.0% to 37.1%, in accordance with the results [2].

Unbalanced and poor nutrition of pregnant women increases the risk of dental caries in the unborn child by forming low caries susceptibility of the embryo tooth tissues, fetal immaturity and enamel hypoplasia of the baby, and as a result, leading to caries of deciduous teeth in children [5]. However, still is not enough studies about particular effects of maternal diet on dental health of child in order to make evidence-based statements. There are studies that show that High fat diet (HFD) has deleterious effect on bone micro-architecture [6 – 7].

A constant component of fatty foods is cholesterol. Cholesterol occurs naturally only in foods of animal origin. The highest concentrations are found in liver and egg yolk, but red meats, poultry (especially the skin), full-fat milk, and cheese make significant contributions to the diet [8]. Dietary cholesterol was implicated in increasing blood cholesterol levels [9].

Bone morphogenetic protein and osteocalcin play a crucial role in stages of odontogenesis and tooth germ

early mineralization. Many studies investigated that BMP-2 is needed to start the differentiation of stem cells from exfoliated deciduous teeth into odontoblasts [10-12]. Some studies show that one of the growth factors for the isolated dental papilla and odontoblasts in future was BMP-2 [13]. Also, BMP-2 has been found to induce differentiation of the cells of follicle into a cementoblasts or osteoblasts phenotype [14]. One more of stimulating effect of BMP-2 is proliferation and then differentiation of pulp cells into odontoblast cells [15], consequently, BMP-2 are able to induce osteodentine and tubular dentine, in turn, growth hormone is able to induce BMP-2 in dental pulp fibroblasts [16].

During tooth development, BMP-2 is initially expressed in the oral epithelial cells at embryonic day 13 (E13), and at later stages of tooth development its expression shifts to the mesenchymal dental papilla and becomes more intense with dentinogenesis [17].

Bone gamma-carboxyglutamate protein (BGLAP, osteocalcin) is protein that is secreted by mature osteoblasts; thus, expression of BGLAP is an indicator for bone function. One of study paid attention to a vitamin K-dependent matrix protein – osteocalcin that is produced by dentine odontoblasts and in bone tissue and has ability to binding to hydroxyapatite. Finding from this study suggest key role of osteocalcin in dentino- and cementogenesis, bone and periodontal ligament remodeling as well as mineralization [18].

Researches said that osteocalcin was expressed only during the later stages – at the maturation stage of enamel formation, and was detected in odontoblasts and their processes within the extracellular matrix. Our data show that osteocalcin and osteonectin, coexpressed in bone and teeth, may diverge in their functional involvement in relation to the binding specificities of the cellular and extracellular components of dental mineralized tissues [19].

There some studies have linked imbalanced maternal nutrition with violated fetal odontogenesis, but it unknown whether excessive cholesterol intake during pregnancy alter teeth germination disorders via influence on expression of two important proteins of tooth development and mineralization, BMP-2 and osteocalcin, that are encoding by BMP-2 and Bglap genes, correspondingly.

THE AIM

The aim of this study was to evaluate the mRNA expression of the key regulators of osteogenesis – osteocalcin and bone morphogenetic protein 2 (BMP-2) in the mouse embryos mandible (17th day of pregnancy) which were borne by females on high-cholesterol

diet for 30 days before fertilization and throughout pregnancy.

MATERIALS AND METHODS

For decades, the laboratory mouse has been the preferred model organism for the study of human biology and diseases. Humans and mice share a very similar genetic background, and around 90% of both genomes can be partitioned into regions of conserved synteny. The extrapolation of the obtained results to humans is theoretically possible due to the great described genes sequence similarity between humans and mice [20].

Forty mature female white mice were housed in cages with controlled conditions and free access to water (20–24°C, 50–60% relative humidity, artificial 12-h light – 12-h dark cycle). Mice were randomly divided to control and experimental groups (20 mice in each group). The control group were fed with standard chow diet, the experimental group with diet with cholesterol enriched diet (with addition of 2 grams of Cholesterol per 100 grams of standard chow).

Adult females after 30 days of experiment in proestrus or estrus cycle phase used in this study were paired overnight with vigorous sexually experienced adult males in proportion 4 to 1. Successful mating was confirmed by the presence of sperm in the vaginal smear the following morning (07:00–08:00) and this day was considered day 1 of pregnancy. Only sperm positive females were used in the study. The pregnant mice were assigned into various groups according to the design of the experiment. Throughout pregnancy females were kept in cages and fed with standard chow for control group or cholesterol-enriched chow for experimental group.

Period of mice embryonic development between E16.5 and E18.5 is known as bell stage. During this stage influence of BMP-2 and Bglap genes is crucial for odontogenesis and mineralization [21]. That is why 17th day of embryonic development was chosen as a day of the end of experiment in order to achieve the aim of this study. 6 pregnant mice from each group were exposed by carbon dioxide on the 17th day of pregnancy (E17). Mandibles of 17 embryonic days old mouse (E17) were excised to provide molecular-genetic investigation. All procedures with animals were in accordance with the «Rules and Regulations for Carrying Out Animal Research Work».

Mandibles of 17 embryonic days old mouse (E17) were used for phenol-chloroform extractions of RNA samples. Sigma-Aldrich (USA) reagents were used for these purposes. Concentration of RNA in samples was analyzed using the Thermo Scientific NanoDrop™

1000 Spectrophotometer (USA) and Thermo Scientific RevertAid First Strand cDNA Synthesis. 200-300 µg of total RNA were used for reverse transcription using First Strand cDNA Synthesis Kit (Fermentas, Lithuania) and (Oligo(dT))₁₈ Primer the oligo(dT)₁₈. PCR amplification of cDNA from RNA was performed.

BMP-2 and Bglap expression was measured and performed by real-time PCR. Next primers were used:

BMP-2 Up: 5'-GTGGAGGAACCTCCAGAGATGA-3',

BMP-2 Dw: 5'-CTGCAGATGTGAGAACTCGTC-3',

Osteocalcin Up: 5'-CAGGAGGGCAATAAGGTAGTGA-3',

Osteocalcin Dw: 5'-CAGGGTTAAGCTCACACTGCTC-3'.

For quantitative analysis, real-time PCR was conducted with a using SYBR Green PCR Master Mix (Thermo Fisher Scientific) – 20 µL with 25 pM of each primer. The thermal cycling conditions (with initial denaturation and AmpliTaq Gold® DNA polymerase activation) were as follows: 10 min at 95°C followed by 50 cycles of 95°C for 15 s, 60°C for 60 s (for osteocalcin 61°C).

In order to distinguish among PCR product melting curve analysis was used. Melting stage consisted of gradual temperature increase from 60 or 61°C to 94°C. By the temperature increase, DNA molecules dissociate and fluorescence of DNA-SYBR Green complex starts to reduce.

7500 Fast Real-time PCR Software by Applied Biosystems was used to perform the data of this investigation.

Statistical analyses. Data were analyzed with Origin 7.0 and Excel 2000. Shapiro-Wilk test was used for checking the data for normality. The study of difference was carried out by Mann-Whitney two-tailed test. Differences were considered significant at $P < 0.05$.

RESULTS

To determine the influence of hypercholesterolemia on BMP-2 and osteocalcin quantity we examined of mRNA expression of encoding genes of these proteins, as a key factor of odontogenesis.

Amplification products (actin, BMP-2 and osteocalcin) concentration relate to curve of amplification cycle number and curve of dissociation amplification products are shown in fig. 1.

The real time PCR analysis showed that in control group with standard chow the expression of BMP-2 and osteocalcin genes in the mandible of mouse embryos genes was at approximately the same level. In control group the relative level of BMP-2 mRNA / actin mRNA was 27.0 ± 2.82 (fig. 2), the relative level of and osteocalcin mRNA / actin mRNA was 30.5 ± 6.28 (fig. 3). However, in the jaws of animals in the experimental group with cholesterol enriched diet, the expression relative level of BMP-2 tended to increase and was 30.9 ± 5.81 ($p=0.53$;

$p>0,05$) that is by 14,4% higher than in control group (fig. 2). Therefore, the expression level of osteocalcin, on the contrary, decreased by 22.3% and was 23.7 ± 5.31 (fig. 3).

The analysis of the obtained data allows us to assume that the hypercholesterol diet to some extent changes the expression of BMP-2 and osteocalcin genes, and these changes are in different directions. According to the function of BMP-2 as a key factor in the differentiation of odontoblasts, it can be assumed that increasing its expression will accelerate odontogenesis. A decrease in the expression of the osteocalcin gene, which ensures mineralization in the tissues of the tooth germ, can lead to insufficient hydroxyapatite saturation of the hard tissues of the forming tooth, and, as a result, the eruption of teeth with a reduced level of mineralization.

DISCUSSION

There is a lot of data on the effect of hypercholesterolemia and of statins on the expression of BMP-2 and osteocalcin. The most indicative data were obtained indirectly through the study of the statins effect (known as hypercholesterolemic agents) on the processes of calcification and ossification. One of the epidemiological studies have shown that statins increase bone mineral density and reduce the risk of bone fractures [22]. Statins can directly stimulate BMP-2 expression and osteoblast differentiation, while inhibiting osteoclast activity and osteoblast apoptosis [23].

There are some studies that show that concentration of osteocalcin in the blood serum depends on the concentration of cholesterol. People with hypercholesterolemia and high blood sugar (metabolic syndrome) tend to have lower osteocalcin level [24]. Also, hypercholesterolemic drugs (rosuvastatin) increase serum osteocalcin levels, although this effect does not depend on lowering cholesterol levels [25].

Unfortunately, these data do not provide a direct answer how exactly hypercholesterolemia influence on tooth development, but lowering cholesterol levels under the influence of statins may suggest that elevated cholesterol levels may adversely affect BMP-2 expression and bone mineral density in general. D.L. Franklin [26] showed that odontoblasts in the early stages of development contain much less cholesterol than fully differentiated cells. Cholesterol content and distribution significantly affect the fluidity of the odontoblast cell membrane, and the low cholesterol content in low-differentiated odontoblasts provides budding of the matrix vesicles required for tooth formation. Earlier, similar data were obtained: the cholesterol content is much higher in permanent teeth compared to the teeth that are formed [27].

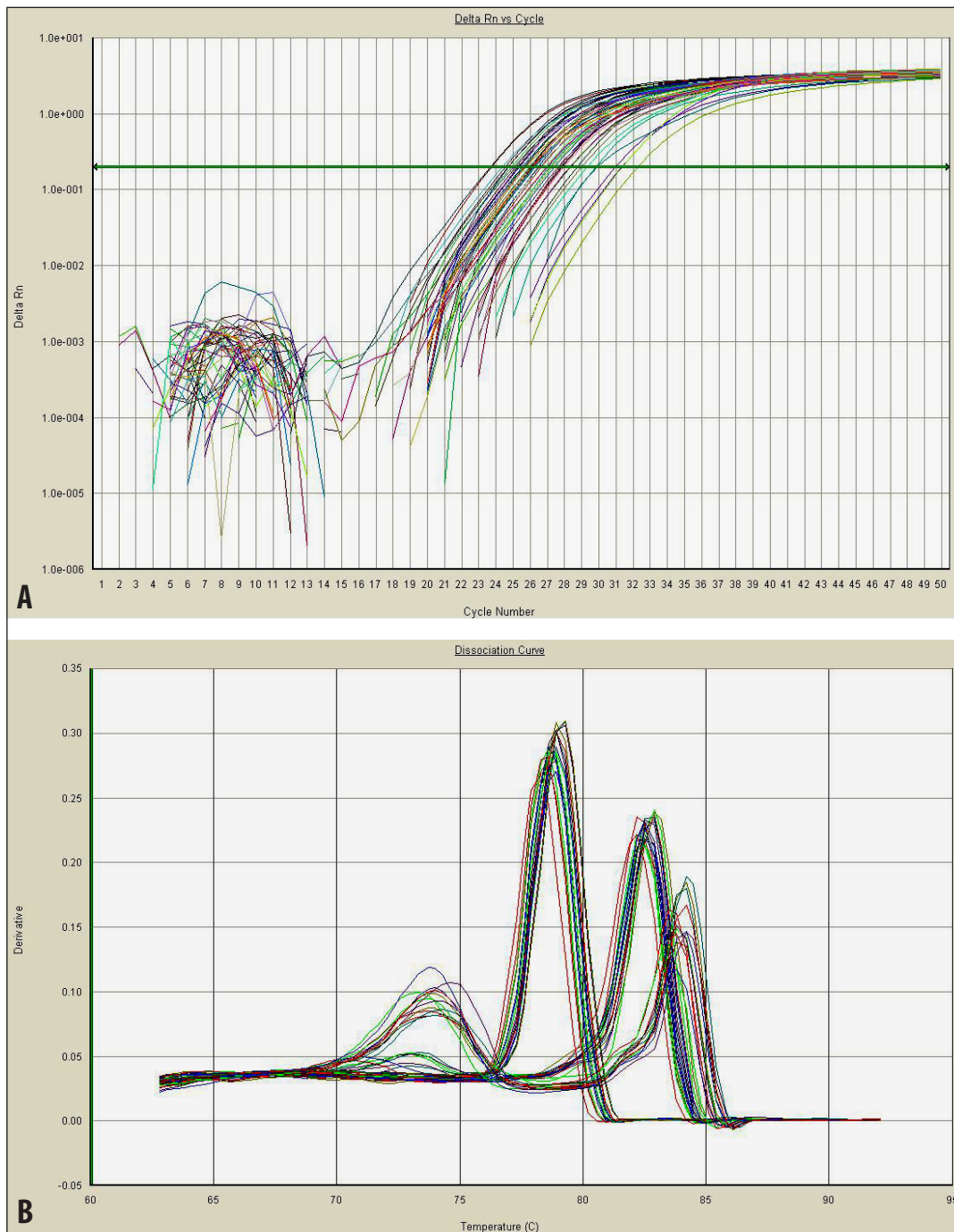


Fig. 1. A: Concentration of amplification products – actin, BMP-2 and osteocalcin correspondingly with curve of amplification cycle number; B: Concentration of amplification products – actin, BMP-2 and osteocalcin correspondingly with curve of dissociation amplification products.

Analysis of the obtained data suggests that the hypercholesterolemic diet alters, to some extent, the expression of BMP-2 and osteocalcin genes, and these changes are multidirectional. Based on the function of BMP-2, as a key factor in the differentiation of odontoblasts and function of osteocalcin as inducer of dentine and enamel mineralization, we can assume that increase of its expression will accelerate odontogenesis. In this case, the decrease in the expression of the osteocalcin gene, which provides mineralization in

tooth tissues, can lead to insufficient saturation with hydroxyapatite of hard tissues of the tooth, which is formed, resulting in the eruption of teeth with low mineralization.

The data from some studies showed that lower levels of osteocalcin are not sufficient to markedly influence dentinogenesis but enamel mineralization is more sensitive to osteocalcin presence. This difference could manifest because of different character of mineralization stages of this two tooth hard tissues – enamel crys-

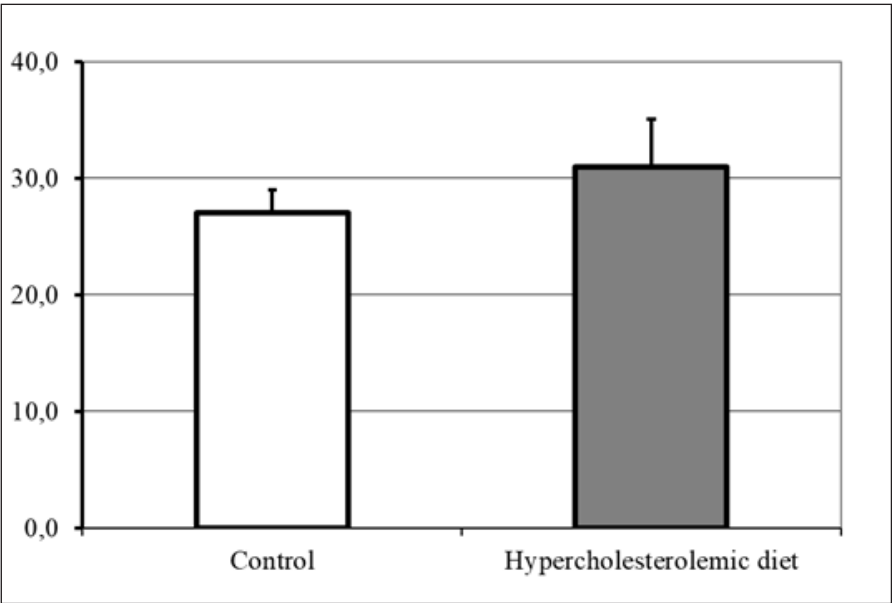


Fig. 2. mRNA expression of BMP-2 relative to actin in mandible of mouse embryos in control with standard chow and experimental cholesterol enriched diet groups. Research groups are represented horizontally, the relative level of BMP-2 mRNA / actin mRNA in are represented vertically

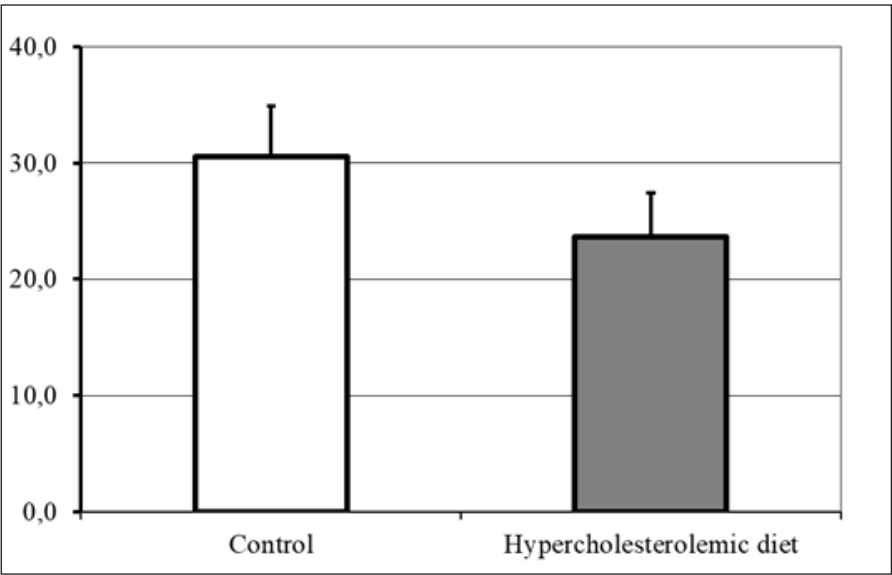


Fig. 3. mRNA expression of osteocalcin relative to actin in mandible of mouse embryos in control and experimental cholesterol enriched diet groups. Research groups are represented horizontally, the relative level of osteocalcin mRNA / actin mRNA in are represented vertically

tals in secretory stage of enamel formation are growing in a non-collagenous enamel matrix contained within a tightly controlled enamel compartment, formed by the mineralized dentin and the layer of secretory ameloblast [28].

In this study we have received evidence that in mandibles of mouse embryos from mice that had high-cholesterol diet for 30 days before fertilization and throughout pregnancy had tendency to increasing BMP-2 expression and decreasing osteocalcin gene expression. Decreased level of osteocalcin gene expression could leads to failure in tooth germ early mineralization.

Results of our study may suggest that maternal hypercholesterolemia has the specific influence on health of the oral cavity of future child through altering saturation of hydroxyapatite and normal process of

tooth mineralization during tooth formation. In turn mineralization defects could result in amelogenesis imperfecta, low level of enamel mineralization and early caries after tooth eruption.

CONCLUSIONS

Our study report influence of the cholesterol enriched diet (2%) on mRNA expression of BMP-2 and osteocalcin encoding genes. The embryos from mouse on cholesterol enriched diet (2%) had increased level of BMP-2 gene expression, however significantly decreased level of osteocalcin gene expression. The histopathological changes investigation in tooth germs in mandible of 17-days mouse embryos allows comparison of genetic and pathomorphological changes and helps to determine the functional significance of changes in expression of the studied genes.

REFERENCES

1. Tanaka K, Miyake Y, Sasaki S, Hirota Y. Dairy Products and Calcium Intake during Pregnancy and Dental Caries in Children. *Nutr. J.* 2012; 11: 33. doi:10.1186/1475-2891-11-33.
2. Savard C, Lemieux S, Weisnagel S et al. Trimester-Specific Dietary Intakes in a Sample of French-Canadian Pregnant Women in Comparison with National Nutritional Guidelines. *Nutrients.* 2018; 10: 768. doi:10.3390/nu10060768.
3. Dubois L, Diasparra M, Bédard B et al. Adequacy of Nutritional Intake from Food and Supplements in a Cohort of Pregnant Women in Québec, Canada: The 3D Cohort Study (Design, Develop, Discover). *Am. J. Clin. Nutr.* 2017;106: 541–548. doi:10.3945/ajcn.117.155499.
4. Blumfield ML, Hure AJ, Macdonald-Wicks L et al. Systematic Review and Meta-Analysis of Energy and Macronutrient Intakes during Pregnancy in Developed Countries. *Nutr. Rev.* 2012; 70: 322–336. doi:10.1111/j.1753-4887.2012.00481.x.
5. Yakubova II. Chapter 38. Ukraine. In *A Compendium on Oral Health of Children around the World: Early Childhood Caries.* New York, USA. 2018, 484p.
6. Adhikary S, Kothari P, Choudhary D et al. Glucocorticoid Aggravates Bone Micro-Architecture Deterioration and Skeletal Muscle Atrophy in Mice Fed on High-Fat Diet. *Steroids.* 2019; 149: 108416. doi:10.1016/j.steroids.2019.05.008.
7. Shen C-L, Han J, Wang S et al. Green Tea Supplementation Benefits Body Composition and Improves Bone Properties in Obese Female Rats Fed with High-Fat Diet and Caloric Restricted Diet. *Nutr. Res.* 2015; 35: 1095–1105. doi:10.1016/j.nutres.2015.09.014.
8. Diet and Health; National Academies Press: Washington, D.C. 1989. doi: 10.17226/1222.
9. Soliman G. Dietary Cholesterol and the Lack of Evidence in Cardiovascular Disease. *Nutrients.* 2018;10: 780. doi:10.3390/nu10060780.
10. Casagrande L, Demarco FF, Zhang Z et al. Dentin-Derived BMP-2 and Odontoblast Differentiation. *J. Dent. Res.* 2010; 89: 603–608. doi:10.1177/0022034510364487.
11. Linde A. Dentin Matrix Proteins: Composition and Possible Functions in Calcification. *Anat. Rec.* 1989; 224: 154–166. doi:10.1002/ar.1092240206.
12. Wise G. Cellular and Molecular Basis of Tooth Eruption. *Orthod. Craniofac. Res.* 2009; 12(2): 67–73. doi:10.1111/j.1601-6343.2009.01439.x.
13. Lesot H, Lisi S, Peterkova R et al. Epigenetic Signals during Odontoblast Differentiation. *Adv. Dent. Res.* 2001; 15: 8–13. doi:10.1177/08959374010150012001.
14. Popowicz T, Foster B.L, Swanson EC et al. Defining the Roots of Cementum Formation. *Cells Tissues Organs.* 2005; 181(3-4): 248–257. doi:10.1159/000091386.
15. About I, Mitsiadis TA. Molecular Aspects of Tooth Pathogenesis and Repair: In Vivo and in Vitro Models. *Adv. Dent. Res.* 2001; 15(1): 59–62. doi:10.1177/08959374010150011501.
16. Li H, Bartold PM, Zhang CZ et al. Growth Hormone and Insulin-Like Growth Factor I Induce Bone Morphogenetic Proteins 2 and 4: A Mediator Role in Bone and Tooth Formation? 1. *Endocrinology.* 1998; 139: 3855–3862. doi:10.1210/endo.139.9.6211.
17. Chen S, Gluhak-Heinrich J, Martinez M et al. Bone Morphogenetic Protein 2 Mediates Dentin Sialophosphoprotein Expression and Odontoblast Differentiation via NF- κ B Signaling*. *J. Biol. Chem.* 2008; 283: 19359–19370. doi:10.1074/jbc.M709492200.
18. Takano-Yamamoto T, Takemura T, Kitamura Y, Nomura S. Site-Specific Expression of MRNAs for Osteonectin, Osteocalcin, and Osteopontin Revealed by in Situ Hybridization in Rat Periodontal Ligament during Physiological Tooth Movement. *J. Histochem. Cytochem.* 1994; 42: 885–896. doi:10.1177/42.7.8014472.
19. Papagerakis P, Berdal A, Mesbah M et al. Investigation of Osteocalcin, Osteonectin, and Dentin Sialophosphoprotein in Developing Human Teeth. *Bone.* 2002; 30: 377–385. doi:10.1016/S8756-3282(01)00683-4.
20. Thesleff I. Tooth Organogenesis and Regeneration. *StemBook.* 2008. doi:10.3824/stembook.1.37.1.
21. 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. doi:10.1002/(SICI)1097-0177(199712)210:4<383::AID-AJA3>3.0.CO;2-C.
22. Mundy G. Stimulation of Bone Formation in Vitro and in Rodents by Statins. *Science (80-).* 1999; 286: 1946–1949. doi:10.1126/science.286.5446.1946.
23. Edwards CJ, Spector TD. Statins as Modulators of Bone Formation. *Arthritis Res.* 2002; 4: 151–153. doi:10.1186/ar399.
24. Zhou M, Ma X, Li H et al. Serum Osteocalcin Concentrations in Relation to Glucose and Lipid Metabolism in Chinese Individuals. *Eur. J. Endocrinol.* 2009; 161(5): 723–729. doi:10.1530/EJE-09-0585.
25. Kanazawa I, Yamaguchi T, Yamauchi M, Sugimoto T. Rosuvastatin Increased Serum Osteocalcin Levels Independent of Its Serum Cholesterol-Lowering Effect in Patients with Type 2 Diabetes and Hypercholesterolemia. *Intern. Med.* 2009; 48: 1869–1873. doi:10.2169/internalmedicine.48.2645.
26. Franklin DL, Arana-Chavez VE, Katchburian E. Cholesterol in the Distal Portions of Differentiating and Fully Differentiated Rat Odontoblasts Observed by Freeze-Fracture. *Arch. Oral Biol.* 1994; 39: 817–819. doi:10.1016/0003-9969(94)90011-6.
27. Antonucci A, Toto N, Di Valerio V, D'Onofrio P. Dental Pulp Lipids from *Bos Taurus* during Odontogenesis. *Arch. Oral Biol.* 1991; 36: 919–922. doi:10.1016/0003-9969(91)90124-D.
28. Bronckers ALJJ, Price PA, Schrijvers A et al. Studies of Osteocalcin Function in Dentin Formation in Rodent Teeth. *Eur. J. Oral Sci.* 1998; 106: 795–807. doi:10.1046/j.0909-8836.1998.eos106306.x.

ORCID and contributionship:

Inessa I. Yakubova: 0000-0003-2780-2460^{B-D}

Volodymyr Ostriancko: 0000-0001-6525-7526^{B,C,E}

Victor Dosenko: 0000-0002-6919-7724^{A,F}

Liliia Bielova: 0000-0001-8546-0222^E

Yurii Skrypnyk: 0000-0003-1196-8204^C

Ganna Viun: 0000-0002-0473-4031^{C,D,F}

Conflict of interest:

The Authors declare no conflict of interest.

CORRESPONDING AUTHOR

Inessa I. Yakubova

Private Higher Educational Establishment

«Kyiv Medical University»

2 Boryspilska St., 02099 Kyiv, Ukraine

tel: +380677132097

e-mail: yakubova.inessa@gmail.com

Received: 29.06.2022

Accepted: 07.06.2023

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

 Article published on-line and available in open access are published under Creative Common Attribution-Non Commercial-No Derivatives 4.0 International (CC BY-NC-ND 4.0)