Effects of between generations changes in nutrition type on vaginal smear and serum lipids in Sprague–Dawley rats

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Abstract

Objective: Previous studies established that between generations changes in feeding protocol can have significant impact on reproductive physiology. The aim of the study was to determine effects of mothers’ nutrition and nutrition of the offspring on the characteristics of vaginal smear and serum lipid content.

Methods: Ten female rats were randomly divided into two groups; first group fed with food containing high content of saturated fatty acids (HFD) and the second with standard laboratory chow (CD). After coupling and lactation period their offspring were further randomly divided into two subgroups fed HFD or CD forming four study groups: (a) CD–CD, (b) CD–HFD, (c) HFD–CD and (d) HFD–HFD. The dams and offspring at the age of 37 and 18 weeks, respectively, were subjected to biochemical analysis of the blood and cytological analysis of the vaginal smears. Additionally body weight was recorded and body mass index (BMI) was calculated.

Results: The HFD–HFD group presented with highest levels of triglycerides and the CD–HFD with the highest levels of cholesterol. Therefore, triglyceride and cholesterol levels were significantly different among the groups (p = 0.001 and p = 0.002, respectively). Vaginal cytological smears analysis showed features of irregular phase interchanges or extended estrous phase in offspring of high-fat fed dams.

Conclusion: Maternal HFD consumption predisposes offspring to increased risk of developing metabolic abnormalities and estrous disorders.

Introduction

Western societies have been encountering obesity epidemic for decades, with incidence of obesity and overweight more than doubled since 1980 [1]. This trend is mirrored in developing nations today and has become a leading problem in the developing world. According to study by Burdge et al., offspring are extremely sensitive to nutritional, hormonal and environmental changes during the gestation and lactation periods, which may result in altered physiology of several systems throughout the life. This phenomenon is named “programming”, and its origins are related to epigenetic modifications which is usually explained as posttranslational modification of the nucleic acids of offspring without changing the genetic sequence [6]. Adult outcomes programmed by infant nutrition include lipid metabolism, blood pressure, obesity, diabetes, arteriosclerosis, behavior and longevity. Such programming occurs in diverse species, including primates [7].

Human and experimental animal studies have highlighted the link between alterations in the early life environment and metabolic disease. This is particularly reflected in the normal functioning of the reproductive system [4]. Indications that the balance of macronutrients in the mother’s diet can have important short- and long-term effects on the offspring came from a series of experimental studies in pregnant rats [5]. These studies revealed the existence of developmental plasticity and alterations in phenotypic outcomes in response to altered environmental conditions during the earliest life period. According to study by Burdge et al., offspring are extremely sensitive to nutritional, hormonal and environmental changes during the gestation and lactation periods, which may result in altered physiology of several systems throughout the life. This phenomenon is named “programming”, and its origins are related to epigenetic modifications which is usually explained as posttranslational modification of the nucleic acids of offspring without changing the genetic sequence [6]. Adult outcomes programmed by infant nutrition include lipid metabolism, blood pressure, obesity, diabetes, arteriosclerosis, behavior and longevity. Such programming occurs in diverse species, including primates [7].

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increased risk of obesity and metabolic disorders later in life. A diet high in calories from excess fat is commonly used to mimic Western societies’ eating habits, which imitate the nutritional environment in which the fetus or infant acquires increased risk of metabolic disorders later in life [8].

In the present study, we have investigated whether the type of food (standard balanced food or food with high saturated fatty acid content) consumed by the dams and their offspring of Sprague–Dawley rats can result in changes of reproductive and metabolic functions, specifically the vaginal smear, estrous and lipid content.

Methods

Experimental design

Ten virgin female Sprague–Dawley rats (Rattus norvegicus) were randomly divided into two groups at the age of 9 weeks. During 6 weeks period, groups of dams were housed in separated cages, in a controlled temperature room (22

HFD – high-fat diet mothers and offspring. Rats were housed with high-fat diet mothers and control diet offspring and (d) HFD–CD – control diet mothers and offspring, (b) CD–HFD – control diet mothers and high-fat diet offspring, (c) HFD–CD – control diet mothers and control diet offspring and (d) HFD–HFD – high-fat diet mothers and offspring. Rats were housed in separated cages, in a controlled temperature room (22 °C), with 12 h light–dark cycle, lights on at 6:00 a.m. Standard laboratory chow (CD) and tap water were available ad libitum. High content of saturated fatty acid food (HFD) was given twice a day (at 9 a.m. and 4 p.m.), 30 g in total per animal, for the prevention of body weight gain and over-nutrition-induced obesity. Experiments were approved by the Ethics Committee of the Faculty of Medicine University of Osijek and carried out according to European Guidelines on Laboratory Animal Care (Directive 2010/63/EU).

Blood collection and biochemical analysis

Blood samples for biochemistry analyses were taken from the tale vein at the age of 37 weeks in dams and 18 weeks in offspring. It was kept at room temperature for 30 min, followed by 15 min centrifugation at 3000 r/min. The supernatant was taken and stored at −20°C until analysis. Cholesterol and triglyceride serum levels were analyzed by commercially available kits at the Department of Medical Biochemistry, University Hospital Centre Osijek, Croatia.

Body weight and body mass index (BMI)
The body weight of dams and offspring was recorded using Beurer KS 22 balance (Beijing, China) at the precise day of blood sampling. BMI was calculated for all groups using a simplified formula: BMI = body weight (g)/total tibia length (mm²) [9]. Tibia length was measured by micrometer caliper from tuberosity of tibia to tibial medial malleolus.

Determination of pregnancy and pup survival

Starting, the pregnancy was monitored every morning between 7.00 and 10.00 a.m. by checking the occurrence of the vaginal plug. The rate of pup survival during first 3 days post-delivery was recorded for each dam.

Determination of estrous cycle in offspring

At the age of 18 weeks, offspring’s vaginal smear collection was initiated and continued during 15 days period, each day at 2 p.m. The estrous cycle was described by vaginal exfoliative cytology in order to assess the presence of ovulation. Different estrous cycle stages were determinate by the presence of typical cytological picture (cell distribution and occurrence) as follows: a cotton swab soaked in saline was inserted into the vagina, rotated and adherent mucous tissue was placed on a glass microscope slide. Samples were air dried and stained by May–Grünewald–Giemsa staining [10]. The morphological features of vaginal exfoliative cells were examined under a light microscope at 100× and 400× magnification (Olympus®; BX50 microscope, Tokyo, Japan) and digital images were captured with Olympus®; C-5050 digital camera. QuickPHOTO PRO imaging software (Promicra s.r.o., Prague, Czech Republic) was used to re-assess the proportion of three cell types: epithelial cells, countrified cells and leukocytes. Estrous stages were determinate as: proestrous, estrous, metestrous and diestrous (Figure 1), with normally total duration of approximately 4–5 days. Estrous phase smear consists of anucleated cornified cells (Figure 1E); a metestrous smear consists of the same proportion among leukocytes, cornified and nucleated epithelial cells (Figure 1M); a diestrous smear is characterized by a predominance of leukocytes (Figure 1D) and a proestrous smear primary consists of nucleated epithelial cells (Figure 1P) [11]. Irregular cycles were characterized by prolonged duration of a single phase, most frequently estrous phase (4–5 days). In addition, cycles in which the interchanges of the phases did not follow regular sequence: proestrous, estrous, metestrous and diestrous (or intermediates) and prolonged cycles (more than 5 days) were also considered as irregular [12].

Statistical analysis

Data analysis was performed using SAS software, version 8.02 (SAS Institute Inc., Cary, NC). Variables were reported as median with interquartile range (Me [Q1–Q3]). Considering small sample size per group (because of the law limitations of animals use in scientific purposes), between-groups analysis was performed using non-parametric tests. Mann–Whitney U-test was used for testing two independent groups, which was extended by Kruskal–Wallis test for comparing three or more samples that are independent. Differences were declared to be statistically significant at p < 0.05.

Results

Biochemical analysis

Feeding with high-fat diet resulted in significant increase of triglyceride concentrations in the HFD dams (1.47 [1.43–1.76])
when compared to the CD dams (0.97 [0.63–1.09]; p = 0.008).

Furthermore, triglyceride levels differed among the offspring (p = 0.001). The highest triglyceride level was found in the HFD–HFD group (0.87 [0.83–1.01]), while offspring of the CD dams exposed to HFD (CD–HFD group) presented with lowest triglyceride levels (0.56 [0.44–0.59]). Post-hoc analysis reveals significant changes between the HFD–HFD versus CD–HFD and HFD–CD groups (p = 0.002, respectively in both) (Figure 2G).

Serum cholesterol concentrations were generally higher in dams and offspring fed with food containing saturated fatty acids than in dams and offspring fed with standard laboratory chow. Higher levels were in the HFD dams compared to cholesterol levels measured in the CD dams (p = 0.008) (Figure 2). Significantly increased level in offspring was only in the CD-HFD group 3.30 [2.94–4.25].

Body weight and BMI
Increased dietary consumption of fatty acids did not have any effect on BMI in dams or offspring (p = 0.46 and p = 0.42, respectively) (Figure 2B and F). However, the CD–CD rats presented with the highest body weight and post-hoc analysis revealed that their body weight was significantly higher compared to the CD–HFD group (p = 0.04) (Figure 2A and E).

Determination of estrous cycle
Cytological analysis of vaginal smears in all offspring of mothers fed with a standard laboratory chow revealed normal estrous cycles (CD–CD, CD–HFD), while offspring of mothers fed with high-fat diet (HFD–CD, HFD–HFD) presented with irregular phase interchanges and extended estrous phase (Figure 3).

Determination of pregnancy and pup survival
Number of days necessary to achieve successful mating was significantly differed between the CD and HFD dams (p = 0.009). Total number of newborns per dam was 78 in the CD group and 44 in the HFD group. The average of survived pups per mother in the CD group after the 3rd day of life was significantly higher than the average of pups per mother in the HFD group (15.6 versus 7.6).

Discussion
In this study, we found that HFD resulted in increased triglyceride and cholesterol levels in the HFD dams and the HFD–HFD and CD–HFD offspring. In addition, dieter changes resulted in alterations of estrous cycle, irregular interchange in cycle phases and prolonged estrous cycle. Our results suggest that a high-fat diet increases maternal triglyceride and cholesterol levels, and this additional energy is probably transferred to the offspring during lactation. Knopp et al. found that the first phase of adipose tissue metabolism is characterized by an increase in food intake, increased plasma insulin levels and increased hepatic conversion of glucose to fatty acids [13]. The overall result of these metabolic changes is to directly ingest fuels to maternal stores. Franco et al. showed that the higher fat and protein concentration in the breast milk seemed to induce early overnutrition in the offspring and in addition to store energy as fat, these offspring had a larger reserve of glycogen and hyperglycemia that may have resulted from increased gluconeogenesis [14].

Serum triglyceride concentrations were the highest in the HFD-HFD group and the lowest in the CD-HFD group suggesting that the offspring were programmed by the hypercaloric HFD intrauterine milieu to be less susceptible to the deleterious effects of an adult HFD.
Figure 2. Body weight, BMI, serum triglyceride and cholesterol levels in dams and offspring. (A) Body weight (g); (B) body mass index (BMI) (g/mm²); (C) triglyceride level (mmol/l); (D) cholesterol level (mmol/l). Data are presented using Whiskers bar graphs (mean, 5–95 percentile). Differences among the groups were tested by Mann–Whitney U-test, \( p < 0.05 \) was considered significant. CD – standard laboratory chow, HFD – high-fat diet.
Our results on the blood lipid profile are in line with previous studies reporting enhanced blood lipid levels upon increased dietary intake of saturated fatty acids [15]. The dams on HFD, as well as both groups of offspring on HFD (CD–HFD, HFD–HFD) presented with significantly increased serum cholesterol levels compared to their respective controls on the control diet. Interestingly, offspring of the HFD dams exposed to HFD had lower cholesterol levels compared to the HFD fed offspring of mothers fed standard laboratory chow. These findings support the “thrifty phenotype” hypothesis that assumes long-term physiological adaptation of fetus in response to environmental changes during intrauterine life, including metabolic changes of mother as preparation to their likely environment in adulthood [16]. Therefore, those who have received appropriate maternal forecast will be better able to cope with their food environment in adulthood, irrespectively of the diet type (groups CD–CD and/or HFD–HFD). This is further supported by the studies demonstrating that population who were not exposed to Western type of diet in the past have higher susceptibility to metabolic diseases [17]. However, up to date this was mostly accounted to genetically inherited susceptibility to the development of obesity and associated diseases or alternatively to rapid changes in lifestyle, but not to genetic programming during intrauterine life [18]. This latter explanation is sometimes referred to as the “thrifty phenotype” hypothesis, to highlight its contrast with the thrifty genotype hypothesis [19].

A HFD is commonly used to investigate the impact of overnutrition during perinatal period. This study utilized an interesting approach to HFD feeding (twice during the daytime – light phase of the day) that prevents body weight gain and overnutrition-induced obesity. In addition, it is supposed that the changes in triglyceride and cholesterol serum levels are exclusively dependent on the type of diet. It is now well accepted that low-grade chronic systemic inflammation in the absence of any systemic or local infection, also called sterile inflammation, is associated with high calorie diet and increased risk of insulin resistance and related disorders [20]. Numerous studies have shown elevated concentration of adipose tissue derived cytokines, suggesting a concept that inflammation may be derived from the accumulation of activated macrophages surrounding enlarged adipocytes in obese subjects [3], especially in visceral fat depots [21]. In addition, it has been shown that some individuals are susceptible to the above-mentioned disorders at a younger age and at a relatively lower BMI, as a result of increased body fat rate, and especially in the presence of visceral adiposity [22]. In our study, BMI did not differ among any of the groups which might be explained by the fact that the BMI was matched based on a single measurement. In general, the BMI of men as well as women increases throughout the life. Therefore, a single BMI measurement may not be an appropriate proxy for lifetime BMI and might be a poor estimate of the long-standing effects of BMI on the reproductive physiology.

It has been well established that obesity impairs the functioning of whole female organism. The hypothalamus, pituitary and/or the ovaries can be affected by nutritional deficiencies, thus lifestyle interventions and weight-loss not only improve body composition and reduce insulin resistance, they also ameliorate reproductive phenotype [23]. In our study, the rate of successful pregnancies among the HFD dams was significantly lower compared to the control dams. In addition, the HFD dams had more trouble in maintaining healthy litters during the perinatal period [24]. The failure of the HFD dams to make the expected metabolic transition from pregnancy to lactation may provide part of the explanation for these reproductive difficulties. Although, the etiology of this relationship has not been established, one possible explanation is that disorders of macronutrient partitioning, such as obesity, lead to energetic inhibition of reproduction [25].

Figure 3. Offspring’s vaginal cytology smears. Vaginal cytology smears of all offspring of mothers fed with a standard diet were normal (CD–CD, CD–HFD), while all vaginal cytology smears of offspring of mothers fed with high content of saturated fatty acid food (HFD–CD, HFD–HFD) showed features of irregular phase interchanges or extended estrous phase. CD – standard laboratory chow, HFD – high-fat diet.
We observed that the offspring of HFD dams were more likely to die during the first 3 days of life. The cause of this excess mortality is not known at present, but could include both biological and behavioral components. Rolls et al. postulated that the young HFD dams are unable to consume sufficient amount of their more energy-dense milk or to metabolize the longer-chain fatty acids that are more abundant in this milk. The effect of these differences in milk composition on the growth of the young has not been established [26]. However, another possible explanation is that the offspring are not able to stimulate appropriate maternal responses, since the HFD dams do not allow adequate nursing, and it is well known that maternal behavior in rats fed with a high saturation of fatty acid food is abnormal, leading to high rates of cannibalism of the young [27].

In this study, we also examined the effect of dams’ and offspring’s diet on cytological changes in vaginal smears of female offspring. In general, vaginal cytological smears of all offspring of mothers fed with a standard diet were normal, while all vaginal cytological smears of offspring of mothers fed with HFD show features of irregular phase interchanges and extended estrous phase. The effects of mother’s diet on the offspring’s reproductive cycle regulation can be explained by the influence of a stressful stimulus on hypothalamic–pituitary–ovary axis. Since the HFD-CD offspring were used to more caloric intake during intrauterine time and lactation period, later transition to standard diet represents a dietary restriction to this group This is in line with a previous study of Schaffer et al., who found that nutrient restriction can result in permanent changes of hypothalamic–pituitary–ovary axis (HPA axis) and exerts a negative effect on fetal ovary development [28]. The same study showed more importantly that female offspring subjected to a bout of maternal nutrient restriction during early development exhibited a marked reduction in fertility. Once again, these findings emphasize the importance of the mother’s diet in the regulation of offspring’s reproductive cycle. However, further investigations are needed to elucidate pathophysiological processes by which disorders of the adipose tissue metabolism can affect reproductive functions.

Considering the global obesity epidemic [29], which affects both the adult and early childhood populations, the importance and necessity of examining the impact of maternal and early childhood type of nutrition on long-term health are apparent. Further research requires a strategy of interdependent clinical, animal and epidemiologic investigations. Such an approach may allow us to use the information outlined here to reduce the prevalence of long-term pathologic effects of obesity.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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