Changes in Biochemical Measurements due to Electrical Stimulation of Ovariectomized Rat Bones

Jitendra Behari[1] and Jayanand[2]

School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India.
Tel: +91-11-2674323, Fax No. +91-11-26717502
[1]jbehari2000@yahoo.co.in, [2]hello_jayanand@yahoo.com

Abstract

The present study was carried out to investigate the effect of capacitively coupled electric field on mineralization over collagen fiber of induced osteoporotic rat bones. Rats were ovariectomized bilaterally to induce the osteoporosis and same age grouped sham operated rats were kept as control. After one month of surgery capacitive coupled pulsed electric field (carrier sinusoidal wave 14 M Hz modulated at 16 Hz pulsed square wave output and the amplitude was 10 Volts) delivered to one leg of experimatal rats and other leg kept as sham exposed. After 60 days of exposure treatment rats were sacrificed and femur and tibia bones were segregated into Control, Ovariectomized (OVX) and Ovariectomized+ Electrical stimulation (OVX+ES). Bone density and mineral content of OVX+ES bones were significantly increased as compared to OVX bones. Biochemical analysis conveyed that exposed rat bones have high amount of collagen I and alkaline phosphatase (ALP) than that of osteoporotic bones.

It is concluded that capacitive pulsed electric field can improve inorganic, organic and micro structural properties of bones.

1. Introduction

Osteoporosis is a condition in which there is deterioration in bone quality and quantity, and is characterized by occurrence of traumatic fractures. At present most of evidences supports pharmacological intervention [1]. However pharmacological treatments are costly and have undesirable side effects [2]. 20% of osteoporotic patients die in the first year after fracture, due to long term hospitalization [3]. Therefore cost-effective and safe alternatives to the pharmacological practices are potentially valuable.

Low level pulsed electromagnetic field can stimulate the bone healing process in different circumstances such as fresh fractures, delayed unions, non unions, osteotomies and congenital psedoarhthrosis. This has been observed in both animals and human studies. Pulsed electromagnetic field exposure treatment can be performed with inductive and capacitive systems. Capacitive coupling system can be optimized by ease of application of dermal electrodes and the size and weight of units are smaller. The therapeutic efficacy of external electric field exposure depends on its magnitude, frequency and waveform.

Bone is composed of two major types of bone cells that execute the function that required to form and maintain bone structure. Osteoblasts, the bone-forming cells and Osteoclasts, the bone-resorbing cells [4]. Osteoblasts perform several functions that result in the formation of bone, they express the membrane protein, alkaline phosphatase and secrete type I collagen. Alkaline phosphatase (ALP) is a membrane-bound metalloenzyme which catalyzes the hydrolysis of phosphomonoesters at an alkaline pH. The activity and localization of ALP are a valuable index for tissue development, differentiation of osteoblast and a histochemical marker. Collagen fibrils secreted by osteoblast are stiffened by integration of the mineral phase [5]. Collagen molecules are staggered within the fiber to provide spaces for nucleation of the calcium apatite crystals for mineralization.

Our purpose was to examine the effect of pulsed electric field in enhancement of osteoblast differentiation, collagen enrichment and mineralization in induced osteoporotic bones.

2. Methods

2.1 Animal Model and induction of osteoporosis

To evaluate the effectiveness of electrical field exposure treatment of postmenopausal osteoporosis, 30 adult female wistar rats (90 days old and 210-220 gm body wt.) were obtained from animal facility of Jawaharlal Nehru University, New Delhi. They were randomly divided into two groups, 10 rats in control and 20 rats in experimental group. The experimental group was subjected to bilateral ovariectomy. All animals were housed in an air-conditioned room where the temperature was maintained at 25°C. They were provided with standard food pellets (prepared by Hindustan Lever ltd., India) and tap water ad libitum.

Bilateral Ovariectomy in experimental group rats were performed using pre-operative anesthetic procedures. Anesthesia was induced by intra-peritoneal (IP) injection of 30 mg of Pentobarbital Sodium (sigma

chemical) per Kg body weight of rat. For each ovary a 1-cm dorsal flank incision, penetrating the abdominal cavity was made. The periovarian fatty tissue was identified and retracted. Using forceps, the periovarian fat was gently grasped and exteriorized. The ovary was dissected out by cutting above the clamped area. After removal of ovary, the uterine horn and other blood capillaries were ligated. Stitches were made on subcutaneous muscles by absorbable thread and on incised skin by nylon thread. Similar process was followed to remove other ovary too. Postoperative care was taken by giving analgesic and antibiotic to prevent against any infection. In Control group, the ovaries were exposed but not removed (sham operation). After 30 days of bilateral ovariectomy, one leg of experimental group rats received exposure treatment.

2.2 Bone stimulator and exposure treatment

Bone stimulator is an external electromagnetic field generator through which two wire leads attached to skin capacitor electrodes. The unit delivers 10 Volts peak-to-peak pulsed square wave at 16 Hz modulated frequency (career frequency 14 MHz). For exposure treatment, output of bone stimulator was given to one hind limb of each experimental rat separately by a pair of copper electrodes. Current density at the target site of application was 80 $\mu A/cm^2$. Each electrode was of 1 cm diameter and 5 cm in length. One leg of ovariectomised rat received electrical stimulation (OVX+ES) and the other leg was tied with same type of electrodes without any connection to stimulator (Sham-exposed or OVX). Exposure treatment lasted for 60 days at the rate of 2 hrs per day.

All rats (control and experimental) were then sacrificed by cervical dislocation after mild anesthesia. Tibia and femora of Control legs, Exposed leg (OVX+ES) and sham exposed leg (OVX) were freed from soft tissues and stored at -20° C for mineralogical and biochemical analysis.

2.2 Mineralogical Analysis

Volume of all bones was measured by submersion of bone in a water filled container with a scale sensitivity of 0.01 ml. Bones (Control, OVX and OVX+ES) were then lyophilized in fridge drier for 8 hrs. After lyophilization, dry bone weight was evaluated and the bone density was calculated.

Lyophilized bones were milled down into fine particles in a mortar. 100 mg of lyophilized dry bone powder from each sample was placed in a pre-weighed clean thermostatic crucible and kept in a muffle furnace (Widson Scientific Works, India) set at a constant temperature of 700° C for 8 hrs. They were then allowed to cool to room temperature and weight of bone ash was measured. Bone mineral content (BMC) and bone mineral density (BMD) were calculated.

2.3 Biochemical Analysis

Collagen type I and Alkaline Phosphatase were estimated following the standard procedure.

3. Results

The results indicate that bilateral ovariectomy was accompanied with significant decrease in femur and tibia dry bone content (BC), dry bone density (BD), BMC and BMD as compared with control partner ($p<0.01$). The decrease in BC and BD were prevented following the exposure treatment. Both femur and tibia BC increased significantly by 14% ($P <0.001$) and 12% ($p<0.05$) respectively after exposure treatment. In case of BD prevention following treatment in femur was 14% ($P < 0.002$) and in tibia was 18% ($P < 0.01$).Whereas exposure increased the BMC of femur by 18% ($P<0.01$) and that of tibia by 16% ($P<0.001$). Similarly BMD also increased in femur and tibia of exposed leg by 16% ($P <0.05$) and 20% ($p<0.001$) respectively. When exposed bones were compared with control, no significant difference was found, which reflects the effectiveness of treatment.

Ovariectomy induced osteoporosis decreased total collagen I content in the femur (13%; $P< 0.001$) as well as tibia (5%; $P <0.05$).Electric field exposure prevented ovariectomy-induced decrease in total collagen I levels in femur (7%; $P <0.01$) and tibia (4%; $P<0.05$). Total collagen I content of treated femur show significant difference when compared with control but tibia exhibited non-significant difference.

Decrease in ALP activity of bones due to ovariectomy is significantly increased by electrical stimulation ($p<0.05$), compared to OVX group in both femur and tibia. In control femur and tibia higher concentration was observed.

Table 1. Effect of ovariectomy and pulsed electric field treatment on bone mineral content (mg), bone mineral density (mg/ml), Total collagen I, ALP activity in femur and tibia bones of rats. (mean±SD)

	Control Bones		OVXed Bones		OVX+ES Bones	
	Femur	**Tibia**	**Femur**	**Tibia**	**Femur**	**Tibia**
Dry bone content (mg)	627.65±94.89	447.37±32.28	507.86±54.86	385.81±66.66	593.62±43.89	439.13±46.49
Dry bone density (mg/ml)	1452.75± 45.26	1161.88±76.93	1182.19±144.26	938.10±178.47	1386.83±129.99	1126.47±148.44
BMC (mg)	415.36±25.87	282.78±20.32	313.89±47.13	225.15±32.24	386.28±63.77	269.40±17.97
BMD (mg/ml)	967.73±140.0	734.39±47.22	731.19±121.81	547.87±93.44	901.44±148.56	690.79±68.50
Total Collagen I (mg)	91.60±4.44	71.77±4.04	79.65±2.38	67.64±4.20	86.25±6.31	70.53±3.88
ALP activity (µmol/min/gm dry bone)	131.25±23.88	108.17±19.02	85±8.31	89.63±9.50	122.79±18.67	92.64±10.72

4. Discussion

Our results suggest that pulsed electric field stimulation can reverse the effect of osteoporosis. Bone density increased significantly after 2 months of exposure treatment. Bone mineral and collagen have long been recognized as the two key components of bone, and mounting evidence supports the view that bone mineral dictates elastic modulus, whereas bone collagen dictates toughness and both together influence strength [6]. In the present work we have found significant increase in mineral content as well as collagen I content in treated leg bones of osteoporotic rats. Although treated bone doesn't return to normal or control level but it checks further damage and some amount of bone formation takes pace. It is possible that further exposure may further improve the bone mechanical properties.

Alkaline phosphatase activity showed that the osteoblast cell differentiation is more in treated bone as compared to the osteoporotic one. Some of the earlier works supports that pulsed electromagnetic field differentiates osteoblast [7]. Osteoblast differentiation is associated with the matrix maturation and mineralization of the bone extracellular matrix [8]. ALP expression considered an early differentiation marker of osteoblast maturation, while collagen formation represents the end of differentiation and function of matured osteoblast. ALP in matrix and osteoblast is a good indicator of bone formation and matrix mineralization [9]. Collagen gives site for mineral precipitation [10]. Previously also we found more mineraliztion in electrostimulated bone of induced osteoporotic rats [11]. Weisemann et al. [12] also observed increased biomineralization by electrical stimulation in osteoblast. In present investigation, the elevation of ALP activity is observed after electrical stimulation which reflects an increased number of osteoblasts and greater degree of osteogenesis. Collagen is a biosynthetic product of osteoblast, so concentration of collagen also increased in electro-stimulated bones. Kurahansi and Yoshiki reported that, elevated ALP releases free inorganic phosphate of phospholipids [13]. Thus intracellular Ca is released out and gets precipitated with inorganic phosphate. Nucleation sites are found within or associated with extracellular collagen fibrils [14]. The kinetics of the precipitation of calcium phosphates from metastable form of calcium and phosphate solution to particular apatite form have been studied in vitro in number of experiments[15-16]. According to Katz et al. [17,] and Glimcher, [18] the majority of bone mineral in bones, resides within the collagen fibrils. It is clear that mineralization occurs in the osteoporotic bone after the exposure treatment, but it is not as much compact as original structure or control.

It seems clear from the present study that electrical exposure can enhance osteogenesis by increasing the osteoblast differentiation and collagen formation which leads to enhancement of mineralization. Elucidation of extent of mineralization by pulsed electromagnetic field which can give enough mechanical strength, may avoid any level of osteoporotic fracture, remains a challenging task for the future.

5. References

1. J.A. Kanis, J.E. Brazier, M. Stevenson, N.W. Calvert, M. Lloyd Jones, "Treatment of established osteoporosis: a systematic review and cost-utility analysis," *Health Technology Assessment,* Winchester, England, **6,** 2002, pp. 1–146,

2. Writing Group for the Women's Health Initiative Investigators, "Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial," *JAMA*, **288,** 2002, pp. 321– 333

3. P. Boyle, M.E. Leon, P. Autier, "Epidemiology of osteoporosis," *J Epidemiol Biostat* **6**, 2001, pp. 185–192

4. S.C. Garner, J. J. B. Anderson, W.W. Ambrose, "Skeletal tissues and mineralization. In: Calcium and Phosphorus in Health and Disease," *CRC Press Inc, Boca Raton, Florida*, 1996, pp. 97-117,

5. V. Ottani, M. Raspanti, A. Ruggeri, "Collagen structure and functional implications," *Micron*, **32,** 2001, pp. 251–260.

6. J. Matthew Silva, D. Michael Brodt, B. Wopenka, S. Thomopoulos, D. Williams, H. M. Maurice, K. O. Wassen Mike, Nozomu Kusano, A. Ruud Bank, "Decreased Collagen Organization and Content Age Associated With Reduced Strength of Demineralized and Intact Bone in the SAMP6 Mouse," **21**, 2006, pp. 78-88

7. Pericles Diniz, K. Shomura, K. Soejima, Gakuji Ito, "Effects of pulsed electromagnetic field (PEMF) stimulation on bone tissue like formation are dependent on the maturation stages of the osteoblasts," *Bioelectromagnetics*, **23**, 2002, pp. 398-405.

8. G. S. Stein, J. B. Lian, "Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype," *Endocr Rev*, **14**, 1993, pp. 424–442.

9. H. E. Gruber, G. J. Marshall, L. M. Nolasco, M. E. Kirchen, D. L. Rimoin, "Alkaline and acid phosphatase demonstration in human bone and cartilage: effects of fixation and methacrylate embedments," *Stain Technol* **63(5)**, 1988 pp. 299-306.

10. V. Andre Frei, B. Chevallay, I. Orly, M. Boudeulle, A. Huc, D. Herbage, "Acellular mineral deposition in collagen-based biomaterials incubated in cell culture media," *Calcif-Tissue-Int*, **66,** 2000, pp. 204-11.

11. Jayanand, J. Behari and R. Lochan, "Effects of low level pulsed radio frequency fields on induced osteoporosis in rat bone," *Ind J of Exp Biol,* **41**, 2003, pp. 587-591.

12. H. Wiesmann, M. Hartig, U. Stratmann, U. Meyer, U. Joos, "Electrical stimulation influences mineral formation of osteoblast-like cells in vitro," *Biochim Biophys Acta,* **1538**, 2001, pp. 28-37.

13. Y. Kurahashi and S. Yoshiki, "Electron microscopic localization of non-specific alkaline phosphatase activity in cells of early developing teeth and bone," *J. Electron Microsc*, **18**, 1969, pp. 219-220.

14. H.C. Anderson, "Biology of disease. Mechanism of mineral formation in bone," *Lab Invest*, **60**, 1989, pp. 320–330

15. J. D. Termine, A. S. Posner, "Calcium phosphate formation in vitro: factors affecting initial phase separation," *Arch Biochem Biophys*, **140**, 1970, pp. 307–317

16. W. G. Robertson, "Factors affecting the precipitation of calcium phosphate in vitro," *Calcif Tissue Res*, **11**, 1973, pp. 311–322.

17. E. P. Katz, E. Wachtel, Y. Yamauchi, G. L. Mechanic, "The structure of mineralized collagen fibrils," *Connect Tissue Res*, **21**, 1989, pp. 149-158.

18. M. J. Glimcher, "The nature of the mineral component of bone and the mechanism of calcification. In Coe FL, Favus MJ (eds) Disorders of Bone and Mineral Metabolism," *Raven Press*, New York, NY, USA, 1992, pp. 265-286.