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EFFECTS OF DIFFERENT INTENSITIES OF COLD STRESS ON CERTAIN PHYSIOLOGICAL PHENOMENA RELATED TO SKELETAL HEALTH IN A HYPOGONADAL RAT MODEL

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Intestinal transference pattern of calcium and associated changes in the activities of intestinal mucosal enzymes, rate of bone turnover and bone metabolism were evaluated in ovariectomized rats exposed to cold stress of various intensities i.e., mild (15°C) or stronger (8°C and 4°C) for 5 min everyday for 7 consecutive days. Except mild cold stress-induced group (15°C), rats of other two groups (8°C and 4°C) showed considerable decrease in the rate of *in situ* intestinal transference of calcium. Likewise, in these groups, the activities of intestinal mucosal enzymes, alkaline phosphatase (AP) and calcium ATPase (Ca^{2+}-ATPase) were decreased significantly in all the segments of small intestine in a descending gradient. Also significant changes in bone turnover and bone resorption were confirmed in these animals by marked alterations in plasma AP activity, urinary calcium and phosphate excretion and urinary calcium to creatinine ratio (Ca: Cr). The skeletal changes were further ascertained by examining other physical and biochemical parameters of bone metabolism viz., body mass, bone density, ratio of mineral to matrix and mineral content of bone ash (calcium and phosphate) in the ovariectomized rat model. All these results suggest that stronger cold stress (8°C and 4°C) may be an important ecological factor in the development of earlier bone loss in hypogonadal rats.

K e y w o r d s: *ovariectomy, cold stress, bone turnover, osteoporosis.*

INTRODUCTION

A sharp decrease in ovarian estrogen production is the predominant cause of rapid hormone-related imbalance of calcium homeostasis and subsequent bone loss during the first decade after menopause (1, 2). In addition, ovarian hormones similar to glucocorticoid and thyroid hormones, have been reported to influence intestinal transference of calcium (3, 4). The effect of chronic cold stress in rat has been reported to cause an increase in ACTH, corticosterone and thyroid hormone secretion (5, 6). Cold stress also has been reported to alter other physiological phenomena viz., alteration in hormone secretion, and decrease in Ca^{2+} absorption. (7). The involvement of alkaline phosphatase and calcium ATPase (Ca^{2+}-ATPase) in calcium absorption has been proposed by many authors, because the activity of these enzymes correlates with the degree of calcium absorption in different parts of the intestinal tract under different

circumstances (8, 9). The activity of both the enzymes are increased by vitamin D (10). A similar dependency of AP activity on vitamin D has also been reported (11, 12). It is suggested that AP and brush border Ca^{2+}-ATPase are expressions of the same molecule (11, 13, 14), since attempts to separate these activities by various biochemical procedures have not been successful (15). Previously, we have reported that chronic cold stress may have positive influence on bone loss for an earlier development of osteoporotic changes in hypogonadal rats (16).

The main objectives of this study were to quantify, in an ovariectomized rat model, relationship between the intensity of cold stress and the changes in various physiological phenomena related to skeletal health. In particular, we investigated (i) *in situ* intestinal transference of calcium in different segments of small intestine and activities of relevant enzymes, and (ii) bone turnover and resorption phenomena.

MATERIALS AND METHODS

All the experiments were approved by the Ethics committee of Presidency College, Calcutta, India,

Animals

Female Wistar rats weighing 120—150 g were used for this study. They were housed in an environmentally controlled laboratory after being divided into five groups consisting six rats (selected randomly) in each group:

Group A : sham-operated control;
Group B : bilaterally ovariectomized;
Group C : ovariectomized + cold stress (15°C);
Group D : ovariectomized + cold stress (8°C);
Group E : ovariectomized + cold stress (4°C).

Under light ether anaesthesia, bilateral (dorsolateral) ovariectomies were performed in the group B, C, D and E and animals of group A were subjected to sham-operation. The animals of all groups were provided with a control diet (17) composed of 71% carbohydrates (equal parts of arrowroot starch and sucrose), 18% protein (casein), 7% fat (groundnut oil) and 4% salt mixture 18), while vitamins were supplied according to Chatterjee *et al.* (19). To overcome the impact of any altered food intake, control (group A) was pair-fed with experimental groups B, C, D and E. The animals of all the groups were maintained on a 12 h light/dark schedule with free access to water supply. The animals of all the groups were maintained in laboratory environment for the next 15 days and were allowed to recover from surgical convalescence. After the recovery period was over, the animals of groups C, D and E were exposed to cold-swim stress according to Shu *et al.* (6). In brief, the animals of groups C, D and E were exposed to cold water of different intensities i. e., 15°C, 8°C and 4°C respectively and were forced to swim for 5 min every day for 7 consecutive days.

Preparation of intestinal loops

After the experimental period was over, body weight of all animals were recorded. They were fasted for 16 h and then anaesthetized with urethane (1.7 mg/g. b. wt). The preparation of intestinal loops for the study of Ca^{2+} transference *in situ* were made following the method of Levine *et al.*

(20). The abdomen of each animal was opened through a midline incision and duodenal, jejunal and ileal segments were located. Two ligatures, one proximal and other distal, were applied tightly on each loop, measuring about 8 cm in all the duodenal, jejunal, and ileal segments. Loops were so selected that each contained 8—10 blood vessels and care was taken so that no major blood vessel was occluded by the ligature.

Measurement of intestinal calcium transference

For the measurement of intestinal calcium transference, 1 ml of Tris-HCl buffer solution containing 0.2 mM $CaCl_2$ was injected into the lumen of each loop with a syringe. Tris-HCl buffer solution was prepared following the method of Singh et al. (3). The intestinal loops were placed in their usual positions and the abdomen was closed. After 1 h, animals were sacrificed, the preselected loops were removed and the fluid from each loop was collected separately, together with a few washings of the lumen with distilled water. The collected fluid was then made up to a definite volume with distilled water. A fraction of this fluid was then used for the estimation of calcium by the method as described by Adeniyi et al. (21) using a Double-Beam Spectrophotometer (Shimadzu, 160 A). The difference between the amount of calcium introduced and the amount of it left unabsorbed was used as an estimate of the amount of calcium absorbed. The intestinal part constituting the loop was dried on a watch glass in an electric oven at 90°C to attain a constant weight, which was recorded as the weight of the dried loop.

Preparation of enzyme extracts and estimation of enzyme activities

For preparation of intestinal mucosal extracts and estimation of enzyme activities, the animals were sacrificed and the whole of the small intestine was quickly removed. The portions comprised of the duodenum, jejunum and ileum were separated and chilled in ice. Intestinal mucosa was collected as described by Maenz and Cheesemann (22), and the scrapings were homogenized according to the method of Koyama et al. (23). Mucosal scrapings were homogenized with 5 volume of Tris-HCl buffer (pH 7.4). The activity of AP was estimated using the p-nitrophenyl phosphate method of Maenz and Cheeseman (22). The protein content of the homogenate, used for the study was determined essentially following the method described by Lowry et al. (24). The activity of Ca^{2+}-ATPase was studied according to the method of Rorive and Kleinzeller (25). Phosphate liberated during enzyme activity was estimated by the method of Lowry and Lopez (26).

Estimation of urinary calcium, phosphate and creatinine

For estimation of urinary content, urine was collected for 24 h (8 a.m. to 8 a.m.) according to the standard laboratory procedure (27) as described elsewhere by Chanda et al. (4). Care was taken so that no urine was lost through evaporation. Total volume was measured. Calcium, phosphate and creatinine content of urine were estimated according to the methods as described respectively by Adeniyi et al. (21), Lowry and Lopez (26) and Nath (28).

Estimation of plasma calcium, AP, corticosterone, T_4 and TSH

Blood was collected directly from the heart under urethane anaesthesia (1.7 mg/g. b. wt.). Heparin was used as anticoagulant. Plasma calcium was estimated by the method of Adeniyi et al. (21). For estimation of plasma AP activity, the method as described by Maenz and Cheeseman (22) was essentially followed. Plasma corticosterone was estimated according to the method of Glick et al. (29) by using a Perkin-Elmer fluorescence spectrophotometer (model 44 B) [excitation wavelength 460 nm and emission wavelength 518 nm]. Plasma concentration of T_4 and TSH were estimated by the ELISA technique using test kit (Enzymun-test T_4 and TSH) of Boehringer Mannheim Immunodiagnostics, India.

Measurement of bone density, bone calcium and phosphate

For the study of physical and biochemical parameters of bone metabolism, one femur and one rib were excised, cleaned of adhering soft tissue, and hydrated for 24 h. Bone volume and density were determined by hydrostatic weighing (30). Briefly, each bone was put in an unstoppered vial filled with deionized water, and the vial was placed under a vacuum for 90 min to ensure that all trapped air diffused out of the bone. Each bone was removed from the vial, blotted with gauze sponge, weighed and returned to the vial containing deionized water. The bone was reweighed in water and the density was calculated. These bones were subsequently dried to constant weight in an oven at 90°C, milled with dry ice, carbonized on an open flame, and ashed in a muffle oven at 600°C for 15 h (31). Ash was dissolved in 20 ml of 20% HCl, after which solutions were analyzed for calcium and phosphate content.

Statistical analysis

Data was expressed as mean ± SEM. Significance was determined using the student's t-test. $P < 0.05$ was considered significant.

RESULTS

Plasma corticosterone, T_4 and TSH

A comparative assessment of plasm concentration of corticosterone, T_4 and TSH of ovariectomized rats subjected to different intensities of cold-swim stress are shown in *Table 1*. An insignificance ($p > 0.05$) in plasma concentrations of corticosterone, T_4 and TSH level were noted in the ovariectomized group, compared with the sham-control group. Note further than in hypogondal state, when rats were exposed to diffrent intensities of cold stress (15°C, 8°C and 4°C), a significant ($p < 0.05$) rise of these hormones were observed compared to hypogonadal (ovariectomized) state alone. However, cold-swim stress at 15°C was not effective in producing significant increase in TSH level.

Table 1. Effects of different intensities of cold stress on plasma corticosterone, T_4 and TSH level of different groups of ovariectomized rats.

Parameters	Group A	Group B	Group C	Group D	Group E	Significant level				% increase			
						AvsB	BvsC	BvsD	BvsE	AvsB	BvsC	BvsD	BvsE
Corticosterone (µg/dl)	22.0±1.36	23.0±1.55	29.0±2.34	38.0±1.80	46.0±2.82	p > 0.05	p < 0.01	p < 0.01	p < 0.01	5.54	26.09	65.22	100.00
T_4 (µg/dl)	2.18±0.02	2.19±0.02	2.24±0.01	2.30±0.02	2.35±0.02	p > 0.05	p < 0.05	p < 0.01	p < 0.01	0.46	2.28	5.02	7.31
TSH µU/ml	0.06±0.01	0.07±0.01	0.09±0.01	0.12±0.02	0.14±0.02	p > 0.05	p > 0.05	p < 0.05	p < 0.01	16.67	28.57	71.43	100.00

Group A = Sham-control; Group B = ovariectomized; Group C = ovariectomized + cold stress [15°C]; Group D = ovariectomized + cold stress [8°C] and Group E = ovariectomized + cold stress [4°C]. Values are expressed as mean ± SEM. (n = 6).

Mucosal calcium transference profiles

A comparative mucosal calcium transference profile of different intestinal segments of ovariectomized rats exposed to different intensities of cold stress are shown in *Table 2*. Calcium transference in the different intestinal segments of ovariectomized rats were significantly reduced ($p < 0.05$) compared to control. Note further that in hypogonadal state, when rats were exposed to stronger cold stress ($8°C$ and $4°C$), a greater decrease in mucosal transference of calcium in all segments were observed compared to hypogonadal state alone, but mild cold stress ($15°C$), however, was not efective in producing any significant change.

Table 2. Effects of different intensities of cold stress on mucosal transference of Ca^{2+} of different groups of ovariectomized rats.

Segments of small intestine	Group A	Group B	Group C	Group D	Group E	Significant level				% decrease			
						AvsB	BvsC	BvsD	BvsE	AvsB	BvsC	BvsD	BvsE
Duodenum	7.12±0.18	6.13±0.17	5.72±0.18	4.85±0.13	4.13±0.20	p < 0.01	p > 0.05	p < 0.01	p < 0.01	13.90	6.69	20.88	32.63
Jejunum	6.98±0.36	5.85±0.49	5.64±0.19	4.80±0.13	4.68±0.18	p < 0.05	p > 0.05	p < 0.05	p < 0.05	16.19	3.59	17.95	20.00
Ileum	5.83±0.23	4.91±0.40	4.75±0.29	3.85±0.37	3.65±0.27	p < 0.05	p > 0.05	p < 0.05	p < 0.05	15.78	3.26	21.59	25.66

Group A = Sham-control; Group B = ovariectomized; Group C = ovariectomized + cold stress [$15°C$]; Group D = ovariectomized + cold stress [$8°C$] and Group E = ovariectomized + cold stress [$4°C$]. Values are expressed as mean ± SEM. (n = 6). Ca^{2+} is expressed in μmol/g dry weight/h.

Mucosal alkaline phosphate activity profiles

Table 3. Effects of different intensities of cold stress on alkaline phosphate activity of intestinal mucosal extracts of different groups of ovariectomized rats.

Segments of small intestine	Group A	Group B	Group C	Group D	Group E	Significant level				% decrease			
						AvsB	BvsC	BvsD	BvsE	AvsB	BvsC	BvsD	BvsE
Duodenum	234.3±12.80	165.2±8.90	156.2±8.90	145.6±5.70	133.2±6.90	p < 0.01	p > 0.05	p < 0.05	p < 0.01	29.44	5.45	11.86	19.37
Jejunum	148.1±8.74	112.2±6.09	107.0±3.54	96.1±5.48	86.0±6.09	p < 0.01	p > 0.05	p < 0.05	p < 0.01	24.24	4.63	14.35	23.35
Ileum	82.8±4.09	62.5±4.32	57.7±4.96	50.6±3.54	47.1±1.82	p < 0.01	p > 0.05	p < 0.05	p < 0.01	24.52	7.68	19.04	24.64

Group A = Sham-control; Group B = ovariectomized; Group C = ovariectomized + cold stress [$15°C$]; Group D = ovariectomized + cold stress [$8°C$] and Group E = ovariectomized + cold stress [$4°C$]. Values are expressed as mean ± SEM. (n = 6). Alkaline phosphatase activity is expressed as p-nitrophenol liberated in μmol/g protein/min at $37°C$.

Shown in *Table 3* are the mucosal alkaline phosphatase activity profile in the different intestinal segments of ovariectomized rats exposed to different

intensities cold stress. Similar to calcium transference results, alkaline phosphatase activity too showed significant ($p < 0.01$) differences between the control and hypogonadal states. Cold stress-induced groups ($8°C$ and $4°C$) were found to be more prone for greater decrease in alkaline phosphatase activity in all segments of hypogonadal rats. Mild cold stress ($15°C$) in such ovariectomized rats showed no significant decrease in alkaline phosphatase activity.

Mucosal calcium ATPase profiles

The mucosal calcium ATPase activity in the different intestinal segments of ovariectomized rats subjected to different intensities of cold stress are shown in *Table 4*. Mucosal calcium ATPase activities also were reduced in stronger cold stress-induced groups ($p < 0.05$ for $8°C$ and $p < 0.01$ for $4°C$), compared to ovariectomized group. Under similar situations, when ovariectomized rats were exposed to mild cold stress ($15°C$), no significant change was observed. Compared to control, a significant ($p < 0.01$) difference of calcium ATPase activity was also observed in all segments of small intestine of ovariectomized group.

Table 4. Effects of different intensities of cold stress on calcium ATPase activity of intestinal mucosal extracts of different groups of ovariectomized rats.

Segments of small intestine	Group A	Group B	Group C	Group D	Group E	Significant level				% decrease			
						AvsB	BvsC	BvsD	BvsE	AvsB	BvsC	BvsD	BvsE
Duodenum	15.9±0.57	13.5±0.64	12.9±0.62	11.4±0.49	10.8±0.49	p < 0.01	p > 0.05	p < 0.05	p < 0.01	15.09	4.44	15.55	20.00
Jejunum	11.1±0.47	8.6±0.36	8.0±0.25	7.5±0.32	6.8±0.25	p < 0.01	p > 0.05	p < 0.05	p < 0.01	24.32	4.76	10.71	19.05
Ileum	7.4±0.22	5.8±0.25	5.6±0.24	5.0±0.27	4.4±0.27	p < 0.01	p > 0.05	p < 0.05	p < 0.01	21.62	3.47	13.79	24.14

Group A = Sham-control; Group B = ovariectomized; Group C = ovariectomized + cold stress [$15°C$]; Group D = ovariectomized + cold stress [$8°C$] and Group E = ovariectomized + cold stress [$4°C$]. Values are expressed as mean ± SEM. (n = 6). Calcium ATPase activity is expressed as P_i liberated in µmol/g protein/min at $37°C$.

Plasma calcium and alkaline phosphatase activity profiles

The plasma calcium and alkaline phosphatase activity profiles of ovariectomized rats exposed to different intensities of cold stress are shown in *Table 5*. Compared to controls, ovariectomized animals showed a significant ($p < 0.01$) increase in plasma alkaline phosphatase (AP) activity. Similar increase in plasma AP activity also were noted when ovariectomized animals were exposed to stronger cold stress ($8°C$ and $4°C$). But no significant change was observed in mild cold ($15°C$) stress-induced rats. Under the conditions of our study, when ovariectomized rats were subjected to stronger cold stress, a significant ($p < 0.05$) alteration in plasma calcium level was observed. However, in the ovariectomized rats, compared with either sham control or

mild stress-induced groups, plasma calcium level did not change significantly (p > 0.05).

Table 5. Effects of different intensities of cold stress on plasma calcium and alkaline phosphatase activity in different groups of ovariectomized rats.

Parameters	Group A	Group B	Group C	Group D	Group E	Significant level				% decrease			
						AvsB	BvsC	BvsD	BvsE	AvsB	BvsC	BvsD	BvsE
Calcium (mg/dl)	7.2±0.07	7.20±0.08	7.24±0.09	7.56±0.18	7.58±0.14	p > 0.05	p > 0.05	p < 0.05	p < 0.05	2.56	0.56	5.00	5.30
AP (U/l)	110.0±7.76	140.0±8.37	152.0±3.92	172.0±9.21	190.0±11.13	p < 0.01	p > 0.05	p < 0.05	p < 0.01	27.27	8.57	22.86	35.71

Group A = Sham-control; Group B = ovariectomized; Group C = ovariectomized + cold stress [15°C]; Group D = ovariectomized + cold stress [8°C] and Group E = ovariectomized + cold stress [4°C]. Values are expressed as mean ± SEM. (n = 6).

Urinary calcium and phosphate excretion and calcium to creatinine ratio

The results of urinary calcium and phosphate excretion and calcium to creatinine ratio (Ca: Cr) of ovariectomized rats exposed to various intensities of cold stress are listed in *Table 6.* Compared to sham-operated control, ovariectomized animals showed a significant (p < 0.01 for calcium; p < 0.05 for phosphate and p < 0.01 for Ca: Cr ratio) increase in all the three parameters. Such an increase in al these parameters was further pronounced when ovariectomized animals were exposed to stronger cold stress (8°C and 4°C). Under similar situations, in mild cold (15°C) stress-induced rats, no significant change was observed in all these parameters.

Table 6. Effects of different intensities of cold stress on urinary excretion of phosphate and calcium, and calcium: creatinine ratio of different groups of ovariectomized rats.

Parameters	Group A	Group B	Group C	Group D	Group E	Significant level				% decrease			
						AvsB	BvsC	BvsD	BvsE	AvsB	BvsC	BvsD	BvsE
Phosphate (mg)	36.64±2.92	46.93±4.05	50.85±3.10	58.23±2.14	60.35±1.84	p < 0.05	p > 0.05	p < 0.05	p < 0.05	28.08	8.35	24.08	28.60
Ca (mg)	3.15±0.25	4.85±0.32	5.72±0.35	6.32±0.35	6.66±0.34	p < 0.01	p > 0.05	p < 0.01	p < 0.01	53.97	17.94	30.31	37.32
Ca:Cr ratio (mg:mg)	1.02±0.07	1.38±0.09	1.48±0.08	1.60±0.04	1.64±0.04	p < 0.01	p > 0.05	p < 0.05	p < 0.01	35.29	7.25	15.94	18.84

Group A = Sham-control; Group B = ovariectomized; Group C = ovariectomized + cold stress [15°C]; Group D = ovariectomized + cold stress [8°C] and Group E = ovariectomized + cold stress [4°C]. Values are expressed as mean ± SEM. (n = 6). Urinary calcium (Ca) and creatinine (Cr) excretion is expressed in mg/24 h urine. Urinary phosphatase excretion is expressed in mg/dl.

Bone density, bone calcium and phosphate profiles

Physical and biochemical parameters of bone metabolism are presented in *Table 7* and *8*. Significant differences in body mass, femoral fresh weight, dry weight and femoral density were found between sham-control and ovariectomized rats. More significant differences in all these parameters were observed when ovariectomized animals were exposed to stronger cold stress (8°C and 4°C). For ribs, a similar change in all these parameters were observed under similar condition. However, mild cold (15°C) stress-induced rats showed no significant change in the physical parameters of femurs and ribs. The ratio of mineral to matrix (percent ash) of femurs and ribs and mineral (calcium and phosphate) content of ash were significantly ($p < 0.05$) different between sham-control and ovariectomized rats. Such an increase in all these parameters were observed when ovariectomized animals were exposed to stronger cold stress (8°C and 4°C). Cold-swim stress at 15°C, however, was not effective in producing any significant change in these parameters.

Table 7. Effects of different intensities of cold stress on body mass and physical parameters of femur and rib of different groups of ovariectomized rats.

Parameters	Group A	Group B	Group C	Group D	Group E	Significant level			
						AvsB	BvsC	BvsD	BvsC
Body mass (gm)	135 ± 3.8	122 ± 2.8	119 ± 3.5	114 ± 3.4	113 ± 2.8	$p < 0.01$	$p > 0.05$	$p < 0.05$	$p < 0.05$
				Femur					
Fresh wt. (mg)	0.334 ± 0.001	0.330 ± 0.002	0.329 ± 0.001	0.326 ± 0.001	0.324 ± 0.002	$p < 0.05$	$p > 0.05$	$p < 0.05$	$p < 0.05$
Dry wt. (gm)	0.222 ± 0.001	0.218 ± 0.001	0.217 ± 0.001	0.215 ± 0.001	0.214 ± 0.002	$p < 0.05$	$p > 0.05$	$p < 0.05$	$p < 0.05$
Volume (ml)	0.191 ± 0.001	0.191 ± 0.001	0.191 ± 0.002	0.191 ± 0.0004	0.191 ± 0.0004	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Density (fresh wt./vol)	1.75 ± 0.005	1.73 ± 0.008	1.73 ± 0.006	1.71 ± 0.006	1.70 ± 0.005	$p < 0.05$	$p > 0.05$	$p < 0.05$	$p < 0.01$
				Rib					
Fresh wt. (mg)	28.9 ± 0.07	28.6 ± 0.11	28.5 ± 0.11	28.2 ± 0.06	28.1 ± 0.11	$p < 0.05$	$p > 0.05$	$p < 0.05$	$p < 0.01$
Dry wt. (mg)	17.6 ± 0.08	17.4 ± 0.07	17.3 ± 0.07	17.2 ± 0.07	17.1 ± 0.09	$p < 0.05$	$p > 0.05$	$p < 0.05$	$p < 0.05$
Volume (μl)	18.6 ± 0.07	18.7 ± 0.07	18.7 ± 0.06	18.7 ± 0.08	18.7 ± 0.07	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Density (fresh wt./vol)	1.55 ± 0.006	1.53 ± 0.005	1.52 ± 0.009	1.51 ± 0.007	1.50 ± 0.006	$p < 0.05$	$p > 0.05$	$p < 0.05$	$p < 0.01$

Group A = Sham-control; Group B = ovariectomized; Group C = ovariectomized + cold stress [15°C]; Group D = ovariectomized + cold stress [8°C] and Group E = ovariectomized + cold stress [4°C]. Values are expressed as mean ± SEM. (n = 6).

Table 8. Effects of different intensities of cold stress on ash percentage and mineral content of dissolved ash of femurs and ribs of different groups of ovariectomized rats.

Parameters	Group A	Group B	Group C	Group D	Group E	Significant level			
						AvsB	BvsC	BvsD	BvsE
Femur									
Ash, % dry wt.	59.6±0.79	57.6±0.62	57.1±0.42	56.2±0.19	55.9±0.40	p < 0.05	p > 0.05	p < 0.05	p < 0.05
Calcium, % ash	38.4±0.91	36.6±0.36	36.3±0.44	35.5±0.40	35.3±0.34	p < 0.05	p > 0.05	p < 0.05	p < 0.05
Phosphate, % ash	15.3±0.34	14.1±0.19	14.1±0.60	13.5±0.19	13.3±0.14	p < 0.01	p > 0.05	p < 0.05	p < 0.01
Rib									
Ash, % dry wt.	46.1±0.22	45.0±0.30	44.8±0.14	44.0±0.28	43.5±0.57	p < 0.01	p > 0.05	p < 0.05	p < 0.05
Calcium, % ash	41.0±1.18	38.0±0.82	37.1±1.53	35.0±1.35	32.5±1.17	p < 0.05	p > 0.05	p < 0.05	p < 0.01
Phosphate, % ash	13.0±0.39	12.1±0.26	11.9±0.33	11.0±0.31	10.6±0.30	p < 0.05	p > 0.05	p < 0.05	p < 0.01

Group A = Sham-control; Group B = ovariectomized; Group C = ovariectomized + cold stress [15°C]; Group D = ovariectomized + cold stress [8°C] and Group E = ovariectomized + cold stress [4°C]. Values are expressed as mean ± SEM. (n = 6).

DISCUSSION

In these studies, we quantified the relationship between the intensities of cold stress and the different bone profiles in ovariectomized rats. The principal finding of this study is that graded cold stress causes variable degree of changes in certain physiological phenomena viz., intestinal transference of calcium, bone turnover and bone loss which earlier (16) we suggested might contribute substantially for a rapid onset of hypogonadal osteoporosis.

To examine whether graded experimental stress could produce effective changes in the secretion of ACTH, corticosterone and thyroid hormones as was reported earlier (5, 6), we verified the plasma levels of corticosterone, T_4 and TSH in all groups of animals in our study (*Table 1*). For further assessment of the effectiveness of graded cold stress, an attempt was made to establish a correlation between these hormones and calcium balance. Our findings and the literature are most consistent with the reports that glucocorticoid and thyroid hormone, both decrease intestinal transference of calcium (8, 9, 32—37) and glucocorticoid only elevates fasting urinary calcium excretion (31, 32). Therefore, it is likely that variable alterations in hormone level as well as in calcium balance found in this study were probably caused by graded stress given to animals.

Calcium transference in ovariectomized rats showed segmental variation, and was seen to have a descending gradient from duodenum to ileum irrespective of groups (*Table 2*). In addition, it was observed that all these changes related to inhibition of calcium transference were more pronounced when ovariectomized rats were subjected to cold stress of different intensities

(15°C, 8°C and 4°C) thus assuming that different intensities of cold stress might have different degree of inhibitory influence over segmental transference of calcium. Literature survey accord our findings, that (i) calcium transference has a descending gradient from duodenum to ileum (38), (ii) ovarian hormone, estrogen and its influence on hydroxylation of vitamin D (39, 40—42) modulate intestinal transference of calcium (43) and in addition, (iii) cold stress also decreases intestinal transference of calcium (7).

In an attempt to elucidate the mechanism of such cold stress-induced alterations in intestinal transference of calcium, an assessment of the activities of relevant enzymes, AP and Ca^{2+}-ATPase was made. Results of our study revealed that, compared to control, activities of both of these enzymes were significantly inhibited in bilaterally ovariectomized rats as well as in rats of different groups exposed to cold-swim stress of different intensities (Table 3 and 4). These results propose that the possible cause of reduction of calcium transference in our study might be because of an inhibition of activities of these enzymes as both the enzymes have been reported to be involved in calcium transference (44, 45). Similar decrease in calcium transference due to cold stress in ovariectomized rats was correlated with intestinal mucosal enzymes in one of our earlier studies (16).

Both the enzymes, AP and Ca^{2+}-ATPase showed similar pattern of activities in our studies which confirmed all previous speculations (11, 13, 14) that both the enzymes are expression of the same molecule. This study might also account for an earlier suggestion that AP activity is under the control of estrogen (3). In addition, a decrease in calcium transference in cold stress-induced rats might be attributed to the decreased formation of active metabolites of vitamin D_3 (46) which eventually reduced AP and Ca^{2+}-ATPase activity.

Compared to sham-controls, we observed a greater loss of urinary calcium and phosphate in bilaterally ovariectomized rats. This loss was progressively enhanced when these animals were exposed to cold stress of various intensities (Table 6). We and other authors (47) did not observe any change in plasma calcium level under similar experimental conditions. In contrast, with stronger cold stress (8°C and 4°C), a significant increase in plasma calcium level was observed (Table 5).

So far as the relevance of this study with calcium homeostasis is concerned, our results strongly argue in favour of the notion that the experimental situations of our study, i.e., ovariectomy and ovariectomy + stronger cold stress (8°C and 4°C) possibly help to set in a hypocalcemic condition. Incidentally, this notion finds its justification since an increased urinary loss in these experimental animals have always been associated with simultaneous decrease in intestinal transference of calcium and these physiological changes are considered as the most important contributing factors for the development of

hypocalcemia and secondary increase in PTH secretion (48, 49). Mild cold stress (15°C) in our study, however, could not produce any significant alteration either in intestinal mucosal enzyme or plasma calcium parameters.

Biochemical markers of bone turnover, namely plasma AP activity and urinary calcium to creatinine ratio (Ca: Cr), were also found to be progressively enhanced when ovariectomized rats were subjected to various intensities of cold stress (*Table 5* and 6). A rise in serum AP level and urinary Ca: Cr ratio has been linked with collagen degradation, bone resorption and osteoporosis by earlier investigators (47, 50—52). Data on changes in body weight of different groups of animals (*Table 7*) suggest that a decrease in body weight might be related with bone loss and osteoporosis. Earlier, similar loss of body weight was attributed to osteoporosis (50) or cold exposure (53) suggesting that cold exposure, in our model of study too, possibly has a positive influence on bone turnover and bone loss. Since bone density in normal rats has been related to body mass (54), the development of osteoporosis in these animals was evident with decreased femoral and rib density and decreased mineral content (*Table 7* and 8). Thus, the positive influence of cold exposure on bone turnover and bone loss, under the conditions of the present study, becomes evident.

In summar, this study quantifies the relationship between the intensities of cold stress and the changes in various physiological phenomena related to skeletal health in a hypogonadal condition.

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