

# Effect of Voltage-Gated Calcium Channels (Cav) Blocker on Ovariectomy Induced Osteoporosis in Rats

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## ABSTRACT

**Objective:** The present study was conducted to investigate the effect of two different doses of two calcium channel blockers i.e. verapamil and nifedipine on genesis and control of osteoporosis by considering various biochemical and bone parameters against ovariectomized (OVX) rat model.

**Methods:** We have used total 42 female albino Wistar rats; one group of 6 animals was kept as Sham operated group. OVX was performed on 36 rats, after 14 days of recovery period; 36 OVX rats were equally and randomly divided into 06 groups, 4 groups for verapamil and nifedipine (two doses for each); one as disease control other is treated as standard. On 41<sup>st</sup> day, various parameters evaluated. **Results:** Low dose of verapamil treated group have P<0.01 significant improvement in bone development; increasing in the bone density, breaking strength and maintaining serum calcium, phosphorous and ALP level. It also reduced the osteoclast count and maintained normal bone architecture. **Conclusion:** From the above study we conclude that there is involvement of specific Cav 1.1 and Cav 1.3 channels

on rat osteoblast cells. Protection offered by low dose of verapamil possibly by controlling entry of Ca<sup>2+</sup> ions through these channels, inhibiting osteoclast formation and extending osteoblast survival.

**Key words:** OVX, Osteoblast, Voltage-gated calcium channels, Verapamil, Nifedipine.

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## INTRODUCTION

Osteoporosis is an emerging and socioeconomic threat characterized by a systemic impairment of bone mass, strength, and micro architecture, which increases the propensity of fragility fractures.<sup>1</sup> Hypertensive patients have an increasing risk of bone fracture compared with normotensive subjects; an increased urinary calcium excretion and a reduced bone density detected in hypertensive patients.<sup>2,3</sup> Pharmacoepidemiological case-control studies indicated that the treatment with thiazide diuretics, beta-blockers, angiotensin-converting inhibitors, calcium channel blockers is associated with a significantly reduced risk of fractures.<sup>4,5</sup> However, basic data supporting these clinical observations are limited. A variety of different Ca<sup>2+</sup> permeable channels that co-exist in the plasma membrane plays major roles in the entry of extracellular Ca<sup>2+</sup>. The free concentration of the Ca<sup>2+</sup> ion in the cytosol involves the regulation of mechanisms necessary for the short-term regulation of cell functions like contraction, excitation, secretion which account for many physiological processes.<sup>6</sup> The Osteoblasts derived from mesenchymal stem cells, plays a pivotal role in bone formation is might be mediated through voltage-gated calcium channels.<sup>7</sup> Intracellular calcium concentration, at least part of which is decreased by calcium channel blockers (CCB'), has been shown to be increased by various bone regulatory factors, such as vitamin D<sub>3</sub>,<sup>8,9</sup> parathyroid hormone<sup>8,10</sup> and prostaglandin E<sub>2</sub>.<sup>8,11</sup> These factors also alter osteoblast differentiation<sup>12,13</sup> thus; it shows that signaling through the L-type calcium channel may be important for osteoblast functions. All these above facts suggests that involvement of calcium channels in the bone remodeling, so this study is undertaken to exploit their detail role and development of new strategies for treatment of osteoporosis.

Ovariectomy induced bone loss in rats is a well-accepted model for investigating agents that could help to prevent osteoporosis because the similar pathophysiology of bone loss caused by ovariectomy in this animal model and by postmenopausal osteoporosis in women have been extensively examined.<sup>14,15</sup> The present study conducted to investigate the

effect of two doses of nifedipine and verapamil on serum biochemical, bone parameters and histopathological observation of bone during postmenopausal osteoporosis in ovariectomized rat model.<sup>16</sup>

## MATERIALS AND METHODS

**Chemicals:** Nifedipine and verapamil HCl were purchased from the Sigma Aldrich, USA. Ketamine, Estradiol valerate, Tramadol, Xylazine, Catguts, EDTA blood collecting vials were procured from the local market while Calcium Kit, Phosphate Kit, Bone specific ALP kit and Tartrate-resistant acid phosphatase (TRAP) were purchased from human Diagnostics worldwide.

**Instruments:** DEXA using a Hologic QDR-1000 X-ray bone densitometer, Vernier calipers, Trinocular microscope, Semi-Autoanalyser (Carex), muffle furnace, Catgut (2.0) etc.

## Animals

Forty two female Wistar albino rats weighing 180-200 g were acquired from the central animal house facility, Northern Border University, Saudi Arabia. Animals are housed in cages in a room maintained under controlled conditions of constant temperature and relative humidity of 21 ± 1°C and 50-55%, 12:12 Hr light/dark cycle. The rats were acclimatized for 10 days, had free access to chow and water. Nations NCBE (National Committee of Bio Ethics) guidelines were strictly followed and the project is approved by the Institutional animal ethical committee (IAEC), (Ref: 5-1436-6-1).

**Animal grouping and dosing:** Out of 42 animals OVX surgery was performed on 36 animals by keeping one group as normal control. Fourteen days after ovariectomy 36 animals were randomly divided in 6 groups by considering two doses for verapamil and nifedipine (high and low dose) and one group as disease control and other group was served standard and treated with estradiol valerate.

Doses of verapamil and nifedipine were selected based upon the FDA guideline on the estimation of the safe starting dose in human and animals.<sup>17</sup>

**Induction of Osteoporosis:** Osteoporosis was induced by bilateral ovariectomy. All operated animals were maintained for 14 days and for initial first three days after surgery animal were treated with gentamicin (10 mg/kg, *i.m.*)<sup>17</sup> and tramadol (25 mg/kg, *s.c.*)<sup>18</sup> to avoid any post-operative infection and pain respectively.

#### Ovariectomy surgery procedure

Ovariectomy was made by two dorso-lateral incisions, approximately 1 cm long above the ovaries. With the use of a sharp dissecting scissors, the skin was cut almost together with the dorsal muscles and the peritoneal cavity was thus accessed. After peritoneal cavity was accessed, the ovary was found, surrounded by a variable amount of fat. The surgery was done under anesthesia, using a Ketamine 80 mg/kg, Xylazine 5 mg/kg intraperitoneally. The connection between the fallopian tube and the uterine horn was cut and the ovary moved out.

Because of muscle bleeding, its incision required suturing.<sup>19</sup> The suturing was performed by using catgut. Recovery period of 14 days, the dosing was started from 15<sup>th</sup> day.

All the drugs were administered once daily for 40 successive days and on 41<sup>st</sup> day various parameters were studied like physical parameters of bone, blood chemistry, histopathological examination and osteoclast count were done. Refer Table 1.

Induction of Osteoporosis in surgically operated animals was confirmed by performing vaginal smear test.

#### Vaginal smear test

The two most commonly used methods of obtaining vaginal cell samples are:

- Lavage or washing with saline or water from a pipette.
- Swab or cotton bud (moistened with saline or water).

a. Vaginal smears were carried out to monitor cellular differentiation and to evaluate the presence of leukocytes, nucleated epithelial cells, or cornified cells. Vaginal smear samples were collected between 08.00 and 10.00 am daily. The vaginal smears were prepared by washing with 10 µl of normal saline (NaCl 0.9%) and were then thinly spread on a glass slide. They were allowed to dry at room temperature and then stained using Methylene blue dripping. The slides were rinsed in distilled water after 30 min and allowed to dry. The smears were studied using the light microscope (40×) and the cell type and their relative numbers were recorded.

Vaginal smear cell counts were performed on 100 cells randomly. The percentage of cornified cells was determined according to following formula:<sup>20</sup>

There was no significant difference in the percentage of cornified cells between the groups; it confirmed a menopausal pattern in OVX rats.

#### Determination of Serum parameters

After 40 days successive treatment on 41<sup>st</sup> various serum parameters like Serum calcium, phosphorus and alkaline phosphatase (ALP) were analyzed as per the method prescribed by Miller, 1994 *et.al.*<sup>21</sup> (Refer Figure 1 and 2).

#### Bone Parameters

$$\text{Percentage of Cornified Cells} = \frac{\text{Cornified Cells}}{\text{Cornified Cells} + \text{Nucleated Cells} + \text{Leucocytes}} \times 100$$

On 41<sup>st</sup> day rats were sacrificed by over doses of CO<sub>2</sub> inhalation and right femur, right tibia and 4<sup>th</sup> lumbar vertebrae were isolated from all rats.

**Table 1: Animal grouping, treatment and doses selected as follows**

Group No	Treatment	Dose	ROA
Group I	Distilled Water	10 ml/kg b.w.	<i>p.o.</i>
Group II	OVX+Distilled Water	10 ml/kg b.w.	<i>p.o.</i>
Group III	OVX+Estradiol valerate	0.1 mg/kg b.w.	<i>i.m.</i>
Group IV	OVX+nifedipine-H	2.11 mg/kg b.w.	<i>p.o.</i>
Group V	OVX+nifedipine-L	1.05 mg/kg b.w.	<i>p.o.</i>
Group VI	OVX+verapamil-H	4.22 mg/kg b.w.	<i>p.o.</i>
Group VII	OVX+verapamil-L	2.11 mg/kg b.w.	<i>p.o.</i>

*p.o.*=per oral, ROA=Route of administration, b.w.=Body weight, H=High dose, L=Low dose.

**2.7.1 Measurement of bone Weight, length and outer diameter of diaphysis right Femur:** Isolated right femur was cleaned to remove all surrounding tissues. Bones were kept in the hot oven at 52°C to achieve constant weight. Dry weight of the right femur was calculated by using 0.01 mg weighing precession digital balance and recorded. The bone length, outer diameter of diaphysis of right femur and tibia were measured using Vernier calipers.<sup>22</sup> (Refer Figure 3, 4, 5).

#### Bone mineral density (BMD) measurement by dual energy X-ray absorptiometry (DEXA):

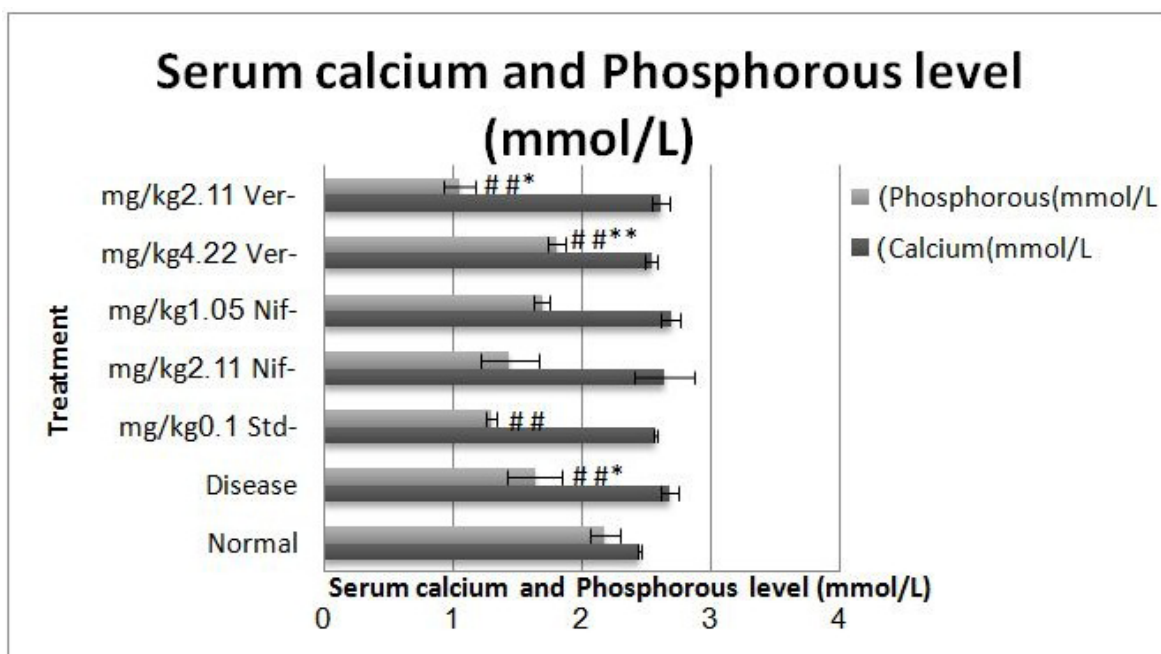
In this study bone mineral density measurements was performed by using *Ex vivo* method at the end of the study in a double blind approach by using a Hologic QDR-1000 X-ray bone densitometer (dual X-ray source of 70 and 140 kVp) and an ultrahigh resolution software program with 0.0254 cm line spacing and a point resolution of 0.0127 cm. This model software enables calculation of mineral content at any desired region, within the image. Right femora of each rat in all groups, were placed on the imaging positioning tray and scanned four times to avoid any errors due to placing position of the sample. The image of the mineral content within the path of this beam is generated by a scanning device. This enables calculation of the total integrated mineral content within the cross-section of the mineralized region, expressed as grams per unit area. For all bone mineral density calculations were done and compared with normal control and disease control groups (Refer Figure 6).

The breaking strength of right femur, tibia and 4<sup>th</sup> lumbar vertebrae were performed by breaking strength apparatus (Monsanto). The fresh bones and vertebrae were placed in hardness compressor, until it fractured. The reading were recorded in Newton's<sup>23</sup> (Refer Figure 7).

**Histopathology of femur bone and osteoclast examination:** The left femur were fixed in 10% neutral formalin for 12 hr at 4°C, decalcified in 5% ethylenediaminetetracetic acid (EDTA) for 7 days, embedded in paraffin and cut into longitudinal section of 5 µm thickness. The section were stained with haematoxylin and eosin and tartrate-resistant acid phosphatase (TRAP), a cytochemical marker for osteoclast and finally counter stained with haematoxylin. The number of positively stained osteoclast in the section of the median portion of the whole femora was enumerated for the all groups.<sup>22,23</sup> (Figure 8 and 9).

#### Statistical Analysis

The results are expressed as mean ± SEM. Comparisons between the treatment groups and positive control; positive control and control were performed by one way analysis of variance (ANOVA) followed by Dunnett test. In all tests the criterion for statistical significance was *p*<0.05 (95% level) and *p*<0.01. The analysis was performed by using Graph pad Prism V.



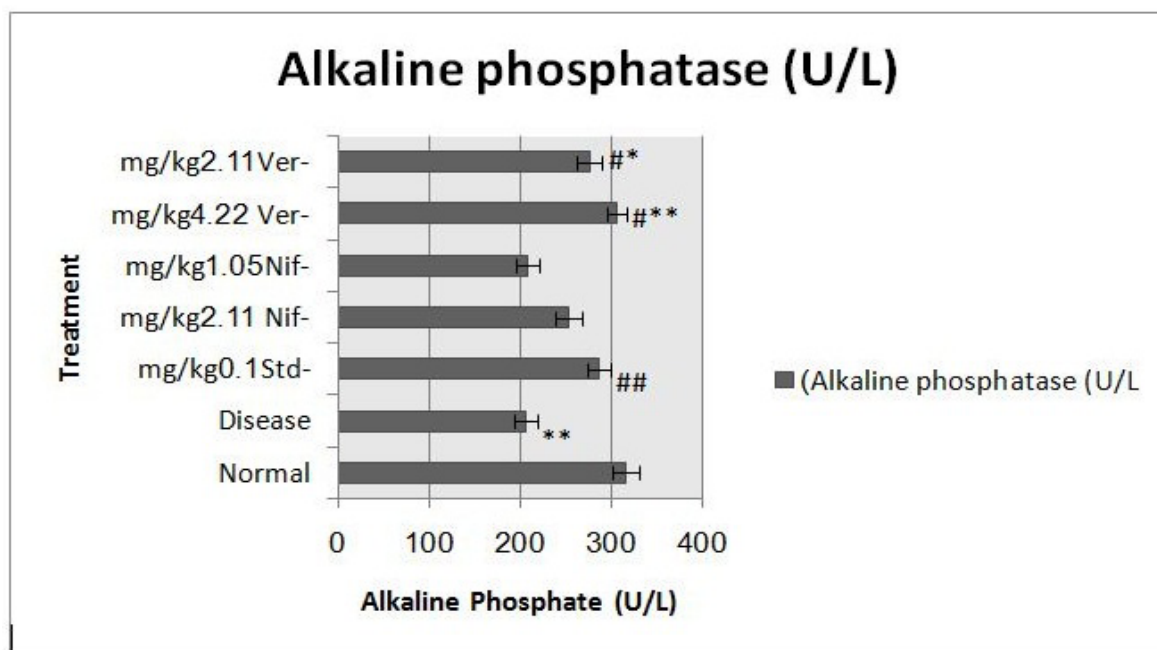
**Figure 1:** Effect of verapamil and nifedipine on Serum Calcium and Phosphorous. level (mmol/L) on OVX Wistar rats.

All of the data obtained from the experimental groups have been compared to Ovariectomized control (Disease) and Normal (Sham-operated) rat groups. The data was analysed statistically by one-way ANOVA followed by Dunnett test using Graph pad prism version 5.0 software.

<sup>#</sup>Comparison of standard and test groups with normal control.

<sup>\*</sup>Comparison of test, standard with disease control.

Values are significant at <sup>\*\*</sup>P<0.05, <sup>\*\*\*</sup>P<0.01 and <sup>\*\*\*\*</sup>P<0.001.

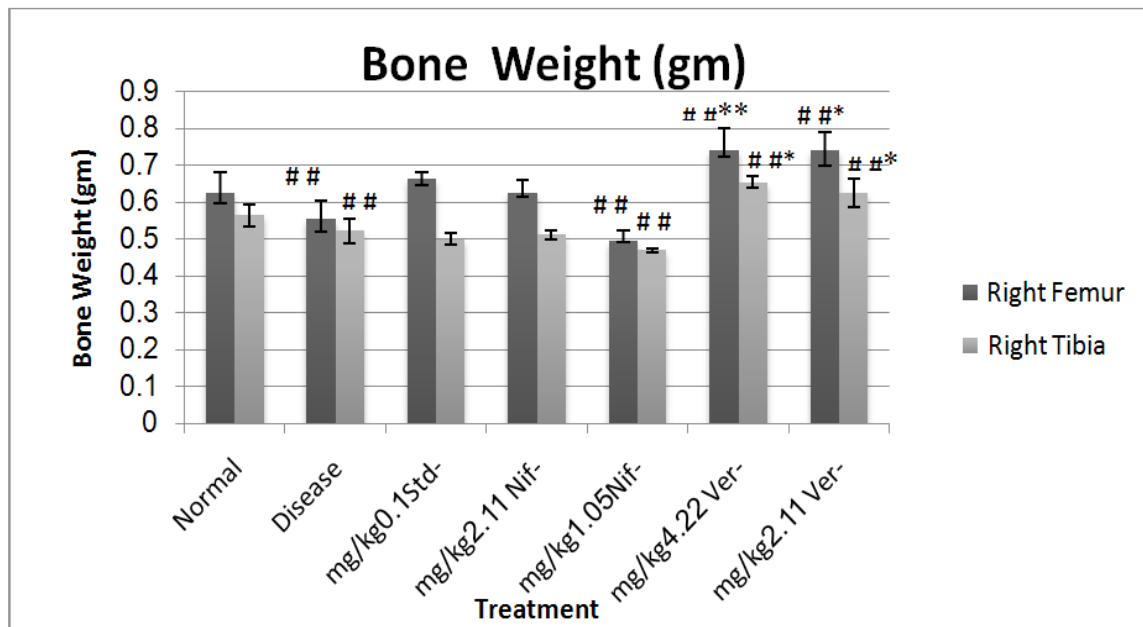


**Figure 2:** Effect of verapamil and nifedipine on Alkaline Phosphate (U/L) on OVX Wistar rats.

All of the data obtained from the experimental groups have been compared to Ovariectomized control (Disease) and Normal (Sham-operated) rat groups. The data was analysed statistically by one-way ANOVA followed by Dunnett test using Graph pad prism version 5.0 software.

<sup>#</sup>Comparison of standard and test groups with normal control.

<sup>\*</sup>Comparison of test, standard with disease control.



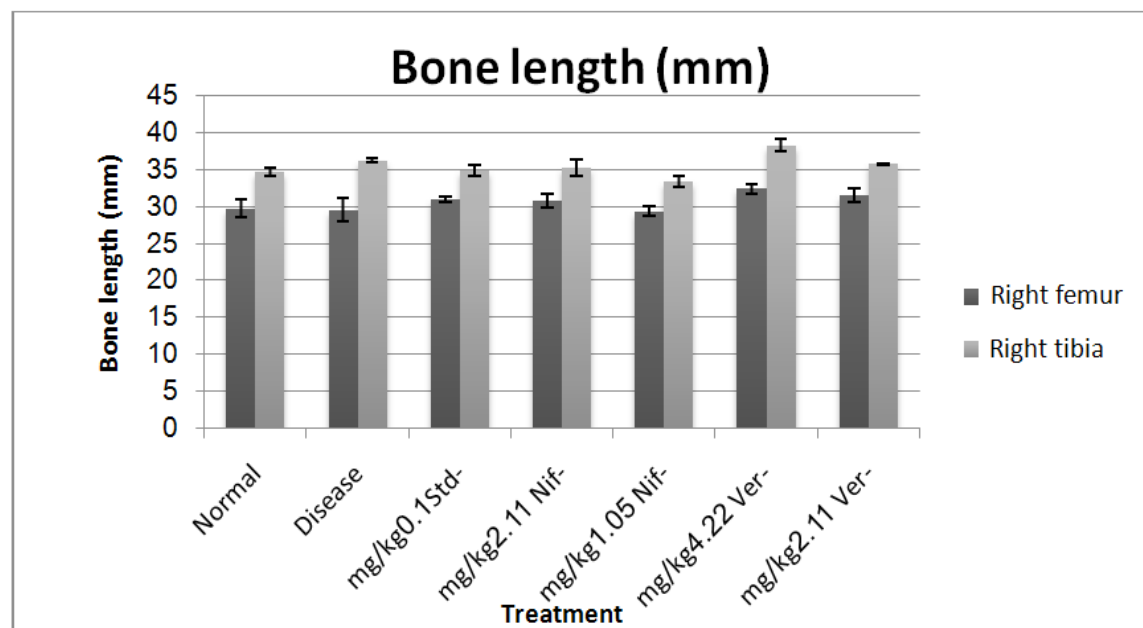
**Figure 3:** Effect of verapamil and nifedipine on Bone Weight (gm) on OVX Wistar rats.

All of the data obtained from the experimental groups have been compared to Ovariectomized control (Disease) and Normal (Sham-operated) rat groups. The data was analysed statistically by one-way ANOVA followed by Dunnett test using Graph pad prism version 5.0 software.

\*Comparison of standard and test groups with normal control.

\*Comparison of test, standard with disease control.

Values are significant at \*\*  $P < 0.05$ , \*\*\*  $P < 0.01$  and \*\*\*\*  $P < 0.001$ .



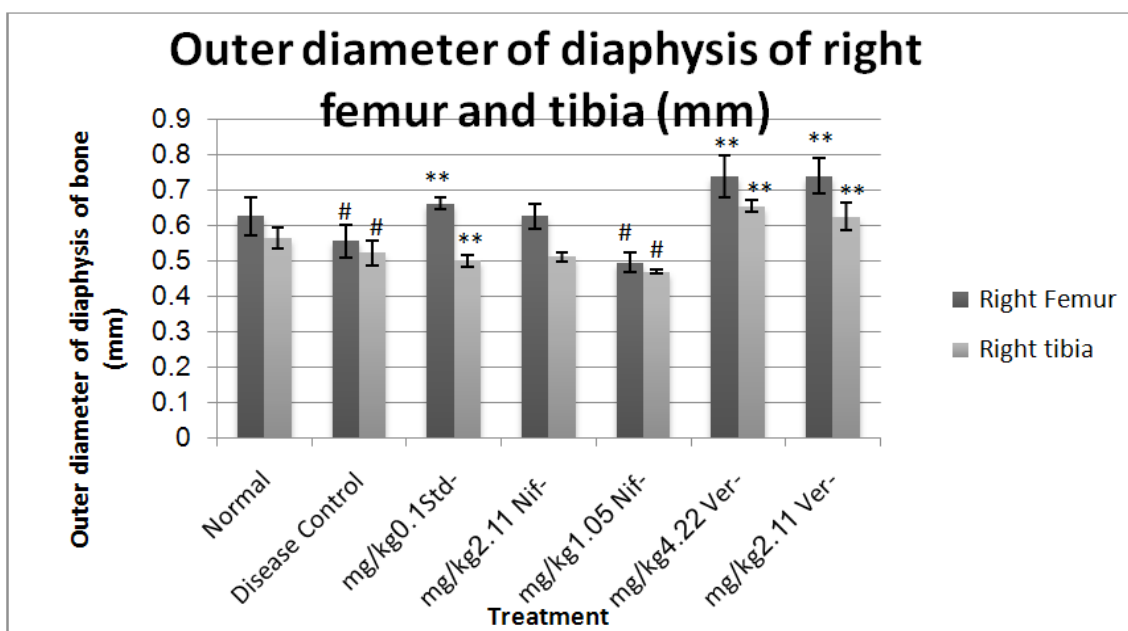
**Figure 4:** Effect of verapamil and nifedipine on Bone length (mm) on OVX Wistar rats.

All of the data obtained from the experimental groups have been compared to Ovariectomized control (Disease) and Normal (Sham-operated) rat groups. The data was analysed statistically by one-way ANOVA followed by Dunnett test using Graph pad prism version 5.0 software.

\*Comparison of standard and test groups with normal control.

\*Comparison of test, standard with disease control.

Values are significant at \*\*  $P < 0.05$ , \*\*\*  $P < 0.01$  and \*\*\*\*\*  $P < 0.001$ .

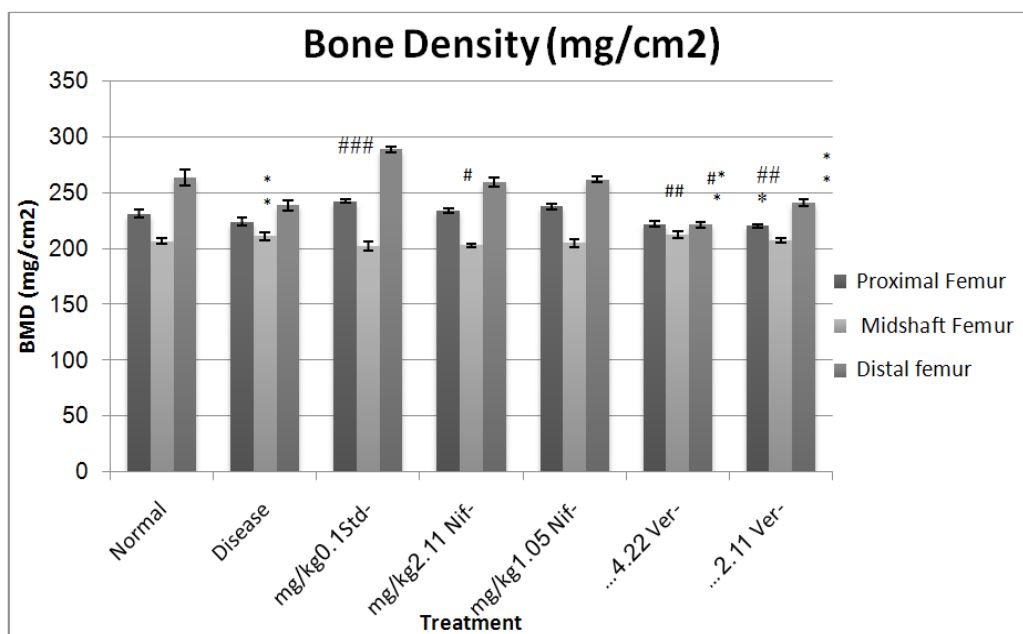


**Figure 5:** Effect of verapamil and nifedipine on Outer diameter of diaphysis of right femur and right tibia on OVX Wistar rats.

All of the data obtained from the experimental groups have been compared to Ovariectomized control (Disease) and Normal (Sham-operated) rat groups. The data was analysed statistically by one-way ANOVA followed by Dunnett test using Graph pad prism version 5.0 software.

<sup>#</sup>Comparison of standard and test groups with normal control.

\*Comparison of test, standard with disease control. Values are significant at <sup>#</sup> $P < 0.05$  and <sup>\*\*\*</sup> $P < 0.01$ .



**Figure 6:** Effect of verapamil and nifedipine on Bone density (mg/cm<sup>2</sup>) on OVX Wistar rats.

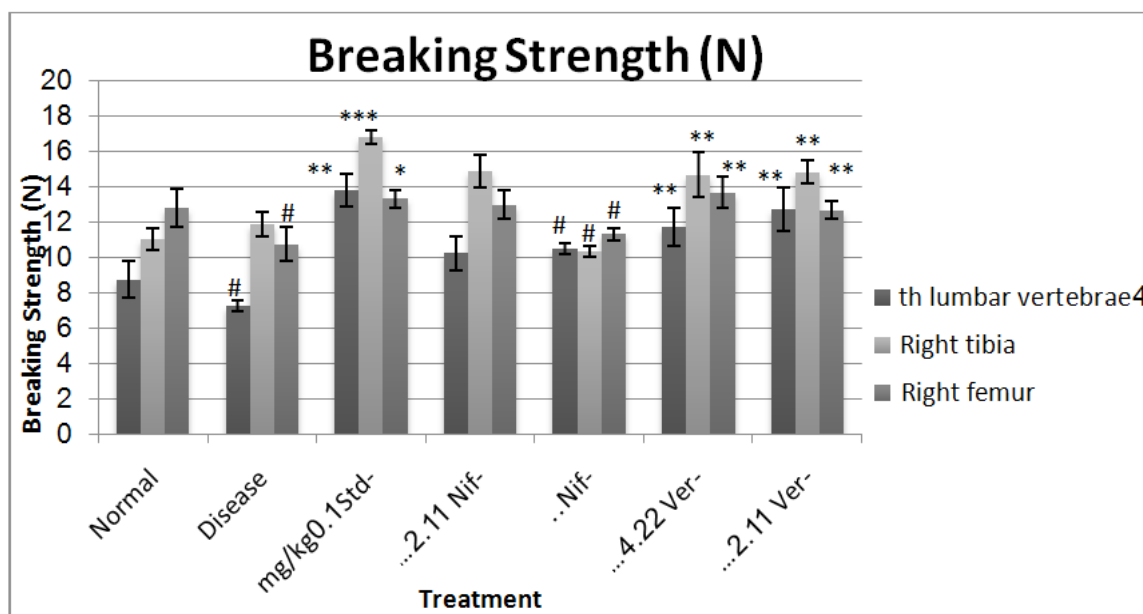
All of the data obtained from the experimental groups have been compared to Ovariectomized control (Disease) and Normal (Sham-operated) rat groups. The data was analysed statistically by one-way ANOVA followed by Dunnett test using Graph pad prism version 5.0 software.

<sup>#</sup>Comparison of standard and test groups with normal control.

\*Comparison of test, standard with disease control.

Values are significant at <sup>#</sup> $P < 0.05$ , <sup>\*\*\*</sup> $P < 0.01$  and <sup>\*\*\*\*\*</sup> $P < 0.001$ .





**Figure 7:** Effect of verapamil and nifedipine on breaking strength (N) of 4<sup>th</sup> lumbar, right tibia and right femur on OVX Wistar rats.

All of the data obtained from the experimental groups have been compared to Ovariectomized control (Disease) and Normal (Sham-operated) rat groups. The data was analysed statistically by one-way ANOVA followed by Dunnett test using Graph pad prism version 5.0 software.

\*Comparison of standard and test groups with normal control.

\*Comparison of test, standard with disease control. Values are significant at \*#P<0.05, \*\*\*#P<0.01 and \*\*\*P<0.001.

## RESULTS

### *Effect of verapamil and nifedipine on Serum Calcium and Phosphorous level (mmol/L) on OVX Wistar rats*

The Serum phosphorous level was significantly ( $P<0.001$ ) declined in Standard estradiol, nifedipine-high dose and verapamil-low dose treated when compared to normal control. The serum level of calcium significantly ( $P<0.01$  &  $P<0.05$ ) reduced in the verapamil high dose and low dose treated groups compared to disease control group respectively. (Figure 1).

### *Effect of verapamil and nifedipine on Alkaline Phosphate (U/L) on OVX Wistar rats.*

The serum level of alkaline phosphatase significantly ( $P<0.01$ ) increased in verapamil treated group compared to disease control groups in dose dependent manner (Figure 2).

### *Effect of verapamil and nifedipine on Bone Weight (gm) on OVX Wistar rats.*

Weight of right femur and tibia were significantly ( $P<0.01$ ) declined by disease control and nifedipine low dose compared with normal control group. verapamil treated and standard estradiol valerate treated groups have shown significant raise in weight of right femur and tibia compared to disease control and normal control groups (Figure 3).

### *Effect of verapamil and nifedipine on Bone length (mm) on OVX Wistar rats*

No significant changes were observed in the bone length in any of the treatment groups (Figure 4).

### *Effect of verapamil and nifedipine on Bone density (m/v) on OVX Wistar rats.*

Bone density was significantly ( $P<0.01$ ) reduced by disease control and nifedipine low dose treated groups compared with normal control

groups. Standard estradiol valerate, verapamil low and high dose treated groups have shown significantly ( $P<0.01$ ) increase in bone density compared to disease control and normal control groups (Figure 5).

### *Effect of verapamil and nifedipine on Outer diameter of diaphysis of right femur and right tibia on OVX Wistar rats.*

Outer diameter of diaphysis of right femur and tibia was significantly ( $P<0.05$ ) reduced by disease control and nifedipine low dose treated groups compared with normal control groups. Standard estradiol valerate, verapamil low and high dose treated groups have shown significantly ( $P<0.01$ ) increase in outer diameter of diaphysis of right femur and tibia compared to disease control and normal control groups (Figure 6).

### *Effect of verapamil and nifedipine on breaking strength (N) of 4<sup>th</sup> lumbar, right tibia and right femur on OVX Wistar rats.*

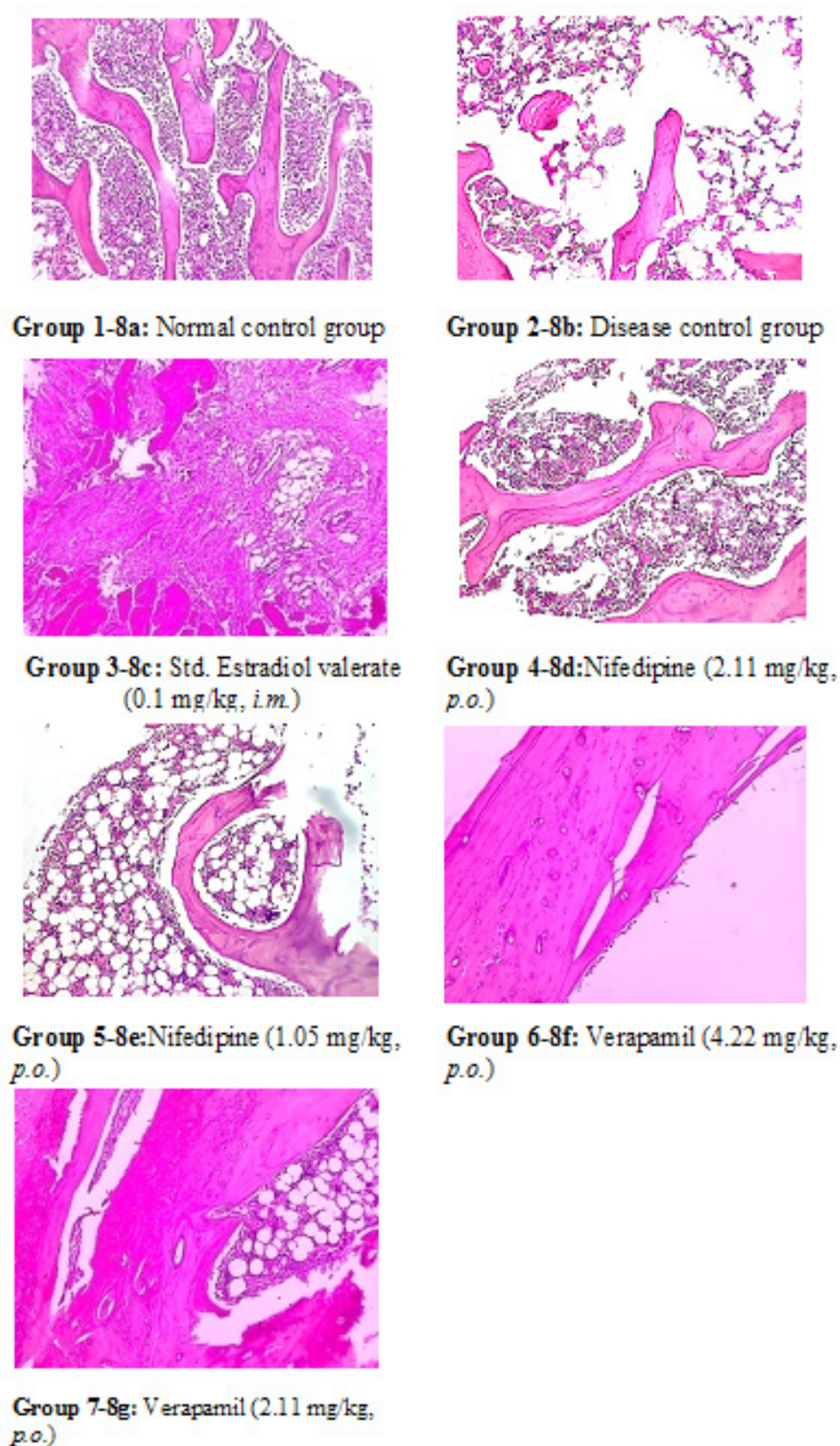
Breaking strength of 4<sup>th</sup> lumbar and right femur significantly ( $P<0.05$ ) reduced by disease control and nifedipine low dose treated group compared with normal control group. Breaking strength of 4<sup>th</sup> lumbar, right tibia and right femur was significantly ( $P<0.01$ ) increased by standard estradiol valerate and verapamil low and high dose (Figure 7).

### *Effect of verapamil and nifedipine on histopathology of left femur on OVX Wistar rats:*

Disease control group showing disturbed microarchitectural of bone. In verapamil treated group have shown protection against estrogen loss and maintained microarchitecture of the bone like standard Estradiol valerate treated group (Figure 8).

### *Effect of verapamil and nifedipine on osteoclast cell count of left femur bone on OVX Wistar rats:*

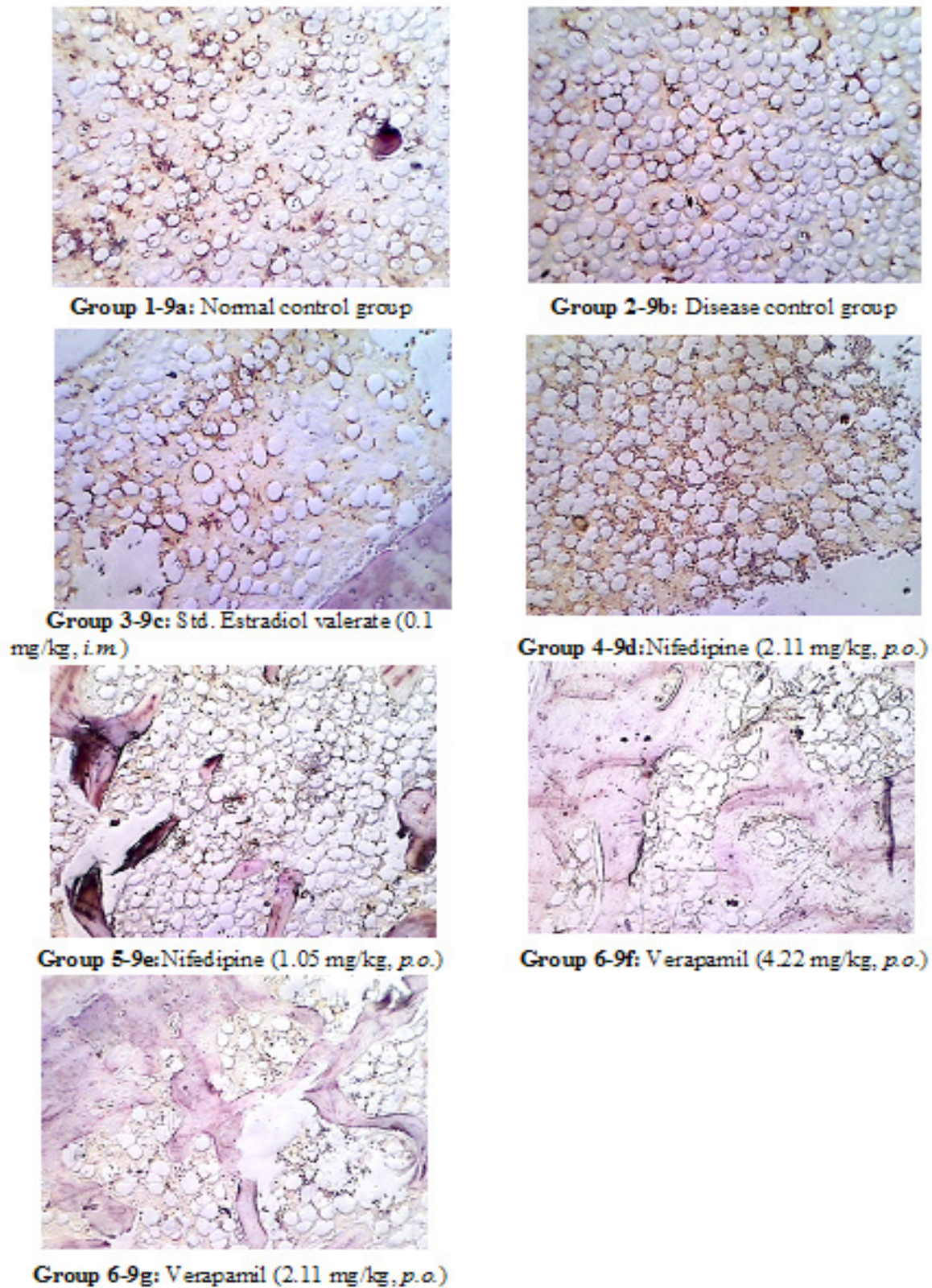
Disease control group showing high osteoclast count indicating bone porosity and resorption. Osteoclast count is reduced by verapamil high and low dose treated group indicating protective effect against estrogen loss OVX Wistar rats (Figure 9).



**Figure 8:** Effect of verapamil and nifedipine on histopathology of left femur on OVX Wistar rats.

The left femur were fixed in 10% neutral formalin for 12 hr at 4°C, decalcified in 5% ethylenediaminetetraacetic acid (EDTA) for 7 days, embedded in paraffin and cut into longitudinal section of 5 µm thickness.

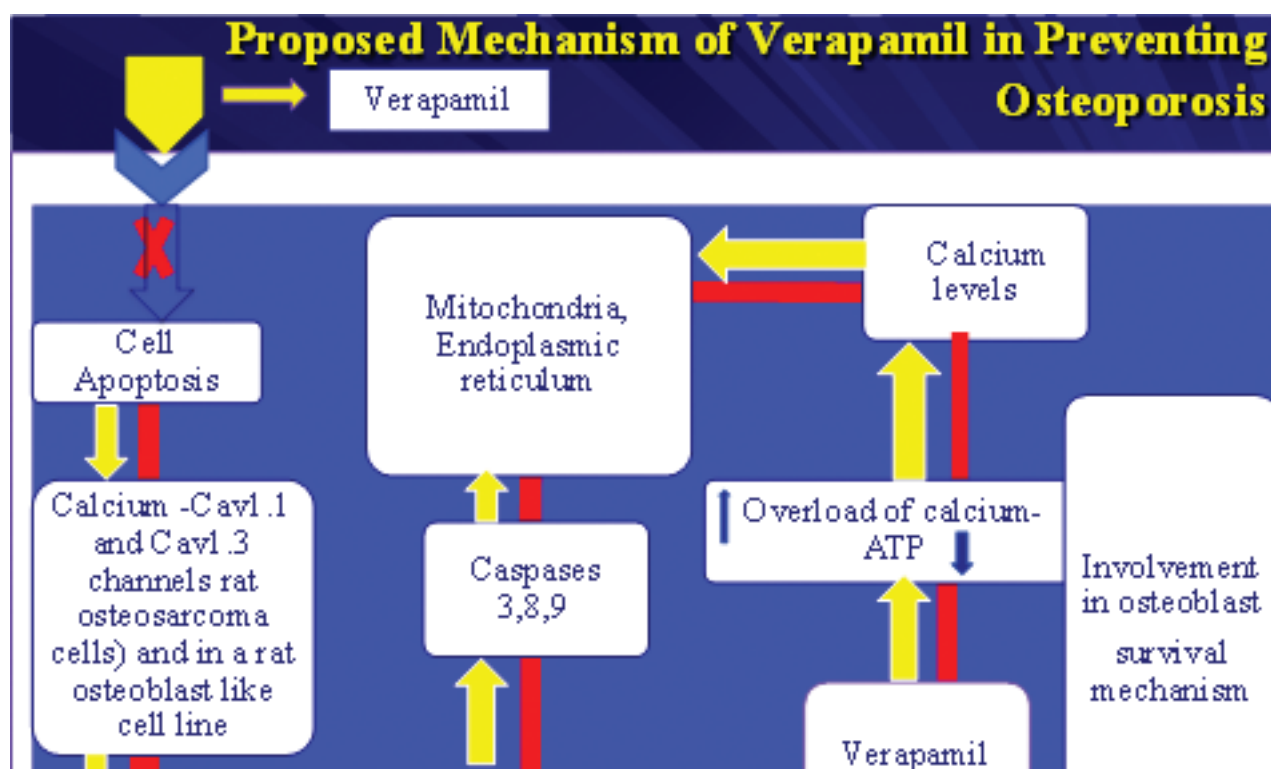




**Figure 9:** Effect of verapamil and nifedipine on osteoclast cell count of left femur bone on OVX Wistar rats (TRAP analysis):

The left femur were fixed in 10% neutral formalin for 12 hr at 4°C, decalcified in 5% ethylenediaminetetracetic acid (EDTA) for 7 days, embedded in paraffin and cut into longitudinal section of 5 µm thickness. The section were stained with haematoxylin and eosin and tartrate-resistant acid phosphatase (TRAP), a cytochemical marker for osteoclast and finally counter stained with haematoxylin. The number of positively stained osteoclast in the section of the median portion of the whole femora was enumerated for the all groups and pictures were taken by using Trinocular microscope and Olympus camera (400 X).





**Figure 10:** Proposed mechanism of verapamil as anti-osteoporosis in the management of post-menopausal osteoporosis.

## DISCUSSION

Calcium is a ubiquitous second messenger involved in multiple signaling pathways mediating diverse physiological functions. The free concentration of the  $\text{Ca}^{2+}$  ion in the cytosol involves the regulation of mechanisms necessary for the short-term regulation of cell functions like contraction, excitation, secretion which account for many physiological processes.<sup>24</sup> In bone,  $\text{Ca}^{2+}$  has a structural role, since osteoblasts deposit an extracellular matrix (ECM) that contains nucleation sites for mineral deposition but their exact role in the control of bone development and resorption is yet to be established, so in the present study we have investigated the role of two L-type  $\text{Ca}^{2+}$  channel antagonists i.e. nifedipine and verapamil in osteoporosis by using OVX rat model.

The test drugs verapamil and nifedipine, two doses of each and standard estradiol valerate were administered successively for 40 days. Tests drugs were administered by *p.o.* route while standard estradiol valerate was administered by *i.m.* route. To avoid circadian changes in the hormonal levels we have administered all drugs in between 9.00 AM to 10.00 AM daily.

To understand the role of these drugs in the bone regulation we have performed parameters like serum calcium, phosphorous and alkaline phosphatase levels; bone parameters like bone density and breaking strength of right femur, right tibia, 4<sup>th</sup> lumbar vertebrae; histopathological examination and osteoclast count of left femur bone on each animal was performed.

Groups treated with verapamil have shown significant decreased level of serum calcium, phosphorous when compared with disease control group (Refer Figure 1). In all treated groups phosphorous levels are sharply decreased when compared to calcium level. The above changes are possibly because of the parathyroid hormone (PTH). Previous study reveals presence of calcium channels on parathyroid cell membrane

that respond to plasma calcium, by blocking calcium channels on parathyroid membrane causes more release of PTH. Parathyroid hormone increases blood calcium level by operates negative feedback system to maintain decreased calcium level by multiple mechanisms i.e. it acts on the respective effectors results in increased number of osteoclasts by increasing bone resorption, kidneys retain calcium in blood, excrete phosphate in urine and also promotes GI absorption of calcium.<sup>25,26</sup>

In our study we have measured serum alkaline phosphatase (ALP) level because elevated ALP indicates that there could be active bone formation occurring as ALP is a byproduct of osteoblast activity.<sup>27</sup> Osteoblasts are specific fibroblasts that secrete and mineralize the bone matrix that's gives strength to the bone. While osteoblast differentiation several extracellular matrix proteins (procollagen I, TGF- $\beta$ , and fibronectin) are produced and for matrix maturation alkaline phosphatase is more expressed by the osteoblast cells, hence increase in the serum ALP level is the clear sign of the bone formation and bone mineralization.<sup>28</sup> In our study serum ALP level was significantly ( $P < 0.01$ ) increased by verapamil low and high doses while nifedipine treated groups there is also increase in ALP level but not significantly compared to disease control group (Refer Figure 2).

Both the test drugs are acting on L-type calcium channels, the difference in their action is may be because of their binding affinity to the specific L-type calcium channels. There are four isoforms of L-VGCCs known and affinity to the same blocker varies. Cav1.3 and Cav1.4, two isoforms of L-VGCCs, has lower affinity for nifedipine, than Cav1.2<sup>29</sup> Cav1.1 and Cav1.3<sup>30</sup> which are expressed on rat osteosarcoma cells<sup>31</sup> and in a rat osteoblast like cell line<sup>32</sup> because of these possible reasons verapamil have shown protective effect compare to nifedipine treated groups.

Verapamil treated groups shown no significant protection against bone length but bone weight ( $P < 0.05$ ) and bone density (proximal femur and

distal femur) was significantly ( $P < 0.01$ ) increased by low and high dose of verapamil compared to disease control group (Refer Figure 3 and 4).

Post-menopausal osteoporotic women mainly affected by fractures occurred in femur bone, tibia, and 4<sup>th</sup> lumbar vertebrae that might be because of these bones are mainly involved in support and bear more strain so we have considered breaking strength of these bones as one of the parameter. In our study verapamil low dose has shown significant ( $P < 0.01$ ) improvement in the breaking strength of right femur bone, right tibia, 4<sup>th</sup> lumbar vertebrae compared with both disease control and sham-operated groups (Refer Figure 7) but not more than Estradiol treated group. Decreasing in the breaking strength in the disease control group indicating bone weakness, it may be because of the more osteoclast (i.e. bone resorption) and less osteoblast (i.e. bone formation) in bone cells. These results were further confirmed by histopathological study and osteoclast count of femur bone, which supports our findings (Refer Figure 8 and 9).

Limitation of our study is that we didn't performed parameters involved in the apoptosis but based upon our finding and pharmacology of test drugs we have postulated overall mechanism. Sustained increase in intracellular calcium has been associated with apoptosis in a variety of cell systems.<sup>33</sup> Cytosolic calcium load is kept under control by  $\text{Ca}^{+2}$  pump, functioning of the  $\text{Ca}^{+2}$  pump is ATP dependent which supplied by mitochondria. Loading of the mitochondrial stores by calcium beyond a certain point, however, disrupts mitochondrial function results in diminish ATP synthesis, thus reducing the energy available for the membrane pumps. This may responsible for increase in free cytosolic  $\text{Ca}^{+2}$  level and possibly activates the caspases proteins to initiates apoptosis. Verapamil has offered protection possibly by controlling the entry of  $\text{Ca}^{+2}$  ions in the osteoblast and osteosarcoma cells via Cav1.1 and Cav1.3 channels.

Based on these observations, we proposed that low dose of verapamil directly inhibited the osteoclast count and also maintained normal bone architecture via its involvement in osteoblast survival mechanism (Figure 10).

## CONCLUSION

On the basis of this study we concluded that low dose of verapamil has potent anti-osteoporotic activity.

The advantage of these finding is that in age old persons many disease like hypertension, arterial fibrillation and osteoporosis co-exist together and to tackle those patient may consume many drugs together, this multiple drugs combination not only gives stress on already weakened body but also interaction in-between them may gives horrifying effects and some time it is fatal too. CCBs have been already approved by FDA and the drugs are in the market from many decades to treat many conditions. Our latest finding suggests that mono therapy with verapamil might be enough to control mild hypertension co-existed with post-menopausal osteoporosis and this mono therapy will also prevent the dreadful drug interactions.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

Authors don't have any conflict of interest.

## ABBREVIATIONS USED

**cALP**: Alkaline phosphatase; **ANOVA**: Analysis of variance; **BMD**: Bone mineral density; **Cav**: Voltage gated Calcium Channels; **CCB**: Calcium channel blockers; **CRAC**:  $\text{Ca}^{+2}$  release-activated  $\text{Ca}^{+2}$  channels; **DEXA**: Dual energy x-ray absorptiometry; **ECF**: Extracellular fluid; **ECM**: Extra Cellular matrix; **EDTA**: Ethylene diamine tetra cetic acid; **FDA**: Food drug approval; **gm**: gram; **IAEC**: Institutional animal ethical committee; **L-VGCC**: L-type of voltage gated calcium channels; **mg**: milligram; **mm**: millimeter; **N**: Nutone; **NCBE**: (National Committee of BioEthics); **Nif**: nifedipine; **OVX**: Ovariectomized; **PTH**: Parathyroid hormone; **RANK**: Receptor activator of nuclear factor  $\kappa$ ?; **RANK-Ligand**: Receptor activator of nuclear factor  $\kappa$ ?-Ligand; **SOCs**: Store operated channels; **TGF- $\beta$** : Transforming growth factor- $\beta$ ; **TRAP**: Tartrate acid resistant phosphatase; **U/L**: units per liter; **Ver**: Verapamil; **VOCs**: Voltage-operated channels.

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