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Morphometric and functional abnormalities of kidneys in the progeny of mice fed chocolate during pregnancy and lactation

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Abstract: Even most commonly consumed beverages like tea, coffee, chocolate and cocoa contain methylxanthines, biogenic amines and polyphenols, among them catechins, that exhibit significant biological activity and might profoundly affect the organism homeostasis. We have previously shown that 400 mg of bitter chocolate or 6 mg of theobromine added to the daily diet of pregnant and afterwards lactating mice affected embryonic angiogenesis and caused bone mineralization disturbances as well as limb shortening in 4-weeks old offspring. The aim of the present study was the morphometric and functional evaluation of kidneys in the 4-weeks old progeny mice fed according to the protocol mentioned above. Progeny from the mice fed chocolate presented considerable morphometric abnormalities in the kidney structure, with the lower number of glomeruli per mm^2 and their increased diameter. Moreover, higher serum creatinine concentration was observed in that group of offspring. No morphometric or functional irregularities were found in the progeny of mice fed theobromine. Abnormalities demonstrated in the offspring of mice fed chocolate are not related to its theobromine content. Consequently, identification of active compound(s) responsible for the observed effects is of vital importance.

Key words: Pregnancy - Progeny - Chocolate - Kidney - Mouse

Introduction

The everyday diet contains abundance of plant-derived compounds that exhibit significant biological activity and might profoundly affect the organism homeostasis. Even most commonly consumed beverages like tea, coffee, chocolate and cocoa are rich in polyphenols, methylxanthines, and biogenic amines [8]. All of them present various functional activities and have been shown to influence a number of physiological and pathological processes in human/animal tissues and organs [4]. Our previous studies have proven that some of these commonly consumed nutritional ingredients might influence the embryonic development when administered on the regular basis during pregnancy and lactation as well as affect both cellular and humoral immune response of mice progeny [5, 20, 21, 22]. Theobromine feeding resulted in a significant inhibition of mice embryo growth (as assessed by their weight) and decreased angiogenic activity [5, 16]. Similarly, chocolate, one of the most popularly consumed products rich in methylxanthines (theobromine) and catechins, caused the reduction of limb and thigh bone relative length paralleled by the suppression of angiogenic factor (*i.e.* VEGF) production [20, 21, 22].

It has been reported that vascular endothelial growth factor plays the key role in mouse kidney development both *in utero* and during the postnatal period [11, 12]. Therefore, its abnormal production during the prenatal

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development, as observed in the progeny of chocolate-fed mothers, might result in disturbed kidney maturation [22]. Both methylxanthines and catechins, the key biologically active chocolate ingredients are known for their significant effect on the kidney tissue. Catechin-induced suppression of glomerular cell proliferation, and stimulation of proximal tubular cell apoptosis have been reported [15, 27]. On the other hand, the role of methylxanthines in the regulation of glomerular filtration as well as their protective effects on the kidney tissue and function have been repeatedly described [6, 10, 19, 23].

Therefore, the aim of the present study was to preliminarily evaluate whether regular administration of chocolate or one of its main compounds, theobromine, to pregnant and lactating mice might influence kidney development and function in their progeny.

Materials and methods

Assessment of theobromine by high performance liquid chromatography (HPLC). HPLC was applied to estimate the theobromine content in chocolate and laboratory chow, according to the method described previously [5, 22]. Samples were diluted with 0.1 M NaOH, shaken on ultrasonic bath, passed through 0.45 μm filter and injected onto HPLC system consisting of the LC-ABZ analytical column (250×4.6 mm I.D., 5 μm, Supelco) with the mobile phase (water-methanol-acetonitryl-acetic acid) delivered at the rate of 1 ml/min,UV-Vis detector SPD-6 AV at 273 nm (Shimadzu, Germany) and a Rheodyne Model 7125 injection valve (Berkeley, USA) equipped with 20 μl loop. Quantitative results (8.8 mg/g in chocolate) were obtained by comparing samples and standard (theobromine, Sigma Aldrich, Germany) peak areas. Some amounts of theobromine were present also in the animals' everyday fare (granulated chow 22.5 μg/g, corn flakes 205 μg/g).

Experimental protocol. Male and female 8-weeks old inbred Balb/c mice, about 20 g of body mass, derived from our breeding colony (breeding material from Oncology Center, Warsaw, Poland) were matched among themselves. Female mice during pregnancy and lactation period (from the first day of fertlization until the fourteen day after delivery) received 6mg/day of theobromine (Sigma Aldrich, Poznan, Poland),"Theo-group", or 400 mg/day of bitter chocolate (Wedel, Poland) "Choc-group", served with wheat flakes, in addition to standard laboratory chow. Control mice, "C-group", were fed wheat flakes and standard laboratory chow only. Everyday consumption of the laboratory chow was monitored and comparable in all experimental groups (5-7 g/day) despite the addition of supplements (chocolate, theobromine).

The chocolate dose of 400 mg/day/mice is comparable to the daily consumption of 200 g by human, applying the counter 7 for differences between mouse and human in relation of the surface to body mass.

Four weeks after birth, progeny of 6 C-mothers: 12 females (F) and 12 males (M), 5 Theo-fed mice (9 F and 9 M) and 5 Choc-fed animals (9 F and 9 M) were sacrificed. In brief, mice were weighed, anesthetized with 3.6% chloral hydrate and bled from the retro-orbital plexus. Sera from all animals were isolated and stored at -80°C for further creatinine evaluation. Kidneys were excised from 6 animals of C-group, 17 animals of Choc-group and 10 animals from Theo- group; weighed, fixed in 4% buffered formalin and processed for light microscopy. Relative weight of each kidney was calculated as a ratio: the weight of organ (mg) divided by body mass (g).

Animals were handled according to the Polish law on the protection of animals and NIH standards. All experiments were accepted and supervised by the local Ethical Committee.

Morphometric evaluation of kidneys. The histological evaluation and quantitative analyses were performed on the hematoxylin and eosin-stained paraffin sections of the kidneys obtained from the progeny. The light microscopic examination (microscope Olympus BX50, Japan) was based on the standard morphological criteria. For the quantitative measurements, image analysis system "analySIS 3.0" (Soft Imaging System GmbH, Munster, Germany) was applied. The whole cut surface of the frontal section of each kidney was analyzed in respect to: (a) the total cortical area and total number of glomeruli, with the results expressed as the number of glomeruli/mm^2 and (b) the glomerular diameter measured in 21 consecutive glomeruli of each sample.

Histotechnical criteria applied for the quantitative analysis of kidney sections were as follows: (a) thickness 3-5 μm, (b) the complete frontal section of the kidney including cortex, medulla and renal pelvis structures and (c) no evidence of traumatic artifacts within sample (e.g.: fragmentation, hemorrhages). The results were presented as a mean ± standard error.

Creatinine measurements. Serum creatinine was measured using the standard Jaffe technique on a Hitachi 747 analyzer (Boehringer Mannheim, Mannheim Germany).

Statistical analysis. The statistical significance of differences between the studied groups and control animals was tested using two-tailed Student's *t*-test.

Results

Morphometric evaluation

No macroscopic abnormalities were noticed in the anatomy of kidneys in both experimental and control groups. Similarly, no differences in kidney's relative weight were observed between the groups.

Kidneys from all groups showed histologically normal, well developed glomeruli and nephron structures. However, in the Choc-group, the glomeruli were less frequent and unevenly distributed in the renal cortex in comparison to the control animals. The morphometric analysis (Table 1) confirmed significant decrease in the quantity of the renal corpuscles/mm^2 in the progeny from chocolate-fed mothers, in comparison to the C- and Theo-groups. The decreased number of glomeruli in Choc-mice was compensated by the glomerular hypertrophy manifested as significantly larger glomerular diameter in comparison to that found in C- and Theo groups.

Creatinine measurements

Kidney function was monitored by creatinine serum concentration which was significantly higher in the progeny of Choc-group than in two other groups (Table 2).

Discussion

Chocolate contains a number of biologically active compounds, however, catechins and methylxanthines are considered the most essential for its overall effect as observed both *in vitro* and *in vivo*.

Table 1. Morphometric analysis of renal glomeruli in control and experimental animals

Groups	Number of analyzed images	Number of glomeruli/mm^2	Statistical significance of differences from the control	Number of analyzed glomeruli	Mean diameter of glomerulus (μm)	Statistical significance of differences from the control
Control	83	8.16 ± 0.51	-	210	48.4 ± 0.4	-
Theo	220	9.39 ± 0.81	n.s.	336	48 ± 0.22	n.s.
Choc	217	6.48 ± 0.23	p<0.01	210	54.2 ± 0.67	p<0.01
Significance of differences between experimental groups		p<0.01			p<0.01	

Table 2. Serum creatinine concentration in control and experimental animals.

Groups	Number of tested sera	Serum creatinine concentration (mg%)	Significance of statistical difference from the control
Control	24	0.42 ± 0.03	-
Theo	18	0.41 ± 0.08	n.s.
Choc	18	0.61 ± 0.03	p<0.01
Significance of differences between experimental groups		p<0.05	

There are conflicting reports whether and to what extent chocolate and its active ingredients might affect the outcome of pregnancy. Potential teratogenicity of theobromine in animals has been observed in several studies in animals, especially for higher drug doses [7, 13, 17, 31], while other authors reported no significant fetotoxicity [24]. On the other hand, the literature concerning catechins in that aspect is non-existing, though their toxicity at high concentrations has been demonstrated [15, 18, 27].

Our previous studies have proven that chocolate administered to pregnant dams in dietary relevant amounts (comparable to 200 g/day) caused evident suppression of the angiogenic activity in embryonic tissue with no apparent fetal malformations, though bone mineralization disturbances were reported [21]. Conversely, in the 4 weeks-old pups fed by lactating mothers from the chocolate- or theobromine-treated groups, a significant shortening of the relative limb length in the offspring, both of the forefeet and hind legs, was observed [5, 22]. It was suggested that the growth retardation was most likely due to the anti-angiogenic effect exerted via VEGF inhibition [9]. Adenosine receptors blockade exerted by theobromine might have also been relevant [3].

In contrast, no differences in relative weights of kidney, spleen, heart and liver between control and experimental progeny were observed in the citied studies. However, since certain abnormalities in organogenesis (osteogenesis, aspermatogenesis, testicular atrophy) as well as higher incidence of pelvic dilatation and renal pelvic microcalculi have been reported by other authors, the morphological evaluation of tissues obtained from experimental groups appeared to be essential [24, 25, 26].

Although macroscopically kidneys did not reveal any abnormalities, there were alterations in glomeruli number and size in the progeny of chocolate-fed but not theobromine-fed group. It might be assumed that VEGF production disturbances, as observed in our previous studies, played essential role in the abnormal kidney tissue structure formation, since this particular cytokine had been proven to mediate glomerulo- and nephrogenesis, both during pre- as well as postnatal periods [11, 12, 22]. In contrast, increased glomerular diameter should be interpreted as a secondary compensative hypertrophy to impaired glomerulogenesis resulting from chocolate-rich experimental diet. Significantly higher serum creatinine concentration in this group of animals, as compared to C and Theo groups further proved that glomerular hypertrophy was functional, albeit not fully adequate in the examined animals.

Since in our previous studies an inhibitory effect of chocolate on the embryo growth and angiogenic activity has been paralleled by similar consequences of theobromine administration to pregnant mice, we assumed that the observed abnormalities in kidney structure might be due to its biological effect on the kidney development. However, in Theo progeny group, no other alterations in glomeruli number or size of were demonstrated. Also, serum creatinine levels were comparable to controls. Therefore, it should be assumed that catechins rather than theobromine might be responsible for the observed effects.

In our previous study, content of three main cocoa catechins in bitter chocolate was estimated by HPLC method [22]. It amounted to 0.22 mg of epigallocatechin, 0.13 mg of catechin and 0.53 mg of epicatechin per 1 g of chocolate used throughout the study.

Both, theobromine and catechins are known for their biological activity towards kidney cells. However, methylxanthines are acknowledged mostly for their pro-

tective effects [19, 23], while catechin toxicity against kidney epithelium has been firmly established [15, 27]. It was shown that epigallocatechin-3-O-gallate, epicatechin-3-O-gallate and gallic acid inhibit the growth of kidney tissue - proximal tubular cells via suppression of their proliferation, induction of apoptosis and some other unidentified mechanisms. Similar effects of catechins towards other cell types have been also described [16, 29, 30]. Importantly, Vance *et al*. have proven that pathophysiological effect of these substances on the kidney cells depended also on the persistence of exposure [27]. The longer tissues were in contact with catechins, the stronger suppressive effect was demonstrated.

In our study, both theobromine and chocolate have been delivered to fetuses/pups for several weeks except that the way of administration was indirect in order to imitate the real-life situation where active ingredients of chocolate and their metabolites are delivered to the newborns and infants via breast milk. The pharmacokinetics of theobromine guarantees the high breast milk/serum concentration ratio (0.829 ± 0.038) providing the effective mechanism of the theobromine delivery to the suckling infants [14]. The detailed data concerning bioavalibility of catechins are scarce, yet certain indirect facts encourage confidence that their bioactivity in breast milk as well as passage through placenta is effective enough to exert functional effects on fetal/neonatal development [1, 2, 28]. In our previous studies, substantial amounts of epigallocatechin in embryos derived from chocolate fed mothers were demonstrated by the HPLC method [21]. Furthermore, significant negative correlation was found between epigallocatechin content in embryonic tissues and their angiogenic activity.

Comprehensive information concerning the effect of active chocolate ingredients methylxanthines (theobromine) and catechins on the prenatal and postnatal development seems to be extremely scarce. Some studies, however, have shown that their dietary uptake considerably affects embryo development as well as suckling maturation. The same conclusion comes from our experiments proving significant effect of bioactive chocolate components, presumably catechins, on renal development and subsequent function. Consequently, particular attention should be paid to the consumption of catechin-rich food during pregnancy and lactation. Moreover, further detailed analysis of the observed effect as well as identification of active chocolate compound(s) is of vital importance.

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