Cold Stress Facilitates Calcium Mobilization from Bone in an Ovariectomized Rat Model of Osteoporosis

Nazrul ISLAM, Shrabani CHANDA, Tapan Kumar GHOSH*, and Chandan MITRA

Department of Physiology, Presidency College, 86/1, College Street, Calcutta 700 073, India; and *Department of Physiology, City College, Rammohan Sarani, Calcutta 700 009, India

Abstract: In an ovariectomized rat model of osteoporosis, the effects of cold stress on intestinal Ca\(^{2+}\) transference and rate of bone turnover were evaluated. In the ovariectomized rats, a significant reduction in intestinal transference of Ca\(^{2+}\) was associated with decreased activities of intestinal mucosal enzymes, alkaline phosphatase (AP), and calcium ATPase (Ca\(^{2+}\)-ATPase) in all the different segments of small intestine in a descending gradient. The development of a high rate of bone turnover and osteoporosis in these animals was confirmed by significant alteration in plasma AP activity and calcium (Ca) level, urinary excretion of Ca and phosphate, and Ca:creatinine ratio. Cold stress in this model, apart from its unique influence in elevating plasma corticosterone and thyroid hormone level, enhanced all the above referred parameters studied in connection with intestinal transference of Ca\(^{2+}\), bone turnover rate, and osteoporosis. The results of this study emphasize that cold stress may have a positive influence on bone loss for an early development of hypogonadal osteoporosis in rats. [Japanese Journal of Physiology, 48, 49–55, 1998]

Key words: cold stress, ovariectomy, bone resorption, osteoporosis.

Under the influence of ecological stresses, various adaptive responses occur in the different biological systems, and if these stresses are frequent and intense and exceed a certain limit, the responses become pathological. It has been well documented that one of the most important stress responses in the biological systems is an alteration in the rate and nature of secretion from classic endocrine glands. The effect of chronic cold stress in rat can be cited as an unique example of this response because it has been reported to cause an increase in ACTH, corticosterone, and thyroid hormone secretion [1, 2]. Cold stress has been reported also to alter other physiological phenomenon, such as a decrease in Ca\(^{2+}\) absorption in young turkeys [3]. A survey of literature has further revealed that stress-responsive hormone glucocorticoid is closely associated with many important physiological responses, viz., a decrease in net intestinal Ca\(^{2+}\) absorption in humans and animals [4–9], suppression of bone formation [5, 6], enhancement of bone resorption [10, 11], increase in urinary Ca excretion, and a compensatory increase in PTH secretion [5, 6]. The survey also suggests that the maintenance of calcium homeostasis is closely linked with thyroid hormone status in the body because hyperthyroidism/thyrotoxicosis has been reported to cause an increased bone resorption and hypercalcemia [12, 13] with subsequent suppression of PTH secretion, activation of vitamin D [12], and absorption of Ca\(^{2+}\) [12, 14]. 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 [15, 16]. Moreover, ovarian hormones similar to glucocorticoid and thyroid hormones have been reported to influence the intestinal transference of Ca\(^{2+}\) [17, 18] and the active role of intestinal mucosal enzymes; viz., AP and Ca\(^{2+}\)-ATPase in such Ca\(^{2+}\) transference phenomenon has also been proposed by other investigators [18–20]. It appears that similar to a situation of sharp decrease in ovarian estrogen production and rapid hormone-related bone loss following surgical or age-related menopause, cold stress too might be highly conducive to initiate bone loss and osteoporotic changes by alter-
ing various physiological and endocrine responses. Information in this field is scanty, and still we have no knowledge of whether cold stress as a circumstantial factor really does influence the development of hypogonadal osteoporosis.

The purpose of this study is to examine all such feasibilities and to assess the individual and cumulative effects of hypogonadism and cold stress in the development of hypogonadal osteoporosis. This study thus has immense implications should this ecological stress (cold stress) be demonstrated to be effective in augmenting osteoporotic changes.

**MATERIALS AND METHODS**

**Animals.** Female Wistar rats weighing 120–150 g were used for this study. They were housed in an environmentally controlled animal laboratory after being divided into three groups consisting of six rats in each group: (A) Sham-operation control; (B) bilaterally ovariectomized; (C) bilaterally ovariectomized + stress-induced. Animals were maintained on a standard laboratory diet [21] composed of carbohydrates (equal parts of arrowroot starch and sucrose) 71%, protein (casein) 18%, fat (groundnut oil) 7%, and salt mixture [22] 4% (in 1,000 g of salt mixture, the calcium and phosphorus contents were Ca citrate·4H₂O 308.2; Ca(H₂PO₄)₂·H₂O 112.8; CaCO₃ 68.5; and K₂HPO₄ 218.7). All vitamins were supplied according to Chatterjee et al. [23], of which the amount of vitamin D specifically was 201.0 U per 100 g diet. The animals of all groups were maintained on a pair-fed diet and 12 h light/dark schedule with free access to water supply. Under light ether anesthesia, bilateral ovariectomies were performed in groups B and C, and the animals in group A were subjected to Sham-operation. The animals of all groups were maintained in a laboratory environment for the next 15 d and allowed to convalesce. After the recovery period, the animals of group C were exposed to cold-swim stress according to the method described by Shu et al. [2]. In brief, the animals were exposed to cold water (8°C) and forced to swim for 5 min every day for 7 consecutive days.

**Preparation of intestinal loops.** After the experimental period, the body weights of all animals were recorded. They were fasted for 16 h and then anesthetized with urethane (1.7 mg/g bw). The preparation of intestinal loops for the study of Ca²⁺ transfer in situ was made following the method of Levine et al. [24]. The abdomen of each animal was opened through a midline incision, and duodenal, jejunal, and ileal segments were located. Two ligatures, one proximal and the 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 a ligature.

**Measurement of intestinal calcium transfer.** For the measurement of intestinal Ca²⁺ transfer, 1 ml of Tris-HCl buffer solution containing 0.2 mM CaCl₂ was injected into the lumen of each loop with a syringe. Tris-HCl buffer solution was prepared following the method of Singh et al. [17]. 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 doubled distilled water. The collected fluid was then expanded to a definite volume with distilled water. A fraction of this fluid was used for the estimation of calcium by the method described by Adeniyi et al. [13], using a Double-Beam Spectrophotometer (Shimadzu, 160 A). The difference between the amount of Ca²⁺ introduced and the amount of it left unasorbed was used as an estimate of the amount of Ca²⁺ 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.** After sacrificing the animal and opening the abdomen, the whole of the small intestine was quickly removed. The portion comprising the duodenum, jejunum, and ileum was separated and chilled in ice. Intestinal mucosa was collected as described by Maenz and Cheeseman [25], and the scrapings were homogenized according to the method of Koyama et al. [26]. Mucosal scrapings were homogenized with 5 volumes of Tris-HCl buffer (pH 7.4).

**Estimation of enzyme activities.** The activity of AP was estimated by using the p-nitrophenyl phosphate method of Maenz and Cheeseman [25]. The protein content of the homogenate used for the study was determined by essentially following the method described by Lowry et al. [27]. The activity of Ca²⁺-ATPase was studied according to the method of Rorive and Kleinzeller [28]. Phosphate liberated during enzyme activity was estimated by the method of Lowry and Lopez [29].

**Estimation of urinary calcium, phosphate, and creatinine.** Urine was collected for 24 h according to the standard laboratory procedure [30] as described elsewhere by Chanda et al. [18]. Care was taken so that no urine was lost through evaporation.
Cold Stress and Hypogonadal Osteoporosis

Table 1. Plasma corticosterone, T₄, and TSH levels of Sham-control (Group A), ovariectomized (Group B), and ovariectomized+cold stress (Group C) rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group A (µg/dl)</th>
<th>Group B (µg/dl)</th>
<th>Group C (µg/dl)</th>
<th>Significant level</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone</td>
<td>22.0±1.36</td>
<td>23.0±1.55</td>
<td>38.0±1.80</td>
<td>A vs. B: p&lt;0.05</td>
<td>A vs. C: p&lt;0.01</td>
</tr>
<tr>
<td>T₄</td>
<td>2.18±0.02</td>
<td>2.19±0.02</td>
<td>2.30±0.02</td>
<td>A vs. B: p&lt;0.05</td>
<td>A vs. C: p&lt;0.01</td>
</tr>
<tr>
<td>TSH</td>
<td>0.06±0.01</td>
<td>0.07±0.01</td>
<td>0.12±0.02</td>
<td>A vs. B: p&lt;0.05</td>
<td>A vs. C: p&lt;0.01</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE (n=6).

Total volume was measured. Ca, phosphate, and creatinine contents of urine were estimated according to the methods described, respectively, by Adeniyi et al. [13], Lowry and Lopez [29], and Nath [31].

**Estimation of plasma calcium and alkaline phosphatase.** Blood was collected directly from the heart under urethane anesthesia (1.7 mg/g bw). Heparin was used as the anticoagulant. Plasma Ca was estimated by the method of Adeniyi et al. [13]. For an estimation of plasma AP activity, the method described by Maenz and Cheeseman [25] was essentially followed.

**Estimation of plasma corticosterone, T₄, and TSH.** Blood collection was done by a method similar to the one described for plasma Ca and AP. Heparin was used as an anticoagulant. Plasma corticosterone was estimated according to the method of Glick et al. [32] by using a Perkin-Elmer fluorescence spectrophotometer (model 44B) (excitation wavelength 460 nm and emission wavelength 518 nm). Plasma concentration of T₄ and TSH were estimated by the ELISA technique by using a test kit (Enzymun-test T₄ and TSH) of Boehringer Mannheim Immuno-diagnostics, India.

**Data.** Data were expressed as mean±SE. Significance was determined by using the one-tail Student’s t-test. Differences were considered significant if p<0.05.

**RESULTS**

**Plasma corticosterone, T₄, and TSH**

The effects of cold stress on plasma concentration of corticosterone, T₄, and TSH are presented in Table 1. Compared with the Sham-control group, rises in plasma concentrations of corticosterone, T₄, and TSH in the ovariectomized group were not significant (p>0.05). But the ovariectomized+stress-induced group showed more significant (p<0.01) rises of these hormones, than in the Sham-control and the ovariectomized groups.

**Mucosal transference of calcium**

The effects of cold stress on mucosal transference of Ca²⁺ in duodenum, jejunum, and ileum of ovariectomized rats are shown in Fig. 1. Compared with the Sham-control group, the mucosal transference of Ca²⁺ in the ovariectomized group was significantly (duodenum, p<0.01; jejunum, p<0.05; ileum, p<0.05) reduced in different intestinal segments, and the percent reductions were 13.90, 16.19, and 15.78, respectively, for duodenum, jejunum, and ileum. But the ovariectomized+stress-induced group showed much higher reductions than in both the Sham-control group and the ovariectomized group, and the percent reductions, compared with the Sham-control group, were 31.88, 31.23, and 33.85, respectively, for duodenum, jejunum, and ileum. The differences in percent reductions between the ovariectomized group and
the ovariectionmated-stress-induced group thus were 20.88, 17.95, and 21.59, respectively, for duodenum, jejunum, and ileum.

Alkaline phosphatase activity

The effects of cold stress on the AP activity of duodenal, jejunal, and ileal mucosal extracts of ovariectionmatized rats are shown in Fig. 2. Compared with the Sham-control group, mucosal AP activity in the ovariectionmatized group was significantly (p<0.05) reduced in different intestinal segments, and the percent reductions were 29.49, 24.24, and 24.52, respectively, for duodenum, jejunum, and ileum. But the ovariectionmatized+stress-induced group showed much higher reductions than the Sham-control group and the ovariectionmatized group did and the percent reductions, compared with Sham-control, were 37.86, 35.11, and 38.89, respectively, for duodenum, jejunum, and ileum. The differences in percent reduction responses between the ovariectionmatized group and ovariectionmatized+stress-induced group thus were 11.86, 14.35, and 19.04, respectively, for duodenum, jejunum, and ileum.

Calcium-ATPase activity

The effects of cold stress on the Ca \(^{2+}\)-ATPase activity of duodenal, jejunal, and ileal mucosal extracts of ovariectionmatized rats are shown in Fig. 3. The results reveal that compared with the Sham-control group, the ovariectionmatized group produced a marked (p<0.01) reduction in Ca \(^{2+}\)-ATPase activity, and the percent reductions were 15.09, 24.32, and 21.62, respectively, for duodenum, jejunum, and ileum. The results of experiments with the ovariectionmatized+stress-induced group reveal further reductions (p<0.01) of Ca \(^{2+}\)-ATPase activity throughout the small intestine, and the percent reductions were 28.30, 32.43, and 32.43, respectively, for duodenum, jejunum, and ileum. The differences in percent reduction responses between the ovariectionmatized group and the ovariectionmatized+

![Fig. 2. Alkaline phosphatase activity of intestinal mucosal extracts of Sham-control (Group A), ovariectionmatized (Group B), and ovariectionmatized+cold stress (Group C) rats. Values are mean±SE (n=6). In all segments, the values between Group A vs. Group B (p<0.01), Group A vs. Group C (p<0.01), and Group B vs. Group C (p<0.05) were significantly different.](image)

![Fig. 3. Calcium-ATPase activity of intestinal mucosal extracts of Sham-control (Group A), ovariectionmatized (Group B), and ovariectionmatized+cold stress (Group C) rats. Values are mean±SE (n=6). In all segments, the values between Group A vs. Group B (p<0.01), Group A vs. Group C (p<0.01), and Group B vs. Group C (p<0.05) were significantly different.](image)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Significant level</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca (mg)</td>
<td>3.15±0.25</td>
<td>4.85±0.34</td>
<td>6.32±0.35</td>
<td>p&lt;0.01</td>
<td>53.97</td>
</tr>
<tr>
<td>Phosphate (mg)</td>
<td>36.64±2.92</td>
<td>46.93±4.05</td>
<td>58.23±2.14</td>
<td>p&lt;0.05</td>
<td>28.08</td>
</tr>
<tr>
<td>Ca:creatinine (mg:mg)</td>
<td>1.02±0.10</td>
<td>1.38±0.10</td>
<td>1.60±0.04</td>
<td>p&lt;0.01</td>
<td>35.29</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE (n=6). Urinary calcium (Ca) and creatinine excretion are expressed in mg/24 h urine. Urinary phosphate excretion is expressed in mg/dl.
Cold Stress and Hypogonadal Osteoporosis

Table 3. Plasma biochemistry of Sham-control (Group A), ovariectomized (Group B), and ovariectomized+cold stress (Group C) rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Significant level</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca (mg/dl)</td>
<td>7.02±0.07</td>
<td>7.20±0.08</td>
<td>7.56±0.18</td>
<td>p&lt;0.05</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>AP (U/l)</td>
<td>110.0±7.76</td>
<td>140.0±8.37</td>
<td>172.0±9.21</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE (n=6).

Table 4. Changes in body weight of Sham-control (Group A), ovariectomized (Group B), and ovariectomized+cold stress (Group C) rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Significant level</th>
<th>% decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>135±3.8</td>
<td>122±2.8</td>
<td>114±3.4</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE (n=6).

stress-induced group thus were 15.55, 10.71, and 13.79, respectively, for duodenum, jejunum, and ileum.

Urinary calcium and phosphate excretion and calcium:creatinine ratio

Table 2 shows the effects of cold stress on urinary Ca and the phosphate excretion and Ca:creatinine ratio of ovariectomized rats. Compared with the Sham-control group, a significant (p<0.01 for Ca and p<0.05 for phosphate) increase in Ca (53.97%) and in phosphate (28.08%) excretions through urine was noted in the ovariectomized group. In the ovariectomized+cold-stress–induced group, these values were further enhanced (p<0.01), and compared with the Sham-control group, they were 100.63 and 58.92% for Ca and phosphate. The differences in percent increase responses between ovariectomized and ovariectomized+cold-stress–induced group thus were 30.31 and 24.08 for Ca and phosphate. Compared with the Sham-control group, the ovariectomized group produced a significant (p<0.01) increase in the Ca:creatinine ratio. This was further enhanced (p<0.01) in the ovariectomized+cold-stress–induced group.

Plasma alkaline phosphatase activity and plasma calcium level

The results of plasma AP activity and plasma Ca level are listed in Table 3. Compared with the Sham-control group, the ovariectomized group showed a significant (p<0.01) increase in plasma AP activity, which was further enhanced (p<0.01) in the ovariectomized+cold-stress–induced group. Similar to other biochemical markers of bone turnover rate, a rise in plasma Ca level in the ovariectomized+cold-stress–induced group is significant compared with either the Sham-control group (p<0.01) or the ovariectomized group (p<0.05).

Changes in body weight

The results of body weight under different experimental conditions are listed in Table 4. Compared with the Sham-control group, a significant decrease in body weight was observed in both the ovariectomized (p<0.01) and the ovariectomized+cold stress (p<0.01) groups.

DISCUSSION

The most encouraging observation of this study is that cold stress has a deteriorating effect on different physiological responses, viz., intestinal transference of Ca²⁺ and biochemical marker of bone turnover, which might be responsible for a faster development of hypogonadal osteoporosis.

To ascertain that our experimental stress was effective to produce changes as a general expectation that chronic cold stress increases the secretion of ACTH, corticosterone, and thyroid hormones [1, 2], we verified the plasma levels of corticosterone, T₄, and TSH in all groups of animals in our study. To make a further assessment of the effectiveness of stress, studies were undertaken to establish a correlation between
these hormones and calcium balance. Our results were quite in agreement with earlier reports that glucocorticoid and thyroid hormone both decrease the intestinal transference of Ca\(^{2+}\) [4–9, 12, 14], and that glucocorticoid only elevates fasting urinary Ca excretion [6, 33], confirming that our stress model was very effective in producing an alteration in hormone level and in calcium balance.

Recently this laboratory reported that the intestinal transference of Ca\(^{2+}\) is reduced in a descending gradient from duodenum to ileum in bilaterally ovariec-tomized rats. The possible cause suggested for this descending gradient response was a segment wise variable inhibition of AP and Ca\(^{2+}\)-ATPase enzymes [18]. The results of our study with bilaterally ovariec-tomized rats also confirmed these observations (Figs. 1–3). It was also observed that all these changes related to the inhibition of Ca\(^{2+}\) transference were more pronounced when ovariec-tomized rats were subjected to cold stress, suggesting that cold stress has a further deteriorating effect in ovariec-tomized rats rather than only a counteracting influence. This is in accord with the earlier report that cold stress decreases Ca\(^{2+}\) absorption in young turkeys [3]. The mechanism of this cold stress–induced reduction in intestinal Ca\(^{2+}\) transference can be attributed to the activities of relevant enzymes, AP and Ca\(^{2+}\)-ATPase, since the activity of both enzymes were significantly reduced in comparison with control or bilaterally ovariec-tomized animals. Although Ca\(^{2+}\) absorption was expected to decrease because of cold stress [3], to our knowledge this is the first time that such an observation in ovariec-tomized rats has been correlated with intestinal mucosal enzymes.

Bilaterally ovariec-tomized rats in our present study had an increase loss of urinary Ca and phosphate. But compared with the bilaterally ovariec-tomized group, this loss was much greater in ovariec-tomized + cold stress subjected rats (Table 2). In accord with an earlier report [34], compared with Sham-control, the bilaterally ovariec-tomized rats did not alter plasma Ca level. But a significant elevation was noted when ovariec-tomized animals were subjected to cold stress (Table 3). Thus with respect to calcium homeostasis, these results suggest that both the experimental conditions of our present study (ovarie-tomized and ovariec-tomized + cold stress) may be highly favorable for the development of a hypocalcemic condition. This suggestion finds its own support as increased urinary Ca losses in these animals are always being associated with simultaneous decreases in the intestinal transference of Ca\(^{2+}\), which are two of the most important factors in the development of hypocalcemia and the secondary increase in PTH secretion [35, 36]. Nevertheless, this observation is of special interest because a cold stress-induced increase in the loss of urinary Ca and an associated impaired calcium homeostasis possibly has not been speculated on before.

Ovariectomy-induced rises in concentrations of biochemical markers of bone turnover, AP and Ca : creatinine ratio, had a more significant rise when these animals were subjected to cold stress (Tables 2 and 3). A rise in serum AP level and urinary Ca : creatinine ratio has been linked with collagen degradation, bone re-absorption, and osteoporosis by earlier investigators [34, 37–39]. Data on body weight changes in different groups of animals (Table 4) suggest that a decrease in body weight may perhaps be related to bone loss and osteoporosis. A similar loss of body weight earlier was attributed to osteoporosis [37] or cold exposure [40], suggesting that cold exposure, in our model of study too, possibly has a positive influence on bone turnover and bone loss.

In conclusion, the observations made in the present study may have far-reaching implications if bone loss and development of osteoporosis in the hypogonadal situation are assessed in the light of ecological stress, which people of menopausal age usually experience in colder parts of the world.

The financial assistance by the University Grants Commission (UGC), New Delhi, India, is gratefully acknowledged. The authors are indebted to Dr. S. K. Dutta for providing assistance in estimating thyroidal parameters.

REFERENCES

6. Suzuki Y, Ichikawa Y, Saito E, and Homma M: Importance of increased urinary calcium excretion in the development of secondary hyperparathyroidism of patients under glucocorticoid therapy. Metabolism 32:
Cold Stress and Hypogonadal Osteoporosis


32. Glick D, Redlich DV, and Levine S: Fluorometric determination of corticosterone and cortisol in 0.02–0.05 milliliters of plasma or submilligram samples of adrenal tissue. Endocrinology 74: 653–655, 1964


