RESULTS & DISCUSSIONS
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In Mineralogical Analysis, Bone Mineral Content (BMC), Bone Mineral Density (BMD), Calcium content, Phosphorous content and Carbon content of all the three groups (control, OVX, OVX+PEMF or exposed group) were quantified. 10 animals were taken in each group.

A significant increase has been found in the femur BMC of OVX+PEMF group as compared to the OVX group (Table.4.1, fig. 4, P<0.05). However when compared to the Control group the increase in BMC of Exposed group was insignificant (P>0.05). BMC of Control group femur was 418.12±27.71, whereas that in exposed group was 388.19±70.33 and OVX group was 313.96±4.77.

Similarly significant increase in femur BMD level of exposed group was found as compared to that of OVX group (Table.4.1, fig. 5, P<0.05). When compared to the control group the increase in BMD of exposed group was insignificant (P>0.05). BMD of femur of exposed group was 918.59±180.68, in OVX group: 738.24±133.72 and in the control group, it was 1016.35±148.28.

A similar trend was observed in the tibia BMC and BMD. BMC and BMD of tibia of exposed group was highly significant as compared with OVX group (Table.4.1, fig. 4 and fig. 5, P<0.001). Whereas when compared with control group it expressed insignificant difference (P>0.05). BMC of tibia of exposed group was 273.71±16.93, OVX group was 220.77±33.79 and in control group it was 284.15±22.07. BMD of tibia of exposed group was found as 714.29±80.53, OVX group was 540.29±83.62 and in control group, it was 751.41±78.14.

This clearly indicates that PEMF accelerate mineralization (increase in BMC) in induced osteoporotic rat bones. This increase is somewhat similar to control group rat bones. Results found not only more mineralization or more mineral precipitation in bone but bone mineral density also increased due to PEMF exposure.
Table 4.1. Mean ± SD values obtained from Mineralogical Analysis

<table>
<thead>
<tr>
<th></th>
<th>Control Rat Bones</th>
<th>OVXed Rat Bone</th>
<th>OVX+PEMF Rat Bones</th>
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<tbody>
<tr>
<td></td>
<td>Femur</td>
<td>Tibia</td>
<td>Femur</td>
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<tr>
<td>Fresh Bone Wt.</td>
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<tr>
<td>(gms.)</td>
<td>0.828 ± 0.077</td>
<td>0.55 ± 0.033</td>
<td>0.675 ± 0.045</td>
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<tr>
<td>Bone Volume</td>
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<tr>
<td>(µl.)</td>
<td>417 ± 47.621</td>
<td>380 ± 27.08</td>
<td>426 ± 17.763</td>
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<tr>
<td>Dry Bone Wt.</td>
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<tr>
<td>(gms.)</td>
<td>0.664±0.044</td>
<td>0.495±0.038</td>
<td>0.599±0.070</td>
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<tr>
<td>BMC (mg.)</td>
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<tr>
<td></td>
<td>418.1±27.71</td>
<td>284.15±22.07</td>
<td>313.96±54.77</td>
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<tr>
<td>BMD (mg./ml.)</td>
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<tr>
<td></td>
<td>1016.35±148.28</td>
<td>751.41±78.14</td>
<td>738.24±133.72</td>
</tr>
<tr>
<td>Total Ca (mg.)</td>
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<tr>
<td></td>
<td>128.44±18.51</td>
<td>86.69±2.86</td>
<td>106.13±4.59</td>
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<tr>
<td>Total P (mg.)</td>
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<td></td>
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<tr>
<td></td>
<td>110.25±5.16</td>
<td>72.95±5.67</td>
<td>82.76±3.13</td>
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<tr>
<td>Total C (%)</td>
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<td></td>
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<tr>
<td></td>
<td>22.40±2.35</td>
<td>19.31±1.63</td>
<td>19.02±1.28</td>
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</tbody>
</table>

BMC= Bone Mineral Content, BMD= Bone Mineral Density,
Fig. 4. Shows bone mineral content of femur and tibia of Control, OVX and OVX+PEMF group. * Statistically significant compared to OVX, P<0.05 in femur and P<0.001 in tibia.

Fig. 5. Shows bone mineral density of femur and tibia of Control, OVX and OVX+PEMF group. * Statistically significant compared to OVX, P<0.05 in femur and P<0.001 in tibia.
Calcium and Phosphorous are the major constituents of bone minerals. Calcium was analysed in Flame Atomic Absorption Spectrometer (FAAS) and phosphorous was evaluated in spectrophotometer by colorometric method. Both showed a significant response to PEMF exposure when compared with OVX group (Table.4.1, fig. 6, P value of femur is <0.05 and tibia <0.05). Whereas when compared with the control group it was found to be insignificant. Calcium content of femur in exposed group was 115±12.84 mg in OVX group: 106.13±4.59 and Control group it was 128.44±18.51mg. Calcium content in tibia of Exposed group was 84.57 ± 3.26, OVX: 69.21+7.65 and in Control rat tibia was 86.69 ± 2.86.

The results of bone Phosphorous content show that PEMF has significant response in ovariectomised rat bones (femur and tibia). In femur P value between OVX and OVX+PEMF is <0.001, and tibia P Value between OVX and OVX+PEMF is <0.001 (Table.4.1, fig. 7). Whereas between controlled and exposed group, no significant results were found. Phosphorus content of femur in exposed group was 98.91 ± 5.42 mg in OVX group: 82.76 ± 3.13 and in Control group it was 110.25 ± 5.16 mg. Phosphorus content in tibia of Exposed group was 70.07 ± 3.8, OVX: 48.62 ± 7.93 and in Control rat tibia was 72.95 ± 5.67.

A significant increase in Carbon % in exposed group was observed as compare to control counterpart (Table.4.1, fig. 8.).

More Ca and P content show positive response of PEMF exposure towards mineralization. Carbon content reflects the organic content (Cellular components and extracellular macromolecules like Collagen).
Fig. 6. Shows total calcium content of femur and tibia in Control, OVX and OVX+PEMF group. * Statistically significant compared to OVX femur and tibia, P<0.05

Fig. 7. Shows total phosphorus content of femur and tibia in Control, OVX and OVX+PEMF group. * Statistically significant compared to OVX femur and tibia, P<0.001
Fig. 8. Shows total carbon content of femur and tibia in Control, OVX and OVX+PEMF group.
Multivariate model was used to compare the induced mineralization by PEMF. Mineralogical data of Control, OVX and OVX+PEMF were fitted in a multivariate model. Here calcium and phosphorous were kept as independent variables. Regression equation and regression coefficient of BMC were evaluated for all the groups of bone samples (control femur and tibia, OVXed femur and tibia and OVX +PEMF femur and tibia).

Figure 9. (a. and b.) display the multivariate model for BMC in Control group. Here the fit is very good for femur.

Here equation for control femur BMC is
\[ y = 12.12 + 0.62 \times Ca + 2.96 \times P, \quad (r^2 = 0.89) \]
The equation of control tibia, BMC is
\[ y = 39.60 - 0.13 \times Ca + 3.51 \times P, \quad (r^2 = 0.79). \]

Figure 10. (a. and b.) display the multivariate model for OVX group. Here fit is fairly good for femur.

The equation for OVX femur BMC is
\[ y = -706.70 + 0.90 \times Ca + 10.02 \times P, \quad (r^2 = 0.81) \]
In case of OVX tibia, BMC equation is
\[ y = 10.31 + 6.07 \times Ca - 4.31 \times P, \quad (r^2 = 0.60). \]

Figure 11. (a and b) displays the multivariate model for exposed group (OVX +PEMF). Fit for BMC is good for both femur and tibia.

BMC equation in case of exposed femur is
\[ y = -991.73 + 2.75 \times Ca + 12.25 \times P, \quad (r^2 = 0.84). \]
BMC equation in exposed tibia is
\[ y = -99.43 + 3.50 \times Ca + 1.10 \times P, \quad (r^2 = 0.80). \]
Fig. 9. Shows the multiple linear regression model graph of BMC in (a) Control Femur and (b) Control Tibia.
Fig. 10. shows the multiple linear regression model graphs of BMC in (a) OVX Femur and (b) OVX Tibia.
Fig. 11. shows the multiple linear regression model graph of BMC in (a) OVX + PEMF Femur and (b) OVX + PEMF Tibia.
**X-Ray diffraction** method show the higher intensity record at an angle of \(2\theta = 32^\circ\), which is the characteristic diffraction angle for calcium hydroxyapatite. The intensity for the control group femur was 1200 counts, for OVX group was 800 counts and for exposed femur it was 950 (fig. 12). Similarly in tibia the intensity was 880 counts for control group, 850 counts for OVX group and 875 counts for OVX + PEMF group (fig. 13). High intensity peak shows higher concentration of calcium hydroxyapatite in more crystalline form.
Fig. 12. Shows the XRD pattern of (a) Control Femur, (b) OVX Femur and (c) OVX + PEMF Femur at 2θ=32° diffraction angle. OVX+PEMF Femur shows high peak as compared to OVX. Control Femur has highest peak.
Fig. 13. Shows the XRD pattern of (a) Control tibia, (b) OVX tibia and (c) OVX + PEMF tibia at $2\theta=32^\circ$ diffraction angle. OVX+PEMF tibia shows high peak as compared to OVX. Control tibia has highest peak but the difference is in lesser extent.
DEXA images obtained with Hologic QDR 1500 system of rat bones (femur and tibia) show an increase in BMD of exposed group as compared to OVX group. PEMF shows recovery in bone mineral density, however the increase is slightly lesser than that of control group (Fig. 14, Table 4.2).

Fig. 14. Shows image of an experimental rat whose right leg is exposed and left leg kept as sham exposed. BMD of right femur (R3) and right tibia (R4) is more than respective sham femur (R2) and tibia (R1).
Table 4.2. Mean ± SD values of Bone Mineral Density (BMD) obtained from DEXA Analysis

<table>
<thead>
<tr>
<th>Bone Type</th>
<th>Control</th>
<th>OVX</th>
<th>OVX+PEMF</th>
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<tbody>
<tr>
<td>Femur</td>
<td>0.13885 ± 0.028</td>
<td>0.108875 ± 0.011</td>
<td>0.117925 ± 0.024</td>
</tr>
<tr>
<td>Tibia</td>
<td>0.142375 ± 0.032</td>
<td>0.128125 ± 0.038</td>
<td>0.138575 ± 0.036</td>
</tr>
</tbody>
</table>

Scanning Electron Microscopy (SEM) was performed in all groups of bone samples to evaluate the changes in cortex and cancellous part of bone. Transverse section of femur bone of Control, OVX and Exposed group were analysed. More compactness in cancellous part and mineral deposition in Control and Exposed bone were found as compared to osteoporotic bone (fig. 15). On the other hand increased cortical thickness in exposed group was more than that of OVXed group (fig. 16 and 17). It was observed that bone marrow was attached to cortex after PEMF exposure and new growth of bone was found in inner side of cortex. Frets (intertrabeculae) of trabecular bone in femur head of OVX group were absorbed. After the PEMF exposure it recovered and intertrabeculae were maintained well. However connectivity was less than control group (fig. 18). Transverse Section (T.S) of acetabular head (ball) images shows more compactness in OVX+PEMF bone as compared to OVX. It indicates that mineralization has taken place due to exposure (fig. 19). Similar study was performed in proximal part of Tibia, which showed that greater porosity in the OVX bone was again refilled in the OVX+PEMF. However the recovery was not as similar as in the control group (fig. 20).
Fig. 15. Shows the SEM images of Cancellous bone of femur head in (a) control, (b) OVX and (c) OVX + PEMF group. Here PEMF exposed bone shows more compactness than OVX but less than control femur (50X).
Fig. 16. Shows the SEM images of cortical part of femur diaphysis in (a) control, (b) OVX and (c) OVX + PEMF group. Here new bone formation shown in PEMF exposed bone as compared to OVX (25X).
Fig. 17. Shows the SEM images of cortical part of Tibia in (a) control, (b) OVX and (c) OVX + PEMF group. Here marrow is attached to inner side part of cortex in PEMF exposed condition as compared to OVX (25X).
Fig. 18. Shows the SEM images of trabecular part of femur head in (a) control, (b) OVX and (c) OVX + PEMF group. Here interconnectivity is more in PEMF exposed bone as compared to OVX (50X).
Fig. 19. Shows the SEM images of acetabular head of femur in (a) control, (b) OVX and (c) OVX + PEMF group. More mineralization taken place in exposed bone as compared to OVX (25X).
Fig. 20. Shows the SEM images of proximal part of Tibia in (a) control, (b) OVX and (c) OVX + PEMF group. Porosity is filled in PEMF exposed bone as compared to OVX (25X).
Light micrograph of Histological analysis were performed in Control, OVX and OVX + PEMF group bone marrow of same place, shown in images (fig. 21). Bone cells are heavily populated in control group whereas in OVX condition they were less populated and osteoclast (multinucleated cells) like cells were clumped together. Bone marrow cells were again proliferated and homogenously placed in marrow cavity of Exposed group. More osteoblasts than osteoclasts were found in exposed group than ovariectomised.

For qualitative study of collagen, Picrosirius Red Assay of histochemistry was performed. The study focused on determining the arrangements of collagen fiber, basically composed of collagen type I before and after the PEMF treatment of osteoporotic rat bone. Sirius red positive extracellular matrix displays the typical birefringence of bone matrix and immuno-positive stain for collagen. The results of collagen in trabecular bones have been presented in figure 22 and 23. In both figures, it is clearly visible that collagen fiber more in exposed bone than OVX but still not up to the level of control bone. Extra cellular matrix of OVX+PEMF image became thicker than OVXed image due to more collagen production in exposed group rat bones. Similar results have been found in case of cortical bones (fig. 24, 25). Collagen production shows the osteoblastic activity of bone.
Fig. 21. Light micrograph stained with H.E. of (a) control, (b) OVX and (c) OVX + PEMF group femur bone marrow. It displays the bone cell Population and arrow indicates the osteoclast-like cells.
Fig. 22. Displays the collagen in light micrograph of the femur trabeculae in (a) control, (b) OVX and (c) OVX + PEMF group. Stained with Sirius Red (40X)
Fig. 23. Displays the collagen in light micrograph of the tibia trabeculae in (a) control, (b) OVX and (c) OVX + PEMF group. Stained with Sirius Red (40X)
Fig. 24 Displays the collagen in light micrograph of the femur cortex in (a) control, (b) OVX and (c) OVX + PEMF group. Stained with Sirius Red (40X)
Fig. 25 Displays the collagen in light micrograph of the tibia cortex in (a) control, (b) OVX and (c) OVX + PEMF group. Stained with Sirius Red (40X)
Biochemical analysis was performed in the femur and tibia of Control, OVX+PEMF and OVX groups. Sirius red assay was done to quantify the collagen type I. Collagen I was specifically stained by sulphonic acid group of dye with basic group present in the collagen. Collagen extracted with acetic acid and pepsin from bone samples of different groups were analysed. It showed that the total concentration of collagen (acetic acid digested collagen + pepsin digested collagen) was more in Exposed group than OVX group bone samples and less than control bone samples (Fig. 26). Total concentration of collagen in Exposed group femur was 2156.42±55.42, in OVX group femur was 1991.64±21.96 and in Control group femur was 2290.04±62.98. Whereas in Exposed tibia it was 1763.41±21.59, in OVX tibia was 1658.84±41.17 and in Control tibia it was 1795.32±33.36 (table 4.3).

| Table 4.3. Mean ± SD values of Collagen Type I and Alkaline Phosphatase from Biochemical Analyses |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | Control Rat Bones | OVXed Rat Bone | OVX+PEMF Rat Bones |
|                                 | Femur | Tibia | Femur | Tibia | Femur | Tibia |
| Acetic Acid Digested Collagen type I | 923.00 ± 35.60 | 732.93 ± 29.46 | 868.70 ± 33.14 | 644.44 ± 63.23 | 971.62 ± 25.74 | 815.30 ± 34.14 |
| Pepsin Digested Collagen type I (µg.) | 1367.04 ± 121.0 | 1062.39 ± 32.34 | 1122.94 ± 29.64 | 1014.40 ± 22.90 | 1184.80 ± 108.56 | 948.11 ± 75.52 |
| Total Alkaline Phosphatase | 230.26 ± 24.58 | 123.79 ± 18.46 | 122.25 ± 18.51 | 89.63 ± 14.22 | 178.42 ± 13.29 | 108.17 ± 17.53 |
The Alkaline Phosphatase is involved in mineralization process and is a good marker of osteoblast. ALP activity was assayed biochemically and histo-immunologically. Biochemical results showed quantitative parameter of ALP activity. PEMF stimulation significantly increased the concentration of ALP (p<0.05), compared to OVX group in both femur and tibia. Whereas in control femur and tibia a more concentration was observed (Table 4.3, fig. 27).

Immuno-histology of ALP was performed and evaluated. The expression of ALP in images of Control, OVX and OVX+PEMF group bones showed more staining in cortex and cancellous bone of OVX+PEMF group bones than that of OVX group (fig.28, 29, 30, 31). Similar trend was found in marrow cells images (fig.32, 33). Control group bones were in even better condition as compared to OVX and OVX+PEMF condition. Here more ALP activity was shown in images.

Fig. 26. Total collagen I concentration is displayed in two fraction, acid digested and enzyme digested.

![Total Collagen Type I](image-url)
Fig. 27 Total ALP concentration in control, OVX and OVX + PEMF groups of bone is displayed.
Fig. 28 Displays the ALP activity in light micrograph of the femur cortex in (a) control, (b) OVX and (c) OVX + PEMF group. Stained with ALP substrate dye (40X)
Fig. 29. Displays the ALP activity in light micrograph of the tibia cortex in (a) control, (b) OVX and (c) OVX + PEMF group. Stained with ALP substrate dye (40X)
Fig. 30. Displays the ALP activity in light micrograph of the femur cancellous bone in (a) control, (b) OVX and (c) OVX + PEMF group. Stained with ALP substrate dye (40X)
Fig. 31. Displays the ALP activity in light micrograph of the tibia cancellous bone in (a) control, (b) OVX and (c) OVX + PEMF group. Stained with ALP substrate dye (40X)
Fig. 32. Displays the ALP activity in light micrograph of the femur bone marrow in (a) control, (b) OVX, and (c) OVX + PFMF group. Stained with ALP substrate dye.
Fig. 33. Displays the ALP activity in light micrograph of the tibia bone marrow in (a) control, (b) OVX and (c) OVX + PEMF group. Stained with ALP substrate dye (40X)
Genotoxic effect

Earlier studies in our laboratory reported the DNA damage (genotoxic effect) on rat brain cells exposed to different low level microwave frequencies by Single Cell Gel Electrophoresis (SCGE or Comet Assay). This suggests that the microwave radiation cause DNA break. So in the present investigation, bone marrow cells were isolated and comet assay was performed [213, 214]. However we didn’t observe any significant difference or DNA damage in exposed marrow as compared to Control group bone marrow samples (fig.34 and 35).

Fig.34. DNA single strand break analysis performed by comet assay shows no damage in bone marrow cells of either group of tibia samples (Control as well as Exposed)

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Fig. 35. DNA single strand break analysis performed by comet assay shows no damage in bone marrow cells of either group of femur samples (Control as well as Exposed).
Many in vitro experiments have showed the potential mechanisms of osteoporosis prevention and treatment by PEMF. They showed the regulation of osteoblast and osteoclast. [215-220,184]. These studies used cells that came from normal animal models or transformed cell lines, whereas we used ovariectomised animal (in vivo). Consequently our results may provide actual situation of induced osteoporotic rat bone treated with capacitively coupled PEMF. Scientists are working to explain how low-level nonionising electromagnetic fields could have convincing effects on living systems, as they do not carry enough energy, either to damage bio molecules, or to cause heating effects. The debate about therapeutic value of weak electromagnetic fields will continue until the mechanism has been clarified. The problem of how weak fields perturb cell function will be understood when the techniques of molecular biology, genetics, biochemistry and biophysics are directed together to answer the question. According to our findings compared to other investigators’, we have tried to discuss a part of probable mechanism. Several other simultaneous pathways may exist.

First step of the mechanism is interaction of electromagnetic field with plasma membrane of exposed cell. Several workers have observed this in different way. Interaction occurs only if energy of an electric field is absorbed by the molecule to change its thermodynamic state, or by the system to shift its chemical equilibrium reported earlier by Teissie et al. [221] and then by Serpersu, and Tsong [222,223] and Chauvin et al. [224]. Dynamic electric fields when coupled to protein, conformational changes can play many useful roles. The concept of electro-conformational coupling is used to explain how an electric signal can affect the activity of a membrane protein. Some workers were observed that the output reaction of an enzyme is directly susceptible to influence by an electric field, energy can also be transduced based on enzyme rectification [225, 226].

Electromagnetic field exposure depolarizes the cell membrane of osteoblast to alter the uptake of calcium ions and increases the concentration of intracellular free calcium in osteoblast cytoplasm [227-231]. Adey was suggested that changes in the Ca$^{++}$ binding receptor (Glycoprotein) play an important role in calcium influx. Calcium transport is coupled to ATPase activity [232].

According to Danon and Korenstein, Calcium influx is driven by induced polarization of plasma membrane when bone cells are exposed to external electric field via a classical
second messenger mechanism and this eventually leads to a mitogenic effect (cell proliferation) [233].

The mitogenic effect was also observed by Berg and Zhang. They reported that, electrical stimulation elevates the transmembrane voltage and results in the elevation of electrical conductivity of the cell membrane. Due to this effect the function of cell membrane protein, lipid as well as expression of genes alters and this leads to cell proliferation [234].

The report carried out by Brighton et al. was focused on biochemical pathways. When signal transduction of various types of electricity (capacitive coupling, inductive coupling, and combined electromagnetic fields) were applied to bone cells (MC3T3-E1), a significant increase in the total bone cells DNA and cell proliferation was observed as compared to controls. This study found that transduction of a capacitively coupled electrical signal is by means of Ca$^{2+}$ ion translocation through voltage-gated calcium channels. This pathway eventually leads to a subsequent increase in bone cell proliferation and mineralization [235].

Hartig et al. also found similar results in sub-confluent cultures and showed biomineralization and bone cell proliferation by capacitively coupled electric fields (CCEF) as compared to the controls. After one week of exposure densely packed osteoblast-like cells group and extracellular matrix was observed [236]. Following these results, the same group further investigated the effects of CCEF on the multistage process of bio-mineralization in vitro [237].

In the present work, mineralization and bone cell proliferation after electromagnetic field exposure have been observed. Analysis of Calcium, Phosphorus (major constituent of Calcium hydroxyapatite), BMC and BMD conveys a significant increase in mineralogical situation of exposed bone with respect to ovariectomised bone. DEXA, XRD and SEM supported the mineralogical analysis. Bone cell proliferation has been observed by histology (HE) of bone marrow, immuno-histology of osteoblast with a marker dye (ALP) and biochemical quantification of Alkaline Phosphtase concentration.

Proliferation of osteoblast causes high alkaline phosphatase (ALP) activity and collagen biosynthesis. To determine how PEMF stimulation modulates osteoblast differentiation, Chang et. al measured ALP activity as a marker of osteoblast differentiation in in vitro. They also found that PEMF stimulation enhanced ALP activity in these cells [238]. ALP present in osteoblast and in matrix vesicle membrane is a very good indicator of
bone formation and matrix mineralization [239-241]. In present investigation, the elevation of ALP is observed after electrical stimulation which reflects an increased number of osteoblasts and greater degree of osteogenesis. Collagen I is a biosynthetic product of osteoblast, so concentration of collagen was also increased in exposed bones. Collagen I was estimated histochemically as well as biochemically with help of Sirius red stain. Collagen provides the site for mineral precipitation.

Kurahansi and Yoshiki reported that, elevated ALP releases free inorganic phosphate of phospholipids [242]. It has been found that, the increased inorganic phosphate causes osteoblast cell death [243]. Thus intracellular Ca is released out and gets precipitated with inorganic phosphate.