Changes in bone histology due to capacitive electric field stimulation of ovariectomized rat

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Background & objectives: Postmenopausal osteoporosis leads to a significant decline in bone mass. That complicates the treatment outcome. The objective of the present study was to find out the effects of pulsed modulated low level electric field capacitively coupled on bone histology of induced osteoporotic rats, for screening the potential therapy for osteoporosis.

Methods: Osteoporosis was induced by performed by bilateral ovariectomy of female Wistar rats. After one month of surgery electric field stimulation was delivered to one leg of experimatal rats while the other was sham exposed. After 60 days of exposure treated rats were sacrificed and femur and tibia bones were segregated into (i) control (CON), (ii) ovariectomized (OVX) and (iii) ovariectomized + electrical stimulation (OVX+ES).

Results: Histopathological analyses showed that capacitively coupled pulsed electric field exposure treatment augmented and restored the bone marrow cell population. Immunohistological localization of alkaline phosphatase (ALP) showed the increased activity of this enzyme after electrostimulation, which showed an enhanced osteoblast differentiation. Collagen histochemistry showed high amount of collagen fiber in exposed rats bones than that of osteoporotic bones. Electron microscopic study revealed the enhancement of microstructural composition and compactness in cortical and trabecular part of treated bones.

Interpretation & conclusions: Our results suggest that capacitively coupled pulsed electric field exposure treatment of specified parameters is efficacious in attenuating the effects of ovariectomy induced osteoporosis and restore the bone loss.

Key words Alkaline phosphatase - capacitively coupled - collagen - electrical stimulation - ovariectomy - postmenopausal osteoporosis

Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk. Osteoporosis is a multifactorial process including demographic and lifestyle factors, morbidity, drug use, medical history and altered hormonal profile. Worldwide, osteoporosis is considered second only to cardiovascular disease as a leading health problem. Osteoporosis is also highly prevalent in India with 1 out of 8 males and 1 out of 3 females in India.

Pharmacologic intervention for osteoporosis includes estrogen, calcitonin, bisphosphonates, selective estrogen receptor modulators (SERMs), parathyroid hormone and strontium ranelate, among others. Most of them are either insufficient to reverse osteoporosis or not well tolerated by the patients.
Therefore, cost-effective and safe alternatives to pharmacological interventions are potentially valuable. Pulsed electromagnetic field (PEMF) therapy is found to be efficacious in peripheral nerve injuries, delayed-union bone fractures, failed joint fusions, and congenital pseudoarthroses (FDA, USA approved). Several cellular mechanisms have been proposed to be affected by PEMF including an increased mineralization, collagen production, endochondral ossification and decreased osteoclastogenesis.

The present study was carried out to determine the efficacy of repeated (2 h/day for 60 days) capacitively coupled electric field (10 V peak-to-peak, 16 Hz modulated output frequency) on histological changes in bones of bilateral ovariectomized rats, a reliable model for postmenopausal osteoporosis.

**Material & Methods**

**Animals:** Adult female Wistar rats (230-250 g) obtained from Experimental Animal Facility of Jawaharlal Nehru University, New Delhi, were individually housed in plastic cages under standard environmental conditions of alternating 12:12 h periods of light dark cycle at 25±1°C room temperature. All rats were allowed ad libitum access to laboratory food pellets and fresh tap water. Equal number of rats (n=12) were utilized for control and experimental groups. The study protocol was approved by Institutional Animal Ethical Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

**Ovariectomy:** Under pentobarbital sodium (Sigma Chemical) anaesthesia (30 mg/kg, bw, ip), bilateral ovariectomy was performed in experimental group of rats. The periovarian fatty tissue was identified and exteriorized. The ovaries were dissected out by cutting above the clamped area, while the uterine horn, other blood vesicles were ligated and muscles and skin were stitched separately. Postoperative care included the systemic administration of analgesics and antibiotics. In control group rats, the ovaries were exposed but not removed (sham operation). Post bilateral ovariectomy (30 days), left leg received exposure treatment.

**Exposure to pulsed electric field:** Rats received exposure to electric field with specially designed instrument by Behari et al. It is an electromagnetic field generator through which two wire leads are attached to skin capacitor copper electrodes, which deliver 10 volts peak-to-peak pulsed square wave at 16 Hz modulated frequency (career frequency 14 MHz). Current density at the target site of application was ~ 80 µA/cm².

All rats (control and experimental) were given mild anaesthesia with sodium phenobarbitone and sacrificed by cervical dislocation. Tibia and femur bones of control legs (CON), ovariectomized sham exposed leg (OVX) and ovariectomized +electrical stimulation exposed leg (OVX+ES) were freed from soft tissues and stored at -20°C for various morphological and histological assays.

**Tissue preparation:** Bone samples were immersed in PLP fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate solution, pH 7.4) at 4°C. These were then subsequently demineralized with 10 per cent EDTA solution and dehydrated with increasing concentration of ethanol before being embedded in paraffin. For alkaline phosphatase (ALP) staining, low melting paraffin was used. The paraffin blocks were then placed in microtome (York Scientific, India) and 5 µm transverse sections were obtained. For scanning electron microscopy (SEM), the fresh bone specimens were trimmed down in 5 mm transverse sections and fixed in 2 per cent glutaraldehyde solution.

**Hematoxylin and eosin (HE):** After deparaffinizing, sections were immersed in the filtered hematoxylin for 3 min and then rinsed with running water until the water became clear. Sections were again immersed in eosin stain for 1-2 min and rinsed until water became clear. Thereafter these were dehydrated in ascending graded alcohol solutions (50, 70, 80, 95 X 2, 100% X 2 times) and cleared with xylene (3 times) and viewed under the light microscope (Nikon, Japan).

**Immunohistochemical demonstration of ALP activity:** ALP histochemistry was performed as described by Miao et al. Briefly; the bone tissue sections were deparaffinized, hydrated through a xylene and graded alcohol series. These were preincubated overnight in 1 per cent magnesium chloride in 100 mm Tris maleate buffer (pH 9.2). Thereafter these were again incubated for 2 h at room temperature with ALP substrate solution (freshly prepared 100 mM Tris-maleate buffer, pH 9.2, containing 0.2 mg/ml naphthol AS-MX phosphate and 0.4 mg/ml Fast Red TR). After washing with distilled water, the sections were counterstained with methyl green nuclear counter stain (0.5% methyl green solution made in 0.1 M sodium acetate buffer at pH 4.2) and mounted with glycerol jelly.
Fig. 1. Images of light micrograph stained with H.E. of femur hypophysis (a-c) and femur epiphysis (d-f) in control, OVX and OVX+ES bone marrow. It displays 20 X image of the bone cell population and arrow indicates the osteoclast-like cells.

Fig. 2. Displays the ALP activity (arrow head) in light micrograph of the femur cancellous bone (a-c), tibia cancellous bone (d-f), femur bone marrow (g-i) and tibia bone marrow (j-l) in control, OVX and OVX+ES bone samples with ALP substrate dye (40X).

Fig. 3. Displays the collagen stained red with Sirius Red in light micrograph of the femur cortex (a-c), femur trabeculae (d-f), tibia cortex (g-i) and tibia trabeculae (j-l) in control, OVX and OVX+ES bone samples. Collagen fibers in cortex are shown with 8 point stars and trabeculae are shown with arrow heads (40X).

Picric acid). Bone sections were then washed twice with acidified water. These were again dehydrated in three changes of 100 per cent ethanol and cleared in xylene and mounted in a resinous medium, and viewed under the light microscope and photographed by using digital camera (Nikon, Japan).

Scanning electron microscopy (SEM): Before doing SEM characterization, the bone samples were dried and mounted on circular disc stubs with adhesive. Carbon coatings were applied at a thickness of about 20 nanometers with the help of sputter coater. SEM images were obtained on ‘low vacuum SEM’ Leo 435 VP (Cambridge, England) at National Facilities of Electron Microscopy, All India Institute of Medical Sciences (AIIMS), New Delhi.

Results

Bone cells population: Images of light micrograph of histological analysis were performed in Control, OVX and OVX+ES bone marrow of the same
region (Fig.1). Bone cells are heavily populated in control whereas in OVX condition these were less and osteoclast (multinucleated cells) like cells were clumped together (Fig. 1b and c). Bone marrow cells were again proliferated and homogeneously placed in marrow cavity of exposed group. More osteoblasts than osteoclasts were found in OVX+ES group than OVX.

**Alkaline phosphatase activity (ALP):** The expression of ALP in control, OVX and OVX+ES bones showed more staining in cortex and cancellous bone of OVX+ES bones than that of OVX (Fig. 2a-f). Similar trend was found in marrow cells images (Fig. 2g-l). Control bones are seen to be better condition as compared to OVX and OVX+ES condition. Here more ALP activity confirmed the more osteoblastic differentiation.

**Collagen:** Picro sirius red staining showed the arrangements of collagen fiber in extracellular matrix of bones. In both types of bones (cortical and cancellous), collagen fibers were more in exposed than in OVX but not up to the level of control sample (Fig. 3). OVX+ES bones had more collagen content in cortex as compared to OVX induced. Similar results were found in case of trabecular part of bones (Fig. 3g-l). Extracellular matrix of OVX+ES image became thicker than OVX image due to more collagen formation in exposed bones.

**Bone electron microscopy:** SEM images in transverse section of bone samples showed the microstructural changes in cortex and cancellous part of bone (Fig. 4). Cortical thickness in exposed bones was more than that of OVX (Fig. 4a-f). On the other hand, more compactness in cancellous part and mineral deposition in Control and OVX+ES bone were found as compared to osteoporotic bone (Fig. 4g-l). It was observed that bone marrow was attached to cortex after electrostimulation and new growth of bone was seen in inner side of cortex. Frets (intertrabeculae) of trabecular bone in femur epiphysis of OVX were absorbed. After the electrostimulation treatment exposure intertrabeculae were recovered. However, connectivity was less than control (Fig. 4g-i). It indicated the mineralization due to exposure treatment. Similar study on the proximal part of tibia, showed that porosity in the OVX bone was refilled in the OVX+ES. However, the recovery process did not reach the control level (Fig. 4j-l).

**Discussion**

The study describes the effect of capacitive pulsed electric field coupling in preventing the deterioration of histological characteristics of ovariectomy induced bone in female Wistar rats. Under normal physiological conditions, bone is a well maintained tissue by a strictly balanced coupling of bone formation and resorption. The end product of remodeling is the maintenance of mineralized bone matrix. Ovariectomy alters the bone turnover i.e., imbalance between bone formation rate and resorption rate, causing loss in bone quality. Bone formation results from a complex cascade of events that involve proliferation of primitive mesenchymal cells, differentiation into osteoblast precursor cells (osteoprogenitor, pre-osteoblast), maturation of osteoblasts, formation of matrix and finally mineralization takes place. Differentiation of osteoblast is one of the key events of bone formation where osteoblast precursor differentiates into the mature cell. Several bone-derived growth factors can cause the appearance of markers of the differentiated osteoblast phenotype, including expression of alkaline phosphatase activity, collagen and osteocalcin.\(^\text{12}\)
Our results suggest that capacitive pulsed electric field stimulation can lead to significant improvement in bone quality after two months of exposure. However, the mechanism by which electromagnetic field stimulation induces cellular proliferation and bone regeneration remains unclear. Selvamurugan et al., showed additive effects of a combined treatment of bone morphogenetic protein (BMP-2) and PEMF on osteoblastic cell proliferation and differentiation. They performed experiments in primary osteoblastic cell culture and treated with two different interventions: BMP-2 and PEMF, for transient (24 h) and continuous treatment extending up to twenty days, and found that the continuous treatment with BMP-2 and PEMF increased expression of osteoblast marker genes (ALP, procollagen, osteocalcin) during early stages of differentiation rather than at a later stage of differentiation and mineralization. Thus, PEMF can stimulate both osteoblastic cell proliferation and differentiation. Our results demonstrated that stimulation distinctly increased osteoblast number in hypophysis as well as in epiphysis. Also osteoclast like multinucleated cells were more in OVX group of rat bones and decreased after electrical stimulation. This is in agreement with results of other workers suggesting that electromagnetic field stimulation affects not only proliferation or differentiation of osteoblasts, but also apoptosis of osteoclasts14-18.

As a confirmation to this alkaline phosphatase activity correspondingly showed an enhancement in osteoblast cell differentiation in treated bones as compared to the osteoporotic ones. Osteoblast differentiation is associated with the matrix maturation and mineralization of the bone extracellular matrix. ALP expression is identified an early differentiation marker of osteoblast maturation and a good indicator of bone matrix mineralization, while collagen formation represents the end of differentiation and gives site for mineral precipitation. Earlier we found increased mineralization in induced osteoporosis due to sciotic denervation, caused by electrical stimulation in bones. Weisemann et al. also observed increased bio-mineralization by electrical stimulation in osteoblast cell line. 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. Electromagnetic field stimulation may lead to increase in cytosolic Ca2+ and activation of calmodulin. Adey has suggested that calcium binding receptor (glycoprotein) of plasma membrane plays important role in calcium influx. In the present study, the elevation of ALP is observed after electrical stimulation which reflects an increase in osteoblasts number and its biosynthetic product, collagen. Kurahansi & Yoshiki reported that elevated ALP releases free inorganic phosphate of phospholipids. Thus intracellular Ca is released out and gets precipitated with inorganic phosphate. Nucleation sites are found within or associated with extracellular collagen fibrils. The kinetics of the precipitation of calcium phosphates from metastable form of calcium and phosphate solution to particular apatite form has been studied in vitro in a number of experiments. Our results showed that mineralization occurred in the osteoporotic bone after the exposure treatment, but it might take longer duration of exposure to reach the level of control.

In conclusion, the present study shows that electrical exposure can enhance osteogenesis by increasing the bone cell density, osteoblast differentiation and collagen formation which leads to restoration in microstructural characteristics. Further study at molecular level should enhance our understanding on mechanism of electrostimulation on bone cells activity.

References


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