The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Letter

Dietary rapeseed (canola) oil suppresses testosterone production and increases plasma aldosterone level in stroke-prone spontaneously hypertensive rats (SHRSP)

Mai Nishikawa[1], Naoki Ohara[1], Yukiko Naito[2], Chihiro Amma[1], Yoshiaki Saito[3], Kenjiro Tatematsu[4], Jinhua Baoyindugurong[5], Daisuke Miyazawa[1], Yoko Hashimoto[6] and Harumi Okuyama[1,†]

[1]College of Pharmacy, Kinjo Gakuin University, 2-1723 Omori, Moriyama-ku, Nagoya, Aichi 463-8521, Japan
[2]School of Allied Health Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
[3]Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 257-8523, Japan
[4]Gifu Pharmaceutical University, 5-6-1 Mitabora, Gifu, Gifu 502-8585, Japan
[5]College of Food Science and Engineering, Inner Mongolia Agricultural University, Zhaowuda Rd. 306, Hohhot, Inner Mongolia, China, 010018
[6]School of Dentistry, Aichi-Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya, Aichi 464-8650, Japan

(Received December 21, 2021; Accepted December 28, 2021)

ABSTRACT — The present study was conducted to survey the influence of canola oil (CAN) ingestion on the steroid hormone production in stroke-prone spontaneously hypertensive rats (SHRSP). Male SHRSP were fed a diet containing 10 wt/wt% soybean oil (SOY, the control) or CAN as the sole dietary fat for 8 weeks. Plasma concentration of luteinizing hormone (LH) was similar in the 2 dietary groups. However, the plasma testosterone level in the CAN group, 1.36 ± 0.271 ng/mL, was lower than in the SOY group, 2.79 ± 0.514 ng/mL ($p < 0.05$, unpaired t-test; $n = 10$), and plasma concentration of aldosterone in the CAN group, 345 ± 79.6 pg/mL, was higher than in the SOY group, 159 ± 33.7 pg/mL ($p < 0.05$, unpaired t-test; $n = 10$). In the testis, the expressions of mRNA for StAR, CYP11A1, CYP17, 3βHSD and 17βHSD and the amounts of the corresponding proteins were significantly decreased. However, in the adrenal gland, the expressions of mRNA for StAR, CYP11A1, 3βHSD and CYP11B1 in the CAN group were not different from those in the SOY group, but the expression of mRNA and the amount of the corresponding protein for CYP11B2 were increased significantly in the CAN group. These findings are indicative of a peripheral, testicular toxicity of CAN. The decreased testosterone and the concomitantly increased aldosterone may play a role in the aggravation by CAN of the genetic diseases (i.e., metabolic syndrome-like complications) in male SHRSP.

Key words: Rapeseed (canola) oil, Testosterone, Aldosterone, Testicular toxicity, Stroke-prone spontaneously hypertensive rat (SHRSP)

INTRODUCTION

Rapeseed oil (canola oil, CAN) is known to shorten the survival of male stroke-prone spontaneously hypertensive rats (SHRSP) compared to animals given soybean oil (SOY, the control) (Huang *et al*., 1996, 1997; Miyazaki

Correspondence: Naoki Ohara (E-mail: oharan@kinjo-u.ac.jp)
[†]Deceased on 24 November 2021

et al., 1998; Ratnayake *et al*., 2000a, 2000b). The life-shortening by CAN is attributable to aggravation of the inherent diseases of SHRSP (i.e., metabolic syndrome-like conditions), including hypertension (Naito *et al*., 2003), increased plasma lipids (Ohara *et al*., 2006; Papazzo *et al*., 2011a), decreased antioxidant enzymes (Papazzo *et al*., 2011b); vascular injuries (Miyazaki *et al*., 1998) and thrombopenia (Ohara *et al*., 2006). On the other hand, CAN is reported to suppress testicular testosterone production in SHRSP compared to control animals given a diet containing SOY (Okuyama *et al.*, 2010). Testosterone deficiency has been known to cause hypertension and cardiovascular disease, which are included in the major symptoms of metabolic syndrome in both, men (Saad and Gooren, 2009; Kelly and Jones, 2013) and in rodents (Hermoso *et al*., 2020; Baik *et al*., 2020; Kelly *et al*., 2016). Since SHRSP, which exhibit a predisposition to the development of complications similar to metabolic syndrome, have been used as a model animal for metabolic syndrome (Hiraoka-Yamamoto *et al*., 2004; de Artiñano and Castro, 2009; Tanaka *et al*., 2013), the suppressed testosterone production by CAN may give rise to an early onset of hypertension and leads to life-shortening. It is unknown if CAN also decreases the testosterone level in men. However, considering those effects are induced by one of the most widely consumed oils in the dietary life of humans, the effects of CAN in SHRSP are worth investigating in more detail.

In addition to the suppression of testosterone production in male SHRSP (Okuyama *et al.*, 2010), CAN elevates plasma aldosterone in male Wistar rats (Ohara *et al*., 2008). These findings make us suspect the influence of CAN on the metabolic pathway of steroids. However, so far, there are no reports referring to the influence of CAN on steroid hormone production. Thus, the present study was conducted to survey the influence of CAN ingestion on the steroid hormone production. That is, the plasma levels of several steroids were measured and compared between male SHRSP given a diet containing CAN and those given a diet containing SOY. Plasma concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were also measured to investigate whether or not dietary CAN also affected the secretion of gonadotropins by the pituitary. The expressions of mRNA for StAR and the enzymes involved in the steroid synthesis pathways were determined in the testis and the adrenal gland. In addition, when the changes in the expressions of mRNA were significant, the amounts of proteins corresponding to the mRNAs were also determined.

Table 1. Fatty acid compositions (%) of in the diets.

Fatty acids		SOY	CAN
14: 0	Myristic acid	0.14	0.22
16: 0	Palmitic acid	9.01	4.37
16: 1	Palmitoleic acid	0.07	0.21
18: 0	Stearic acid	4.38	1.93
18: 1	Oleic acid	23.64	63.67
18: 2 n-6	Linoleic acid	54.50	19.56
18: 3 n-3	Linolenic acid	7.23	8.00
20: 0	Arachidic acid	0.39	0.59
20: 1	Eicosenoic acid	0.20	1.15
22: 0	Behenic acid	0.45	0.32
Total fatty acids (g/100 g diet)		9.88	9.22

SOY, 10 w/w% soybean oil diet (control); CAN, 10 wt/wt% canola oil diet.

MATERIALS AND METHODS

Diets

SOY and CAN were gifts from the Japan Oilseed Processors Association (Tokyo, Japan). A fat-free AIN-93G powder diet supplemented with 10 wt/wt% (24.8 energy percentage) SOY and the same diet containing, instead, 10 wt/wt% CAN were prepared in CLEA Japan (Tokyo, Japan). The fatty acid compositions of the diets are shown in Table 1.

Animal husbandry

Twenty male SHRSP (Izm), at 4 weeks of age were purchased from Japan SLC (Shizuoka, Japan). Two to three animals were housed per plastic cage, with a bedding of wood chips, and acclimatized for 7 days in a room with a conditioned temperature of 23 ± 1ºC and a relative humidity of 50 ± 5% and with lighting from 8:00–20:00. After acclimation, the animals were divided into 2 groups of 10 animals each. The animals in each group were given, *ad libitum*, the SOY diet or the CAN diet and tap water for 8 weeks. The diets were replaced with fresh ones every 3 days. The SOY diet was used as the control, since general rat chows contain SOY as the major fat ingredient, and the life-span of SHRSP fed a SOY diet is in the mid-range of that of SHRSP fed one of several different diets, each of which contained a single oil or fat as the sole dietary fat (Huang *et al*., 1996).

All the animals were used following the requirements set by the Committee for Ethical Usage of Experimental Animals in Kinjo Gakuin University.

Blood and tissue samples

At the end of the feeding period, the animals were anes-

thetized by sevoflurane (Fujifilm Wako, Osaka, Japan) inhalation, and blood was drawn from the abdominal vein using heparin sodium (Nacalai Tesque, Kyoto, Japan) as an anticoagulant. The blood samples were centrifuged at 150 x g for 15 min at 4ºC, and the plasma samples obtained were kept under −80ºC until use. After the blood sampling, the testes and the adrenal glands were removed. The left testis and bilateral adrenal glands from every animal were frozen in liquid nitrogen and stored under −80ºC until use for the determination of steroid contents in the testis, and for qRT-PCR and Western blotting in the testis and the adrenal glands. The right testes were fixed in 0.1 M phosphate buffer containing 10% formalin, embedded in paraffin, sectioned, stained and examined under a microscope. Plasma and testicular tissue levels of steroids were determined using all the animals. Plasma gonadotropin levels, the expressions of mRNA for StAR and enzymes involved in the steroid metabolism, the amounts of the corresponding proteins and Leydig cell numbers were examined in 6 out of 10 animals in each group.

Determination of plasma FSH and LH

Plasma concentrations of FSH and LH were measured by commercially available ELISA kits, E-EL-R0391 with a detection range of 3.31–200 ng/mL (Elabscience Biotechnology, Houston, TX, USA) and ENZ-KIT107 with a detection range of 0.14–32.9 ng/mL (ENZO Life Sciences, Lausen, Switzerland), respectively, following the instructions provided in the kits, and the results were obtained by subjecting 96-well plates to colorimetric detection at 450 nm.

Determination of steroids in plasma and testis

The concentrations of steroids were determined using a method described previously (Takagi *et al.*, 2010). Briefly, 2H- or 13C-labelled steroids were added to the plasma as internal standards, and steroids were extracted in diethyl ether. The standards used were as follows: testosterone-13C3 and aldosterone-d7 (IsoSciences, King of Prussia, PA, USA). The extracts were then purified using an InertSep pharma column (GL Sciences, Tokyo, Japan) and then derivatized with picolinyl ester. The resulting derivatives were placed in an InertSep SI column (GL Sciences), and the eluted fractions were injected into liquid chromatography-mass spectrometry/mass spectrometry (LC/MS/MS) equipment, comprising a Nexera UHPLC system (Shimadzu, Kyoto, Japan) and an API4000 mass spectrometer (SCIEX, Framingham, MA, USA).

Quantitative analysis of mRNA expression

The total RNA was extracted from the frozen testicular and adrenal tissues using Trizol™ reagent (Thermo Fisher Scientific, Tokyo, Japan) and chloroform-isopropanol with phase-separation by centrifugation. The concentration of the total RNA obtained was determined by absorbance at 260 nm using an Ultra-micro spectrophotometer NanoDrop™ 1000 (Thermo Fisher Scientific). Two μg of total RNA was reverse-transcribed to cDNA using a High Capacity RNA-to cDNA™ kit (Thermo Fisher Scientific). The cDNA obtained was diluted (1:2) and used with EagleTaq universal MMX™ (Roche Diagnostics, Tokyo, Japan) and a probe/primer kit, TaqMan Assay™ (Thermo Fisher Scientific). The target genes and TaqMan Assays™ used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Rn01775763_g1; steroidogenic acute regulatory protein (StAR), Rn00580695_m1; CYP11A1, Rn00568733_m1; CYP11B1, Rn02607234_g1; CYP11B2, Rn01767818_g1; CYP17, Rn00562601_m1; 3βHSD, Rn01789220_m1; 17βHSD, Rn00562601_m1.

Real-time PCR was performed using a 7300 Real-Time PCR System (Applied Biosystems-Thermo Fisher Scientific, Tokyo, Japan) programmed as follows: a 10-min holding period at 95ºC, followed by 40-cycle amplification for 15 sec at 95ºC, and 1-min at 60ºC. Expression values were calculated according to the ΔΔCT method. The expression of each mRNA relative to that for glyceraldehyde-3-phosphate dehydrogenase was compared between the 2 diets.

Western blotting

The expression of protein corresponding to every mRNA whose expression was significantly different between the 2 dietary groups was investigated by Western blotting. Aliquots of the tissues of the testes and adrenal glands were homogenized in 4 volumes of 10 mmole/L Tris-HCl buffer (pH 7.4) containing 137 mmole/L NaCl, 0.1 mmole/L EDTA, 20% glycerol and protease inhibitors cocktail (Thermo Fisher Scientific) using Ultla-Turrax™ (Ika, Staufen, Austria). The homogenates were centrifuged at 1,000 x g for 15 min at 4°C and the supernatants were obtained. The protein concentrations of the supernatants were determined by BCA protein assay reagents (Nacalai Tesque) and each sample was diluted to a protein concentration of 1 mg/mL. The sample was mixed with an equivalent volume of Laemmli buffer (pH 6.8), consisting of 62.5 M Tris, 2% SDS, 12% 2-mercaptoethanol, 25% glycerol and 0.01% bromphenol blue and denatured at 95°C for 5 min. A 15 μL aliquot of each sample was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane

using a Trans-Blot™ semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, Tokyo, Japan). The membrane was soaked in Bullet Blocking One™ (Nacalai Tesque) for 10 min at room temperature and washed in 20 mmole/L Tris-HCl buffer containing 137 mmole/L NaCl, 0.1% Tween 20 (TBS-T buffer, pH = 7.6). Then, the specimens were incubated in a primary antibody to ß-actin, diluted to 1: 5000~20000 (8457, Cell Signaling Technology, Danvers, MA, USA), GAPDH, diluted to 1: 2000 (sc-32233, Santa Curz Biotechnology, Dallas, TX, USA), StAR, diluted to 1: 100~500 (8449, Cell Signaling Technology), CYP11A1, diluted to 1: 1000 (AB1244, Sigma-Aldrich Merck, Tokyo, Japan), CYP17, diluted to 1:200~1000 (sc-66850, Santa Curz Biotechnology), 3βHSD, diluted to 1:1000 (sc-515120, Santa Curz Biotechnology), 17βHSD diluted to 1:100~200 (sc-32872, Santa Curz Biotechnology) or to CYP11b2 diluted to 1;1000 (PA-5-67694, Invitrogen, Thermo Fisher) overnight at 4°C. The membrane was rinsed in TBS-T buffer and incubated with a suitable secondary IgG conjugated to horseradish peroxidase (Goat Anti-Mouse IgG HRP Conjugate 71045 or Goat Anti-Rabbit IgG Antibody or HRP-conjugate 12-348, Sigma-Aldrich Merck) for 1 hr at room temperature or Simple Stain MAX-PO MULTI™ (Nichirei Biosciences, Tokyo, Japan) for 15 min at room temperature. Antibody binding to the membrane was visualized using Western Blot Hyper HRP Substrate (Takara Bio, Tokyo, Japan). A range of exposure times for each immunoblot assured that signal intensity was proportional to protein concentration. An Amersham AI680 lumino image analyzer (GE Healthcare, Tokyo, Japan) was used to quantify the blots and the values were normalized to the values of β-actin or GAPDH.

Leydig cell number

The Leydig cell number was examined in 6 animals for each dietary group. The right testis of each animal was fixed in 0.1 M phosphate buffered 10% formalin, and paraffin blocks were prepared, routinely. Sections, 4 μm in thickness, were cut from each block and immunohistochemically stained for calretinin in Leydig cells (Strauss *et al*., 1994; Augusto *et al*., 2002). Briefly, the sections were deparaffinized with xylene, dehydrated through graded ethanol solutions, and washed with phosphate buffered saline (PBS). The specimens were placed in citrate buffer at pH 6.0 and incubated for 20 min in a 95°C water bath to induce epitope retrieval. After washing with PBS, the specimens were covered with 2 drops of Blocking One Histo™ (Nacalai Tesque) for 15 min. After removing Blocking One Histo™ one drop of a primary antibody against calretinin (A9807 Abclonal, Tokyo, Japan) diluted to 1:50 in Immuno Shot™ (Takara Bio) was applied for 1 hr at room temperature. The specimens were rinsed with PBS containing 0.05% Tween 20 and endogenous peroxidase was blocked using methanol containing 3.3% hydrogen peroxide. Then the specimens were covered with 2 drops of an HRP-conjugated secondary antibody, Simple Stain MAX-PO MULTI™ (Nichirei Biosciences) and incubated for 30 min at room temperature. After washing with PBS containing 0.005% Tween20 the specimens were covered with one drop of 3,3'-diaminobenzidine, tetrahydrochloride, DAB solution (peroxidase stain DAB kit, Nacalai Tesque) and incubated strictly for 10~12 seconds. The specimens were washed with distilled water followed by PBS containing 0.005% Tween20, and nuclear staining was done using hematoxylin. The DAB-stained preparations were placed under a BZ X-800 microscope (Keyence, Osaka, Japan) and the total number of Leydig cells within 10 microscopic fields at a magnification of 200 were counted for each specimen.

Statistical methods

The results are presented as means ± S.E. The differences of the variables between the dietary groups were compared by unpaired t-test when the data sampled from two populations followed a Gaussian distribution or by Mann-Whitney test when the distribution was non-Gaussian. In all cases significance was set at $p < 0.05$. Prism v.9.10 (GraphPad Software, San Diego, CA, USA) was used for the statistical analyses.

RESULTS

Plasma FSH and LH

Plasma concentrations of FSH and LH are shown in Table 2. Neither of the gonadotropins was different between the SOY diet and the CAN group.

Steroids in plasma and testis

Plasma concentrations of pregnenolone, progesterone, corticosterone, 11-dehydrocorticosterone, deoxycorticosterone and dehydroepiandrosterone in the CAN group tended to be increased but were not significantly different from those in the SOY group. In contrast, the plasma concentrations of both, aldosterone and testosterone, were significantly different between the dietary groups (i.e., the aldosterone concentration was significantly higher and the testosterone concentration significantly lower, in the CAN group than in the SOY group). In the testis, the tissue concentrations of both, pregnenolone and testosterone, were significantly decreased in the CAN group compared to the SOY group. Plasma androstenedione and

Table 2. Plasma FSH and LH levels in male SHRSP given SOY diet or CAN diet for 8 weeks.

Gonadotropins	SOY	CAN	p value	Difference
FSH (pg/mL)	351 ± 19.2	347 ± 50.0	0.9366	n.s.
LH (ng/mL)	18.7 ± 1.88	19.5 ± 0.634	0.6831	n.s.

SOY, 10 wt/wt% soybean oil diet (control); CAN, 10 wt/wt% canola oil diet.
Values are means ± S.E. of 6 animals.
n.s., Group mean values are not significantly different (unpaired t-test).

Table 3. Plasma and testicular tissue concentrations of steroids in male SHRSP given SOY diet or CAN diet for 8 weeks.

Steroids	SOY	CAN	p value	Difference	
Pregnenolone (ng/mL plasma)	0.373 ± 0.0731	0.554 ± 0.175	0.3128	n.s.	
Progesterone (pg/mL plasma)	965 ± 299	1600 ± 816	0.4755	n.s.	
Corticosterone (ng/mL plasma)	305 ± 57.2	332 ± 52.0	0.7315	n.s.	
11-Dehydrocorticosterone (ng/mL plasma)	29.4 ± 5.25	33.4 ± 3.67	0.5402	n.s.	
Deoxycorticosterone (ng/mL plasma)	2.66 ± 0.840	3.67 ± 1.53	0.5700	n.s.	
Dehydroepiandrosterone (pg/mL plasma)	3.77 ± 1.79	2.61 ± 0.22	0.2241	n.s.	
Androstenedione (pg/mL plasma)	512 ± 94.3	301 ± 58.0	0.0729	n.s.	
Aldosterone (pg/mL plasma)	159 ± 33.7	345 ± 79.6	0.0433	s.d.	Mann-Whitney test
Testosterone (ng/mL plasma)	2.79 ± 0.514	1.36 ± 0.271	0.0247	s.d.	Unpaired t-test
Pregnenolone (ng/g testis)	36.4 ± 1.98	27.2 ± 3.20	0.0251	s.d.	Unpaired t-test
Dehydroepiandrosterone (ng/g testis)	1.63 ± 0.386	0.784 ± 0.172	0.0630	n.s.	
Testosterone (ng/g testis)	167 ± 28.1	92.2 ± 21.4	0.0492	s.d.	Unpaired t-test

SOY, 10 wt/wt% soybean oil diet (control); CAN, 10 wt/wt% canola oil diet.
Values are means ± S.E. of 10 animals.
s.d., Group mean values are significantly different ($p < 0.05$ by Man-Whitney test or unpaired t-test).
n.s., Group mean values are not significantly different.

testicular dehydroepiandrosterone concentrations in the CAN group tended to be lower than in the SOY group.

mRNA expression for proteins involved in steroid metabolism in the testis and the adrenal gland

In the testis, the expressions of mRNA for StAR, CYP11A1, CYP17, 3βHSD and 17βHSD in the CAN group were significantly depressed without exception compared to the SOY group (Fig. 1A). In the adrenal gland, the expressions of mRNA for StAR, CYP11A1 and 3βHSD in the CAN group tended to be increased but were not significantly different compared to those in the SOY group. Interestingly the expression of mRNA for CYP11B2 in the CAN group was significantly increased and 3 times as high as that in the SOY group. The expressions of mRNA for CYP11B1 in the 2 dietary groups were similar (Fig. 1B).

Expressions of proteins involved in steroid metabolism

The expression of every protein corresponding to mRNA whose expression was significantly different

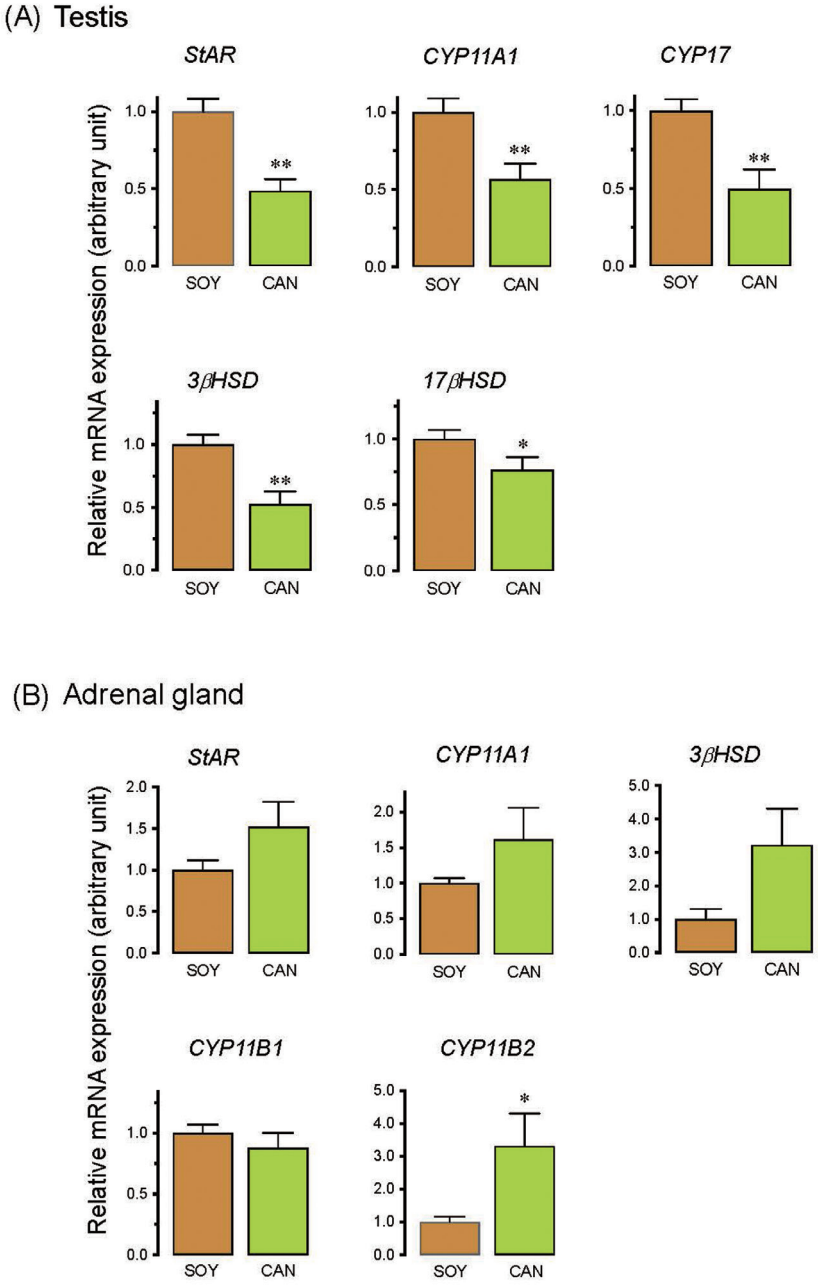

Fig. 1. Expressions of mRNA for StAR and enzymes involved in the steroid metabolism in male SHRSP given SOY diet or CAN diet for 8 weeks. (A) Testis, (B) Adrenal gland. SOY, 10 wt/wt% soybean oil diet (control) group; CAN, 10 wt/wt% canola oil group. *P < 0.05, **P < 0.01, significantly different from the values in the soybean oil group (unpaired t-test).

between the 2 dietary groups was investigated by Western blotting. The protein expressions of StAR, CYP11A1, CYP17, 3βHSD and 17βHSD in the testis in the CAN group were significantly depressed compared to those in the SOY group. In the adrenal gland, the expression of CYP11B2 in the CAN group was significantly increased compared to that in the SOY group (Fig. 2).

Leydig cell number

Leydig cell numbers were comparable in the 2 die-

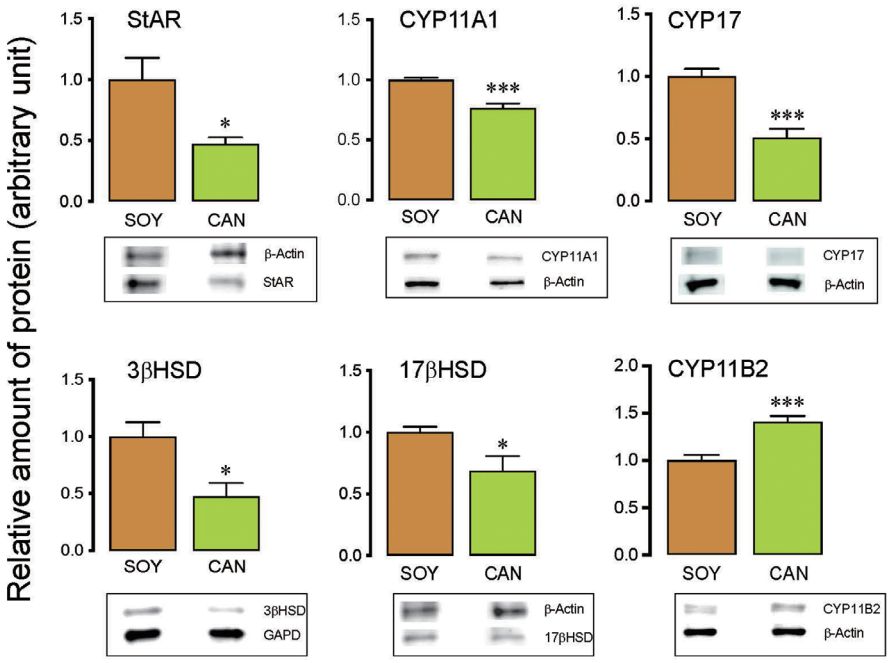

Fig. 2. Amounts of protein of StAR and enzymes involved in the steroid metabolism in the testis and the adrenal gland (CYP11B2) of male SHRSP given SOY diet or CAN diet for 8 weeks. SOY, 10 wt/wt% soybean oil (control) group; CAN, 10 wt/wt% canola oil group. *P < 0.05, ***P < 0.001, significantly different from the values in the soybean oil group (unpaired t-test).

tary groups (Fig. 3). The cell counts within 10 microscopic fields at a magnification of 200 were 1167 ± 36.6 and 1176 ± 34.7 in the SOY group and the CAN group, respectively (N = 6, p = 0.8519).

DISCUSSION

Male SHRSP given the CAN diet showed a significant overall suppression of the gene expression and protein production in the testis for StAR, CYP11A1, CYP17, 3βHSD and 17βHSD, all of which are responsible for testosterone production. In these animals, the concentrations of pregnenolone and testosterone in the testicular tissue were significantly decreased compared to those in the animals given the SOY diet. The decreased production of testosterone resulted in a significantly lower plasma testosterone level in the animals given the CAN diet. In contrast to the testis, the CAN diet did not affect the gene expression for StAR, CYP11A1or 3βHSD in the adrenal gland. Therefore, CAN appears to affect the 2 organs differently, although they both possess similar steroid metabolism pathways. The CAN effect may not involve central but only peripheral action (i.e., suppression of the steroid metabolism in the testis), because the decrease in testosterone level was not accompanied by changes in plasma LH concentration. Since Leydig cell numbers were comparable in the animals given the 2 different diets, the CAN-induced suppression might not to be due to organic or pathological changes but to a functional change in the Leydig cells.

Since StAR is the rate-determining protein in the steroid metabolism and responsible for the transport of cholesterol into mitochondria (Clark *et al*.,1994; Stocco and Clark, 1996; Christenson and Strauss, 2000), a shortage or a malfunction of StAR by CAN may result in the suppressed expressions of the enzymes (i.e., CYP11A1, CYP17, 3βHSD and 17βHSD), and in the suppressed production of the intermediate products which are regulated by these enzymes in the steroid metabolism pathway. Thus, StAR and transcription factors for StAR (Rust *et al*.,1998; Tremblay *et al*., 2002; Manna *et al*., 2003) cannot be ruled out as possible targets in CAN toxicity. However, even though cholesterol is the immediate precursor of pregnenolone in the steroid metabolism pathway in both, the testis and the adrenal gland, and the role of StAR is common to the pathways in both organs, CAN-induced suppression of the gene expressions of the molecules were found only in the testis. If StAR or a tran-

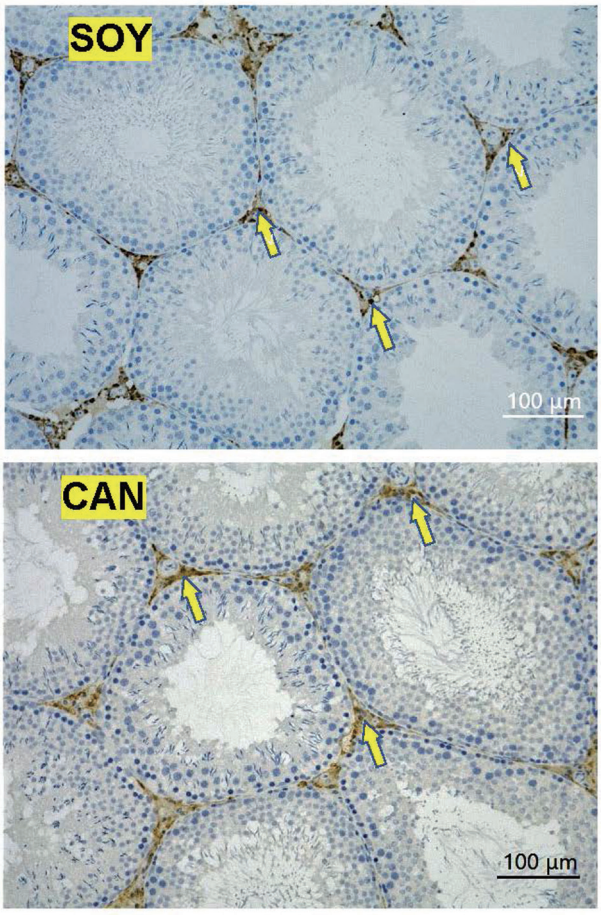

Fig. 3. Leydig cells in male SHRSP given SOY diet or CAN diet for 8 weeks. SOY, 10 wt/wt% soybean oil (control) group; CAN, 10 wt/wt% canola oil group. Yellow arrows, Leydig cells. Calretinin in Leydig cells was stained using an anti-calretinin primary antibody, an HRP-conjugated secondary antibody and DAB reagent.

scription factor for StAR is a target in CAN toxicity, the depressed gene expression for StAR should have been seen not only in the testis but also in the adrenal gland. Although concentrations of steroids in the adrenal gland were not determined in the present study, plasma concentrations of pregnenolone, progesterone, corticosterone, deoxycorticosterone and 11-dehydrocorticosterone, all of which might be associated with the amounts of the intermediate products in the pathway for gluco- and mineralocorticoid production in the adrenal glands, and the expressions of mRNAs for StAR, CYP11A1, CYP17 and 3βHSD in the adrenal gland in the CAN group were not decreased but, rather, tended to be increased compared to those in the SOY group. On the basis of these findings, the adrenal gland is not, but the testis is, likely to be a target organ of CAN toxicity.

In the CAN group, plasma aldosterone concentration was significantly greater than in the SOY group, and the expressions of mRNA and the protein for CYP11B2 in the adrenal gland in the CAN group were also increased significantly compared to the SOY group. Since there were no significant increases in precursors of aldosterone nor in the expressions of mRNA for the enzymes involved in producing the precursors upstream in the metabolic pathway, the increased plasma aldosterone along with the increased expressions of mRNA and the protein for CYP11B2 should have been induced by some stimulus from outside of the adrenal gland. One of the possible causes is an increased plasma renin level owing to the CAN-induced vascular injuries in the kidney. In fact, it was reported that an increased incidence of nephropathy with thickening in the arterioles was found in male SHRSP given a 10 wt/wt% CAN diet compared to those given a 10 wt/wt% SOY diet (Ohara *et al*., 2006). Moreover, severer renal injury accompanied by a significantly increased expression of mRNA for renin was found in male SHRSP after a 9-week ingestion of a diet containing 10% CAN as compared to animals given a 10% SOY diet (Miyazaki *et al*., 2000). Thus, increased renin release due to renal vascular injuries is a possible cause of the increased plasma aldosterone by CAN in male SHRSP.

The existence of another possible cause for the CAN-induced increase in plasma aldosterone concentration may be indicated by the fact that the CAN-induced decrease in testosterone was accompanied by an increase in aldosterone. It is possible that the decreased plasma testosterone directly inhibited aldosterone production in the adrenal gland. According to the report by Kau *et al*. (1999), plasma aldosterone level was elevated in orchiectomized rats, and the aldosterone level was restored by testosterone replacement. They also reported that in primary cultured rat zona glomerulosa cells, testosterone in the range of 10^{-9}–10^{-7} mol/L concentration-dependently inhibited aldosterone production. In the present study, the plasma testosterone concentration in the SOY group was approximately 0.97 x 10^{-9} mol/L, but that in the CAN group was 0.47 x 10^{-9} mol/L. These findings are suggestive of an inhibition by the CAN diet of the regulation via the suppression of testosterone production in the testis. In other words, plasma testosterone in male SHRSP may physiologically negatively regulate the production of aldosterone at the adrenal gland, and the CAN-induced decrease in testosterone would inhibit the negative regulation of aldosterone production. The testosterone deficiency is associated with metabolic syndrome in men and

induces metabolic syndrome-like symptoms in rodents (Hermoso *et al*., 2020; Baik *et al*., 2020; Kelly *et al*., 2016). In addition, SHRSP exhibits a predisposition to the development of complications similar to metabolic syndrome and is therefore used as a model animal of metabolic syndrome in men (Hiraoka-Yamamoto *et al*., 2004; de Artiñano and Castro, 2009; Tanaka *et al*., 2013). Thus, the suppressed testosterone production is a possible trigger for the early onset of metabolic syndrome-like symptoms and life-shortening in male SHRSP given the CAN diet.

In conclusion, it is demonstrated that the CAN diet decreases plasma testosterone concentration in male SHRSP, and the lowered testosterone level is accompanied by an increased plasma aldosterone level. The CAN diet suppresses the expressions of mRNA for StAR, CYP11A1, CYP17, 3βHSD and 17βHSD, the amounts of corresponding proteins and the intermediate products, which are regulated by those molecules in the steroid metabolism pathway. Since plasma luteinizing LH level was not affected, the decrease in testosterone with the suppression of the expressions of the molecules involved in the steroid metabolism may be due to the testicular toxicity of CAN. Although the relevancy between the 2 steroid hormones and the role of each one in the CAN-induced adverse events still remain to be elucidated, both steroids are presumed to be involved in the aggravation of metabolic syndrome-like conditions in male SHRSP.

ACKNOWLEDGMENTS

The authors are grateful to Mr. Nobuo Taguchi in Japan Oilseed Processors Association for providing the soybean and canola oils. The authors would also like to thank Mr. David L. Carleton in Cis Pacific Co., Ltd. for his help with the English text. This study was supported by the Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research (C) #26350133), Aichi Health Promotion Foundation and by Nagono Foundation for Promotion of Medical Sciences.

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

REFERENCES

Augusto, D., Leteurtre, E., De La Taille, A., Gosselin, B. and Leroy, X. (2002): Calretinin: a valuable marker of normal and neoplastic Leydig cells of the testis. Appl. Immunohistochem. Mol. Morphol., **10**, 159-162.

Baik, M., Jeong, J.Y., Park, S.J., Yoo, S.P., Lee, J.O., Lee, J.S., Haque, M.N. and Lee, H.-J. (2020): Testosterone deficiency caused by castration increases adiposity in male rats in a tissue-specific and diet-dependent manner. Genes Nutr., **15**, 14.

Christenson, L.K. and Strauss, J.F. III. (2000): Steroidogenic acute regulatory protein (StAR) and the intramitochondrial translocation of cholesterol. Biochim. Biophys. Acta, **1529**, 175-187.

Clark, B.J., Wells, J., King, S.R. and Stocco, D.M. (1994): The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). J. Biol. Chem., **269**, 28314-28322.

de Artiñano, A.A. and Castro, M.M. (2009): Experimental rat models to study the metabolic syndrome. Br. J. Nutr., **102**, 1246-1253.

Hermoso, D.A., Bizerra, P.F., Constantin, R.P., Ishii-Iwamoto, E.L. and Gilglioni, E.H. (2020): Association between metabolic syndrome, hepatic steatosis, and testosterone deficiency: evidences from studies with men and rodents. Aging Male, **23**, 1296-1315.

Hiraoka-Yamamoto, J., Nara, Y., Yasui, N., Onobayashi, Y., Tsuchikura, S. and Ikeda, K. (2004): Establishment of a new animal model of metabolic syndrome: SHRSP fatty (fa/fa) rats. Clin. Exp. Pharmacol. Physiol., **31**, 107-109.

Huang, M.Z., Naito, Y., Watanabe, S., Kobayashi, T., Kanai, H., Nagai, H. and Okuyama, H. (1996): Effect of rapeseed and dietary oils on the mean survival time of stroke-prone spontaneously hypertensive rats. Biol. Pharm. Bull., **19**, 554-557.

Huang, M.Z., Watanabe, S., Kobayashi, T., Nagatsu, A., Sakakibara, J. and Okuyama, H. (1997): Unusual effects of some vegetable oils on the survival time of stroke-prone spontaneously hypertensive rats. Lipids, **32**, 745-751.

Kau, M.M., Lo, M.J., Wang, S.W., Tsai, S.C., Chen, J.J., Chiao, Y.C., Yeh, J.Y., Lin, H., Shum, A.Y., Fang, V.S., Ho, L.T. and Wang, P.S. (1999): Inhibition of aldosterone production by testosterone in male rats. Metabolism, **48**, 1108-1114.

Kelly, D.M. and Jones, T.H. (2013): Testosterone: a metabolic hormone in health and disease. J. Endocrinol., **217**, R25-R45.

Kelly, D.M., Akhtar, S., Sellers, D.J., Muraleedharan, V., Channer, K.S. and Jones, T.H. (2016): Testosterone differentially regulates targets of lipid and glucose metabolism in liver, muscle and adipose tissues of the testicular feminised mouse. Endocrine, **54**, 504-515.

Manna, P.R., Eubank, D.W., Lalli, E., Sassone-Cors, P. and Stocco, D.M. (2003): Transcriptional regulation of the mouse steroidogenic acute regulatory protein gene by the cAMP response-element binding protein and steroidogenic factor 1. J. Mol. Endocrinol., **30**, 381-397.

Miyazaki, M., Huang, M.Z., Takemura, N., Watanabe, S. and Okuyama, H. (1998): Free fatty acid fractions from some vegetable oils exhibit reduced survival time-shortening activity in stroke-prone spontaneously hypertensive rats. Lipids, **33**, 655-661.

Miyazaki, M., Takemura, N., Watanabe, S., Hata, N., Misawa, Y. and Okuyama, H. (2000): Dietary docosahexaenoic acid ameliorates, but rapeseed oil and safflower oil accelerate renal injury in stroke-prone spontaneously hypertensive rats as compared with soybean oil, which is associated with expression for renal transforming growth factor-beta, fibronectin and renin. Biochim. Biophys. Acta, **1483**, 101-110.

Naito, Y., Nagata, T., Takano, Y., Nagatsu, T. and Ohara, N. (2003): Rapeseed oil ingestion and exacerbation of hypertension-related conditions in stroke prone spontaneously hypertensive rats. Toxicology, **187**, 205-216.

Ohara, N., Naito, Y., Nagata, T., Tatematsu, K., Fuma, S., Tachibana, S. and Okuyama, H. (2006): Exploration for unknown substances in rapeseed oil that shorten survival time of stroke-prone spontaneously hypertensive rats. Effects of super critical gas

extraction fractions. Food Chem. Toxicol., **44**, 952-963.

Ohara, N., Naito, Y., Nagata, T., Tachibana, S., Okimoto, M. and Okuyama, H. (2008): Dietary intake of rapeseed oil as the sole fat nutrient in Wistar rats --lack of increase in plasma lipids and renal lesions. J. Toxicol. Sci., **33**, 641-645.

Okuyama, H., Ohara, N., Tatematsu, K., Fuma, S., Nonogaki, T., Yamada, K., Ichikawa, Y., Miyazawa, D., Yasui, Y. and Homma, S. (2010): Testosterone-lowering activity of canola and hydrogenated soybean oil in the stroke-prone spontaneously hypertensive rat. J. Toxicol. Sci., **35**, 743-747.

Papazzo, A., Conlan, X. A., Lexis, L. and Lewandowski, P.A. (2011a): Differential effects of dietary canola and soybean oil intake on oxidative stress in stroke-prone spontaneously hypertensive rats. Lipids Health Dis., **10**, 98.

Papazzo, A., Conlan, X., Lexis, L. and Lewandowski, P. (2011b): The effect of short-term canola oil ingestion on oxidative stress in the vasculature of stroke-prone spontaneously hypertensive rats. Lipids Health Dis., **10**, 180.

Ratnayake, W.M., Plouffe, L., Hollywood, R., L'Abbé, M.R., Hidiroglou, N., Sarwar, G. and Mueller, R. (2000a): Influence of sources of dietary oils on the life span of stroke-prone spontaneously hypertensive rats. Lipids, **35**, 409-420.

Ratnayake, W.M., L'Abbé, M.R., Mueller, R., Hayward, S., Plouffe, L., Hollywood, R. and Trick, K. (2000b): Vegetable oils high in phytosterols make erythrocytes less deformable and shorten the life span of stroke-prone spontaneously hypertensive rats. J. Nutr., **130**, 1166-1178.

Rust, W., Stedronsky, K., Tillmann, G., Morley, S., Walther, N. and Ivell, R. (1998): The role of SF-1/Ad4BP in the control of the bovine gene for the steroidogenic acute regulatory (StAR) protein. J. Mol. Endocrinol., **21**, 189-200.

Saad, F. and Gooren, L. (2009): The role of testosterone in the metabolic syndrome: a review. J. Steroid Biochem. Mol. Biol., **114**, 40-43.

Stocco, D.M. and Clark, B.J. (1996): Regulation of the acute production of steroids in steroidogenic cells. Endocr. Rev., **17**, 221-244.

Strauss, K.I., Isaacs, K.R., Ha, Q.N. and Jacobowitz, D.M. (1994): Calretinin is expressed in the Leydig cells of rat testis. Biochim. Biophys. Acta, **1219**, 435-440.

Takagi, K., Miki, Y., Nagasaki, S., Hirakawa, H., Onodera, Y., Akahira, J., Ishida, T., Watanabe, M., Kimijima, I., Hayashi, S., Sasano, H. and Suzuki, T. (2010): Increased intratumoral androgens in human breast carcinoma following aromatase inhibitor exemestane treatment. Endocr. Relat. Cancer, **17**, 415-430.

Tanaka, S., Kojiguchi, C., Yamazaki, T., Mitsumoto, A., Kobayashi, D., Kudo, N. and Kawashim, Y. (2013): Altered fatty acid profile in the liver and serum of stroke-prone spontaneously hypertensive rats: reduced proportion of cis-vaccenic acid. J. Oleo Sci., **62**, 933-948.

Tremblay, J.J., Hamel, F. and Viger, R.S. (2002): Protein kinase A-dependent cooperation between GATA and CCAAT/enhancer-binding protein transcription factors regulates steroidogenic acute regulatory protein promoter activity. Endocrinology, **143**, 3935-3945.