Ovariectomy, but not orchiectomy, exacerbates metabolic syndrome after maternal high-fructose intake in adult offspring

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ABSTRACT
High fructose diet is associated with the global metabolic syndrome (MtS) pandemic. MtS develops in early life, depending on prenatal and postnatal nutritional status. We hypothesized that ovariectomy increases the chances of developing MtS in adult offspring following high fructose intake by the mother. Pregnant C57BL/6J mouse dams drank water with or without 20% fructose during pregnancy and lactation. After weaning, the pups were fed regular chow. The offspring were evaluated until they were 7 months of age after the mice in each group, both sexes, were gonadectomized at 4 weeks of age. The offspring (both sexes) of the dams who had high fructose intake developed MtS. In the offspring of dams who drank tap water, orchiectomy increased the body weight gain and body fat accumulation, while ovariectomy increased the body fat accumulation as compared to the sham controls. In the offspring of dams with high fructose intake, orchiectomy decreased the body weight gain, body fat accumulation, visceral adiposity, and glucose intolerance, while ovariectomy exacerbated all of them as compared to the sham operations. These data indicate that ovariectomy encourages the development of MtS in adult offspring after maternal high fructose intake, while orchiectomy prevents the development of MtS. The sex difference indicates that male and female sex hormones play contradictory roles in the development of MtS.

INTRODUCTION
The global increase in the prevalence of obesity during the previous 30 years has occurred in parallel with the rise in the use of high fructose corn syrup that was first introduced just before 1970 [1,2]. The increasing incidence of this contributing factor of metabolic syndrome (MtS) is related to the increasing presence of fructose in the diet, partially owing to the introduction of high fructose corn syrup as a sweetener in soft drinks and other foods [3,4]. High fructose intake, particularly if combined with a high calorie intake, has been linked to diet-induced health problems [5-8]. In animal models, several studies have addressed the effects of diets supplemented with fructose or sucrose on the development of MtS. In Wistar Kyoto rats, at 21 weeks of age, high fructose intake (20%–25%)-induced MtS, including hypertension, insulin resistance, and dyslipidemia, but not type 2 diabetes [9]. Further, we found that high fructose intake induced the activation of RAS, resulting in hypertension and MtS [10]. High fructose diets (HFrDs) lead to several adverse metabolic and cardiovascular effects, including dyslipidemia, insulin resistance, hypertension, hyperuricemia, and weight gain [11,12]. The deleterious effects of high fructose intake in adults have been studied in detail; however, limited data are available on the long-term effects of high fructose exposure during gestation, lactation, and infancy.

Maternal high fructose intake can negatively affect the metabolic health of the offspring [13]. Several studies have examined...
the evidence linking early life fructose exposure during critical periods of development and its implications on long-term cardio-metabolic health in offspring [14-16] and have shown the impact of maternal high fructose intake on the offspring during and after pregnancy [17,18]. Although maternal high fructose intake induces cardiometabolic syndrome in the offspring, whether maternal high fructose intake induces MtS in successive generations has not been studied. Further, maternal HFrD induces differential vulnerability MtS in the offspring [19]. In our previous study, the expression of Na’-K’-2Cl’ co-transporter 1 (NKCC1) was epigenetically regulated during the postnatal development of hypertension [20]. It is possible that maternal fructose intake induces hypertension and other cardiometabolic diseases via epigenetic modification of the offspring [21]. Some studies have shown that MtS in pregnancy is associated with an increased risk of obesity and hypertension in the offspring [22,23]. Furthermore, maternal fructose intake induces insulin resistance, oxidative stress, and MtS [24,25]. A HFrD during pregnancy induces fatty liver and glucose intolerance in rats [26]. Therefore, high fructose intake during pregnancy leads to adverse responses in mothers and provides a fetal environment that may lead to programming that influences the health of the offspring.

The development of MtS due to fructose intake depends on the sex of the offspring. A HFrD is significantly associated with low serum testosterone in men aged 20–39 years old in the United States [27]. Maternal fructose intake disrupts ovarian estradiol synthesis in rats. Sex-specific changes in the expression of the renin-angiotensin-aldosterone system in male rats were induced by a high fructose diet [28]. Further investigations are required for a comprehensive understanding of the underlying mechanisms of sex-specific fructose-induced cardiovascular pathologies [29]. Fructose intake increases the blood pressure; this can be prevented by estrogen treatment in intact male rats, but not in gonadectomized rats [30]. Androgen is necessary for the development of hypertension in fructose intake rats and gonadectomy prevents hypertension in fructose intake rats [31]. The presence of testosterone is essential for the development of endothelial dysfunction and increased blood pressure [32]. Fructose drinking (65% w/v) for 3 months increases the renal cortical levels of the proteins involved in metabolism in male mice, but not in female mice. However, only female mice display increased urine volume and plasma K⁺, decreased plasma Na⁺, and a reduction in the expression of NKCC2 in the kidneys [33]. The developed MtS is regulated not by diet alone but by its interplay with sex. In this study, we hypothesized that ovariectomy increases the chances of development of MtS in adult offspring after high fructose intake by the mother.

METHODS

Animals

This study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals after approval by the Institutional Review Board of Kyungpook National University (2016-0157). Every effort was made to minimize both the number of animals used and their suffering. We randomly assigned eight-week-old pregnant C57BL/6j mice (F0) into groups provided with drinking water with or without 20% fructose [9,34,35] over the course of the pregnancy and lactation periods. Breeding was performed between 1 male and 3 female representing each litter to yield the subsequent next generations. After weaning, the pups were started on regular chow. At four weeks of age, half of each group, both male and female, were gonadectomized and the mice were evaluated at seven months of life. The food intake amount was the same among the offspring groups (Fig. 1A). The fructose was purchased from Millipore (Billerica, MA, USA) and the mice were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). The tissues were frozen in liquid nitrogen and stored at –80°C until further study. We calculated the appropriate number of rats for this experiment by using the G*power software.

Glucose tolerance test (GTT)

GTT was performed on the offspring at 7, 11, 16, and 28 weeks of age. The mice were fasted for 16 h before the experiments. Fasting glucose levels were determined using Accu-Chek Performa (Roche, Berlin, Germany). Glucose (20% solution, 2 g/kg) was injected intraperitoneally and the blood glucose levels were measured at 30, 60, and 120 min.

Histological analysis

For Oil Red O and hematoxylin & eosin (H&E) stains, liver and white adipose tissues were fixed in 4% formalin overnight, then dehydrated and embedded in paraffin. The paraffin-embedded samples were sectioned at a thickness of 3 mm. The slides were examined using light microscopy [36].

Blood chemistry

Whole blood was collected from the tail vein. Serum was isolated by centrifugation at 2,000 rpm for 10 min. Blood chemistry was evaluated by the Pohang Technopark Foundation.

In vivo imaging

Mice were imaged after seven months (n = 4–6/group). The protocol for micro-computed tomography (CT) imaging was
performed at the Pohang Center for Evaluation of Biomaterials, Pohang Technopark in Pohang, Korea using a Siemens Inveon Trimodality Image system (Inveon; Siemens, Washington, DC, USA). The CT slice images were reconstructed using Siemens Inveon Acquisition Workplace Software (Inveon; Siemens). During the scanning, mice were supplemented with nasal cannula oxygen. Each session lasted an average of 15 min. Transverse views of the CT images (one per animal) at the level of the fifth lumbar vertebra were selected for the analysis of visceral adipose tissue (VAT). The cross-sectional total body area and adipose tissue area were measured using software (Inveon Research Workplace). Percent VAT was calculated as the proportion of VAT to the cross-sectional total body area.

**Western blot**

For protein expression analysis, frozen tissues were homogenized in RIPA buffer containing protease inhibitors. Protein-matched samples (Bradford assay) were electrophoresed (SDS-PAGE) and then transferred to nitrocellulose (NC) membranes. The NC membranes were blocked with 5% skim milk in TBS (25 mmol/L Tris base and 150 mmol/L NaCl) for 2 h at room temperature and then incubated with the following primary antibodies (1:1,000 diluted) at 4°C overnight: SREBP1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ac-K antibody (Cell Signaling Technology, Beverly, MA, USA), ACC antibody (Thermo Fisher, Waltham, MA, USA), FAS antibody (Thermo Fisher), GAPDH antibody (Thermo Fisher), and SCD1 antibody (Abcam, Cambridge, UK). The membranes were incubated with second-
ary antibodies (1:5,000 diluted) at room temperature for 1 h and then washed three times for 10 min each in TBST. The target proteins were detected with ECL plus detection reagents (Amersham, Pittsburgh, PA, USA). The expression levels were quantified using optical densitometry and the ImageJ software (http://rsbweb.nih.gov).

Statistics

The results were expressed as mean ± standard error (SE). Data were analyzed with the Kruskal-Wallis test, one-way ANOVA or two-way ANOVA followed by a post-hoc Tukey’s comparison test. Differences were considered significant at p < 0.05. A Student’s t-test was used to analyze differences in means between two groups. Statistical procedures were performed using SPSS software (release 19.0; IBM Co., Armonk, NY, USA).

RESULTS

Gonadectomy influenced weight gain in adult offspring

At the start of the experiment, the body weight of the male and female mice were comparable (Fig. 1). The body weight and weight gain were affected by maternal high fructose intake in offspring at 7 months. In order to determine whether sex influenced weight gain in mice whose mothers consumed HFrD, we gonadectomized offspring from both groups after weaning (Fig. 1). In the offspring of dams who drank tap water, orchiectomy increased the body weight gain. In the offspring of dams with a high fructose intake, orchiectomy attenuated body weight gain, while ovariectomy exacerbated body weight gain as compared to the sham operations. We observed that mice whose mothers had high fructose intake who had undergone orchiectomy had significantly decreased weight gain after 28 weeks of weaning. It is noteworthy that while the weight gain in male mice decreased, that in female mice offspring increased with gonadectomy.

Gonadectomy had effects on glucose tolerance development in adult offspring

Although maternal high fructose exposure induced glucose intolerance at 7 months of age in both the sexes, each showed a different amount of glucose tolerance (GTT). Maternal high fructose exposure elevated the blood glucose levels with no significant change after gonadectomy (Table 1). In order to assess the effect of high fructose intake by the mother on the glucose intolerance of the offspring, we performed GTT on the offspring at 7, 11, 16, and 28 weeks of age (Figs. 2, 3). Maternal high fructose-intake caused glucose intolerance in male mice at 11 weeks of age (Fig. 2A) and in female mice at 28 weeks of age (Fig. 3A). In the offspring of dams with high fructose intake, orchiectomy suppressed glucose intolerance, while ovariectomy promoted glucose intolerance as compared to sham operations. However, there was no significant difference in the fasting blood glucose levels between the sham and gonadectomy groups. Maternal fructose intake resulted in significantly higher area under the curve (AUC) values compared to the control diet attenuated by orchiectomy after 28 weeks in male mice (Fig. 2B). Maternal fructose intake with ovariectomy resulted in significantly higher AUC values compared to the control diet and fructose diet after 16 weeks in female mice (Fig. 3B). These findings suggested that high fructose intake induced-glucose intolerance is determined by the sex of the offspring.

Table 1. Effect of maternal high fructose exposure on serum biochemical parameters at 28 weeks in male and female offspring

|                  | Control Sham | Ovariectomy Sham | Fructose Sham | Control Sham | Ovariectomy Sham | Fructose Sham | Control Sham | Ovariectomy Sham | Fructose Sham |
|------------------|--------------|-------------------|---------------|--------------|------------------|---------------|--------------|------------------|---------------|
| **ALT (U/L)**    | 22.2 ± 3.2   | 55.3 ± 2.1**      | 35.0 ± 5.0*   | 73.0 ± 2.8*  | 10.5 ± 3.0       | 20 ± 3.5*     | 20.5 ± 2.0*   | 115.3 ± 9.2*    | 105.2 ± 7.3   |
| **AST (U/L)**    | 60.0 ± 5.2   | 101.2 ± 12.2**    | 129.2 ± 20**  | 95.0 ± 11.5  | 40.5 ± 7.2       | 45.2 ± 10.5   | 60.2 ± 5.0*   | 85.3 ± 3.3*     |               |
| **Glucose (mg/dl)** | 72.0 ± 5.6   | 113.5 ± 10.3*     | 120 ± 8.1**   | 133.5 ± 9.5  | 72.3 ± 8.5       | 85.2 ± 11.5   | 115.3 ± 9.2*  | 105.2 ± 7.3     |               |
| **Triglyceride (mg/dl)** | 90.5 ± 20.4  | 63.2 ± 18.3*      | 141.5 ± 28.0* | 181.0 ± 25.6* | 50.3 ± 12.4     | 76.0 ± 15.0   | 118.2 ± 10.3** | 130.3 ± 16.5    |               |
| **Total cholesterol (mg/dl)** | 61.8 ± 8.2   | 95.3 ± 20.2*      | 125.2 ± 25.0* | 125.8 ± 7.5  | 45.3 ± 8.4       | 50.0 ± 9.5    | 131.5 ± 20.1** | 145.8 ± 9.0     |               |
| **Uric acid (mg/dl)** | 6.8 ± 0.5    | 6.6 ± 1.2         | 6.3 ± 1.8     | 6.5 ± 2.2    | 6.4 ± 1.0        | 6.8 ± 2.5     | 6.6 ± 1.2     | 6.2 ± 3.5       |               |

ALT, alanine aminotransferase; AST, aspartate aminotransferase. Blood was obtained from mice (each group, n = 6) (*p < 0.05 and **p < 0.01, control sham vs. control gonadectomy. *p < 0.05 and **p < 0.01, control sham vs. fructose sham. *p < 0.05, fructose sham vs. fructose gonadectomy).
Gonadectomy does not affect the maternal fructose-induced steatosis in adult offspring

Assessment of liver morphology and Oil Red O staining showed that fructose-induced steatosis in offspring at 7 months of age (Fig. 4). When more fat accumulated in the liver, darker the Oil Red O stain and more the vacuoles on H&E stain. In our study, the maternal fructose intake caused a greater increase in the vacuoles on liver Oil Red O stain in male offspring than in the female offspring. Maternal fructose-induced male offspring considerably increased in steatosis compared to female offspring. In order to determine whether sexes affect steatosis in vivo, we gonadectomized the offspring. There are no significant differences after gonadectomy in both the sexes. It is noteworthy that maternal high fructose intake caused steatosis but was unaffected by gonadectomy.

Gonadectomy influences maternal fructose-induced lipogenesis in adult offspring

Fig. 5A and C were representative Western blot in the livers of male and female offspring. Maternal high fructose exposure increased the expression of lipogenesis proteins in both the sexes (Fig. 5); these were attenuated by gonadectomy. These results suggest that gonadectomy suppresses lipogenesis.

Gonadectomy affects maternal fructose-induced fat accumulation in the body

Then, we performed micro-CT scanning to measure fat storage (Fig. 6). The CT transaxial images showed that male and female mice born to mothers with high fructose intake had higher body fat accumulation than the control mice. Moreover, gonadectomy with control mice both had higher body fat accumulations than the sham (Fig. 6A). We observed that maternal fructose mice that had undergone orchiectomy exhibited significant decrease in visceral adipose tissue (Fig. 6B). However, maternal fructose-exposed mice with ovariectomy showed significant increase in the visceral adipose tissue (Fig. 6C).

Gonadectomy influences maternal fructose-induced adipocyte hypertrophy

Gonadectomy has no effect on adipocyte hypertrophy in both
In the offspring of dams with high fructose intake, orchiectomy attenuated adipocyte hypertrophy (Fig. 7A, B), while ovariectomy showed no change as compared to the sham operations (Fig. 7A, C). These results suggest that gonadectomy in maternal fructose males suppresses adipocyte hypertrophy.

**DISCUSSION**

In the present study, we hypothesized that ovariectomy increases the chances of developing MiS in adult offspring following high fructose intake by the mother. We found that both, male and female offspring of dams with high fructose intake developed MiS. In the offspring of dam drunk tap water, orchiectomy increased body weight gain and body fat accumulation, while ovariectomy caused greater increase in body fat accumulation than in sham controls. In order to determine whether sex influenced the maternal fructose-exposed mice, we gonadectomized both the offspring after weaning. In the offspring of dams with high fructose intake, orchiectomy attenuated the body weight gain, body fat accumulation, visceral adiposity, and glucose intolerance, while ovariectomy exacerbated all of these as compared to sham operations. To our knowledge, this is the first report to show that male and female sex hormones play contradictory roles in the development of MiS.

Several studies have investigated the evidence that has linked fructose exposure during early life and critical periods of development on the implications on long-term MiS in the offspring [14-16]. Our previous study showed that maternal fructose exposure induces multigenerational activation of the renin-angiotensin-aldosterone system and hypertension [37]. Further, maternal fructose intake is linked to epigenetic modifications in the offspring [38]. We found that maternal high fructose exposure induced the development of MiS in the offspring. However, MiS in the male offspring more severe than that in the female offspring. The development of MiS with high fructose intake is dependent on sex [27-32]. However, the long-term effects of high fructose exposure during the maternal period are largely unknown. As per a previous trial, gonadectomy induced weight gain in cats [39]. Therefore, in order to determine whether sex affects the offspring phenotypes after maternal fructose exposure in mice, we gonadectomized the offspring after weaning.

In our study, gonadectomized mice exhibited greater increase in body weight than the fructose sham group (Fig. 1C). It is noteworthy that gonadectomy prevented obesity in maternal fructose-
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exposed male mice, but not in female mice. Orchiectomy prevents the development of obesity in otsuka-long-evans-tokushima fatty (OLETF) rats [40] and attenuates hypertension induced by fructose treatment [30]. In contrast, ovariectomy induces hyperglycemia, especially in combination with a high-fat diet [41]. In our study, orchiectomy suppressed glucose intolerance development in the maternal fructose-exposed group (Fig. 2), while ovariectomy promoted glucose intolerance (Fig. 3).

Chronic high-fat high fructose diet (HFFD) induces hepatic steatosis in females, with significant increases in the proteins that are involved in hepatic lipogenesis, while HFFD significantly induces liver injury, inflammation, and oxidative stress only in males [42]. Several conditions show different phenotypes in males and females despite the consumption of the same foods [24,33,42,43]. In our study, maternal high fructose intake caused steatosis but showed no sex-based differences (Fig. 4). Maternal high fructose exposure increased the expression of lipogenesis in both the sexes, but only gonadectomy in maternal high fructose-exposed female suppressed lipogenesis (Fig. 5). To our knowledge, this is the first study to show that the offspring of dams with high fructose intake developed MtS in both the sexes.

The nuclear estrogen receptor (ER) was characterized in adipocytes in males and females. ER is also expressed in other organs that are associated with satiety and feeding, such as the hypothalamus and pituitary gland in males. Estrogen signaling through ERα in the liver helps prevent whole body and hepatic insulin resistance that is associated with high-fat diet feeding in males [44]. ERα absence results in marked increases in WAT, but not in brown adipose tissue (BAT), increased insulin resistance, and impaired glucose tolerance in both males and females, as well as altered energy expenditure in males [45]. Our results also showed that in the offspring of dams with high fructose intake, orchiectomy attenuated body weight gain, visceral adiposity, and glucose intolerance, while ovariectomy exacerbates all of them as

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**Fig. 4. Effects of gonadectomy on maternal high fructose exposure induced steatosis.** Representative images of offspring livers are shown (each group, n = 6). Liver sections were stained with Oil Red O or H&E (bar = 50 μm, stain magnification ×100). Maternal high fructose intake induced steatosis.
Fig. 5. Effects of gonadectomy on maternal high fructose exposure induced lipogenesis in offspring livers. The expression of lipogenesis proteins was detected using Western blotting in male offspring (A) and female offspring (C). Maternal high fructose exposure increased the expression of lipogenesis in both the sexes. All the samples were run in the same condition. (B, D) Relative protein expression was quantified using optical densitometry (ImageJ software, http://rsbweb.nih.gov). The sample was obtained from mice liver (each group, $n = 6$). The graphs show the mean ± standard error for 3 independent experiments (*p < 0.05 and **p < 0.01, control sham vs. fructose sham; #p < 0.05 and ##p < 0.01, fructose sham vs. fructose gonadectomy).

Fig. 6. Effects of gonadectomy on maternal high fructose exposure increased fat mass. (A) Representative micro-computed tomography image of body fat mass at 28 weeks. Maternal high fructose exposure increased body fat. (B, C) Percentages of visceral adipose tissue (VAT) in male and female mice. Data are expressed as mean ± standard error for 6 mice (*p < 0.05, control sham vs. control gonadectomy; *p < 0.05, control sham vs. fructose sham; #p < 0.05, fructose sham vs. fructose gonadectomy).
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Compared to sham operations (Fig. 6). Moreover, gonadectomy in males suppressed adipocyte hypertrophy (Fig. 7).

The ovarian estrogens and central 17β-estradiol (E2) play a major role in the regulation of energy balance and glucose homeostasis [46,47]. In particular, E2 controls fat distribution, differentiation, and fibrosis in WAT, and induces thermogenesis in BAT through proopiomelanocortin (POMC) and neuropeptide Y neurons in the arcuate nucleus, melanin-concentrating hormone cells in the lateral hypothalamic area, and food intake [48]. Longitudinal population studies have shown that low testosterone status in men is a risk factor for the later development of MtS [49]. Androgen deficiency is associated with obesity, MtS, and type 2 diabetes mellitus in men. Subcutaneous fat and total fat of androgen receptor null male mice were significantly heavier than those of wild male mice at 40 weeks of age [50]. In another study, orchietomized wild-type mice exhibited increasing body weight than sham-operated wild-type mice [51].

Maternal over-nutrition induces long-term epigenetic alterations in the offspring’s hypothalamic POMC promoter that predisposes the offspring to metabolic disorders in later life [52]. Maternal intake of the peroxisome proliferator-activated receptor α ligand during lactation promotes epigenetic modulation of the fibroblast growth factor-21 gene in the maternal mouse liver that ameliorates diet-induced obesity in adulthood [42]. This explains why the liver and adipose tissue show different patterns. It is possible that maternal fructose exposure causes epigenetic modifications in the offspring. Therefore, future studies will focus on the mechanism underlying maternal fructose-induced MtS.

In summary, we found that the offspring of mice on high fructose diets developed obesity, steatosis, hepatic lipogenesis, hyperglycemia, and hypertension in both the sexes. Our results showed that orchietectomy encourages the development of MtS in adult offspring following high fructose consumption by mothers, while orchietectomy prevented MtS. In particular, our study suggests that the sex difference implicates that male and female sex hormones play contradictory roles in the development of MtS.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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