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Original Article

Sex differences in the effects of high-fat diet on mouse sciatic nerves

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ABSTRACT — Obesity is caused by a chronic positive energy balance, which not only increases the amount of lipid in adipose tissue, but also, in non-adipose tissue. Excessive accumulation of lipids in tissues may lead to cell dysfunction or cell death, a phenomenon known as lipotoxicity. The aim of this study was to investigate the effects of high-fat diet (HFD) feeding on the sciatic nerves of both male and female mice. HFD feeding induced increased mRNA levels of *Bax* and *Bcl2* in HFD group of males only, although the accumulation of fatty acids in the sciatic nerves was induced by HFD feeding in the both sexes. To determine whether estrogen was involved in the inhibitory effects of HFD feeding-induced increased expression of apoptosis-related genes, ovariectomized (OVX) females were fed a normal diet (ND) or HFD with or without daily ethinylestradiol treatment for 1 week. In OVX mice, the mRNA levels of *Bax* and *Bcl2* in HFD group were higher than in ND group. In contrast, in OVX mice treated with ethinylestradiol, there was no significant between ND and HFD groups. In conclusion, HFD induced apoptosis in the sciatic nerves of males, but not females, and estrogen had inhibitory effects on HFD-induced apoptosis in the sciatic nerves of females. Long-term feeding studies are needed to investigate the pathological effects on sciatic nerves of mice fed HFD as pathological findings were not observed under our study condition.

Key words: High-fat diet, Apoptosis, Lipotoxicity, Fatty acid, Ovariectomy, Estrogen

INTRODUCTION

Obesity is caused by a chronic positive energy balance, which increases the amount of lipid in adipose tissue. Lipid accumulation is well-known and the harmful effects of lipid accumulation in non-adipose tissue such as liver, skeletal muscle, pancreas, and heart has been reported. Previous study reported that the percentage of patients with peripheral neuropathy was more frequent in groups with a BMI ≥30 compared to BMI < 30 and BMI 30 to < 35 groups, but not significantly so (Herrera-Rangel *et al.*, 2014). Free fatty acids mediate dysfunction in peripheral nerves (Perez-Matos *et al.*, 2017). Thus, it is assumed that HFD feeding has adverse effects on peripheral nerves.

Excessive accumulation of lipids may lead to cell dysfunction or cell death, a phenomenon known as lipotoxicity. The ingestion of excessive amounts of fatty acids is considered to be a risk factor for cardiovascular diseases, insulin resistance, dyslipidemia, and obesity (Mensink *et al.*, 2003; Lopez-Garcia *et al.*, 2005; Mozaffarian *et al.*, 2006). Interestingly, high-fat diet (HFD) feeding resulted in an induction of hepatic expression of *de novo* lipogenic genes (*Fasn*, *Elovl6*, and *Scd1*), and the accumulation of fatty acids in liver and adipose tissues (Oosterveer *et al.*, 2009; Park and Mun, 2013; Crescenzo *et al.*, 2017). Hepatic *de novo* lipogenesis contributes to the pathogen-

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esis of non-alcoholic fatty liver disease (Donnelly *et al.*, 2005), and is involved in the development of type 2 diabetes (Ameer *et al.*, 2014). However, it is unclear whether HFD feeding promotes *de novo* lipogenesis and induces injury in peripheral nerves.

Apoptosis is a genetically programmed mechanism of cell death that is triggered in response to cellular stress. BCL2 family proteins, such as BAX and BCL2, are well-known as key regulators of apoptosis. Many studies reported that HFD feeding induced apoptosis in several tissue types (Wang *et al.*, 2008; Moraes *et al.*, 2009; Hsu *et al.*, 2016). The excess accumulation of fatty acids causes apoptosis in cardiomyocytes, renal tubular cells, and pancreatic β-cells (Weinberg, 2006). In neural cells, treatment of fatty acids induced protein expression of BAX and the number of apoptotic cells (Yuan *et al.*, 2013).

Sex hormones, such as estrogen and androgen, contribute to the sex differences in body weight and metabolism between males and females and are thought to be responsible for sex-specific differences (Ogawa *et al.*, 2015; Boese *et al.*, 2017; True *et al.*, 2017). Multiple studies have shown that estrogen prevented cell death in many cell types (Kanda and Watanabe, 2003; Kim *et al.*, 2006; Vasconsuelo *et al*., 2008; Bailey *et al*., 2012). A recent study indicated that male mice fed HFD enhanced the pathogenesis of neuropathy in the sciatic nerves, however, the effects of HFD feeding on peripheral nerves was not investigated in females (Rumora *et al.*, 2019).

The aim of this study was to investigate the effects of HFD feeding on the sciatic nerves of both sexes. We demonstrated that HFD induced the apoptosis in the sciatic nerves of males, but not females, and estrogen had the inhibitory effects of HFD-induced apoptosis in the sciatic nerves of females.

MATERIALS AND METHODS

Animals

Male and female Crl:CD1(ICR) mice (Charles River Laboratories Japan, Inc., Kanagawa, Japan) were obtained at 7 weeks old. The animals were used for the study after 7 days of acclimation. Prior to start dosing, the animals were checked for any diseases or injuries, and those without weight or feeding abnormalities were used.

Male and female mice randomly divided into 2 group: the normal diet (ND) group and HFD group (5 animals per group), and then the ND group and HFD group were maintained on D12450J (EPS Ekishin Co., Tokyo, Japan) and D12492 (60 kcal% lard-based fat; EPS Ekishin Co.) for 7 days, respectively.

All animal experiments were conducted in accordance with the animal study protocol approved by the institutional animal care and use committee of the testing facility. Animals were kept at controlled temperature (22.0 ± 2.0°C), humidity (60 ± 10%) and lighting (a 12 hr light: 12 hr dark cycle with lights on at 0800 hr) with *ad libitum* food and water.

Ovariectomy and E2 treatment

Twenty female mice at 7 weeks of age were ovariectomized (OVX) and five female mice at 7 weeks of age were sham-operated (Sham). OVX were randomly divided into four groups (n = 5 per group). 1) OVX-control group: OVX mice fed control diet, 2) OVX-HF group: OVX mice fed high fat diet, 3) E2-control group: OVX-control mice treated ethinylestradiol (EE2), 4) E2-HF group: OVX-HF mice treated EE2. After 1 week, E2-control and E2-HF groups were administered with EE2 (0.01 mg/kg body weight; Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) by oral gavage once a day for 7 consecutive days. The other two groups were orally administered with vehicle (corn oil). OVX mice were fed ND or HFD. All mice were sacrificed by exsanguination under anesthesia.

Analysis of fatty acids in sciatic nerves

The amount of fatty acids was analyzed by gas chromatography mass spectrometry (GC/MS) as previously described (Tanetani *et al.*, 2009). In brief, the sciatic nerves were homogenized in 47.5 mL of MeOH/$CHCl_3$/H_2O (2:1:0.8) containing 0.1 mg of 10-undecenoic acid (C11:1) as an internal control using a Physcotron (NITION Co., Ltd., Tokyo, Japan). Homogenate was filtered through a filter paper, and lipids were extracted into the $CHCl_3$ phase after addition of 100 mL of $CHCl_3$. Extracted lipids were saponified in EtOH/25% KOH (3:2) at 65°C for 1 hr. After the reaction, the mixture was acidified with 10% HCl. Fatty acids were extracted into *n*-hexane phase after addition of 60 mL of *n*-hexane. The *n*-hexane phase was evaporated and dissolved in 2 mL of toluene/MeOH (4:1). Fatty acids were methylated at 80°C for 1 hr after addition of three drops of conc. H_2SO_4. Fatty acid methyl esters were extracted into the organic phase after addition of 5 mL of sat. $NaHCO_3$ aq. Fatty acid methyl esters were analyzed using a GC (Agilent 6890 series GC System; Agilent Technologies, Santa Clara, CA) fitted with a fused silica capillary column (Omegawax TM250 capillary column; Supelco, Bellefonte, PA), and equipped with a flame ionization detector. The GC conditions were: injector and detector

temperature, 250°C; running temperature program, 100°C for 5 min, then increasing at 4°C/min to 260°C and holding this temperature for 25 min. Injection volume was 2 μL. Each fatty acid was identified by the retention times of authentic methyl esters (Merck KGaA, Darmstadt, Germany) and confirmed by GC/MS.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from sciatic nerves and reverse transcribed with PrimeScript reverse transcription Reagent Kit (Takara Bio Inc., Shiga, Japan). The resultant cDNA was subjected to quantitative real-time RT-PCR (qRT-PCR) using the following specific primers: *Srebp1* (forward primer 5′-GCAGCCACCATCTAGCCTG-3′ and reverse primer 5′-CAGCAGTGAGTCTGCCTTGAT-3′), *Fasn* (forward primer 5′-GGAGGTGGTGATAGCCGGTAT-3′ and reverse primer 5′-TGGGTAATCCATAGAGCCCAG-3′), *Elvol6* (forward primer 5′-GAAAAGCAGTTCAACGAGAACG-3′ and reverse primer 5′-AGATGCCGACCACCAAAGATA-3′), *Scd1* (forward primer 5′-TTCTTGCGATACACTCTGGTGC-3′ and reverse primer 5′-CGGGATTGAATGTTCTTGTCGT-3′), *Bax* (forward primer 5′-TGAAGACAGGGGCCTTTTTG-3′ and reverse primer 5′-AATTCGCCGGAGACACTCG-3′), *Bcl2* (forward primer 5′-ATGCCTTTGTGGAACTATATGGC-3′ and reverse primer 5′-GGTATGCACCCAGAGTGATGC-3′), *Actb* (forward primer 5′-CAGCCTTCCTTCTTGGGTAT-3′ and reverse primer 5′-GCTCAGTAACAGTCCGCCTA-3′). The PCR profiles consisted of denaturation at 95°C for 1 min, primer-annealing at 55°C for 1 min, and primer extension at 72°C for 30 sec. The final primer extension was performed at 72°C for 10 min. The final primer extension was performed at 72°C for 10 min. The PCR in qRT-PCR was performed with SYBR Premix Ex TaqII (Takara Bio Inc.) on Thermal Cycler Dice, TP-900 (Takara Bio Inc.). Ct values were transformed into relative quantification data by $2^{-\Delta\Delta}$Ct method, and data ere normalized to the *Actb*.

Statistical analyses

Statistical analysis for comparing 2 groups was performed using an *F*-test, followed by Student's *t*-test or Aspin-Welch's *t*-test. And statistical analysis for comparing 4 groups was performed using Bartlett's test, followed by Tukey-Kramer test or Steel-Dwass test. Pharmaco Basic (version 15.0; Scientist Press Co., Ltd., Tokyo, Japan) was used to perform the statistical analysis. Data are expressed as mean ± S.D., and differences were considered statistically significant at a p value of < 0.05.

RESULTS

The effects of HFD on the sciatic nerves of male and female mice

In both male and female mice, there were no significant changes in body weight or body weight gain between the ND and HFD groups (Tables 1, 2). To determine whether HFD feeding induced the accumulation of fatty acids in the sciatic nerves, the amount of palmitic acid, stearic acid, and oleic acid was analyzed. HFD increased the levels of stearic acid and oleic acid, but not palmitic acid in the sciatic nerves of both male and female mice (Tables 1, 2).

We investigated whether fatty acid synthesis-related genes were increased by HFD feeding in the sciat-

Table 1. Body weight and fatty acids amount in the sciatic nerves of male mice.

Group		Body weight (g)			Fatty acids amount (μg)		
		Initial	Final	Gain	C16:0	C18:0	C18:1
Male	Mean	32.8	35.1	2.3	78.6	74.7	177.1
ND	S.D.	1.7	2.8	2.6	12.3	6.7	4.8
Male	Mean	32.5	35.1	2.6	67.4	91.4*	185.0*
HFD	S.D.	0.7	1.3	1.5	10.7	13.0	3.3

ND = normal diet; HFD = high-fat diet, *$p < 0.05$ vs Male ND (Student *t*-test, n = 5).

Table 2. Body weight and fatty acids amount in the sciatic nerves of female mice.

Group		Body weight (g)			Fatty acids amount (μg)		
		Initial	Final	Gain	C16:0	C18:0	C18:1
Female	Mean	24.9	26.4	1.4	64.1	82.2	177.2
ND	S.D.	2.0	2.3	0.6	5.8	7.6	5.8
Female	Mean	24.1	26.1	2.0	60.1	95.0*	191.4*
HFD	S.D.	1.8	1.7	1.9	6.7	5.8	9.9

ND = normal diet; HFD = high-fat diet, *$p < 0.05$ vs Female ND (Student *t*-test, n = 5).

ic nerves. The mRNA levels of *Srebp1*, *Fasn* and *Elvol6* were significantly elevated compared with ND (Fig. 1A, B, C) in the both males and females. *Scd1* mRNA expression tended to be increased in both sexes ($p = 0.06$ in males and $p = 0.08$ in females) (Fig. 1D). To determine the effects of HFD on apoptosis-related genes in the sciatic nerve, *Bax* and *Bcl2* mRNA were evaluated by qPCR analysis. In male mice, the mRNA levels of both *Bax* and *Bcl2* in HFD group were higher than in ND group. Conversely, in females, there was no significant difference between ND and HFD groups (Fig. 2).

The effects of HFD on the sciatic nerve of OVX mice

To determine whether estrogen was involved in the inhibitory effects of HFD feeding increased expression of apoptosis-related genes, OVX mice were fed ND or HFD with or without daily EE2 treatment for 1 week. There were no significant differences between the groups in body weight (Fig. 3A). Uterine weight was significantly decreased by OVX but was restored by E2 replacement; HFD had no effects on uterine weight (Fig. 3B). In OVX mice, the mRNA levels of both *Bax* and *Bcl2* in HFD group were higher than in the ND group (Fig. 3C, D). In contrast, in OVX mice treated with EE2, there was no significant difference between ND and HFD groups (Fig. 3C, D).

DISCUSSION

In our study, HFD feeding for 1 week had no effects on body weight similar to previous studies (Winzell and Ahrén, 2004; Gladding *et al.*, 2018). In the sciatic nerve of males however the apoptosis markers (*Bax* and *Bcl2* mRNA expression) were increased by HFD feeding for 1 week. Therefore suggesting that HFD feeding induced injury of sciatic nerves before weight gain in males.

In the sciatic nerves of both sexes, the amount of fatty acids was increased by HFD feeding, and HFD induces the increased expression of *Srebp1*, *Fasn*, and *Elvol6* genes in the sciatic nerve. SREBP1, a transcription factor, is a key regulation of *de novo* lipogenesis in the liver (Xu *et al.*, 2016). SREBP1 binds to the sterol regulatory element in the nucleus, and activates the transcription

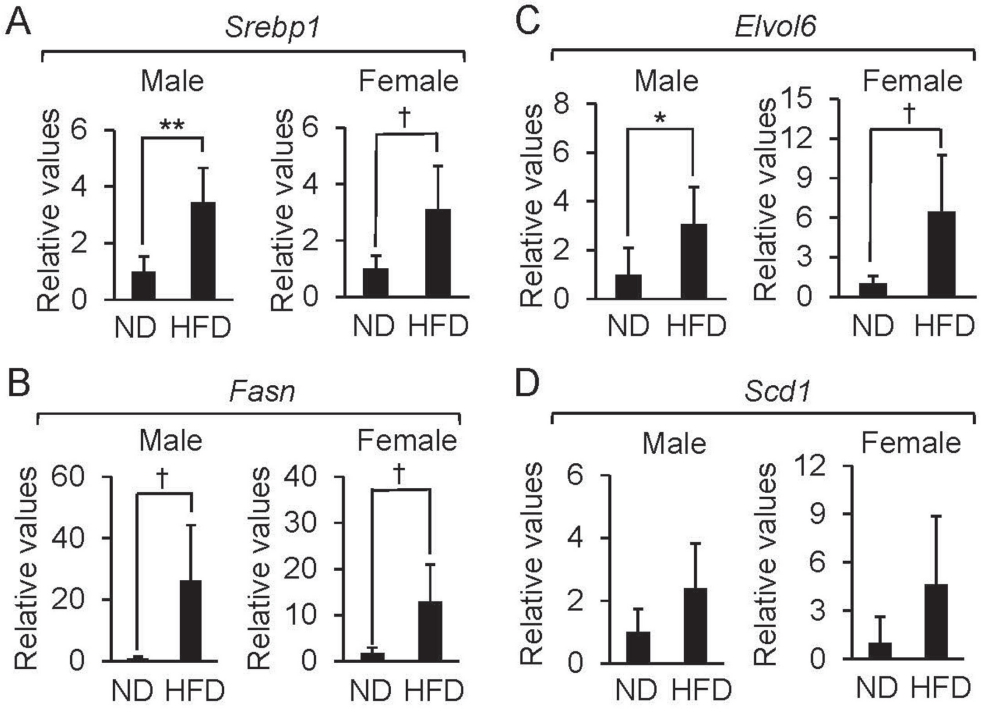

Fig. 1. The levels of fatty acid synthesis-related genes in sciatic nerves of mice fed high diet. Male and female mice were fed normal diet (ND) or high-fat diet (HFD) for 1 week. The mRNA levels of *Srebp1* (A), *Fasn* (B), *Elvol6* (C), and *Scd1* (D) were normalized with *Actb* mRNA level in sciatic nerves in males and females. Values are indicated as means S.D. (n = 5), and statistically significant differences are indicated by **; $p = 0.01$, *; $p = 0.05$ in Student's *t*-test or †; $p < 0.05$ in Aspin-Welch's *t*-test.

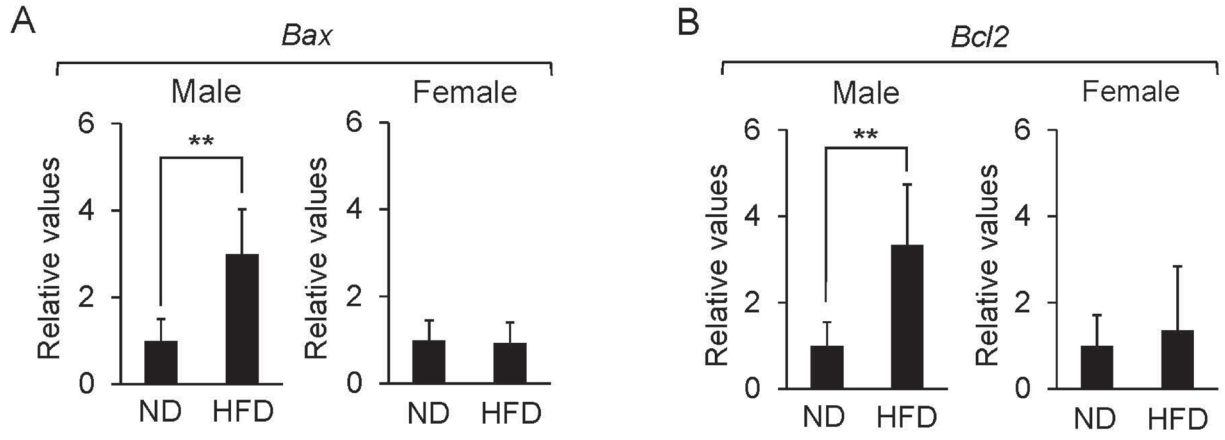

Fig. 2. The levels of apoptosis-related genes in sciatic nerves of mice fed high-fat diet. Male and female mice were fed normal diet (ND) or high-fat diet (HFD) for 1 week. The mRNA levels of *Bax* (A) and *Bcl2* (B) were normalized with *Actb* mRNA level in sciatic nerves in males and females. Values are indicated as means S.D. (n = 5), and statistically significant differences are indicated by **; $p = 0.01$ in Student's *t*-test.

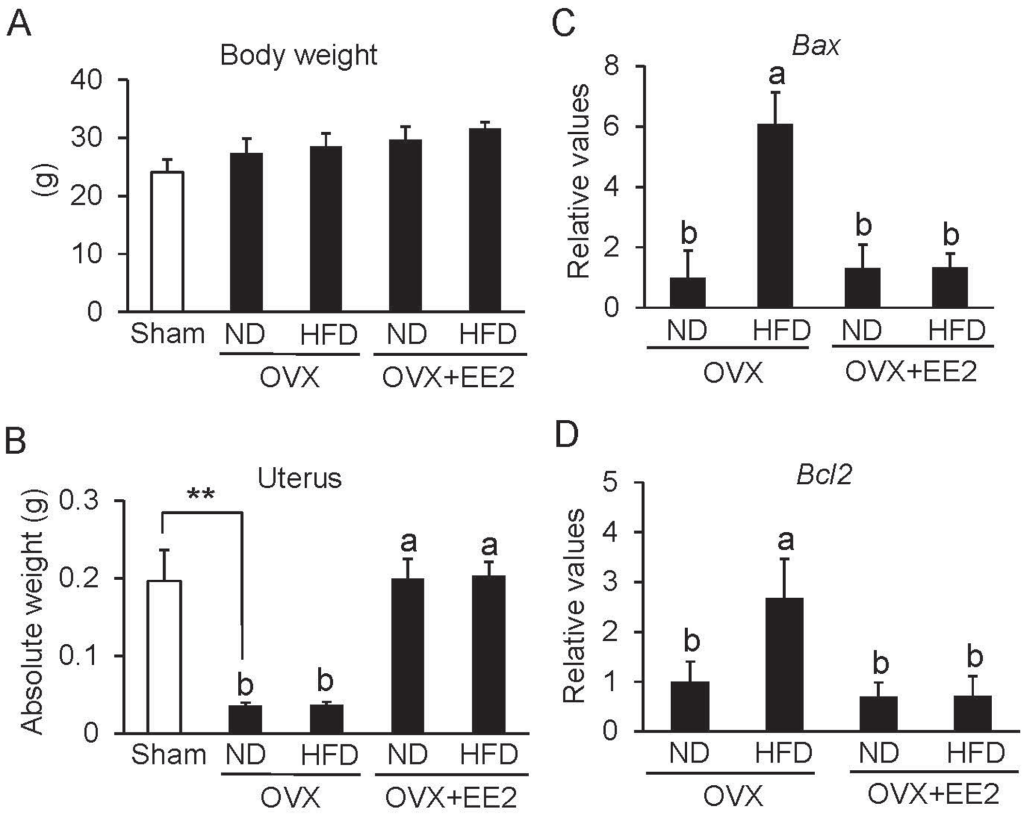

Fig. 3. The levels of apoptosis-related genes in the sciatic nerve of ovariectomized mice fed high-fat diet. OVX mice were fed normal diet (ND) or high-fat diet (HFD) with or without daily ethinylestradiol (EE2) treatment for 1 week. Sham-operated mice (Sham) fed normal diet for 1 week. The mRNA levels of *Bax* (A) and *Bcl2* (B) were normalized with *Actb* mRNA level in sciatic nerves in males and females. Values are indicated as means S.D. (n = 5), and statistically significant differences are indicated by different letter in Tukey-Kramer test.

of target genes, such as *Fasn*, *Elvol6* and *Scd1*, associated with fatty acid synthesis (Xu *et al.*, 2016). Several studies indicated that the lipid accumulation was caused by modulating the activation of SREBP1 signaling in hepatocytes isolated from HFD-fed rodent (Jung *et al.*, 2012; Jo *et al.*, 2014; Kim *et al.*, 2019). Therefore, suggesting that HFD feeding might induce *de novo* lipogenesis *via* the activation of SREBP1 signaling in the sciatic nerves similar to the liver.

In OVX females fed HFD, the levels of the apoptosis-related genes were increased compared to OVX mice fed ND. In contrast, EE2 replacement into OVX mice abolished the HFD-induced mRNA levels of two apoptosis-related genes. Several studies suggested that estrogen may ameliorate lipotoxicity-induced adverse effects in various cell types, such as hepatocytes and hippocampal astrocytes (Frago *et al.*, 2017; Galmés-Pascual *et al.*, 2020). We recently found that estrogen prevented fatty acid-induced cell death in neural cells (Ogawa *et al.*, 2020). This suggests that estrogen could have inhibitory effects on cell damage in the sciatic nerves, however the protective mechanisms induced by estrogen in sciatic nerves are still unknown.

HFD feeding increased the expression of apoptosis-related genes in the sciatic nerves of males, but not females. Male mice are more vulnerable than the females to the impacts of HFD on weight gain, metabolic alterations and deficits of learning, and hippocampal synaptic plasticity (Hwang *et al.*, 2010). In contrast, HFD feeding decreases cell proliferation and the number of immature neurons in the dorsal subregion of the hippocampus of females only (Robison *et al.*, 2020). HFD-induced lipotoxicity in the central nerves was less in females than in males (Morselli *et al*., 2016). Thus, the results so far are controversial. We would expect that estrogen attenuated lipotoxicity in the sciatic nerve of females, although further studies are needed to more closely examine the effects of HFD feeding on central nerves and peripheral nerves.

In conclusion, HFD feeding damaged the sciatic nerves of males only, although the accumulation of fatty acids in sciatic nerves was induced by HFD feeding in the both sexes. In females, estrogen attenuated HFD feeding damaged the sciatic nerves. Pathological findings in sciatic nerves were not observed under our study condition. The long-term HFD feeding study is considered necessary to investigate the pathological effects on sciatic nerves.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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