ORIGINAL ARTICLE

CONTENTS

Influence of maternal cholesterol-enriched diet on chemical composition of teeth enamel in offspring of mice

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ABSTRACT

Aim: To determine the chemical composition of the tooth enamel of two-day-old mice from hypercholesterolemic mothers by energy dispersive X-ray spectroscopy.

Materials and Methods: Forty mature female mice were randomly assigned (n = 20/group) to either a standard chow vivarium diet (control group) or a cholesterol-enriched chow diet (experimental group). After fertilization, pregnancy and birth, on postnatal day 2, the incisor segments of 6 pups form each group were used for energy dispersive X-ray spectroscopy.

Results: Influence of maternal hypercholesterolemic diet on tooth development and mineralization was examined, which revealed changes in enamel chemical composition. First, the results indicate the presence of seven elements (Na, Cl, Ca, P, Mg, S, Fe) in the enamel of both the hypercholesterolemic and normal offspring, but the content of element Ca2+ decreased, the content of elements P5+, Na+, Cl- tended to increase in pups from hypercholesterolemic mice. Second, the initial level of mineralization according to the atomic (%) Ca / P in hypercholesterolemic pups ratio was 1.26, comparing with normal pups where level of mineralization was 1.34. Taking into account that irreversible changes in the structure of the enamel were observed when the Ca / P ratio was below 1.33, we can suggest that the eruption of teeth with an imperfect structure could be because of maternal hypercholesterolemic diet.

Conclusions: Results of this study suggest that hypercholesterolemic diet during gestation and lactation leads to altered enamel mineralization in mice because of changes in chemical composition and may link to the early childhood caries.

KEY WORDS: the tooth enamel mice, cholesterol-enriched diet, chemical composition of teeth enamel

Wiad Lek. 2024;77(2):299-304. doi: 10.36740/WLek202402117 DOI

INTRODUCTION

Nutrition during pregnancy and lactation is perhaps the most influential but often overlooked non-genetic factor in fetal development [1, 2]. A maternal diet containing adequate quantities of micro- (vitamins, macro- and microelements) and macronutrients (proteins, fats, carbohydrates) is a key point for maintaining the health of a pregnant woman and plays an important role in the growth and development of the fetal oral cavity and the teeth [3, 4]. A low-protein diet during gestation-lactation results in impaired odontogenesis that may increase susceptibility of dental anomalies [5]. Studies have suggested that enamel hypoplasia, salivary gland hypofunction and saliva compositional changes may be the mechanisms through which the malnutrition is associated with caries, while an altered eruption timing may create a challenge in the analysis of the age specific caries rates [6]. Not only a deficiency, but also an excess of nutrients can negatively affect the course of pregnancy and fetal organism, as well as the fetal teeth condition in the pre-eruption phase, as it can affect the cellular architecture of the organic matrix and enamel maturation processes [7, 8]. Maternal high-fat diet together with embryonic *Cited2* deficiency significantly reduces the expression of the left-determining gene *Pitx2*, with a dramatic increase in the penetrance of left–right patterning defects, and the appearance of novel defects including cleft palate [9]. Cholesterol intake increases during pregnancy because pregnant women are advised to consume more eggs and other foods rich in cholesterol [10]. However, higher dietary cholesterol from eggs intake during pregnancy was associated with greater risk of gestational diabetes mellitus [11]. The result of a hypercholesterolemic diet, on the one hand, is an increase in the expression of BMP2, which accelerates odontogenesis, on the other hand, a decrease in the expression of the osteocalcin gene leads to insufficient saturation of the tooth hard tissues with hydroxyapatite, which results in the eruption of teeth with low mineralization [12].

AIM

The aim of the study was to determine the chemical composition of the tooth enamel of two-day-old mice from hypercholesterolemic mothers by energy dispersive X-ray spectroscopy. We hypothesized that hypercholesterolemia during gestation and lactation would result in altered mineralization of the enamel.

MATERIALS AND METHODS

ANIMALS AND DIET

Forty mature female mice were purchased and housed in the vivarium of the Bogomolets Institute of Physiology National Academy of Science of Ukraine (Kyiv, Ukraine) in stainless steel cages under controlled conditions (20–24 °C, 50–60% relative humidity, 12-h light–dark cycle) with free access to water. Mice were randomly assigned (n = 20/group) to either a standard chow vivarium diet (24% protein, 11% fat, 48% carbohydrates, 5.5% fiber, 6% vitamin, 5.5% ash) (control group) or a cholesterol-enriched chow diet (a standard chow vivarium diet with the addition of 0,2% Cholesterol (manufactured by Merck, Germany) (experimental group). Female mice were given ad libitum access to their respective diet throughout the study duration. Females in proestrus or estrus phase were mated overnight with male breeders (4 females with 1 male) and separated the following morning. The presence of spermatozoa in the vaginal smear was considered as an indicator of fertilization and first day of pregnancy (gestational *day (GD) 0)*. Pregnant females had spontaneous birth, and day of birth was recorded as day 0 of the mouse's life. Throughout the suckling period the dams remained on their respective diets.

TISSUE COLLECTION

At wearing (postnatal day 2) 6 pups form each group (appearance features were nubs in ear area, visible milk spot and manifesting pigment on skin) were by inhalation overdose of carbon dioxide. CO2 exposure time is recommended 60 minutes for euthanasia of two day old mice (D-2) [13]. The lower jaws were removed and all soft tissue carefully cleaned by dissection. All samples were stored in test tubes (10% streptomycin solution), tightly closed, at a temperature of +2...+4°C. Before the study, the samples were washed with hands wearing rubber gloves in distilled water and passively dried. The incisor segments were used for scanning electron microscopy–Energy dispersive X-ray analysis. The mice used in this experiment were cared for in accordance with the guidelines established by the «Rules and Regulations for Carrying Out Animal Research Work» [14]. All procedures were reviewed and approved by the Private Higher Educational Institution «Kyiv Medical University».

SCANNING ELECTRON MICROSCOPY–ENERGY DISPERSIVE X-RAY ANALYSIS

When jaws were passively dried, the samples were placed in a vacuum apparatus (Ion Sputter JFC-1600, Jeol, Japan) for 5 min until the residual moisture completely evaporated. The samples were affixed to the scanning electron microscopy (SEM) stubs and sputter-coated with a thin layer of Pt (~25 nm), in order to make the material conductive (Fig. 1). examined with a JSM-6100 JEOL SEM operating at 15 kV and 15–20 mm working distance.

SEM analysis was performed by JSM-6100 JEOL SEM under the standard high vacuum mode, operating at 5–10 kV throughout the analysis and 15–20 mm working distance. Measurements took place on 19 areas of enamel in the control group and on 21 areas in the experimental group. The morphology of each sample was imaged with a variety of magnifications. The size of the areas of enamel spectroscopy ranged from 50x50 µm to 250x250 µm.

The chemical composition of the enamel was obtained using an energy dispersive X-ray spectral analyzer INCA Energy 450 (Oxford Instruments Nanoanalysis, UK). EDX analysis was performed at 10 kV and a 10 mm working distance. The count was conducted on the vestibular surface of incisors (19 areas of enamel in the control group and on 21 areas in the experimental group).The elements quantified were Oxygen (O), Natrium (Na), Chlorine (Cl), Calcium (Ca), Phosphorus (P), Magnesium (Mg), Sulfur (S), Ferrum (Fe). All analyzed elements were normalized to the factory calibration provided by Oxford Instruments Aztec/INCA software and an Oxford Instruments X-Max 50 mm^2 detector system. C, O, F, Na, Mg, P, and Ca were calibrated based on $CaCO_3$, SiO_2, MgF_2, albite, MgO, GaP and wollastonite. The element content was calculated as the relative weight percentage of the total element content (100%).

STATISTICAL ANALYSIS

Statistical analysis of the digital data was performed using Excel 2000 and Origin 7.0. Results were expressed mainly as means +/– standard error of the mean. Probability distribution of mean ($P<0.05$) was calculated using Student's t-test.

Table 1. Offspring characteristics of experimental and control groups

Groups	Gestation length, days ± SD	Average litter size, ± SD	Male/female (ratio)	Birth weight ± SE, g	Body length at birth ± SE, mm	Survival rate at 2 PNDs
Experimental, n=20	18.9 ± 0.6*	7.21 ± 1.1*, n=144	74/70 (1.06)*	1.22 ± 0.01*	25.00 ± 0.16*	95 %*, n=137
Control, n=20	19.0 ± 0.3	7.14 ± 2.7, n=142	75/67 (1.12)	1.28 ± 0.01	24.93 ± 0.13	97 %, n=139

1. SD standard deviation
2. PND – postnatal days
3. *p ≥ 0.05.

Table 2. Chemical (elemental) composition of D-2 mouse teeth enamel by the EDX method of the control group

	Chemical element	The number of examined samples	Number of samples containing a chemical element		Qualitative composition of samples, %
			abs	%	
Atomic	O	19	19	100	61.65
	Na	19	19	100	1.15
	Cl	19	19	100	0.31
	Ca	19	19	100	21.82
	P	19	19	100	14.35
	Ca/P				**1.34**
	Mg	19	1	5,26	0.89
	S	19	1	5,26	0.36
	Fe	19	9	47,37	0.45
Weight	O	19	19	100	41.82
	Na	19	19	100	1.13
	Cl	19	19	100	0.47
	Ca	19	19	100	36.55
	P	19	19	100	19.28
	Mg	19	1	5,26	0.92
	S	19	1	5,26	0.5
	Fe	19	9	47,37	1.06

RESULTS

OFFSPRING PHENOTYPE

Each dam was between 6–9 pups with an average size of 7.2 ± 1.2 in experimental group and 7.1 ± 2.7 in control group (means ± standard deviations). There were no other significant differences in the following parameters between the pups of experimental and control groups: gestation length, survival rate, average litter size, male/female ratio, birth weight, and birth body length (Table 1).

SEM-EDX ANALYSIS

Fig. 1. shows the photo of enamel surface on the vestibular area of incisor of a two-day-old mouse, that was made by scanning electron microscopy.

Characteristic X-ray spectra were obtained on the vestibular area of incisor of a two-day-old mouse with the help of an energy dispersive X-ray spectrum analyzer (Fig. 2).

Different distribution of the trace elements concentration in tooth enamel was revealed using energy-dispersive spectral X-ray diffraction analysis. In the enamel of the experimental and the control groups were found seven elements – Na, Cl, Ca, P, Mg, S, Fe. It was found that the chemical elements Ca2+, P5+, Na+, Cl- were found in 100% of the samples in the control group when studying the chemical composition of the D-2 mouse teeth enamel using the EDX method (Table 2).

Elements Mg2+, S were found in 5.26% of the samples, Fe3+ – in 47.37%. The Ca2+ content was 36.55%, the P5+ content was 19.28%. The initial level of mineralization according to the atomic (%) Ca / P ratio was 1.34. It was found that the chemical elements Ca2+, P5+, Na+, Cl- were found in 100% of the samples in experimental group, which received a diet with an increased content of Cholesterol (Table 3).

DISCUSSION

In this study, influence of maternal hypercholesterolemic diet on tooth development and mineralization was

Fig. 1. A section of the tooth enamel (vestibular surface of incisor) of a two-day-old mouse (D-2), obtained by SEM where the chemical (elemental) composition was measured.

Table 3. Chemical (elemental) composition of D-2 mouse teeth enamel by the EDX method of the experimental group

	Chemical element	The number of examined samples	Number of samples containing a chemical element		Qualitative composition of samples, %
			abs.	%	
Atomic	O	21	21	100	61.64
	Na	21	21	100	1.23
	Cl	21	21	100	0.33
	Ca	21	21	100	20.29
	P	21	21	100	16.08
	Ca/P				**1.26**
	Mg	21	3	14,29	0.92
	S	21	3	14,29	0.36
	Fe	21	11	52,38	0.47
Weight	O	21	21	100	41.86
	Na	21	21	100	1.20
	Cl	21	21	100	0.50
	Ca	21	21	100	34.51
	P	21	21	100	21.14
	Mg	21	3	14,29	0.92
	S	21	3	14,29	0.50
	Fe	21	11	52,38	1.12

examined, which revealed changes in enamel chemical composition. We found out few findings that warrant further discussion. First, the results of this study indicate the presence of seven elements (Na, Cl, Ca, P, Mg, S, Fe) in the enamel of both the hypercholesterolemic and normal offspring, but the content of element Ca2+ decreased, the content of elements P5+, Na+, Cl- tended to increase in pups from hypercholesterolemic mice. Second, the initial level of mineralization according to the atomic (%) Ca / P in hypercholesterolemic pups ratio was 1.26, comparing with normal pups where level of mineralization was 1.34 (at the lower limit (1.33), that suggests that the enamel of the teeth of mice that have just erupted is immature). Taking into account that irreversible changes in the structure of the enamel were observed when the Ca / P ratio was below 1.33 [5], we can suggest that the eruption of teeth with an imperfect structure could be because of maternal hypercholesterolemic diet during gestation and lactation.

Although previous work suggests that maternal high-fat diet during pregnancy and lactation can alter fetal cholesterol metabolism and predispose adult offspring to metabolic abnormalities [15, 16] , no investigation has assessed the relationship between maternal hyper-

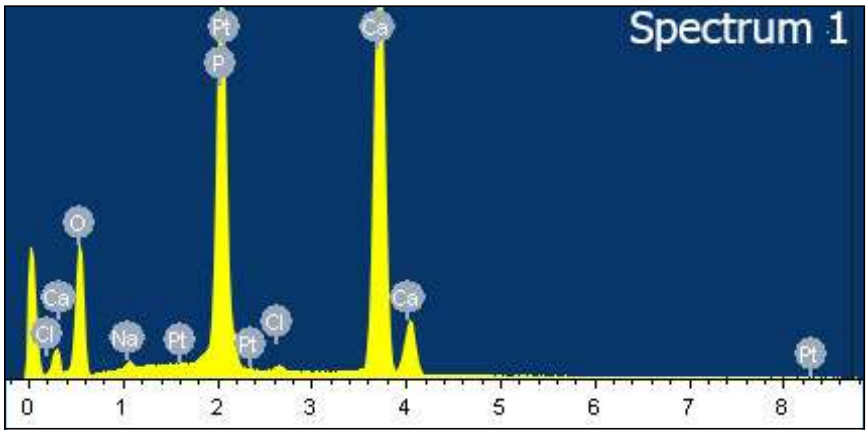

Fig. 2. X-ray characteristic spectrum of the tooth enamel surface (vestibular surface of incisor) of a two-day-old mouse (D-2 mouse) obtained using an energy dispersive X-ray spectrum analyzer.

cholesterolemia and chemical composition of tooth enamel. In our study we have found that a hypercholesterolemic diet during pregnancy and lactation is associated with enamel maturation violation.

The study of the chemical composition of the surface layers of enamel is relevant due to the fact that the variability of the elemental composition of teeth allows to detect metabolic disorders in the process of tooth development and after eruption [17]. The large amounts of free fatty acids, diglycerides, cholesterol and phospholipids as intrinsic components can influence on enamel maturation [18]. A limited number of studies reported significant associations between molar incisor hypomineralization and pre- and perinatal factors such as maternal illness and medication use in pregnancy [19] There are data in the literature that the intensity of caries of temporary teeth in children is affected by the amount of sweet food consumed by a pregnant woman [20]. Evidence suggests that cholesterol intake increases during pregnancy, so maternal hypercholesterolemia could be one of the factor that leads to hypomineralization of the enamel and in its turn manifested in early caries [21].

CONCLUSIONS

In summary, results of this study suggest that hypercholesterolemic diet during gestation and lactation leads to altered enamel mineralization in mice because of changes in chemical composition. Based on the rise of cholesterol intake during pregnancy and the early childhood caries because of the eruption of teeth with an imperfect structure mechanism of maternal hypercholesterolemia influence on enamel maturation needs further investigation.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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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

RECEIVED: 15.09.2023
ACCEPTED: 28.12.2023