*Original Article*

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# Regulatory Role of PPARγ Agonist Pioglitazone in Osteoclastogenesis of Type2 Diabetic Postmenopausal Women

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## Abstract

Pioglitazone, a class of thiazolidinedione (TZDs) is a ligands for peroxisome poliferator activated receptor gamma (PPARγ). Activation of PPARγ receptors negatively regulates bone haemostasis. The present study focuses mainly into two categories firstly generation of functional osteoclasts and second, to study the impact of pioglitazone and estrogen on bone health. whole blood were taken from diabetic and non diabetic postmenopausal women to measures the baseline parameters and for isolation of peripheral blood mononuclear cells (PBMC) and cultured in osteoclasts medium for 14 days into following five groups: PBMCs from non diabetic postmenopausal women, PBMCs from type 2 diabetic postmenopausal women, PBMCs from type 2 diabetic Postmenopausal + Pioglitazone, PBMCs from type 2 diabetic Postmenopausal + Pioglitazone + 17 beta estradiol and PBMCs from type 2 diabetic Postmenopausal + 17 beta estradiol. mRNA expression was analysed for Cathepsin K. Increase in the numbers of TRAP positive multinucleated cells was observed in pioglitazone treated cells of type2 diabetic postmenopausal women (88.12 ± 12.25, $p<0.001$) as compared to type2 diabetic (46.46 ± 11.54), non diabetic (40.85 ± 9.45) co treated (68.44 ± 15.12, $p<0.01$) and estrogen treated type 2 diabetic postmenopausal women (39.12 ± 9.11, $p <0.0001$). In addition the expression ratio of Cathepsin K mRNA was significantly greater in pioglitazone treated type2 diabetic postmenopausal women as compared to other groups ($p<0.05$). We found that, PPARã agonist pioglitazone induced formation of TRAP positive multinucleated cells and upregulates the expression of bone resorption gene Cathepsin K leading to osteoclastogenesis in type2 diabetic postmenopausal women.

**Keywords:** Pioglitazone; Osteoclast; Type2 Diabetes Mellitus.

## Introduction

Bone fracture is one of the most important metabolic bone disease observed in women with diabetes mellitus [1-3]. A prospective cohort survey study shows that type 2 diabetes mellitus postmenopausal women (T2DPW) have 1.7 fold higher risks of bone fracture as compared to postmenopausal women without type2 diabetes mellitus [4]. Pioglitazone, a class of thiazolidinedione (TZDs) is an anti-diabetic drug used for management of type 2 diabetes mellitus (T2DM). TZDs are the ligands for peroxisome poliferator activated receptor gamma (PPARγ), activation of PPARγ receptors leads to increased glucose utilization and insulin sensitivity as well as decreased hepatic glucose [5-10]. Clinical studies suggested that, PPARγ signaling negatively regulates bone haemostasis [11,12] leading to unbalance between bone formation and bone resorption [13]. PPARã initiate the binding of macrophage colony stimulating factor (M-CSF) and receptor activator of NFkB ligand (RANKL) to their respective receptors c-fms and RANK on mononuclear osteoclast precursors triggering the process of osteoclastogenesis and give rise to multinucleated osteoclasts [14]. It is well know that estrogen plays an important role in bone remodelling. Decreased quantities of sex hormones estrogen by any external factors are thought to be one

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of the important factors, responsible for increased resorption of bones [15]. Estrogens protect the adult skeleton against bone loss by slowing down the rate of bone remodelling and by maintaining a focal balance between bone formation and resorption [16, 17]. The present study is focus mainly into two category firstly we generates functional osteoclasts from type 2 diabetic postmenopausal women (T2DPW) using an in vitro technique from peripheral blood monocytes (PBMCs) in the presence of human M-CSF and soluble RANKL. Second we study the impact of pioglitazone and estrogen on bone resorption cells (osteoclasts). We found that, PPARγ agonist pioglitazone induced formation of multinucleated positive cells (osteoclasts) and upregulates the expression of bone resorption gene Cathepsin K leading to osteoclastogenesis in peripheral blood mononuclear cells (PBMCs) of type 2 diabetic postmenopausal women.

## Materials and Methods

Demographic and anthropometric details like age, height, weight, duration of diabetes, medical history, body mass index (BMI) (kg/m2) and blood pressure were obtained from the medical records of the study subjects (all data not shown). 7 ml of whole blood were taken from diabetic (n= 13) and non diabetic postmenopausal women (n = 13) to measures the baseline parameters. All study subjects who are at the risk factors for osteoporosis and patients who are on corticosteroids, estrogen, statin etc and those with a history of bone fractures or bone disease are excluded from the study. The diagnosis of diabetes was based on previous history of diabetes or on the criteria of World Health Organization. Inform consent were taken from the study subjects and approved by institutional ethical committee (Rajiv Gandhi Hospital, Chennai, India: Ref. No. 15338/ME5/2012). Mononuclear cells were isolated from peripheral blood mononuclear cells (PBMCs) and cultured in osteoclasts medium for 14 days into following groups

*Group 1:* PBMCs derived from non diabetic postmenopausal women (NDPW)

*Group 2:* PBMCs derived from type 2 diabetic postmenopausal women (T2DPW)

*Group 2a:* PBMCs derived from type 2 diabetic Postmenopausal + Pioglitazone (T2DPWPIO)

*Group 2b:* PBMCs derived from type 2 diabetic Postmenopausal + Pioglitazone + 17 beta estradiol (T2DPWPIOE).

*Group 2c:* PBMCs derived from type 2 diabetic Postmenopausal + 17 beta estradiol (T2DPWE)

### *Isolation of Mononuclear Cells from Whole Blood*

Whole blood was drawn for isolation of peripheral blood mononuclear cells (PBMCs). Blood was diluted in 1:1 with phosphate- buffered saline (PBS, without Ca2+ and Mg2+). Two parts of diluted blood were layered on top of one part ficoll and centrifuged at 1800 rpm, for 30 min at room temperature. The cell layer on top of the Ficoll-Paque was collected, resuspended in phosphate buffer saline and centrifuged at 2500 rpm for 10 minutes for three times. Mononuclear cells were counted in a hemocytometer, and used immediately.

### *Culture of Human Osteoclasts*

Sorted population of PBMCs were culture in 24 well plate at 2 $\times 10^5$ cells per plate in α- minimal essential medium (MEM) containing pen-strep and 10% FBS. Medium was supplemented with 25ng/ml human M-CSF, 40 ng/ml human RANKL and 1 ìM dexamethasone and L-glutamine. The culture was incubated in a humidified atmosphere of 5% $CO_2$ and 37 °C. The cells were re-fed twice weekly by semi-depletion. On 14 days, cells were fixed and stained for tartarate resistance acid phosphates (TRAP).

### *Cell Proliferation Assay*

The cell proliferation assay was determined using the 3-(4, 5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Osteoclast precursor cells ($1\times10^5$ cells/well) were incubated with different concentrations of pioglitazone (0-10μM) and 17 beta estradiol (0-10 nM) for 48 hrs in 96 well plates and were incubated at 37°C in a humidified mixture of 5% $CO_2$ and 95% air in an incubator. 25 µl of MTT reagent (5 mg/ml in PBS), was added to each well and incubated at 37°C for 3 hrs. At the end of the incubation period, the supernatants were removed by tilting the plate completely without disturbing the cell layer and added 150µl of DMSO to each well. After 15 min of shaking, the readings were recorded as absorbance at 590 nm on a microplate reader.

### *Tartrate-Resistant Acid Phosphatase Staining*

Cells were washed with PBS and fixed with 37 % formaldehyde for 30 second. After washing with PBS, cells were incubated with 0.1% Triton X-100 for 10 min, washed, and then incubated for 60 minutes at 37°C in dark room with TRAP staining (sigma, Aldrich), following the manufacturer's instruction and counter stain for 2 minutes with hexamatoxylin solution. Cells were washed with water and TRAP-

positive multinucleated cells containing three or more nuclei which were osteoclasts and counted under a light microscope. The data were expressed as mean ± S.D.

*Isolation of RNA and RT-PCR*

Total RNA was isolated from cell cultured plates using TRIZOL reagent. The quantity and integrity of the isolated RNA was assessed by optical density measurements, obtaining the ratio of absorbance values at 260 nm and 280 nm using a Bio-photometer (Eppendorf, Germany). A total of 0.5 µg RNA was used to synthesis cDNA (Qiagen, Germany). Polymerase chain reaction was performed for Cathepsin K for 30 cycles, 94 °C for 1 minute, 60 °C for 1 minute and 72 °C for 1 minute and endogenous control β-actin for 30 cycles 94 °C for 35 second, 64 °C for 45 second and 72 °C for 1 minute. Primer sequence for Cathepsin K was sense 5′-CCGCAGTAATGA CACCCTTT-3′ and antisense 5′-AAGGCATTGG TCATGTAGCC -3′, 258 bp and β-actin sense 5′ TCACCCACACTGTGCCCATCTACGA-3′ and antisense 5′CAGCGGAACCGCTCATTGCCA ATGG-3′, 285 bp. The PCR product was visualized on 2% agarose gels.

*Statistical Analysis*

Data were expressed as mean ± SD and percentage wherever appropriate. A p value of <0.05 was considered statistically significant.

**Results**

*Blood and Serum Biochemistry*

Table 1 shows the baseline clinical and biochemical characteristics like demographic, anthropometric and biomedical details of study groups of non diabetic postmenopausal women (NDPW) and diabetic postmenopausal women (T2DPW). Significant differences were observed in systolic blood pressure (P<0.05), fasting plasma glucose (P<0.001) and HbA1C (P<0.001) [Table 1].

*Osteoclasts Cell Viability Assay*

The effect of pioglitazone and estradiol on cell proliferation was assessed by a 3-[4,5-dimethylthiazo l-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Pioglitazone treated cells shows the half maximal effective concentration at 4.0 ìM and estradiol half minimal inhibitory concentration is at 7nM. Hence the predicted concentration of pioglitazone and estradiol was used for cell culture.

*Pioglitazone Induced TRAP Positive Multinucleated Osteoclasts in PBMCs of type2 Diabetic Postmenopausal Women*

To determine the effect of pioglitazone on differentiation and activation of osteoclasts, osteoclast precursor cells were stimulated with RANKL and M-CSF following pioglitazone treatment. The formation of TRAP positive multinucleated cells (more than 3 nuclei) was analyzed. Increased in numbers of TRAP positive multinucleated cells was observed in pioglitazone treated cells of type2 diabetic postmenopausal women (T2DPWPIO; Group 2a: 88.12 ± 12.25) as compared to type2 diabetic postmenopausal women (T2DPW; Group 2; 46.46 ± 11.54) and non diabetic postmenopausal women (NDPW; Group 1; PBMCs: 40.85 ± 9.45). Significant difference between TRAP positive cells in T2DPWPIO (group 2a) and T2DPW (group 2) was observed (p <0.0001), no significant differences were observed between the expression of TRAP positive multinucleated cells of T2DPW and NDPW.

Postmenopausal women usually experience a reduced level of estrogen due to natural physiological process. Depletion of estrogen by any other factors is serious health risk for postmenopausal women. To determine the impacts of pioglitazone on estrogen function, osteoclasts cells were co -treatment with pioglitazone + estradiol (Group 2b). Slight reduction in TRAP positive multinucleated cells were observed in co-treated cells of multinucleated (T2DPWPIOE; Group 2b: 68.44 ± 15.12) as compared to T2DPWPIO treated cells and significant differences were observed between the groups (p<0.01). Further we also observed marked reduction of multinucleated osteoclasts in estrogen treated cells of T2DPW (group 2c; T2DPWE: 39.12 ± 9.11) as compared to other groups. Significant reduction of TRAP positive multinucleated was observed as compared to co- treated cells (p <0.0001) [Figure 1 and 1a].

*Pioglitazone Up-Regulates Osteoclastic Marker Gene Cathepsin K in Type2 Diabetic Postmenopausal Women*

The mRNA expressions of osteoclasts marker Cathepsin K was assessed by RT-PCR in osteoclasts of NDPW and T2DPW. The expression ratio of Cathepsin K mRNA was significantly greater in T2DPWPIO as compared to other groups. Significant differences were observed between T2DPW vs. T2DPWPIO (P< 0.01), T2DPWPIO vs. T2DPWPIOE (p< 0.001) and T2DPWPIOE vs. T2DPWE (p< 0.05) [Figure 2].

**Table 1:** Blood and Serum biochemistry

| Variables | NDPW, n = 13 | T2DPW, n=13 |
|---|---|---|
| Ages (yrs) | 57.44 ± 3.15 | 55.38 ± 4.11 |
| Duration of diabetes | - | 7.84 ± 5.4 |
| Duration of postmenopausal (yrs) | 7.89 ± 2.42 | 8.11 ± 1.55 |
| BMI (kg/m²) | 20.76 ± 3.15 | 19.44 ± 4.16 |
| SBP (mmhg) | 118 ± 4.82 | 121.72 ± 6.13 * |
| DBP (mmhg) | 73.1 ± 5.4 | 74 .11 ± 5.5 |
| FBG (mmol/l) | 80.94 ± 6.32 | 156.71 ± 10.38 *** |
| HbA1c (%) | 5.2 ± 1.38 | 7.7 ± 1.43 *** |
| Urea (µl/ml) | 19.12 ± 3.23 | 20 ± 7.64 |
| Creatinine (mmol/l) | 0.99 ± 0.782 | 1.1 ± 0.89 |
| T.Cho (mmol/l) | 3.44 ± 0.8 | 4.2 ± 1.83 |
| TG (mmol/l) | 1.68 ± 0.81 | 1.82 ± 0.54 |

BMI: Body mass index, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, FPG: Fasting plasma glucose, HbA1c: Glycated hemoglobin A1c, T.Cho: Total cholesterol, TG: Total glyceraldehydes. Values are expressed as mean ± SD for the study group. (* P < 0.05, ** P < 0.01, *** p<0.001).

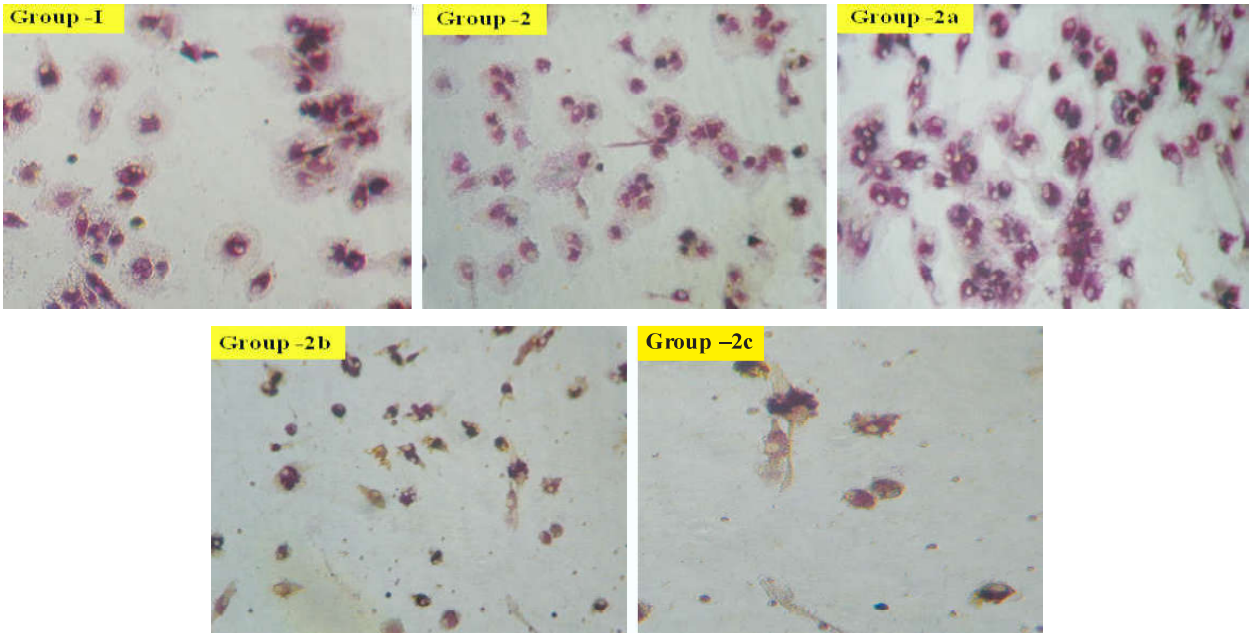


**Fig. 1:** Representative photographs showing TRAP positive multinucleated osteoclasts and quantitative comparison between the numbers of TRAP positive cells in study groups

**Group 1:** PBMCs derived from non diabetic postmenopausal women, **Group 2:** PBMCs derived from type 2 diabetic postmenopausal women, **Group 2a:** PBMCs derived from type 2 diabetic Postmenopausal + Pioglitazone, **Group 2b:** PBMCs derived from type 2 diabetic Postmenopausal + Pioglitazone + 17 beta estradiol and **Group 2c:** PBMCs derived from type 2 diabetic Postmenopausal + 17 beta estradiol

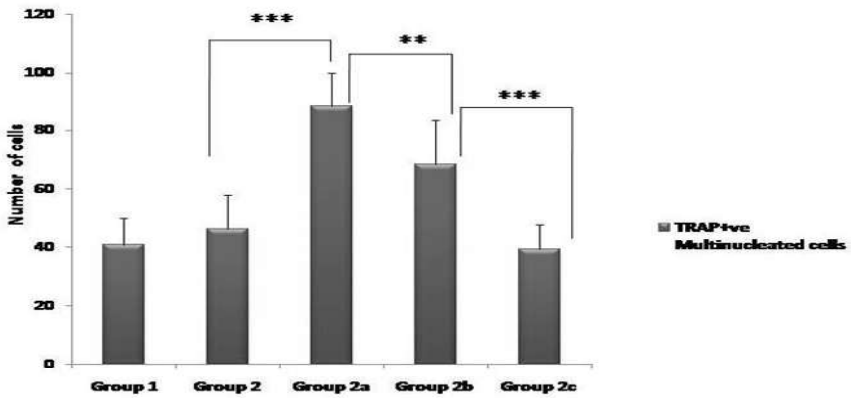


**Fig. 1a:** Quantitative comparison between the numbers of TRAP positive cells in study groups

Quantitative comparisons of TRAP positive multinucleated cells among different groups. Values are expressed as mean ± SD for the study group. (* P < 0.05, ** P < 0.01, *** p<0.001).

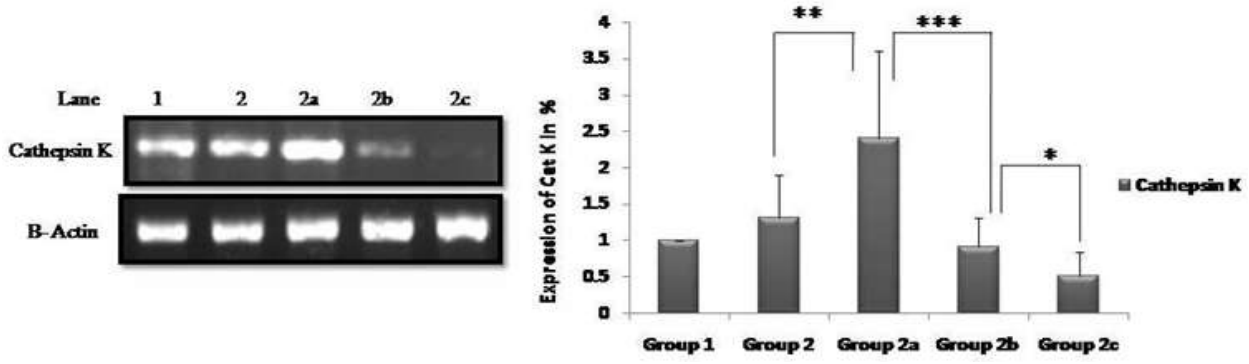


**Fig. 2:** mRNA gene expression of osteoclasts marker Cathepsin K

**Fig. 2:** Cathepsin K mRNA gene expression in osteoclast cells Lane 1: NDPW, 2: T2DPW, 2a: T2DPWPIO, 2b: T2DPWPIOE and 2c: T2DPWE. Comparisons were made between the groups. A p value were represent as $p < 0.05$*, $p < 0.01$** and $p < 0.001$***

## Discussion

Several clinical data found an association between pioglitazone and increased risk of fractures especially in older females [18-20]. Our present study reveals that T2DPW were the group at the greatest fracture risk from pioglitazone (TZD) treatment. To our knowledge no studies have experimentally proved that pioglitazone induces bone resorption cell osteoclasts in T2DPW.

The present finding shows a relatively increased in the number of TRAP positive multinucleated osteoclasts in T2DPW subjects (Group-2: 46.46 ± 11.54) as compared to NDPW subjects (Group-1: 40.85 ± 9.45) [Figure 2]. It may be due to hyperglycemia that directly induces osteoclast mediated bone resorption by promoting osteoblast to adipocyte shift [21] and increases fat accumulation in the marrow cavity [22]. Other reasons which could account for increased osteoclasts differentiation in T2DPW subjects may be due to decreased levels of leptin and increased advanced glycation end products [23-25].

As pioglitazone has an important role in regulation of osteoclasts through the activation of PPARã signalling (26-30), the effects of pioglitazone on osteoclasts was analysed. We found that Pioglitazone increases the propensity of the osteoclast precursor cells in PBMCs of T2DPW (group 2a) in In vitro condition. This is indicated by an increased in the number of TRAP positive multinucleated cells in T2DPWPIO osteoclasts (Group 2a: 88.12 ± 12.25) as compared to T2DPW (Group-2: 46.46 ± 11.54) and NDPW osteoclasts [Figure 1]. The possible reason would be PPARγ agonist pioglitazone osteoclasts, may prime osteoclast-committed precursors into mature active osteoclasts from hematopoietic stem cells through binding to and an activation of the nuclear receptor PPARγ [31] and undergoes a conformational change and activates the transcription of genes involved in osteoclastogenesis [32]. We next evaluate the effect of osteoclasts number co-treated with pioglitazone and estrogen (group 2b). Significant reduction (group 2b: 68.44 ±15.12) in the number of TRAP positive multinucleated was observed (figure 1a) as compared to T2DPWPIO treated cells ($p<0.01$). In spite of estrogen treatment, it didn't achieved greater inhibition of osteoclasts formation when co treated with pioglitazone. These finding suggested potential contributing mechanism for TZD-induced osteoclast activation and bone loss, which may involve the suppression of estrogen function and production. A study from human granulosa cells study shows that TZDs inhibits estrogen synthesis by interfering with androgen binding to aromatase [33]. Inhibition of aromatase activity by TZDs explained their negative effect on bone density in postmenopausal women [34,35]. Further we found marked reduction in estrogen treated cells of T2DPW (39.12 ± 9.11) as compared to all other group. Studies from cell line [36], and animal [37] shows that estrogen treatment suppressed formation of multinucleated osteoclasts induced by M-CSF and RANKL and decrease cfms and calcitonin receptor in postmenopausal women.

Cathepsin K a cystein proteinase expressed potently in osteoclasts and plays an important role in degrading the organic phase of bone during bone resorption. Deficient of Cathepsin K in mice display an impaired resorptive activity and inhibition of osteoclast activity. This underlines the importance of Cathepsin K in bone remodelling (38). Our present data observed an increased Cathepsin K gene expression ratio in pioglitazone treated cells of T2DPWPIO (group 2a) as compared to NDPW and T2DPW (group 1 and group 2) [Figure 2]. Significant

increased in the expression of Cathepsin K was observed in T2DPWPIO osteoclasts ($p < 0.01$). Activation of M-CSF and RANKL by TZDs may be one of the major reasons that trigger the proosteoclastogenic transcription factors, leading to osteoclasts differentiation and cell fusion by up-regulating osteoclast functional genes such as cathepsin K [39-41]. Cathepsin K concertedly regulates the differentiation, maturation and activation of osteoclasts during bone remodelling [42-44]. Cathepsin K deleted mice shows marked reduction in bone resorption and increases number of osteoclasts and bone formation rate as compared with that of wild-type mice. The bone formation phenotype observed in Cathepsin K knockout mice suggests that this enzyme involved in mediating changes in osteoclast-derived coupling factors that regulate bone formation [45]. Hence the study concluded that pioglitazone has more potent effect on the adult skeleton in the context of low estrogens level which is more susceptible to increased fracture risk.

Some of the limitations in the present work are, firstly the number of subjects enrolled in the study is less, second the effect of pioglitazone and estrogen on osteoclasts function was evaluated only with TRAP staining and Cathepsin K gene. Additional study in larger study population is needed to study the effect of Pioglitazone on osteoclasts associated molecular markers is needed to confirm the role of pioglitazone on signalling cascade of bone physiology. Further cellular and molecular study is highly recommended to establish the role of pioglitazone on osteoclasts function and estrogen signalling.

*Conflicts of Interest*

There are no conflicts of interest in the study

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