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Dose- and time-related effects of caffeine on the testis in immature male rats

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Abstract: We previously showed that prepubertal chronic caffeine exposure adversely affected the development of the testes in male rats. Here we investigated dose- and time-related effects of caffeine consumption on the testis throughout sexual maturation in prepubertal rats. A total of 80 male SD rats were randomly divided into four groups: controls and rats fed 20, 60, or 120 mg caffeine/kg/day, respectively, via gavage for 10, 20, 30, or 40 days. Preputial separation was monitored daily before the rats were sacrificed. Terminal blood samples were collected for hormone assay, and testes were grossly evaluated and weighed. One testis was processed for histological analysis, and the other was collected to isolate Leydig cells. Caffeine exposure significantly increased the relative weight of the testis in a dose-related manner after 30 days of exposure, whereas the absolute testis weight tended to decrease at the 120 mg dose of caffeine. The mean diameter of the seminiferous tubules and height of the germinal epithelium significantly decreased in the caffeine-fed groups after 40 days of caffeine exposure, which was accompanied by a reduced BrdU incorporation rate in germ cells. In addition, caffeine intake significantly reduced *in vivo* and *ex vivo* testosterone production in a dose-related manner. Our results demonstrate that caffeine exposure during sexual maturation alter the testicular microarchitecture and also slow germ cell proliferation even at the 20 mg dose level. Furthermore, caffeine may act directly on Leydig cells and interfere with testosterone production in a dose-related manner, consequently delaying onset of sexual maturation.

Key words: caffeine, germ cell, Leydig cell, sexual maturation, testosterone

Introduction

The consumption of high caffeine-content energy drinks has increased markedly in recent years, and 28% of children and 31% of adolescents are reported to be regular consumers [16, 26]. Accordingly, cases of toxicity secondary to energy drink consumption increased more than 10-fold from 2005 to 2009 in the United States [23, 26]. Moreover, there is growing concern about a decrease in male reproductive health, because a number

of animal studies and human cases have pointed to toxic effects of caffeine on the male reproductive system [5, 7, 20, 21, 29]. For instance, a number of studies suggest that prenatal caffeine exposure impairs male gonadal development and thus later gonadal function [5, 21]. On the other hand, chronic caffeine intake had no adverse effects on male reproductive function in men and adult animal studies [15]. However, data on the effect of caffeine on pubertal development are relatively sparse, and most studies have focused on prenatal expo-

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sure. Cardiovascular responses and motor activity in response to the same dose of caffeine were different in boys from in men [30]. Considering that puberty is a period of rapid development of reproductive capacity, its vulnerability to insults seems to be greater than that in adults [27]. Indeed, our previous study in young rapidly growing rats established that chronic high-dose caffeine, when administered during sexual maturation, reduced testis weight and the secretory activity of Leydig cells [17].

The aim of the present study was to investigate the effects of a range of caffeine doses and exposure times on the onset of sexual maturation and the testis and to determine the lowest dose and duration that has negative effects on the testis in male rats. The *in vivo* and *ex vivo* testosterone secretory activity of the testis were also analyzed to determine the dose-related effects of caffeine. In addition, 5-bromo-2'-deoxyuridine (BrdU) incorporation in the testes was examined as a marker of germ cell proliferation.

Materials and Methods

Animal

Eighty immature male Sprague-Dawley rats were obtained at 17 days of age along with their mothers from Samtako Biokorea (Kyunggi, South Korea) and were allowed to acclimate under controlled humidity (40–50%), temperature (22–24°C), and light conditions (12-h light-dark cycle). Animal care was consistent with institutional guidelines, and the Hanyang University ACUC committee approved all procedures involving animals (HY-IACUC-2013-0110A). All animals were housed individually the day after weaning at 21 days of age and were fed standard rat chow *ad libitum*. The experiment was started when the rats were 22 days of age, as postnatal days (PD) 22–25 are considered the beginning of sexual maturation in rats [8].

Experimental design

Twenty animals were assigned to each of four groups based on their mean body weights to obtain an even distribution. Caffeine (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in distilled water (10 ml/kg) at concentrations calculated to deliver 20, 60, and 120 mg/kg body weight/day (designated as CF1, CF2, and CF3, respectively) and administered by gavage to ensure complete consumption of the established daily dose in the

morning (9 to 11 a.m.). The control group (CT) received distilled water. Five animals from each group were sacrificed on days 10, 20, 30, and 40 of the experiment. We chose 120 mg/kg as the highest dose because the effects of the high doses (120 and 180 mg/kg) used in our previous study on the testes in immature rats were similar [17]. The lowest dose chosen here (20 mg/kg), which was below the upper limit of safety for fetal development in an animal study [33], was comparable to human consumption of approximately two cups of coffee. Using body surface area (BSA) for dose conversion [22], the dosages employed in this study were equivalent to approximately 3.2, 9.6, and 19.4 mg/kg in humans.

Animals were examined for any treatment-related clinical signs and weighed on a daily basis, and food intake was also monitored. Body weight was measured to the nearest 0.1 g with an electronic scale (Dretec Corp., Seoul, South Korea) and recorded from the day before the start of feeding of caffeine until the end. Preputial separation (PPS) was checked daily throughout the experimental period, and the age when PPS was achieved was recorded. All the animals were killed 24 h after their last treatment, using established protocols and ethical procedures. Terminal blood samples were collected by heart puncture from the 40-day group, and the sera were stored at –70°C.

Weighing the testes

The testes were dissected and cleaned of fat and connective tissue. They were then weighed to the nearest 0.001 g with an electronic scale (Adventurer™ electronic balance, AR1530, OHAUS Corp., Parsippany, NJ, USA) and their morphology was grossly evaluated. Then, one testis from each male rat was fixed in 10% buffered formalin (pH 7), and the other was processed for cell preparation.

Histological analysis of testis

Immediately after removal, one testis from each animal was processed for paraffin embedding and sectioning. Serial sections of 5 µm thickness were taken from the midportion of the testis and stained with hematoxylin and eosin. All histomorphometric evaluations were performed by the same trained, calibrated, and blinded examiner using an image analysis system (Leica LAS software) coupled to a light microscope (DM4000B, Leica, Heidelberg, Germany) with final magnifications of ×100 or ×200. Four serial sections were traced for

each testis, and eight measurements per section were made of the number of seminiferous tubules within two defined regions (1.23 mm²) at 100-fold magnification; these measurements were combined to obtain a mean value per animal. For analyzing the diameters of the seminiferous tubules, we defined regions (0.30 mm²) ($\times 200$) that had round or nearly round cross sections of tubules as possible. Then, the longest diameter and its perpendicular diameter of each cross section of tubules were measured, and a mean value per animal was calculated from eight measurements per section. Also, the areas of the intertubular spaces within the same defined regions were analyzed, and mean values were calculated. The height of the germinal epithelium was obtained by subtracting the luminal diameter from the tubule diameter [9] for the same seminiferous tubules. For measuring the luminal diameter, a line was made along the luminal border of germinal cells within the same defined region as mentioned above, and two perpendicular diameters of the tubular lumens were measured to obtain a mean value per animal.

BrdU incorporation assay

To detect germ cell proliferation, BrdU-specific immunohistochemistry was performed. Animals were intraperitoneally injected with BrdU (100 mg/kg) (Sigma B5002) 24 h before they were sacrificed following 40 days of feeding of caffeine-containing distilled water or distilled water only, and one testis was prepared for sectioning. After embedding in paraffin, serial sections (5 μ m thickness) were obtained from the midportion of the testis. Dehydrated tissue slides were incubated in 2N HCl at 37°C for 30 min and washed twice with 0.2% Triton X in PBS (pH 7.4). They were then incubated with PBS containing 1% normal goat serum (NGS) for 1 h at room temperature, followed by incubation with an anti-BrdU antibody (Sigma, B2531) at a dilution of 1:500 in 1% NGS at 4°C overnight. They were washed with PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG (Life technologies, A11029) (diluted 1:200) for 1 h at 37°C. They were then washed again with buffer, and nuclear DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories, Inc., Burlingame, CA, USA). The number of BrdU-positive cells was counted with a fluorescence microscope (Leica, Heidelberg, Germany). Three serial sections were traced per animal, nine tubules per section, which had relatively increased numbers of BrdU-positive

cells, were selected at 200-fold magnification, and the average number of BrdU-positive cells per tubule was measured. Proliferation rate (%) was estimated as the percentage of BrdU-positive cells per tubule.

Preparation and primary culture of Leydig cells

To measure testosterone production *ex vivo*, one testis from each male rat exposed for 40 days was sliced into four pieces weighing approximately 50 to 100 mg each. Leydig cells were isolated and purified as previously described [10]. Briefly, the testis was minced under sterile conditions, and the tissue was then dissociated in buffer containing collagenase D (Roche Applied Science, Indianapolis, IN, USA), DNase I (Roche), and Dispase II (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min. Following digestion, the seminiferous tubules were allowed to settle, and the supernatant was removed. The tubules were then rinsed in DMEM/F12 and allowed to settle, and the supernatant was filtered through 100 μ m nylon mesh and centrifuged in Percoll gradient buffer. The resulting pellet, containing Leydig cells, was suspended in DMEM/F12, and the purity of Leydig cells was determined by staining of 3 β -HSD-positive cells as previously described [18]. Cells were resuspended in DMEM/F12 containing 2% BSA, placed in 24-well plates (3 $\times 10^5$ cells/well), and cultured with or without luteinizing hormone (200 ng/ml) (NIDDK, NIH, Baltimore, MD, USA) for 24 h at 37°C in a 5% CO₂ incubator. After 24 h, the medium was collected for testosterone assay, and cells were used for immunofluorescence detection of 3 β -HSD protein to ensure the purity of cultured Leydig cells again (Supplemental Fig. 1).

Testosterone measurement

Testosterone levels were analyzed in serum samples and in the conditioned media collected from the cultured Leydig cells using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Biotech Co., Ltd., Wuhan, China). The intra- and inter-assay coefficients of variance were less than 15%, and the limit of detection was 0.06 ng/ml under the conditions of our test. Absorbance was read at 450 nm within 15 min against a blanking well in an ELISA Reader (Bio-Rad, Hercules, CA, USA). All samples were run in duplicate.

Statistical analysis

Data for each group are expressed as means with stan-

Table 1. Effects of caffeine on body weight gains and food efficiency ratios

Experiment (days)		10	20	30	40		
Body weight gain (g)	CT	59.1 ± 5.21	162.6 ± 12.44	257.0 ± 24.4	341.4 ± 42.85		
	CF1	56.5 ± 4.38	158.0 ± 11.07	241.1 ± 22.03	332.0 ± 18.41		
	CF2	52.0 ± 1.60	149.9 ± 5.92*	238.7 ± 16.63	299.6 ± 30.38		
	CF3	48.7 ± 2.33* [†]	144.5 ± 9.14* [†]	201.4 ± 15.14* [†]	246.1 ± 46.31*		
Experiment (weeks)		1	2	3	4	5	6
FER	CT	0.50 ± 0.05	0.48 ± 0.04	0.33 ± 0.08	0.35 ± 0.02	0.24 ± 0.06	0.24 ± 0.01
	CF1	0.46 ± 0.08	0.47 ± 0.05	0.33 ± 0.07	0.33 ± 0.03	0.31 ± 0.03	0.23 ± 0.07
	CF2	0.44 ± 0.06*	0.48 ± 0.05	0.33 ± 0.08	0.29 ± 0.04*	0.29 ± 0.05	0.18 ± 0.02*
	CF3	0.43 ± 0.05*	0.41 ± 0.08* [†] [‡]	0.31 ± 0.05	0.25 ± 0.08* [†]	0.24 ± 0.10	0.20 ± 0.07

Values are given as means ± SD of five rats per group at each time point. FER, food efficiency ratio = weight gain (g/day)/food intake (g/day); body weight gain (g) = terminal body weight – initial body weight. CT, control; CF1, 20 mg caffeine; CF2, 60 mg caffeine; CF3, 120 mg caffeine. * $P < 0.05$ vs. CT. [†] $P < 0.05$ vs. CF1. [‡] $P < 0.05$ vs. CF2.

standard deviations (SD). All data were analyzed using SPSS ver. 10.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical significance was determined by Kruskal-Wallis one-way analysis of variance for multiple group comparisons and the Mann-Whitney *U*-test for two-group comparisons. Significance was accepted at $P < 0.05$.

Results

Body weight gain and food efficiency ratio

The body weights of the rats were controlled at the beginning of the experiment, and no difference between the groups was observed (CT, 58 ± 4.6 g; CF1, 57.7 ± 4.8 g; CF2, 57.7 ± 4.7 g; CF3, 57.9 ± 4.4 g). Throughout the experimental period, the dose levels induced no treatment-related clinical signs such as ungroomed appearance, decreased fecal output, altered fecal consistency, and excess salivation. The data are summarized in Table 1. The body weights of all animals increased greatly during the investigation period. The body weights of control animals increased approximately 2.2-, 3.9-, 5.4-, and 6.8-fold after 10, 20, 30, and 40 days, respectively. However, the caffeine-fed rats had lower body weight gains at all times during the experiment, the effects being dose- and time-dependent.

In order to investigate if the decreased weight gains were relevant to reduced food intake, average daily food intake was recorded. During the period of exposure to the caffeine, food consumption in all the caffeine-fed groups relatively decreased compared with that of the control (data not shown). Food efficiency ratios (FERs) were calculated by dividing the body weight gain by the food intake at one-week intervals. All the caffeine-fed groups had lower FERs than the controls, especially the

CF3 group. The CF2 and CF3 groups also showed decreases during the experiment.

Age at PPS

The progress of PPS (detachment of the prepuce from the glans penis) as an index of sexual maturation was examined daily from PD 30. Mean age at complete PPS was PD 52.3 ± 1.2 for the control, and those for CF1, CF2, and CF3 were PD 54.6 ± 1.5, PD 57.5 ± 2.4, and PD 58.5 ± 0.6, respectively (Fig. 1A). The delays were statistically significant in CF2 and CF3 compared with the control ($P < 0.05$).

Weights of the testes

The weights of the testes are summarized in Fig. 2. Absolute testis weight was slightly lower in CF3 than in the controls on and after 20 days of exposure, but we observed no differences between the control and the other caffeine-fed groups (Fig. 2A). Testis weight relative to body weight significantly increased in the caffeine-fed groups relative to the control, increasing in a dose-related manner on and after 30 days of exposure (Fig. 2B). These results show that the reductions in absolute testis weights were not proportional to the body weights. Similar data were obtained from an analysis of individual testes.

Histological findings in the testis

The histomorphometric parameters are summarized in Table 2. Representative testicular sections obtained from the animals after 40 days of exposure are shown in Fig. 3. Seminiferous tubule diameter increased progressively with age in all groups. There was a sharp increment in tubule diameter between 10 and 20 days, espe-

cially in the control. No treatment-related difference in tubule diameter was observed between the control and any of the caffeine-fed groups throughout the first 30 days of exposure, but a significant reduction was noted in CF2 and CF3 after 40 days ($P < 0.05$ vs. CT). In paral-

lel with the reductions in tubule diameter, the height of the germinal epithelium was also significantly reduced in CF2 and CF3 at 40 days ($P < 0.05$ vs. CT), and the density of seminiferous tubules but not intertubular areas was significantly higher in CF2 and CF3 at 40 days of exposure ($P < 0.05$ vs. CT). In addition, the seminiferous tubules in the caffeine-fed groups, especially CF2 and CF3, were of irregular size and distorted shape (Fig. 3).

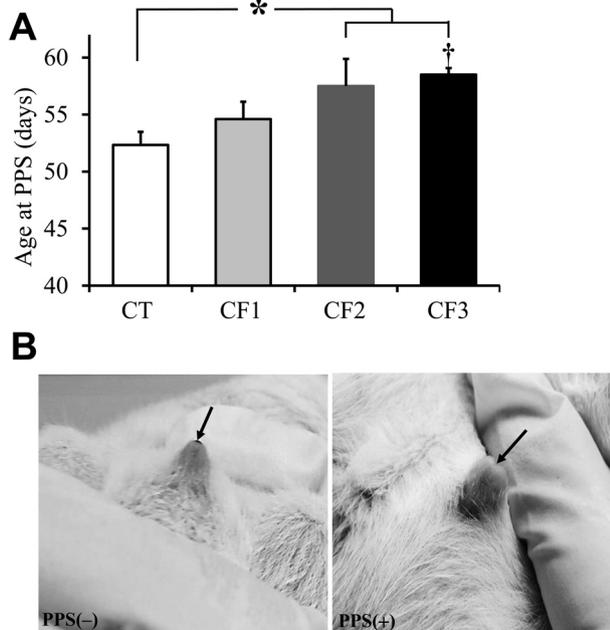


Fig. 1. Effect of caffeine on preputial separation in immature male rats. (A) Effect of caffeine exposure from weaning on the mean age at preputial separation (PPS). (B) Representative picture of a penis before (left) and after (right) PPS. CT, control; CF1, 20 mg caffeine; CF2, 60 mg caffeine; CF3, 120 mg caffeine. * $P < 0.05$ vs. CT. † $P < 0.05$ vs. CF1.

Germinal epithelial cell proliferation

To determine whether the changes in the microarchitecture of the testis in the caffeine-fed animals were related to epithelial cell proliferation, we measured BrdU incorporation in the basal epithelial layer of the seminiferous tubules after 40 days of exposure. As shown in Fig. 4, BrdU incorporation was variable between tubules even in the same section. We also found a similar trend in all sections examined. This most likely resulted from the difference in germ cell proliferation between tubules, and this needs to be investigated further. Here, nine tubules per section, which had a relatively increased number of BrdU-positive cells compared with others, were selected for the analysis. The percentage of BrdU-positive cells in the control group was $75.0 \pm 9.4\%$, and the percentage was significantly lower in the caffeine-fed groups, with the percentage decreasing in a dose-related manner (CF1, $62.5 \pm 12.2\%$; CF2, $52.9 \pm 11.1\%$; CF3, $48.4 \pm 5.5\%$) ($P < 0.01$ vs. CT; $P < 0.05$ vs. CF1; $P < 0.001$ vs. CF2).

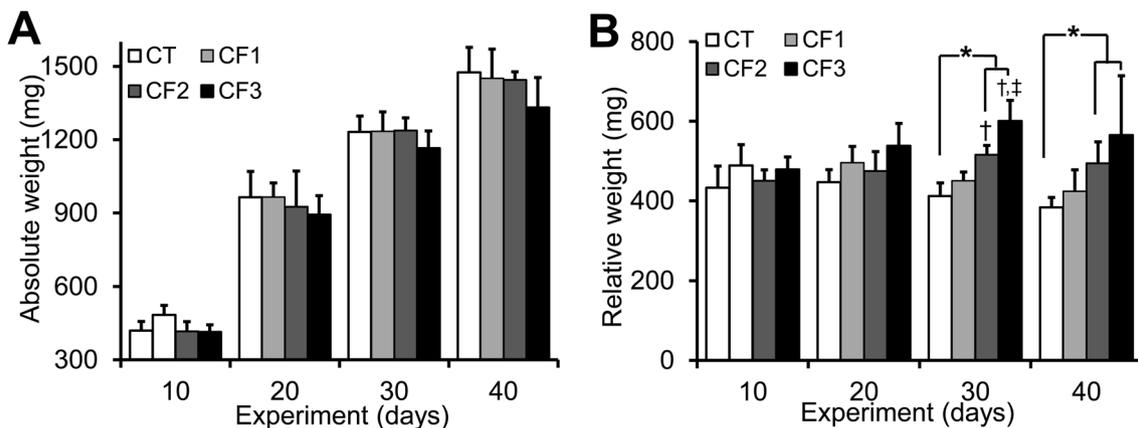


Fig. 2. Effect of caffeine on the weights of the testes. (A) Absolute testis weight (mg) and (B) testis weight relative to body weight (mg/100 g body weight) in the control and caffeine-fed groups at each designated time point. Values are expressed as the mean \pm SD. CT, control; CF1, 20 mg caffeine; CF2, 60 mg caffeine; CF3, 120 mg caffeine. * $P < 0.05$ vs. CT. † $P < 0.05$ vs. CF1. ‡ $P < 0.05$ vs. CF2.

Table 2. Histomorphometric parameters in the testes of the control and caffeine-fed groups

Experiment (days)		10	20	30	40
Seminiferous tubule diameter (μm)	CT	162.5 \pm 14.0	226.0 \pm 16.4	244.4 \pm 11.8	263.2 \pm 14.1
	CF1	176.9 \pm 16.0	223.8 \pm 17.6	240.5 \pm 16.3	274.3 \pm 15.9
	CF2	172.2 \pm 15.7	223.7 \pm 11.8	240.6 \pm 15.4	250.4 \pm 15.8* \ddagger
	CF3	175.9 \pm 16.2	223.1 \pm 12.0	246.6 \pm 15.5	250.9 \pm 13.5* \ddagger
Germinal epithelium height (μm)	CT	108.9 \pm 12.6	114.6 \pm 13.1	129.3 \pm 13.5	138.7 \pm 15.9
	CF1	108.6 \pm 16.0	118.8 \pm 17.6	123.9 \pm 14.9	147.6 \pm 15.6
	CF2	109.2 \pm 22.9	118.7 \pm 13.5	121.0 \pm 14.7	121.4 \pm 14.1* \ddagger
	CF3	116.0 \pm 15.3	115.8 \pm 11.2	129.3 \pm 13.8	119.0 \pm 12.6* \ddagger
Seminiferous tubule number	CT	229 \pm 9.0	90 \pm 9.2	83 \pm 1.9	64 \pm 6.6
	CF1	215 \pm 23.8	89 \pm 11.0	82 \pm 6.0	68 \pm 4.0
	CF2	219 \pm 19.6	87 \pm 5.8	84 \pm 10.1	74 \pm 4.2*
	CF3	209 \pm 24.2	93 \pm 1.8	79 \pm 5.7	76 \pm 3.9*
Intertubular area (mm^2)	CT	0.10 \pm 0.04	0.14 \pm 0.04	0.14 \pm 0.02	0.12 \pm 0.02
	CF1	0.11 \pm 0.02	0.14 \pm 0.03	0.12 \pm 0.02	0.11 \pm 0.02
	CF2	0.09 \pm 0.02	0.14 \pm 0.03	0.13 \pm 0.02	0.13 \pm 0.02
	CF3	0.09 \pm 0.02	0.12 \pm 0.03	0.13 \pm 0.02	0.12 \pm 0.01

Values are represented as means \pm SD. of five rats per group at each designated time point. Four serial sections were traced for each testis, and eight measurements per section were made of the number of seminiferous tubules within two defined regions (1.23 mm^2) at 100-fold magnification. Eight measurements per section were made for analyzing the diameter of seminiferous tubules or area of intertubular space within the same defined regions (0.307277 mm^2) at 200-fold magnification. CT, control; CF1, 20 mg caffeine; CF2, 60 mg caffeine; CF3, 120 mg caffeine. * P < 0.05 vs. CT. $\ddagger P$ < 0.05 vs. CF1. $\ddagger P$ < 0.05 vs. CF2.

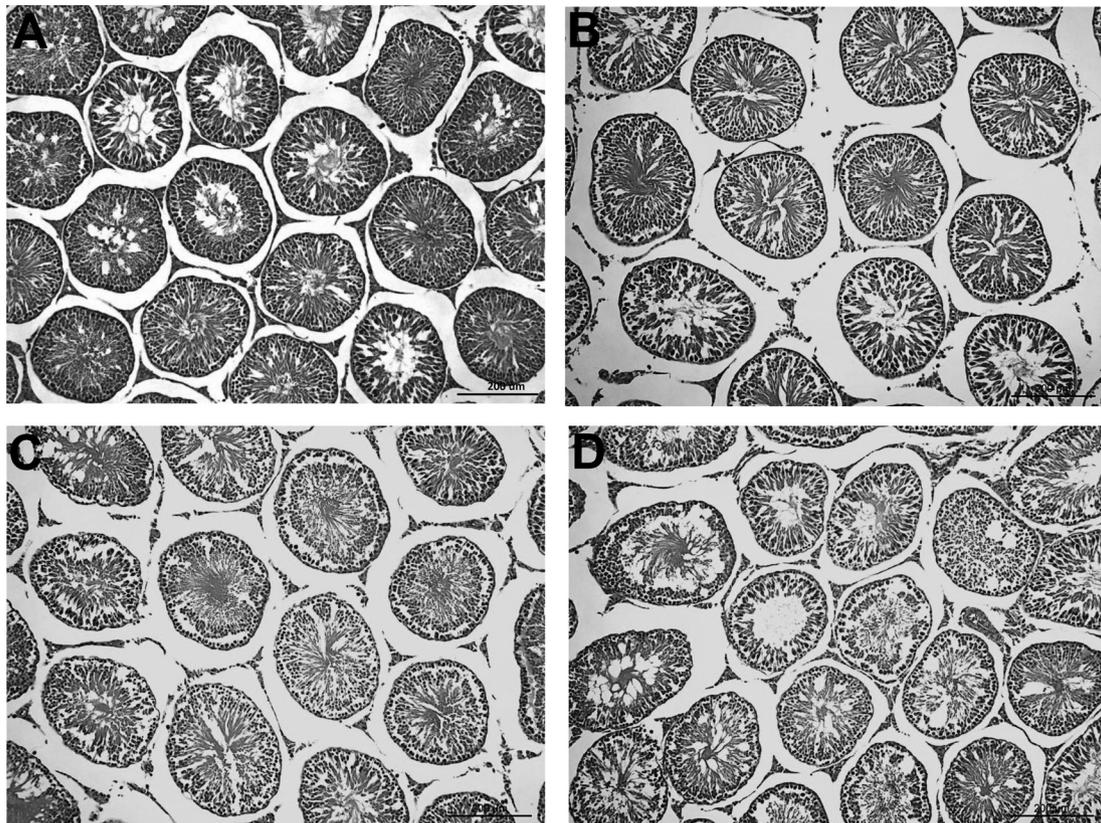


Fig. 3. Representative sections of the testes from the control and caffeine-fed groups at 40 days of caffeine exposure. The tissues were stained with hematoxylin and eosin. Rat testicular sections ($\times 100$) from the control (A) and caffeine-fed animals, CF1 (B), CF2 (C), and CF3 (D). Reductions in the diameter of seminiferous tubules and germinal epithelium height were evident in the high-dose caffeine-fed groups. In addition, loose interstitial tissue was observed in the caffeine-fed groups. CF1, 20 mg caffeine; CF2, 60 mg caffeine; CF3, 120 mg caffeine. Scale bar = 200 μm .

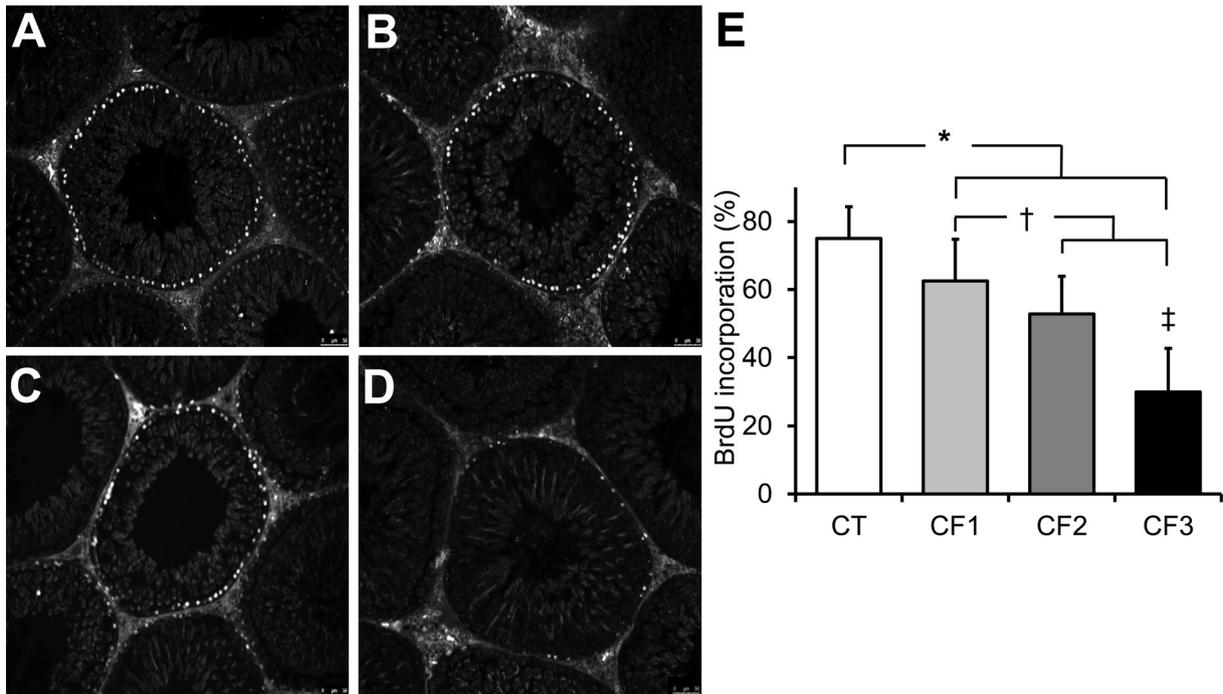


Fig. 4. Effects of caffeine on *in vivo* germinal epithelial cells proliferation at 40 days of caffeine exposure. Representative fluorescence confocal microscopy images ($\times 200$) of 5-bromo-2'-deoxyuridine (BrdU)-labeled germinal epithelial cells in the seminiferous tubules of control (A) and caffeine-fed animals, CF1 (B), CF2 (C), and CF3 (D). Cells were stained with BrdU antibody to visualize BrdU-labeled cells (green) and counterstained with DAPI to confirm nuclear status (blue). Scale bar=50 μm . (E) Proliferation indices of germinal epithelial cells of the control and caffeine-fed groups. The DNA labeling index was determined by scoring the number of BrdU-positive nuclei per total nuclei in the germinal epithelium. Three serial sections were traced per animal, nine tubules per section, which had relatively increased amounts of germ cells staining, were selected at 200-fold magnification, and the average number of BrdU-positive cells per tubule was measured. Proliferation rate (%) was estimated as the percentage of BrdU-positive cells per tubule. Data are expressed as the mean \pm SD of 5 rats per group. CT, control; CF1, 20 mg caffeine; CF2, 60 mg caffeine; CF3, 120 mg caffeine. * $P < 0.01$ vs. CT. † $P < 0.05$ vs. CF1. ‡ $P < 0.001$ vs. CF2.

Testosterone concentrations

Changes in testosterone have a substantial impact on the overall maturation of the reproductive organs [12]. In addition, because histological changes in the testes of the caffeine-fed groups were noted after 40 days of exposure, we analyzed the effects of caffeine consumption on the *in vivo* and *ex vivo* production of testosterone (Fig. 5). The serum levels of testosterone after 40 days of exposure are depicted in Fig. 5A, and they tended to be lower in the caffeine-fed groups, although a significant decrease was seen only in CF2 (CT, 25.03 ± 3.03 ng/ml; CF2, 7.02 ± 2.47 ng/ml) ($P < 0.05$ vs. CT). No significant effect was observed in CF3 because of great variability between individuals (19.38 ± 11.25 ng/ml) (Fig. 5A). Serum levels of testosterone may reflect the androgenic status of male rats, but they can be highly variable due to the release of large pulses of testosterone from the testes into the blood [28]. Therefore, we measured the

amounts of testosterone produced by primary Leydig cells retrieved from the animals after 40 days of exposure (Fig. 5B). Basal testosterone production was comparable in the control and all caffeine-fed groups, but a large increase in testosterone production in response to LH stimulation was only observed in the Leydig cells of the controls (an approximately 10-fold increase relative to the basal level).

Discussion

Our findings clearly demonstrate that prepubertal caffeine exposure interferes with sexual maturation including developmental changes in microarchitecture as well as secretory activity of the testis in a dose- and time-related manner and that these effects could occur after only a brief exposure at more than the 60 mg dose level. We are unaware of any reports in the literature examin-

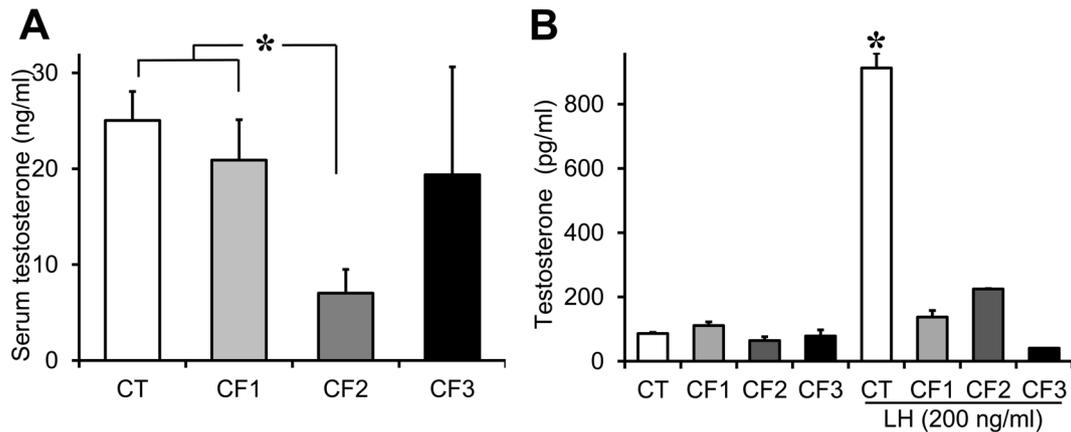


Fig. 5. Effects of caffeine on serum testosterone levels and Leydig cell testosterone production. (A) Serum concentration of testosterone in the control and caffeine-fed groups at 40 days of caffeine exposure. Data are presented as the mean \pm SD of five rats per group. CF1, 20 mg caffeine; CF2, 60 mg caffeine; CF3, 120 mg caffeine. * P <0.05 vs. CT or CF1. (B) Testosterone production in the presence or absence of LH (200 ng/ml) in cultured rat primary Leydig cells from control and caffeine-fed animals at 40 days of caffeine exposure. Data are presented as the mean \pm SD. LH, luteinizing hormone; CT, Leydig cells retrieved from control animals; CF1, CF2, and CF3, Leydig cells retrieved from caffeine-fed animals (20 mg, 60 mg, and 120 mg/kg/day). * P <0.01 vs. CT.

ing the effects of a range of caffeine doses for different times during the rapid growth and maturational period of reproductive organs.

The choice of dose levels was based on the literature and our previous studies [2, 17]. To achieve the typical developmental delays in gonad growth and to avoid sublethal effects, 120 mg/kg/day was used as the highest dose level in this study. The lowest dose (20 mg/kg) was comparable to the intake of approximately two cups of coffee [3]. Considering that most energy drinks provide more than 1 mg/ml of caffeine, one or two energy drinks can easily provide the above doses of caffeine.

The available experimental animal and human data support a possible effect of caffeine on body size [25, 29]. Similarly, our results indicated that caffeine decreased weight gain, with inhibitory effects noted after only 10 days of exposure (Table 1). In addition, caffeine-fed animals consumed less food on average than the controls (data not shown). There seemed to be a causal relationship between body weight and food consumption. However, the decreased FER observed in the caffeine-fed groups suggests that the reduced body weight was not simply due to the reduced food intake but was possibly due to altered metabolic activities resulting from caffeine consumption.

Usually, PPS in the male rat is assessed as the first visual sign of sexual maturation [11], and we also used this external sign as a measure of the onset of sexual

maturation. As shown in Fig. 1, caffeine exposure delayed PPS by more than 5 days, specifically in CF2 and CF3, compared with the control. Given the fact that there is no evidence for a critical body weight at the time of PPS [4], the relatively reduced body weight in the caffeine-fed groups may not have been a main contributor to delayed PPS. In fact, we did not observe a constant body weight at PPS (body weights at PPS were 335 ± 37 g in the control, 315 ± 13 g in CF1, 295 ± 34 g in CF2, and 249 ± 41 g in CF3).

As sexual maturation progresses, increases in testicular volume reflect both gonadotropin- and testosterone-mediated events, and the volume continues to increase until the testes reach adult size and shape [13]. To ensure an accurate assessment of the effects on male gonadal development, we fed caffeine during the male rat's juvenile (PD 21–35) and peripubertal (PD 35–55) periods [13]. Evidence from human reports and animal studies has raised concern that high caffeine intake may alter reproductive development. Indeed, prenatal caffeine exposure caused dose-related reductions in the testis weight of male offspring, reducing testis weight by 11.09% and 22.56% at low (25 mg/kg) and high dose (45 mg/kg), respectively [5]. However, treatment of adult male rats with caffeine (40 or 80 mg/kg/day, gavage) for 3 weeks did not have any negative effects on testis weight or fertility [32]. On the other hand, we previously showed that high doses of caffeine (120 or 180 mg/kg) reduce

the absolute testis weight in immature male rats [17]. Similar to this, absolute testis weight was slightly reduced in CF3 (Fig. 2A), but there was no difference between the control and the other dosage groups. Therefore, prepubertal caffeine exposure may cause a reduction in absolute testis weight at high doses, i.e., more than 120 mg/kg. On the other hand, increases in testis weight relative to body weight in the caffeine-fed animals (Fig. 2B) suggest that increases in testis weight during sexual maturation seem to be less dependent on body weight gain. Similarly, other researchers have reported that dose levels of 5, 20, and 100 mg/kg did not cause any differences in absolute testis weight in immature male rats but that increases in relative testis weight were observed beginning at the 20 mg dose level [29].

The weight of the testis is largely dependent on the mass of the differentiated spermatogenic cells [1, 14], and hence changes in testis weight in the caffeine-fed animals might be due to a decreased number of germ cells. In agreement with the above, caffeine treatment led to a reduction in germ cell proliferation, as shown in Fig. 4, and this most likely would lead to a decrease of spermatogenic cells, although it was not possible to include a sperm analysis in this study due to the age of the rats (PD 62). In general, coordinated and regular spermatogenic cycles begin after about 75 days of age in the rat [24]. Previous animal studies demonstrated that prenatal caffeine exposure inhibited differentiation of the seminiferous cords [20] and decreased the diameter of seminiferous tubules [5]. In addition, adult animal studies have reported similar changes in the seminiferous tubules [6], along with a breakdown of the germinal epithelium [7], although the doses and duration of caffeine treatment differed between studies. Based on the changes in testis weights between groups according to exposure duration (Fig. 2), changes in the seminiferous tubules could be expected, as shown in tissues obtained from animals fed 60 and 120 mg/kg after 40 days of exposure (Table 2, Fig. 3). In parallel with the reduced tubule diameter, significant decreases in germinal epithelium height were observed. On the other hand, tubule number increased in CF2 and CF3 compared with the control at 40 days of exposure. In general, each testicle has approximately 20 to 30 tightly coiled seminiferous tubules [24], and decreases in tubule diameter lead to reductions in testis weight. The absolute testis weights of CF2 or CF3 were not significantly different relative to the controls, even though the tubule diameter was

reduced. This might be explained by an increased number of tubules.

It has been variously suggested that caffeine treatment increases, decreases, or has no effect on serum testosterone depending upon the stage of development and upon the duration and dose of treatment. For instance, increased testosterone levels were reported in men and adult animals with high caffeine intake [6, 19, 25, 31], whereas maternal caffeine exposure led to a subsequent decrease of serum testosterone levels in male rat offspring [5, 21]. On the other hand, no clear effect was reported following treatment of immature male rats with any doses of caffeine (up to 100 mg/day) for 33 days, which corresponded to an age of 55 days [29]. The different results from our study may be related to differences in animal age, because serum testosterone levels vary with time, but there is less diurnal variation after 60 days of age in the male rat [28]. Thus, we chose serum samples obtained from animals at 40 days of exposure (corresponding to 62 days of age). As shown in Fig. 5A, caffeine-fed animals generally had reduced serum testosterone levels, but the level was highly variable between individuals, particularly in CF3, and this probably resulted from pulsatile release of this hormone from the testis into the bloodstream. To directly assess secretory activity of Leydig cells, testosterone levels were analyzed in *ex vivo* cultured Leydig cells. As shown in Fig. 5B, *ex vivo* testosterone productions induced by LH was markedly reduced in cells from the caffeine-fed animals even at the 20 mg dose level, demonstrating that the reduction in serum testosterone was due to impaired synthesis, possibly through modification of cell responsiveness to LH stimulation. Likewise, prenatal caffeine exposure significantly reduced testosterone biosynthesis in the fetal rat testes by inhibition of Leydig cell differentiation along with decreased steroidogenic enzyme activity [20]. On the other hand, *in vitro* cultured Leydig cells increased testosterone secretion in the presence of caffeine [29]. This may not reflect physiologic processes.

Our results demonstrate that caffeine exposure during sexual maturation alters the testicular microarchitecture and also slows germ cell proliferation even at the 20 mg dose level. Furthermore, caffeine may act directly on Leydig cells and interfere with testosterone production in a dose-related manner, consequently delaying onset of sexual maturation. As the lowest dose adopted in this study also affected parameters related to sexual maturation, further studies using a larger number of animals,

as well as females, are required to determine the minimal safe dose of caffeine.

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References

- Boockfor, F.R. and Blake, C.A. 1997. Chronic administration of 4-tert-octylphenol to adult male rats causes shrinkage of the testes and male accessory sex organs, disrupts spermatogenesis, and increases the incidence of sperm deformities. *Biol. Reprod.* 57: 267–277. [Medline] [CrossRef]
- Brent, R.L., Christian, M.S., and Diener, R.M. 2011. Evaluation of the reproductive and developmental risks of caffeine. *Birth Defects Res. B Dev. Reprod. Toxicol.* 92: 152–187. [Medline] [CrossRef]
- Butt, M.S. and Sultan, M.T. 2011. Coffee and its consumption: benefits and risks. *Crit. Rev. Food Sci. Nutr.* 51: 363–373. [Medline] [CrossRef]
- Cameron, J.L. 1991. Metabolic cues for the onset of puberty. *Horm. Res.* 36: 97–103. [Medline] [CrossRef]
- Dorostghoal, M., Erfani Majd, N., and Nooraei, P. 2012. Maternal caffeine consumption has irreversible effects on reproductive parameters and fertility in male offspring rats. *Clin. Exp. Reprod. Med.* 39: 144–152. [Medline] [CrossRef]
- Ezzat, A.R. and el-Gohary, Z.M. 1994. Hormonal and histological effects of chronic caffeine administration on the pituitary-gonadal and pituitary-adrenocortical axes in male rabbits. *Funct. Dev. Morphol.* 4: 45–50. [Medline]
- Friedman, L., Weinberger, M.A., Farber, T.M., Moreland, F.M., Peters, E.L., Gilmore, C.E., and Khan, M.A. 1979. Testicular atrophy and impaired spermatogenesis in rats fed high levels of the methylxanthines caffeine, theobromine, or theophylline. *J. Environ. Pathol. Toxicol.* 2: 687–706. [Medline]
- Hansson, L.I., Menander-Sellman, K., Stenström, A., and Thorngren, K.G. 1972. Rate of normal longitudinal bone growth in the rat. *Calcif. Tissue Res.* 10: 238–251. [Medline] [CrossRef]
- Ma, Y.H., Hu, J.H., Zhou, X.G., Mei, Z.T., Fei, J., and Guo, L.H. 2000. γ -aminobutyric acid transporter (GAT1) over-expression in mouse affects the testicular morphology. *Cell Res.* 10: 59–69. [Medline] [CrossRef]
- Klinefelter, G.R., Hall, P.F., and Ewing, L.L. 1987. Effect of luteinizing hormone deprivation in situ on steroidogenesis of rat Leydig cells purified by a multistep procedure. *Biol. Reprod.* 36: 769–783. [Medline] [CrossRef]
- Korenbrod, C.C., Huhtaniemi, I.T., and Weiner, R.I. 1977. Preputial separation as an external sign of pubertal development in the male rat. *Biol. Reprod.* 17: 298–303. [Medline] [CrossRef]
- Mann, T. 1974. Secretory function of the prostate, seminal vesicle and other male accessory organs of reproduction. *J. Reprod. Fertil.* 37: 179–188. [Medline] [CrossRef]
- Marty, M.S., Chapin, R.E., Parks, L.G., and Thorsrud, B.A. 2003. Development and maturation of the male reproductive system. *Birth Defects Res. B Dev. Reprod. Toxicol.* 68: 125–136. [Medline] [CrossRef]
- Mruk, D.D. and Cheng, C.Y. 2004. Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocr. Rev.* 25: 747–806. [Medline] [CrossRef]
- Nawrot, P., Jordan, S., Eastwood, J., Rotstein, J., Hugenholz, A., and Feeley, M. 2003. Effects of caffeine on human health. *Food Addit. Contam.* 20: 1–30. [Medline] [CrossRef]
- Oddy, W.H. and O’Sullivan, T.A. 2009. Energy drinks for children and adolescents. *BMJ* 339: b5268. [Medline] [CrossRef]
- Park, M., Choi, Y., Choi, H., Yim, J.Y., and Roh, J. 2015. High Doses of Caffeine during the Peripubertal Period in the Rat Impair the Growth and Function of the Testis. *Int. J. Endocrinol.* 2015: 368475. [Medline] [CrossRef]
- Payne, A.H., Downing, J.R., and Wong, K.L. 1980. Luteinizing hormone receptors and testosterone synthesis in two distinct populations of Leydig cells. *Endocrinology* 106: 1424–1429. [Medline] [CrossRef]
- Pollard, I. 1988. Increases in plasma concentrations of steroids in the rat after the administration of caffeine: comparison with plasma disposition of caffeine. *J. Endocrinol.* 119: 275–280. [Medline] [CrossRef]
- Pollard, I., Williamson, S., and Magre, S. 1990. Influence of caffeine administered during pregnancy on the early differentiation of fetal rat ovaries and testes. *J. Dev. Physiol.* 13: 59–65. [Medline]
- Ramlau-Hansen, C.H., Thulstrup, A.M., Bonde, J.P., Olsen, J., and Bech, B.H. 2008. Semen quality according to prenatal coffee and present caffeine exposure: two decades of follow-up of a pregnancy cohort. *Hum. Reprod.* 23: 2799–2805. [Medline] [CrossRef]
- Reagan-Shaw, S., Nihal, M., and Ahmad, N. 2008. Dose translation from animal to human studies revisited. *FASEB J.* 22: 659–661. [Medline] [CrossRef]
- Rockville, M.D. 2011. Emergency Department Visits Involving Energy Drinks. Network DAW.
- Russell, L. 1992. Normal development of the testis. *Pathobiology of the aging rat.* 395–405.
- Sarobo, C., Lacorte, L.M., Martins, M., Rinaldi, J.C., Moroz, A., Scarano, W.R., Delella, F.K., and Felisbino, S.L. 2012. Chronic caffeine intake increases androgenic stimuli, epithelial cell proliferation and hyperplasia in rat ventral prostate.

- Int. J. Exp. Pathol.* 93: 429–437. [Medline] [CrossRef]
26. Seifert, S.M., Schaechter, J.L., Hershorin, E.R., and Lipshultz, S.E. 2011. Health effects of energy drinks on children, adolescents, and young adults. *Pediatrics* 127: 511–528. [Medline] [CrossRef]
 27. Sharpe, R. 1988. Endocrinology and paracrinology of the testis. *Physiology and toxicology of male reproduction*. 71–102.
 28. Smith, E.R., Damassa, D.A., and Davidson, J.M. 1977. Feedback regulation and male puberty: testosterone-luteinizing hormone relationships in the developing rat. *Endocrinology* 101: 173–180. [Medline] [CrossRef]
 29. Tinwell, H., Colombel, S., Blanck, O., and Bars, R. 2013. The screening of everyday life chemicals in validated assays targeting the pituitary-gonadal axis. *Regul. Toxicol. Pharmacol.* 66: 184–196. [Medline] [CrossRef]
 30. Turley, K.R., Desisso, T., and Gerst, J.W. 2007. Effects of caffeine on physiological responses to exercise: boys versus men. *Pediatr. Exerc. Sci.* 19: 481–492. [Medline] [CrossRef]
 31. Wedick, N.M., Mantzoros, C.S., Ding, E.L., Brennan, A.M., Rosner, B., Rimm, E.B., Hu, F.B., and van Dam, R.M. 2012. The effects of caffeinated and decaffeinated coffee on sex hormone-binding globulin and endogenous sex hormone levels: a randomized controlled trial. *Nutr. J.* 11: 86. [Medline] [CrossRef]
 32. Whitby, K.E., Collins, T.F., Welsh, J.J., Black, T.N., O'Donnell, M., Gray, G.C., Green, S., and West, W.L. 1986. Reproduction study of caffeine administration to male Osborne-Mendel rats. *Food Chem. Toxicol.* 24: 277–282. [Medline] [CrossRef]
 33. Wink, C.S., Rossowska, M.J., and Nakamoto, T. 1996. Effects of caffeine on bone cells and bone development in fast-growing rats. *Anat. Rec.* 246: 30–38. [Medline] [CrossRef]