JBM J Bone Metab 2024;31(3):196-208
https://doi.org/10.11005/jbm.2024.31.3.196
pISSN 2287-6375 eISSN 2287-7029

Original Article

Effects of Psychogenic Stress Frequency during the Growth Stage on Oxidative Stress, Organ and Bone Development

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Received: October 27, 2023
Revised: May 10, 2024
Accepted: May 15, 2024

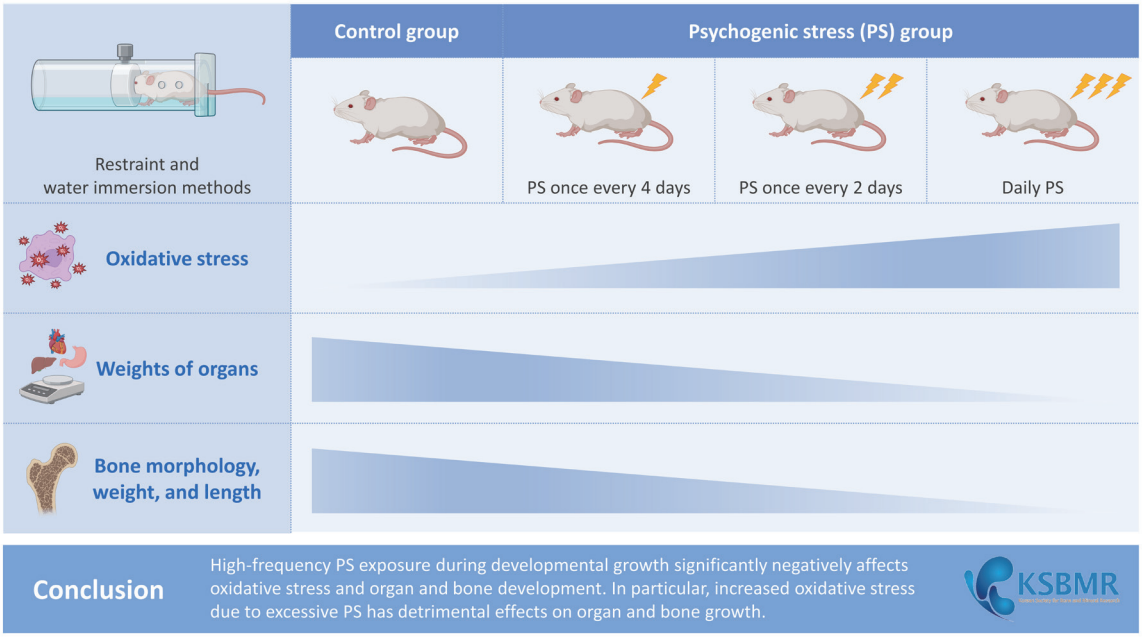
Background: This study aimed to examine the effects of psychogenic stress (PS) frequency on oxidative stress and organ development during growth and to gain fundamental insights into developmental processes during this period. **Methods:** Four-week-old male Wistar rats were randomly assigned to a control and three PS groups according to PS frequencies. PS was induced using restraint and water immersion techniques once daily for 3 hr at a time for a period of 4 weeks. **Results:** Oxidative stress increased with increasing PS frequency. The weights of organs other than the adrenal glands significantly decreased with increasing PS frequency, indicating growth suppression. Furthermore, bone morphology, weight, and length significantly decreased with increasing PS frequency. **Conclusions:** High-frequency PS exposure during developmental growth significantly negatively affects oxidative stress and organ and bone development. In particular, increased oxidative stress due to excessive PS has detrimental effects on organ and bone growth.

Key Words: Bone development · Oxidative stress · Stress, psychological

INTRODUCTION

Recently, complex changes in social organization and behavioral restrictions due to the coronavirus disease pandemic have aggravated various types of stress.[1-4] The incidence of diseases caused by psychogenic stress (PS) has increased in adolescents and teenagers. There are concerns regarding its ill-effects on health, growth, and development during growth spurts.[2,4-6] Stress leads to the loss of homeostasis and decline in the functioning of the endocrine system.[7,8] This is particularly evident in the brain and immune cells. It also negatively impacts various organs during the growth stage. Adrenal enlargement, thymic atrophy, and decreased renal weight and volume have also been reported.[9,10] Kidneys syn-

Graphical Abstract



thesize active vitamin D, and hence, they are important regulatory organs for bone metabolism. A decline in renal function promotes bone resorption owing to hypocalcemia and increased parathyroid hormone (PTH) activity.[11-13] Additionally, changes in steroid hormone levels, secreted from the adrenal cortex, affect the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system. This suppresses growth hormone levels, leading to a decrease in bone mass.[14,15]

Herrera-Covarrubias et al. [16] and Araujo et al. [17] demonstrated the stress-induced effects of hormones and suggested a link between stress and the endocrine system. In addition, these hormones are affected by oxidative stress, which fluctuates depending on the *in vivo* conditions such as stress and immune function.[17-19]

In summary, stress induces oxidative stress and hormonal imbalances related to biological functions. Thus, stress can negatively affect normal growth and biological functions. The peak bone mass is closely related to bone disease later in life. Therefore, investigating the effects of PS on the bones and associated organs during the rapid growth stages is necessary.[20,21]

The present study aimed to examine the effects of PS exposure on oxidative stress at the biochemical level during growth. Additionally, this study sought to determine the morphological effects of different frequencies of PS exposure on bone and organ development, with the objective of gaining insight into healthy growth and development.

METHODS

The animal experiments were conducted in accordance with the "Guidelines related to Animal Experimentation at the Aomori University of Health and Welfare (Approval no. 19001)."

1. Experimental animals

Four-week-old male Wistar rats (CLEA Japan, Inc., Tokyo, Japan) were randomly assigned to the control (C, N=6) and PS groups. To further examine the effects of PS frequency, rats were classified into the following three groups: PS once every 4 days (PS-I, N=6), PS once every 2 days (PS-II, N=6), and daily PS (PS-III, N=6). Throughout the rearing period, the room temperature was maintained at 22±2°C, humidity at 55±5%, and darkness maintained from 7 PM to 7 AM. During the rearing period, the experimental animals were fed on CE-2 (CLEA Japan, Inc.), and tap water

was freely provided.

2. Protocol of PS exposure

PS was administered using the restraint and water-immersion stress (RWIS) methods, as previously described by Sakamoto et al. [22] and Ohta et al. [23] PS was performed at the same room temperature and humidity conditions as in the usual animal care environment. PS was performed for 4 weeks, once daily for 3 hr. RWIS used small animal fixation adjusters with adjustable lengths to account for individual differences in body size. Rats were placed in the "rat fixation adjuster" that adjusts to two sizes of experimental rats (small, Φ31×20–80 mm; medium, Φ60×60–170 mm). The intake port at the front of the fixation adjuster was moved to the tail to the maximum setting, and the rats were restrained. Afterwards, the restrained rats were transferred to a water tank and submerged in 2 cm of water level.

3. Body weight and body length

Body weights were measured at the same time before the experiment and every week thereafter. On the final day of the experiment, three types of mixed anesthetics were injected intraperitoneally, and body and tail lengths were measured. The body was placed in the dorsal position and measured from the end of the snout to the anterior margin of the anus. The tail length was measured from the posterior margin of the anus to the tip of the tail.

4. Blood collection and anesthesia

Blood sample was collected from the caudal vein of the rats immediately before the start of the experiment and 4 week later after the end of the experiment. The experimental animals were immobilized using a rat fixation adjuster both before and after the experiment, and 1 mL of blood was collected from the tail vein, respectively. Blood was collected from the tail vein both before and after the experiment and centrifuged at 3,000 rpm for 10 min. Anesthesia was prepared with 25 mL of three types of mixed anesthetic agents (Medetomidine Hydrochloride 1.875 mL+Midazolam 2 mL+Butorphanol Tartrate 2.5 mL+Normal Saline 18.625 mL) and administered intraperitoneally (0.5 mL/BW100 g) after blood collection at the end of the experiment.

5. Biochemistry

The sampled blood was separated into plasma and blood cells. The oxidative stress and antioxidative capacity were then measured using Free Radical Analytical System 4 (FRAS4; H&D srl, Parma, Italy).[24,25] We referred to our previous studies for the explanation and methodologies utilized for the reactive oxygen metabolites-derived compounds (d-ROMs) and biological antioxidant potential (BAP) measurements.[24]

1) Oxidant stress

To measure d-ROMs, 10 μL of thawed plasma was placed into an Eppendorf tube containing acetate buffer with pH 4.8 and mixed by inversion. The mixture was then transferred to a cuvette containing dry chromogen and mixed by inversion. The new mixture was subsequently separated by centrifugation at a constant temperature of 37°C for 60 sec, placed into a photometer, and exposed to light at a wavelength of 505 nm for 5 min to determine the color change.

2) Antioxidant capacity

BAP measurement is a method for evaluating the reduction power of reducing substances in plasma. In total, 50 μL of iron (III) chromogen was mixed into a thiocyanate solution, producing a red color. The solution was then placed in a 505 nm photometer, and the ferric ion concentration was measured for 3 sec. Subsequently, 10 μL of plasma was mixed into this solution, and the color change was measured for 5 min at a constant temperature of 37°C. In addition, oxidation stress index (OSI) (BAP/d-ROMs; relative tolerance to oxidative stress) was calculated using BAP and d-ROMs.

6. Morphology

1) Organs

Subsequently, the chest was opened under anesthesia, and the animals were sacrificed by perfusion with saline solution through the left ventricle. The thymus, heart, spleen, liver, right and left adrenal glands, and kidneys were removed. The removed organs were immersed in 10% neutral buffered formalin solution and stored until analysis. For analysis, they were washed in saline, trimmed, and wiped dry before wet weight was determined. The organs were then oven-dried at 110°C for 24 hr (NDO-420;

https://doi.org/10.11005/jbm.2024.31.3.196

Tokyo Rikakikai Co., Ltd., Tokyo, Japan), and the dry weight was measured.[21]

2) Bones

The femur and tibia were wet-weighed after removing the muscle tissue and ligaments around bones and the water from bones surface. After measuring the wet weight of the bone, the maximum bone length (50% of the maximum bone length) was measured using an ABS Digimatic Caliper CD-20APX (Mitutoyo Co., Kanagawa, Japan) in 0.1 mm increments. The bone center was then cut vertically, and bone cross-sections were photographed using a digital camera and stored on a computer until further analysis. The photographed bones were oven-dried at 110°C for 24 hr (NDO-420; Tokyo Rikakikai Co., Ltd.), similar to that performed for the organs. The dry weights of bones were determined.[22]

7. Image analysis

The total cross-sectional area, dense bone area, and circumference were measured using WinROOF (Mitani Co., Tokyo, Japan), a general-purpose image-processing package for Windows, to analyze the cross-sectional areas of the central bone.

8. Statistical analyses

The mean and standard deviation were calculated using IBM SPSS Statistics version 27 software (SPSS Inc., Chicago, IL, USA), and relevant *t*-tests were employed to compare results obtained before and after the experiments for each group. One-way analysis of variance was used for comparisons among the four groups, followed by Scheffe's test for multiple comparisons. Pearson's correlation was employed to ascertain the relationship between oxidative stress on organs and bone morphology. The statistical significance level was set at a *P* value less than 0.05.

RESULTS

1. Body weight and length

Prior to the commencement of the experiment, no significant differences were observed in body weights among the four groups. However, a significant difference was observed from the second week of the experiment ($P<0.01$). Subsequently, significant differences were observed among the groups at the third week and at the end of the experiment ($P<0.001$ for each comparison). In comparison to the pre-experimental weight, the final weights of groups C, PS-I, PS-II, and PS-III exhibited a significant increase of 319.9, 262.5, 239.7, and 214.6%, respectively (all $P<0.001$) (Fig. 1).

Significant differences were observed in the total length and body length among the four groups at the end of the experiment ($P<0.001$). The total length and body length of group C were significantly lower than those of the other groups (all $P<0.001$) (Table 1).

2. Organs

1) Thymus

Compared to the wet and dry weights of the thymus gland in group C, those in the PS-III group significantly decreased by 27.8% and 23.5%, respectively (all $P<0.05$) (Table 2).

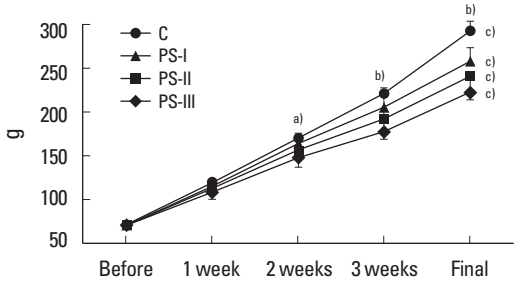


Fig. 1. Change in body weight. [a]$P<0.01$, [b]$P<0.001$ among the groups. [c]$P<0.001$ vs. before weight. C, control; PS, psychogenic stress.

Table 1. Body length (mm)

	C	PS-I	PS-II	PS-III	*P*-value
Total	385.7±7.34	360.8±8.01[a]	362.5±7.82[a]	350.7±5.89[a]	0.001
Body[b]	211.5±2.74	188.3±11.7[a]	191.3±4.97[a]	187.5±6.12[a]	0.001
Tail	174.2±8.35	172.5±6.12	171.2±6.62	163.2±10.01	0.111

The data is presented as mean±standard deviation. *P*-value obtained from one-way analysis of variance.
[a]$P<0.001$ vs. control.
[b]Tip of the nose to anal margin.
C, control; PS, psychogenic stress.

Table 2. Wet and dry weights of organ

	C	PS-I	PS-II	PS-III	*P*-value
Wet weight					
Thymus (mg)	581.9±95.07	524.0±54.20	454.3±75.94	420.3±72.83[a]	0.007
Spleen (mg)	912.1±96.42	815.0±128.3	657.3±55.11[c],[d]	587.5±35.60[c],[e]	0.001
Adrenal gland (mg)					
Right	25.21±1.769	26.08±2.128	25.62±1.859	26.46±1.994	0.706
Left	26.09±1.993	26.47±2.132	26.22±2.200	28.00±2.855	0.472
Kidney (g)					
Right	1.567±0.037	1.318±0.108[c]	1.316±0.102[c]	1.120±0.077[c],[e],[f]	0.001
Left	1.440±0.097	1.257±0.156	1.249±0.069	1.143±0.177[b]	0.008
Heart (g)	1.088±0.043	1.022±0.069	1.040±0.064	0.960±0.057[a]	0.010
Liver (g)	16.17±0.997	13.93±0.969[c]	12.97±0.603[c]	12.03±0.460[c],[e]	0.001
Dry weight					
Thymus (mg)	89.67±13.80	81.41±10.26	71.50±12.85	68.62±10.16[a]	0.023
Spleen (mg)	170.8±16.89	150.8±25.47	123.0±10.94[c]	113.8±7.00[c],[e]	0.001
Adrenal gland (mg)					
Right	5.373±0.592	5.713±0.580	5.610±0.371	6.057±0.378	0.147
Left	5.543±0.357	5.628±0.619	5.603±0.622	6.237±0.402	0.099
Kidney (g)					
Right	0.246±0.019	0.230±0.016	0.209±0.012[b]	0.198±0.009[c],[e]	0.001
Left	0.245±0.012	0.228±0.015	0.206±0.009[c]	0.203±0.016[c],[d]	0.001
Heart (g)	0.197±0.005	0.188±0.008	0.182±0.008	0.181±0.009	0.168
Liver (g)	3.608±0.222	2.963±0.207[c]	2.811±0.115[c]	2.600±0.098[c],[d]	0.001

The data is presented as mean±standard deviation. *P*-value obtained from one-way analysis of variance.
[a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ vs. control. [d]$P<0.05$, [e]$P<0.01$ vs. PS-I. [f]$P<0.01$ vs. PS-II.
C, control; PS, psychogenic stress.

2) Spleen

The spleen wet weights were significantly lower in groups PS-II and PS-III than in group C by 27.9% and 35.6%, respectively (all $P<0.001$). Compared to the spleen wet weight in the PS-I group, those in the PS-II and PS-III groups were significantly decreased by 19.3% ($P<0.05$) and 27.9% ($P<0.01$), respectively. In contrast, the spleen dry weight was significantly lower in the PS-II and PS-III groups than in group C by 28.0% and 33.4%, respectively (all $P<0.001$). It was significantly lower in the PS-III group than in the PS-I group by 24.5% ($P<0.01$) (Table 2).

3) Adrenal gland

No significant differences were observed in the wet and dry weights of the right and left adrenal glands among the groups (Table 2).

4) Kidney

The wet weight of the right kidney was significantly lower in the PS-I and PS-II groups than in group C by 15.9% and 28.7%, respectively (all $P<0.001$). The wet weights of the right kidney in the PS-I and PS-II groups were significantly higher than in the PS-III group by 15.2% (all $P<0.01$). Dry weights of the right kidney was lower by 15.0% ($P<0.01$) and 19.5% ($P<0.001$) in the PS-II and PS-III groups, respectively, than in group C. The PS-III group had a significantly lower dry weight (5.3%; $P<0.01$) than the PS-I group.

The wet weight of the left kidney was significantly lower in the PS-III group than in group C by 20.8% ($P<0.01$). In contrast, the dry weights of the left kidney were significantly lower in the PS-II and PS-III groups than in group C by 15.9% and 17.1%, respectively (all $P<0.001$). It was significantly lower in the PS-III group than in the PS-I group by 5.3% ($P<0.05$) (Table 2).

5) Heart

The wet weight of the heart was significantly lower by

https://doi.org/10.11005/jbm.2024.31.3.196

11.9% in the PS-III group than in group C ($P<0.05$) (Table 2).

6) Liver

The wet weights of the liver were significantly lower in the PS-I, PS-II, and PS-III groups than in group C by 14.2%, 19.8%, and 25.9%, respectively (all $P<0.001$). It was significantly lower in the PS-III group than in the PS-I group by 13.7% ($P<0.01$). In contrast, the dry weights of the liver were significantly lower in the PS-I, PS-II, and PS-III groups than in group C by 18.0%, 22.2%, and 28.0%, respectively (all $P<0.001$). It was significantly lower in the PS-III group than in the PS-I group by 12.2% ($P<0.05$) (Table 2).

7) Bones

(1) Weight

The wet weights of the femurs were significantly lower in the PS-I group 7.5% ($P<0.05$), PS-II group 11.8% ($P<0.001$), and PS-III group 17.9% ($P<0.001$) than in group C. The PS-III group had a significantly lower wet weight (11.2%; $P<0.001$) than the PS-I group. Moreover, the wet weights of tibias were 11.1% ($P<0.01$) and 16.5% ($P<0.001$) lower in the PS-II and PS-III groups, respectively, than in group C (Fig. 2).

The dry weights of femurs were significantly lower in the PS-I, PS-II, and PS-III groups than in group C by 10.7%, 12.9%, and 18.5%, respectively ($P<0.001$). The femur dry weight in the PS-III group was significantly lower than that in the PS-I group by 8.6% ($P<0.05$). In contrast, the tibia dry weight was significantly lower in the PS-1 group by 8.6% ($P<0.01$), PS-II group by 10.6% ($P<0.001$), and PS-III group by 15.7% ($P<0.001$) than in group C. The tibia dry weight was significantly lower in the PS-III group than in the PS-I group (7.8%; $P<0.05$) (Fig. 2).

(2) Length

Femurs were significantly shorter in the PS-I, PS-II, and PS-III groups than in group C by 4.5%, 5.9%, and 7.0%, respectively (all $P<0.001$). The femur was significantly shorter in the PS-III group than in the PS-I group by 2.9% ($P<0.01$). In addition, the tibias were significantly shorter in the PS-I, PS-II, and PS-III groups than in group C by 2.6% ($P<0.01$), 4.3% ($P<0.001$), and 7.2% ($P<0.001$), respectively. Tibias were significantly longer in PS-I and PS-II than in PS-III by 4.6% and 3.0%, respectively (all $P<0.001$) (Fig. 3).

(3) Circumference

The PS-III group had a significantly lower bone circumference of the femur than group C by 8.5% ($P<0.01$) (Fig. 3).

(4) Cross-sectional area

The total cross-sectional area of the central half of the femur was significantly lower in the PS-III group than in groups C (12.6%) and PS-I (11.1%, $P<0.05$). The cross-sectional area of the dense bone was significantly lower in the PS-III group than in group C (20.8%, $P<0.01$) (Fig. 3).

3. Oxidative stress and antioxidant capacity

No significant difference was observed in the pre-experimental d-ROMs, BAP, and OSI among the four groups. A post-experimental comparison among the four groups revealed that d-ROMs were significantly lower in the C Group than in the PS-III Group, (33.7%, $P<0.001$), PS-I Group

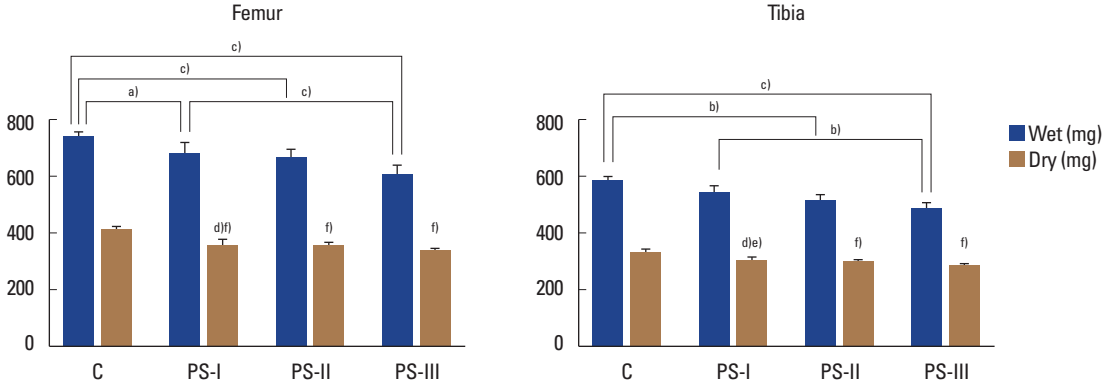


Fig. 2. Weight of bones wet and dry. The psychogenic stress (PS) affected bone weight, with more pronounced growth retardation in the femur, which has greater bone mass. In addition, PS showed different effects on bone mass depending on the frequency of PS. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$. [d]$P<0.05$ vs. PS-III. [e]$P<0.01$ vs. control. [f]$P<0.001$ vs. control. C, control.

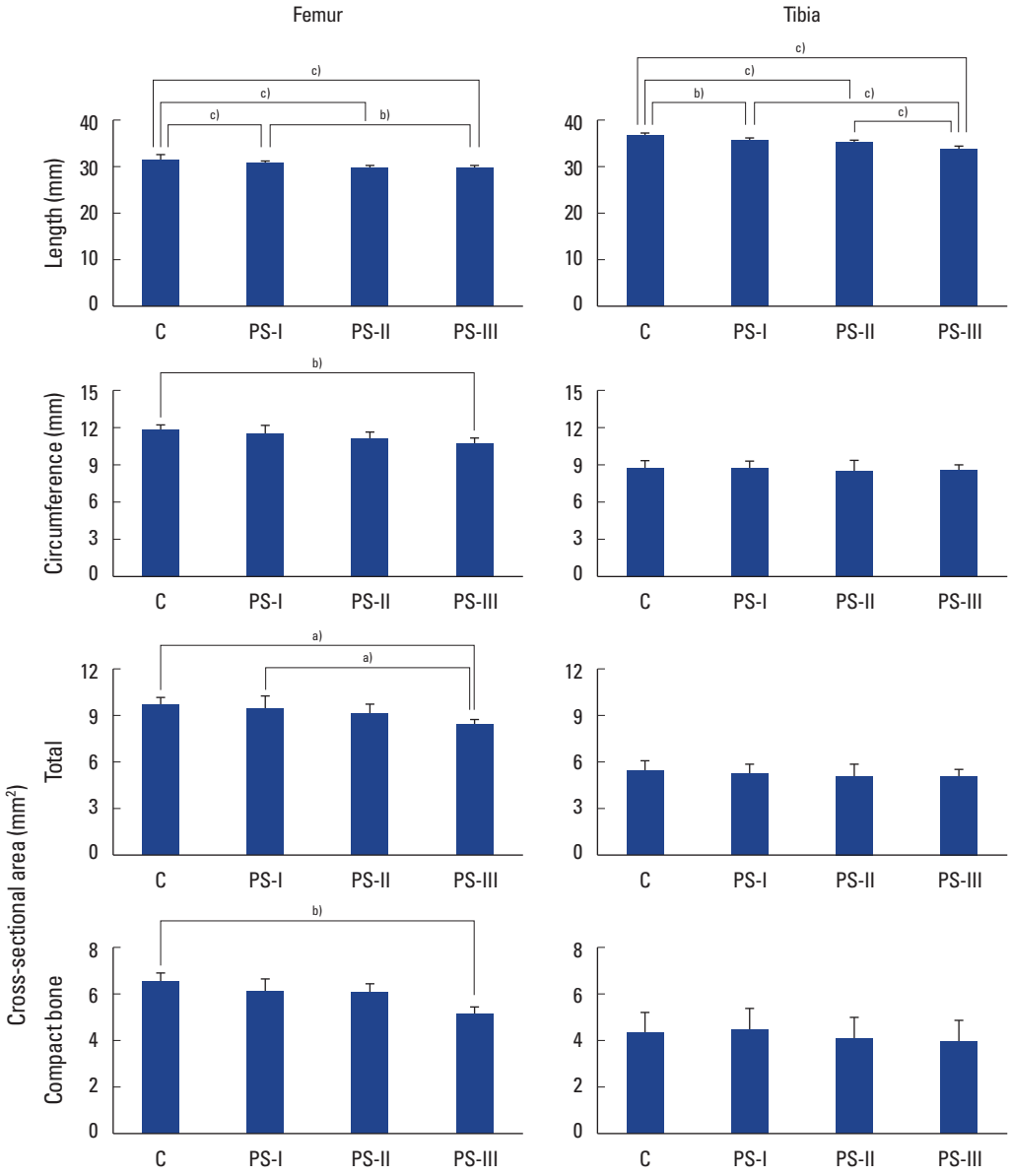


Fig. 3. Bone morphology. This suggests that psychogenic stress (PS) has a significant effect on bone length growth and that the growth inhibition is greater with the frequency of PS. On the other hand, only the femur showed significant growth inhibition in terms of perimeter and bone cross-sectional area. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$. C, control.

(32.1%, $P<0.001$), and PS-II Group (21.9%, $P<0.001$). Additionally, BAP was significantly lower (13.3%, $P<0.01$) in the C Group than in the PS-III Group. However, the post-experimental OSI was significantly lower in group C (24.6%, $P<0.05$) and group PS-I (28.1%, $P<0.01$) than in the PS-III (Fig. 4).

4. Correlation between oxidative stress and organ weight

The correlations between the wet weight of organs and the OSI d-ROM were all significantly negative (all $P<0.05$). The correlation coefficients were as follows: r=-0.517 for the thymus, r=-0.608 for the spleen, r=-0.715 for the right kidney, r=-0.441 for the left kidney, and r=-0.582 for the liver. The right kidney demonstrated a significant negative correlation with the OSI for BAP (r=-0.471), as did the liver (r=-0.567). In contrast, the left adrenal showed a positive correlation (r=0.417) (all $P<0.05$). In addition, the thymus, spleen, and right kidney demonstrated significant negative correlations with OSI, with r values of -0.431, -0.455,

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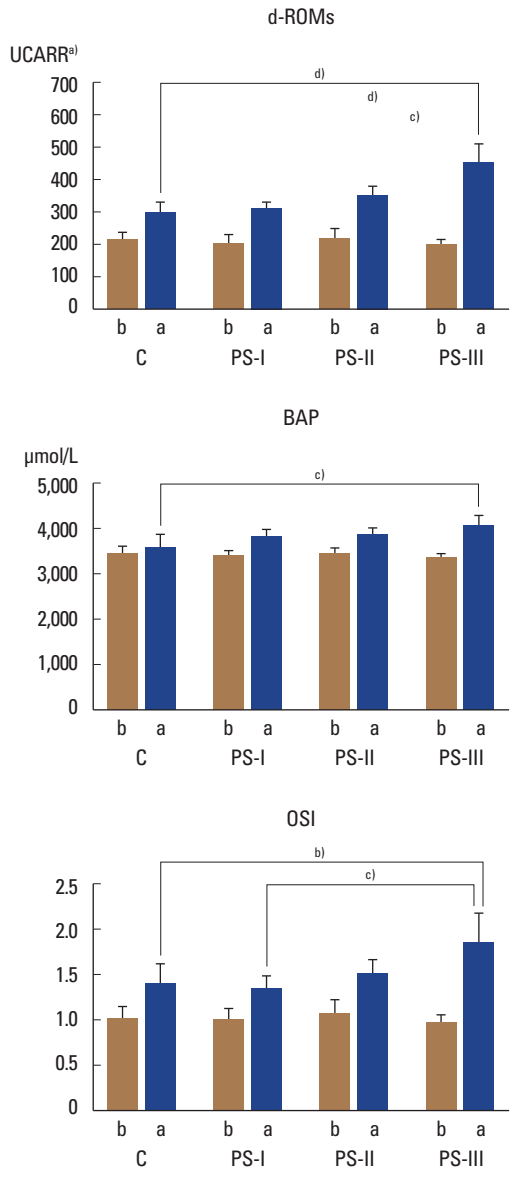

Fig. 4. Oxidative stress and antioxidant capacity. No significant difference was observed in the pre-experimental reactive oxygen metabolites-derived compounds (d-ROMs), biological antioxidant potential (BAP), and oxidation stress index (OSI) among all four groups. Oxidative stress increased when the frequency of psychogenic stress (PS) increased, and the antioxidant capacity and OSI increased in response to the changes in oxidative stress. [a)]1 UCARR=0.08 mg/dL H_2O_2. [b)]$P<0.05$, [c)]$P<0.01$, [d)]$P<0.001$. C, control; UCARR, carratelli units; a, after; b, before.

and -0.572, respectively (all $P<0.05$) (Table 3). Conversely, among the dry weights of the organs, the thymus exhibited a negative correlation of r=-0.480, the spleen r=-0.573, the right kidney r=-0.663, the left kidney r=-0.526, and the liver r=-0.584 for d-ROM (all $P<0.05$). For BAP, the spleen exhibited a negative correlation of r=-0.478, and the liver exhibited a negative correlation of r=0.533. The left adrenal gland exhibited a positive correlation of r=0.423 (all $P<0.05$). Moreover, for OSI, the thymus exhibited a negative correlation of r=-0.424, the spleen r=-0.423, the right kidney r=-0.576, the left kidney r=-0.417, and the liver r=-0.414 (all $P<0.05$) (Table 3).

Table 3. Correlation between oxidative stress and organ

	d-ROM	BAP	OSI
Wet weight			
Thymus	-0.517[b)]	-0.302	-0.431[a)]
Spleen	-0.608[b)]	-0.491[a)]	-0.455[a)]
Adrenal gland			
Right	0.081	0.290	-0.028
Left	0.023	0.417[a)]	-0.132
Kidney			
Right	-0.715[c)]	-0.471[a)]	-0.572[b)]
Left	-0.441[a)]	-0.334	-0.336
Heart	-0.361	-0.392	-0.230
Liver	-0.582[b)]	-0.567[b)]	-0.399
Dry weight			
Thymus	0.480[a)]	-0.207	-0.424[a)]
Spleen	-0.573[b)]	-0.478[a)]	-0.423[a)]
Adrenal gland			
Right	0.284	0.388	0.155
Left	0.240	0.423[a)]	0.094
Kidney			
Right	-0.663[c)]	-0.303	-0.576[b)]
Left	-0.526[b)]	0.361	-0.417[a)]
Heart	-0.187	-0.223	-0.113
Liver	-0.584[b)]	-0.533[b)]	-0.414[a)]

[a)]$P<0.05$, [b)]$P<0.01$, [c)]$P<0.001$ vs. organ wet weight or dry weight.
d-ROM, reactive oxygen metabolite-derived compounds; BAP, biological antioxidant potential; OSI, oxidation stress index.

5. Correlation between oxidative stress and bone morphology

The femur exhibited a significant negative correlation with d-ROM for wet weight (r=-0.760), dry weight (r=-0.622), bone length (r=-0.611), circumferential length (r=-0.546), bone cross-sectional area (r=-0.567), and dense bone area (r=-0.658). The results were statistically significant at the P value less than 0.01 level for each variable. The correlation coefficients were as follows: -0.458 for wet weight, r=-0.469 for dry weight, r=-0.528 for bone length, r=-0.414 for perimeter, and r=-0.453 for dense bone area.

Table 4. Correlation between oxidative stress and bone morphology

	Oxidative stress items			Bone morphology items					
	d-ROM	BAP	OSI	Wet	Dry	Length	Circ	Area	CB
d-ROM	-	0.277	-0.940[c]	-0.760[c]	-0.662[c]	-0.611[b]	-0.546[b]	-0.567[b]	-0.658[c]
BAP	0.277	-	-0.066	-0.485[a]	-0.469[a]	-0.528[b]	-0.414[a]	-0.353	-0.453[a]
OSI	0.940[c]	-0.066	-	-0.615[c]	-0.517[b]	-0.448[a]	-0.423[a]	-0.468[a]	-0.515[b]
Wet	-0.720[c]	-0.411[a]	-0.598[b]	-	0.938[c]	0.900[c]	0.738[c]	0.746[c]	0.813[c]
Dry	-0.676[c]	-0.388	-0.559[b]	0.973[c]	-	0.908[c]	0.715[c]	0.722[c]	0.817[c]
Length	-0.763[c]	-0.543[b]	-0.598[b]	0.930[c]	0.916[c]	-	0.622[c]	0.597[b]	0.660[c]
Circ	-0.180	0.163	-0.246	0.544[b]	0.507[a]	0.353	-	0.946[c]	0.877[c]
Area	-0.141	-0.114	-0.111	0.511[a]	-0.509[a]	0.335	-0.743[c]	-	0.889[c]
CB	-0.344	-0.126	-0.401	0.633[c]	0.604[b]	0.500[a]	-0.892[c]	-0.633[c]	-

Pearson's correlation coefficient (r value) between oxidative stress and bone morphology in femur (above the diagonal) and tibia (below the diagonal).
[a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$.
d-ROM, reactive oxygen metabolite-derived compounds; BAP, biological antioxidant potential; OSI, oxidation stress index; Circ, circumference; CB, compact bone.

All of these correlations were negative and statistically significant at the *P* value less than 0.05 level for each variable. For OSI, the correlations between the variables were as follows: wet weight (r=-0.615), dry weight (r=-0.517), bone length (r=-0.448), circumferential length (r=-0.423), transverse bone area (r=-0.468), and dense bone area (r=-0.515). All of these correlations were significant at the 0.05 level. Conversely, significant positive correlations of r=0.715 to 0.938 were observed between bone morphology items for wet and dry weight of the femur (all $P<0.001$) (Table 4).

The tibia exhibited a significant negative correlation of r=-0.720 for wet weight, r=-0.676 for dry weight, and r=-0.763 for bone length for d-ROM (all $P<0.001$), as well as a significant negative correlation of r=-0.411 for wet weight and r=-0.543 for bone length for BAP (all $P<0.05$). Furthermore, for OSI, significant negative correlations were observed for wet weight, dry weight, and bone length, with correlation coefficients of r=-0.598, r=-0.559, and r=-0.598, respectively (all $P<0.01$). Conversely, the bone morphology items demonstrated significant positive correlations between r=0.507 and 0.930 for the wet and dry weight of the tibia (all $P<0.05$) (Table 4).

DISCUSSION

In this study, we used growing male Wistar rats to investigate the effects of the presence and frequency of PS exposure on oxidative stress. Furthermore, the effects of PS-induced changes in oxidative stress on organ and bone development were examined from a morphological perspective.

The rats showed a rapid increase in body weight and organ development during the early growth stages. However, the growth slows after physical maturation.[26,27] The increase in body weight in the control group observed in this study corroborates the findings of previous studies. However, this increase was not observed in PS-treated groups. Sekine et al. [28] reported that long-term PS in adult rats reduces body weight by 10 to 20 g. Additionally, Flak et al. [29] and Saegusa et al. [30] suggested that this phenomenon is associated with appetite loss due to stress burden. Although the findings of the present study corroborate those of previous studies,[26-30] suppression in body weight increase was greater in our study than in those reported earlier, and it was affected by PS frequency. Thus, the results suggest that the presence or frequency of PS used in this study affects growth in all cases, and that the frequency of PS has a significant effect on the degree of inhibition in development.

Du Ruisseau et al. [31] reported that adult rats subjected to PS for 8 hr per day for 15 days demonstrate thymic atrophy and adrenal enlargement. Therefore, PS induces hormonal changes in various organs. Kapitonova et al. [32] reported that PS causes thymic atrophy during growth. Thymic and hepatic atrophy due to PS was also observed in the present study. This finding corresponds with the results of previous studies;[27,32] however, no significant adrenal

https://doi.org/10.11005/jbm.2024.31.3.196

enlargement due to PS was observed. Based on the changes observed in body weight and organs (except for the adrenal gland), the present results validate the biological response to PS. The adrenal glands secrete numerous hormones, such as those secreted during growth spurts, that are critical for maintaining biological functions.[33] Hence, the adrenal glands play a critical role in growth by maintaining the homeostasis of the endocrine system. PS-related oxidative stress may affect hepatic function; therefore, consideration of the biological responses to PS is necessary.[9,10,34,35] Rats show rapid growth from the growth stage until maturity, and increase in the weight of various organs manifests as an increase in body weight.[26] The body and organ weights observed in the present study suggest that the presence and frequency of PS are key factors in suppressing development during the growth stage. Therefore, PS may have a greater effect on rats during the growth stage, when the organs and functions are immature, than on rats that have reached maturity.

Foertsch et al. [36] exposed seven-week-old mice to PS for 19 days and observed that PS suppresses bone growth. Cells at the bone growth sites showed an increase in tyrosine hydroxylase expression. This finding suggests that the effects of PS on local adrenaline signal transmission adversely affect the endochondral ossification. Sakai et al. [37] reported that PS can reduce the rate of increase in bone mineral density in six-week-old rats. PS, also, markedly suppressed the growth of the femur and tibia compared to the control group. These findings are comparable to the PS and biological results presented in previous studies.[36,37] In particular, PS caused atrophy of the femur, as demonstrated by measuring bone circumference, cross-sectional area, and compact bone area at a position exactly halfway along the length of the femur. The measurements clearly showed that the PS frequency had a major effect on bone growth during the growth stage. Stress results in a decline in renal function and morphology,[13,34,35] which are associated with bone disease Furthermore, stress-induced PTH overproduction results in aberrant calcium metabolism,[38] and organ growth is highly correlated with bone growth.

In the present study, the PS group showed markedly lower growth rates in all organs compared to the control group. In summary, PS-related growth suppression in various organs influences bone morphology. Bone develops rapidly during growth. However, after attaining peak bone mass in youth, bone mass gradually decreases with age. Thus, bone growth is crucial. The results of the present study on the bones of the subjects were more striking than those of previous studies. The biological responses and physical changes differ between growth and adulthood, with more pronounced changes occurring during the growth phase.[26-30] The definition of the growth phase used in the growth phase experiments is broad, and one-two weeks can result in significant physiological changes in laboratory animals. Furthermore, the type of experimental animals and the intensity of PS were considered as possible reasons for the remarkable results of our experiments. The experimental animals used in this study were "weanling rats," which are extremely developed during the growth phase. In other words, even during the growth phase, the biological responses and developmental status differ depending on age, which may be reflected in changes in bone mass and different levels of biological responses to PS. Animals showed a decrease in metabolic levels when subjected to stress, as shown by a decrease in the expression levels of enzymes in DNA-synthesizing and energy-generating systems.[39] Therefore, the suppression of all-organ development due to PS exposure in this study was thought to affect bone morphology.

Stress affects the thymus, adrenal glands, and hypothalamus, which are involved in hormone-secretory functions,[40] while hormones affect oxidative stress. Reactive oxygen species (ROS) contribute to immune responses against bacteria and foreign substances that invade the body.[41] However, excessive ROS production and accumulation *in vivo* leads to increased oxidative stress and oxidative damage to DNA and even normal cells.[41] *In vivo* oxidative stress is regulated by antioxidants, such as superoxide dismutase and glutathione peroxidase, which are influenced by hormones and other factors.[42] In this study, oxidative stress was significantly increased in the high-frequency PS exposure group compared to the control and low-frequency PS exposure groups, and the response of antioxidant capacity to oxidative stress was significantly increased in the high-frequency PS exposure group. Furthermore, only the left adrenal gland showed a positive correlation with oxidative stress, whereas all other organs showed negative correlations. In addition, all parameters negatively correlated with bone morphology in response

to oxidative stress. This suggests that high-frequency PS exposure induces excessive oxidative stress *in vivo* and has a pronounced effect on the growth of organs, bones, and other organisms.

In this investigation, oxidative stress was significantly higher in the daily high-frequency PS group than in the control and low-frequency PS groups. Antioxidant capacity responded to an increase in oxidative stress. Daily PS exposure induced excessive oxidative stress *in vivo*, affecting the growth of organs, bones, and other organisms. Bone metabolism, which plays a central role in bone morphology, is constantly regulated by the balance between bone formation and resorption. In particular, bone metabolism is affected by *in vivo* factors, such as hormones and oxidative stress.[43,44] Stress increases the plasma corticosterone and estradiol levels,[45,46] which are factors that affect bone metabolism. Furthermore, bone-related factors act synergistically or conjugatively in response to hormonal secretion and other internal conditions, forming a complex mechanism for bone metabolism.[47] These findings suggest that, during growth, bones are affected by organ development and oxidative stress conditions, which contribute to bone metabolism. Thus, PS in immature organisms increases oxidative stress, which is reflected in the PTH and HPA system functions during organ development, and a high frequency of PS leads to bone undergrowth and atrophy. These findings suggest that bone is susceptible to organ development and oxidative stress during growth, which affects the body's internal environment and contributes to its effects on bone metabolism.

In this study, we investigated the morphological changes caused by PS exposure on organ and bone development during growth. We found increased oxidative stress associated with the frequency of PS exposure, suggesting a link between oxidative stress and growth. Thus, the suppression of PS exposure or PS-induced oxidative stress production during growth is important for healthy growth.

Increased oxidative stress with PS in the growth stage has negative effects on organ and bone development, and high frequencies of PS have a greater inhibitory effect on organisms. In other words, removal or suppression of the frequency of PS is important for the healthy development of immature organisms.

DECLARATIONS

Funding

This work was supported by the Aomori University of health and welfare (Research Foundation AUHW-2020-22).

Ethics approval and consent to participate

Animal handling and use were in accordance with the guidelines related to animal experimentation at the Aomori University of Health and Welfare (Approval no. 19001).

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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REFERENCES

1. Lahti A, Rosengren BE, Dencker M, et al. Socioecological and biological associations of lower levels of physical activity in 8-year-old children: a 2-year prospective study. BMJ Open Sport Exerc Med 2019;5:e000597. https://doi.org/10.1136/bmjsem-2019-000597.
2. Thein E, Mahlouly J. Stress fractures. Rev Med Suisse 2019;15:2293-7.
3. Dubey S, Biswas P, Ghosh R, et al. Psychosocial impact of COVID-19. Diabetes Metab Syndr 2020;14:779-88. https://doi.org/10.1016/j.dsx.2020.05.035.
4. Hallal PC, Victora CG, Azevedo MR, et al. Adolescent physical activity and health: a systematic review. Sports Med 2006;36:1019-30. https://doi.org/10.2165/00007256-200636120-00003.
5. Zong Y, Tang Y, Xue Y, et al. Depression is associated with increased incidence of osteoporotic thoracolumbar fracture in postmenopausal women: a prospective study. Eur Spine J 2016;25:3418-23. https://doi.org/10.1007/s00586-

https://doi.org/10.11005/jbm.2024.31.3.196

015-4033-8.
6. Godoy LD, Umeoka EHL, Ribeiro DE, et al. Multimodal early-life stress induces biological changes associated to psychopathologies. Horm Behav 2018;100:69-80. https://doi.org/10.1016/j.yhbeh.2018.03.005.
7. Balyan R, Cai M, Zhao W, et al. Repeated restraint stress upregulates rat sulfotransferase 1A1. J Basic Clin Physiol Pharmacol 2018;30:265-73. https://doi.org/10.1515/jbcpp-2016-0038.
8. Huda MN, Ahmad SM, Alam MJ, et al. Infant cortisol stress-response is associated with thymic function and vaccine response. Stress 2019;22:36-43. https://doi.org/10.1080/10253890.2018.1484445.
9. Marchon RG, Ribeiro CT, Costa WS, et al. Immediate and late effects of stress on kidneys of prepubertal and adult rats. Kidney Blood Press Res 2018;43:1919-26. https://doi.org/10.1159/000496004.
10. Díaz-Aguila Y, Cuevas-Romero E, Castelán F, et al. Chronic stress and high sucrose intake cause distinctive morphometric effects in the adrenal glands of post-weaned rats. Biotech Histochem 2018;93:565-74. https://doi.org/10.1080/10520295.2018.1499961.
11. Wakasugi M, Kazama JJ, Taniguchi M, et al. Increased risk of hip fracture among Japanese hemodialysis patients. J Bone Miner Metab 2013;31:315-21. https://doi.org/10.1007/s00774-012-0411-z.
12. Yokota K. Inflammation and osteoclasts. Nihon Rinsho Meneki Gakkai Kaishi 2017;40:367-76. https://doi.org/10.2177/jsci.40.367.
13. Shigematsu T. Programs for continuing medical education 2016: B session: 3. The linkage between kidney and other organs as heart, vessel, bone and anemia. Nihon Naika Gakkai Zasshi 2017;106:521-7.
14. Izawa S, Shirotsuki K, Sugaya N, et al. The application of saliva to an assessment of stress: procedures for collecting and analyzing saliva and characteristics of salivary substances. Jpn J Complement Altern Med 2007;4:91-101. https://doi.org/10.1625/jcam.4.91.
15. Azuma K, Adachi Y, Hayashi H, et al. Chronic psychological stress as a risk factor of osteoporosis. J Uoeh 2015;37:245-53. https://doi.org/10.7888/juoeh.37.245.
16. Herrera-Covarrubias D, Coria-Avila GA, Aranda-Abreu GE, et al. Prepuberal stress and obesity: effects on serum corticosterone, prolactin, testosterone and precancerous prostate lesions in adult rats. Exp Oncol 2019;41:130-7. https://doi.org/10.32471/exp-oncology.2312-8852.vol-41-no-2.13093.
17. Araujo NC, Neto AMM, Fujimori M, et al. Immune and hormonal response to high-intensity exercise during orienteering. Int J Sports Med 2019;40:768-73. https://doi.org/10.1055/a-0970-9064.
18. Ren J, Chung SH. Anti-inflammatory effect of alpha-linolenic acid and its mode of action through the inhibition of nitric oxide production and inducible nitric oxide synthase gene expression via NF-kappaB and mitogen-activated protein kinase pathways. J Agric Food Chem 2007;55:5073-80. https://doi.org/10.1021/jf0702693.
19. Antoncic-Svetina M, Sentija D, Cipak A, et al. Ergometry induces systemic oxidative stress in healthy human subjects. Tohoku J Exp Med 2010;221:43-8. https://doi.org/10.1620/tjem.221.43.
20. Shirato N. Pathophysiology of bone and calcium metabolism in young women. Clin Calcium 2019;29:27-34.
21. Orito S, Kuroda T, Onoe Y, et al. Age-related distribution of bone and skeletal parameters in 1,322 Japanese young women. J Bone Miner Metab 2009;27:698-704. https://doi.org/10.1007/s00774-009-0094-2.
22. Sakamoto Y, Ogawa T, Ogawa M, et al. Effects of 15-day chronic stress on behavior and neurological changes in the hippocampus of ICR mice. Yakugaku Zasshi 2015;135:151-8. https://doi.org/10.1248/yakushi.14-00180.
23. Ohta Y, Yashiro K, Ohashi K, et al. Effect of dietary vitamin E supplementation on liver oxidative damage in rats with water-immersion restraint stress. J Nutr Sci Vitaminol (Tokyo) 2015;61:113-22. https://doi.org/10.3177/jnsv.61.113.
24. Lee S, Hashimoto J, Suzuki T, et al. The effects of exercise load during development on oxidative stress levels and antioxidant potential in adulthood. Free Radic Res 2017;51:179-86. https://doi.org/10.1080/10715762.2017.1291939.
25. Seki Y. Evaluation of total oxidative stress by d-ROMs testing. J Anal Biosci 2009;32:301-6.
26. Tadokoro S, Kurihara Y, Kurihara N, et al. On body weight and organ weight in rats. Kita Kanto Igaku 1962;12:250-65. https://doi.org/10.2974/kmj1951.12.250.
27. Nobunaga T, Uchida T, Nakamura K, et al. Studies on the growth of the Donryu rat: Increase of body and organ weights and general observations of male rats. Exp Anim 1963;12:13-26. https://doi.org/10.1538/expanim1957.12.1_13.

28. Sekine I, Kishikawa M, Takaki Y, et al. Experimental hypertension inducesed by long-term psychogenic stress and vascular changes in rats. J Jpn Atheroscler Soc 1983;11:159-65. https://doi.org/10.5551/jat1973.11.1_159.
29. Flak JN, Jankord R, Solomon MB, et al. Opposing effects of chronic stress and weight restriction on cardiovascular, neuroendocrine and metabolic function. Physiol Behav 2011;104:228-34. https://doi.org/10.1016/j.physbeh.2011.03.002.
30. Saegusa Y, Takeda H, Muto S, et al. Decreased plasma ghrelin contributes to anorexia following novelty stress. Am J Physiol Endocrinol Metab 2011;301:E685-E96. https://doi.org/10.1152/ajpendo.00121.2011.
31. Du Ruisseau P, Taché Y, Selye H, et al. Effects of chronic stress on pituitary hormone release induced by combined hemi-extirpation of the thyroid, adrenal and ovary in rats. Neuroendocrinology 1977;24:169-82. https://doi.org/10.1159/000122707.
32. Kapitonova M, Kuznetsov SL, Klauchek SV, et al. Accidental thymic involution in the growing body under the effect of different types of stressors. Morfologiia 2006;130:56-61.
33. Barinov EF, Sulaeva ON. Mechanisms of adrenal embryogenesis. Usp Fiziol Nauk 2001;32:99-112.
34. Maksymchuk O, Chashchyn M. The impact of psychogenic stressors on oxidative stress markers and patterns of CYP2E1 expression in mice liver. Pathophysiology 2012;19:215-9. https://doi.org/10.1016/j.pathophys.2012.07.002.
35. Hidalgo J, Armario A, Flos R, et al. Restraint stress induced changes in rat liver and serum metallothionein and in Zn metabolism. Experientia 1986;42:1006-10. https://doi.org/10.1007/bf01940708.
36. Foertsch S, Haffner-Luntzer M, Kroner J, et al. Chronic psychosocial stress disturbs long-bone growth in adolescent mice. Dis Model Mech 2017;10:1399-409. https://doi.org/10.1242/dmm.030916.
37. Sakai F, Suzuki H, Miyai Y, et al. Effects of restraint stress on ovalbumin-specific antibody titers and bone density in brown norway rats. J Jpn Soc Nutr Food Sci 2014;67:87-94. https://doi.org/10.4327/jsnfs.67.87.
38. Takeuchi Y. Endocrine disease: progress in diagnosis and treatment. Topics: II. Progress in treatment; 5. Role of parathyroid hormone in bone metabolism and its clinical implication. Nihon Naika Gakkai Zasshi 2012;101:1007-14. https://doi.org/10.2169/naika.101.1007.
39. Kuhn CM, Schanberg SM. Responses to maternal separation: mechanisms and mediators. Int J Dev Neurosci 1998;16:261-70. https://doi.org/10.1016/s0736-5748(98)00034-3.
40. Solomon MB, Jones K, Packard BA, et al. The medial amygdala modulates body weight but not neuroendocrine responses to chronic stress. J Neuroendocrinol 2010;22:13-23. https://doi.org/10.1111/j.1365-2826.2009.01933.x.
41. Eguchi H, Fujiwara N, Ookawara T, et al. Oxidative stress and health. J Anal Biosci 2009;32:247-56.
42. Fujiwara N, Nakano M, Kato S, et al. Oxidative modification to cysteine sulfonic acid of Cys111 in human copper-zinc superoxide dismutase. J Biol Chem 2007;282:35933-44. https://doi.org/10.1074/jbc.M702941200.
43. Lee S, Kumagai T, Hashimoto J, et al. A change of osteocalcin (OC) and tartrate resistant acid phosphatase 5b (TRACP-5b) with the menstrual cycle. Horm Metab Res 2012;44:699-703. https://doi.org/10.1055/s-0032-1311606.
44. Sindler AL, Reyes R, Chen B, et al. Age and exercise training alter signaling through reactive oxygen species in the endothelium of skeletal muscle arterioles. J Appl Physiol (1985) 2013;114:681-93. https://doi.org/10.1152/japplphysiol.00341.2012.
45. Lee JH, Kimm S, Han JS, et al. Chasing as a model of psychogenic stress: characterization of physiological and behavioral responses. Stress 2018;21:323-32. https://doi.org/10.1080/10253890.2018.1455090.
46. MacNiven E, deCatanzaro D, Younglai EV. Chronic stress increases estrogen and other steroids in inseminated rats. Physiol Behav 1992;52:159-62. https://doi.org/10.1016/0031-9384(92)90446-9.
47. Matsuo K, Irie N. Osteoclast-osteoblast communication. Arch Biochem Biophys 2008;473:201-9. https://doi.org/10.1016/j.abb.2008.03.027.

https://doi.org/10.11005/jbm.2024.31.3.196