

***In vitro* regulation of osteoclast generation: A cost-effective strategy to combat osteoporosis with natural antioxidants and polyphenols like EGCG**

WASIL HASAN

SABA AHMAD

HAMIDA THAKUR

Department of Biochemistry
Faculty of Medicine, Jawaharlal Nehru Medical College
Aligarh Muslim University, Aligarh
India

MAZHAR ABBAS

Department of Orthopaedic Surgery
Faculty of Medicine, Jawaharlal Nehru Medical College
Aligarh Muslim University, Aligarh
India

ABBAS ALI MAHDI

Department of Biochemistry
King George Medical University, Lucknow, U. P.
India

NAJMUL ISLAM¹

Department of Biochemistry
Faculty of Medicine, Jawaharlal Nehru Medical College
Aligarh Muslim University, Aligarh
India

Abstract:

*Osteoporosis has been shown to be associated with elevated levels of TNF- α and intracellular reactive oxygen species (ROS). ROS is actively related to production of TNF- α , which in turn, leads to activation of bone markers, thereby resulting in accelerated osteoclast differentiation leading to osteoporosis. (-)-Epigallocatechin-3-gallate (EGCG), one of the most potent polyphenol, an active ingredient of green tea (*Camellia sinensis*), has earlier been reported to have potent*

antioxidant and free radical scavenging properties along with strong immunomodulatory activity. EGCG is found to suppress osteoclasts differentiation. EGCG is also found to exert immunomodulatory and antioxidative effects on monocytes of osteoporotic patients. Secreted TNF- α were found to be suppressed using varied doses of EGCG. EGCG, dose dependently found to enhance the glutathione peroxidase (GPx) activity. Therefore, EGCG has the potential to be used as an adjunct in the chemoprevention and treatment of osteoporosis.

Key words: Epigallocatechin-3-gallate (EGCG), Glutathione peroxidase (GPx), Reactive oxygen species (ROS), TNF- α

Introduction

Bone is the primary site of haematopoiesis. The complex interplay between the bone organ system and the immune system is continuously emerging with the advancement of research (Bab and Einhorn, 1994; Horowitz and Jilka, 1992; Wein *et al.*, 2005). Osteoporosis is a disease of bones that leads to an increased risk of fracture (Alldredge *et al.*, 2009), where the bone mineral density (BMD) is reduced, bone micro architecture deteriorates, and the amount and variety of proteins in bone is altered. Osteoporosis is defined by the World Health Organization (WHO) as a bone mineral density that is 2.5 standard deviations or more below the mean peak bone mass (WHO, 1994). The underlying mechanism in all cases of osteoporosis is an imbalance between bone resorption and bone formation (Frost and Thomas, 1963). Bone is resorbed by osteoclast cells, after which new bone is deposited by osteoblast cells (Raisz, 2005). Osteoclasts having the unique property of dissolving bone are the cells of hematopoietic origin, whose inhibition leads to treatment of diseases of bone loss such as osteoporosis.

¹ Corresponding author: nxi7@hotmail.com

There are various peculiar characteristics of osteoclasts, such as tartrate-resistant acid phosphatase (TRAP) staining, multinuclearity, formation of actin ring structure and a polar cell body during resorption, and contraction in response to calcitonin. Osteoclasts express a number of molecular markers, such as calcitonin receptor, RANK (receptor of RANKL, receptor activator of NF κ B ligand), c-fms (receptor of M-CSF, macrophage-colony stimulating factor), cathepsin K, c-src, fosL1 and the vitronectin receptor (integrin α v β 3) (Nijweidi *et al.*, 1986; Susa *et al.*, 2004; Netter, 1987).

The activation of osteoclasts is regulated by various molecular signals, of which RANKL is one of best studied. This molecule is produced by osteoblasts and other cells (e.g. lymphocytes), and stimulates. Osteoprotegerin (OPG) binds RANKL before it has an opportunity to bind to RANK, and hence suppresses its ability to increase bone resorption. RANKL, RANK and OPG are closely related to tumor necrosis factor and its receptors (Raisz, 2005). Therefore, the OPG/RANKL/RANK cytokine system is essential for osteoclast biology. Various studies suggest that human metabolic diseases such as osteoporosis are related to alterations of this system (Hofbauer *et al.*, 2004). Furthermore, local production of eicosanoids and interleukins is thought to participate in the regulation of bone turnover, and excess or reduced production of these mediators may underlie the development of osteoporosis (Raisz, 2005).

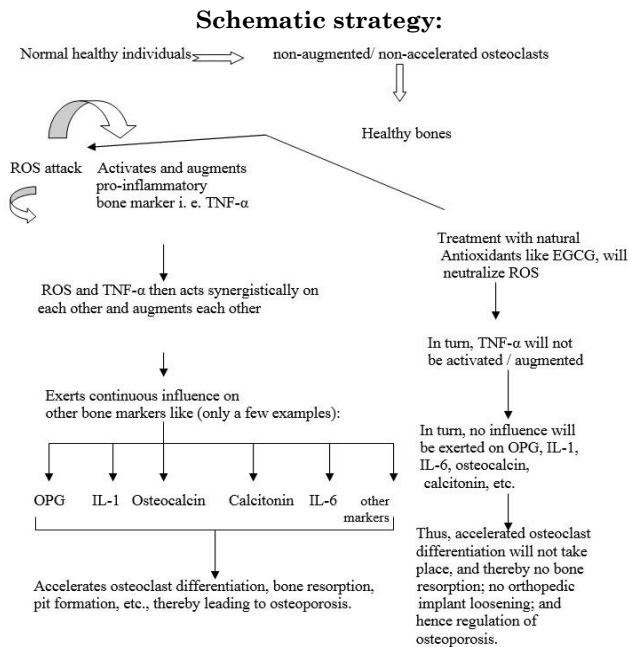
Tea [*Camellia sinensis* (Theaceae)] is second only to water in terms of worldwide popularity as a beverage. Green tea is chemically characterized by the presence of large amounts of polyphenolic compounds known as catechins, which includes epicatechin (EC), epicatechin-3-gallate (ECG), and EGCG account for 30–40% of the dry weight of green tea (Lambert and Yang, 2003; Yang *et al.*, 2006). The compound (-)-epigallocatechin-3-gallate (EGCG) is the most abundant and

biologically active catechin contained in green tea (Lambert and Yang, 2003). It is devoid in black tea (Lorenz *et al.*, 2009). EGCG has been shown to be 25 to 100 times more potent than vitamins C and E in terms of antioxidant activity (Doss *et al.*, 2005). Although catechins have been shown to reduce bone resorption, the mechanisms of action of EGCG on bone metabolism have not been well studied (Delaisse *et al.*, 1986). The protective action of green tea polyphenols (GTPs) against bone loss in ovariectomized (OVX) rats through their antioxidant capacities to scavenge reactive oxygen species (ROS) has been documented [Shao *et al.*, 2011].

Recent evidence showed that EGCG could increase the formation of mineralized bone nodules by human osteoblast-like, SaOS-2 cells (Vali *et al.*, 2007), enhance osteogenesis in a bone marrow mesenchymal stem cell line (Chen *et al.*, 2005), and induce apoptotic cell death of osteoclasts differentiated from RAW 264.7 cells *in vitro* (Yun *et al.*, 2007). EGCG may improve osteoporotic condition by inhibiting progressive bone loss due to both increased osteoclastic bone resorption and a decrease in osteoblastic bone formation. EGCG is also reported to suppress osteoclast differentiation and ameliorate experimental arthritis in mice over the short term (Morinobu *et al.*, 2008). Furthermore, EGCG inhibits matrix metalloproteinases (MMPs) expression and activity, which plays an important role in degeneration of the matrix associated with bone and cartilage. Regulation of osteoclast activity is essential in the treatment of bone-disease, including osteoporosis and rheumatoid arthritis (Oka *et al.*, 2012). Recently, it has been reported that EGCG at a low concentration can slightly enhance the osteogenic effect *in vivo*, whereas at a higher concentration it can prevent the osteogenic differentiation of human alveolar bone-derived cells (hABCs) both *in vitro* and *in vivo* (Mah *et al.*, 2014). EGCG can also be used as a proosteogenic agent for the stem-cell-based therapy of

osteoporosis (Jin *et al.*, 2014). The inhibitory effect of EGCG to osteoclastogenesis is reported to be associated with a down regulation of RANKL/RANK signal, and increased apoptosis of preosteoclasts (Zhao *et al.*, 2014).

Thus, in the present study, attempts have been made to probe the effects of natural antioxidant EGCG on bone markers and osteoclast generation in cultures of PBMC's from patients with osteoporosis. Dietary supplementation in anti-bone resorptive therapy is relevant as a safe and economic option, where EGCG has been proposed as an effective herbal therapeutic, as it is a strong anti-oxidant and anti-inflammatory agent having beneficial effects on bone metabolism. Thus, it is hoped that this cost-effective strategy would be of immense help in the better understanding of osteoporosis management. In order for clarity, the overall cost-effective schematic strategy to combat osteoporosis with natural antioxidants like EGCG is summarized below.



2. Materials and methods

2.1. Materials

The study has prior institutional ethical clearance. Blood from patients with osteoporosis (n=30-40) was obtained from those admitted to the orthopedic wards and from the corresponding out patient section of J.N. Medical College Hospital, A.M.U., Aligarh. Blood donated by healthy normal volunteers (n=30-40) served as controls. EGCG was from Sigma–Aldrich (St. Louis, MO, USA). Ficoll-Paque was from Pharmacia (LKB Biotechnology Piscataway, NJ). RANKL and M-CSF were from R & D Systems, USA. All other chemicals were of the highest purity grade available.

2.2. Isolation of peripheral blood mononuclear cells (PBMC) from blood of normal and osteoporosis patients

Blood samples were drawn from appreciable number of patients (n=30-40) with osteoporosis as well as normal healthy volunteers which served as controls (n=30-40) for isolation of peripheral blood mononuclear cells (PBMC), using a Ficoll-Hypaque density gradient method as described by us previously (Hasan *et al.*, 2006; Hasan *et al.*, 2007; Islam *et al.*, 2004).

2.3. Culture Conditions for Osteoclastogenesis

The culture conditions for osteoclastogenesis have been described elsewhere (Faust *et al.*, 1999; Buckley *et al.*, 2005). This has also been employed in our earlier communication elsewhere. The isolated PBMCs were centrifuged at 400g for 30 minutes at 21°C. The resulting buffy layer was removed with a Pasteur pipette. Then the remaining cells were washed twice in PBS. These isolated mononuclear cells were cultured at a density of 2×10^6 cells/cm² in 10 cm Petri dishes in α -MEM culture medium supplemented with 10% FCS, 100U/ml penicillin, 100 μ g/ml streptomycin, 50ng/ml M-CSF and

25ng/ml RANKL (Osteoclastogenic medium) at 37°C in a humidified 5% CO₂ atmosphere. After 24 h of culture, the non-adherent cell fraction was discarded. The adherent population was washed with PBS. Thereafter, trypsin was added to the culture and incubated at 37°C for approximately 6 min. Subsequently, cells were scrapped off and reseeded. The culture duration was 1, 3 and 5 days and the cells were subjected to phenotypic characterization by TRAP staining and were identified as committed multinucleated preosteoclasts (pOCs).

2. 4. Tartrate Resistant Acid Phosphatase (TRAP) Staining and Quantification of TRAP-positive Multinucleated Cell Number

TRAP staining of adherent cultures was done with a kit (Sigma–Aldrich, USA) on a 96-well culture plate, exactly according to manufacturer's instruction. This has also been employed in our earlier communication elsewhere. The stained cells developed red color of different intensity. The number of TRAP-positive multinucleated (>2 nuclei per cell) preosteoclasts cells was measured using the 1 × 1-mm grid placed in the ocular of the microscope. Five sites were measured in a well of a 96-well plate, and a mean value was calculated.

2.5. Dose Response Effect of Epigallocatechin gallate (EGCG)-a green tea polyphenol and natural antioxidant, on generation of osteoclasts from PBMCs

To investigate the effect of natural antioxidants like epigallocatechin-3-gallate (EGCG) on osteoclast generation from PBMCs, varying doses of EGCG (0-25 µg/ml) were added to the PBMC cultures seeded at a cell density of 2x10⁵ cells/cm² in a 96-well plate in a osteoclastogenic medium as described above and incubated for 24 h (1 day), 72 h (3 day) and 120 h (5 days) at 37°C in a humidified atmosphere of 5% CO₂. Thereafter, the cells were analysed with TRAP staining.

2.6. Isolation of total RNA/messenger RNA

As per manufacturer's specifications (Qiagen, Valencia , CA), total RNA or messenger RNA (mRNA) was isolated from cells of normal healthy controls as well as cells from patient with osteoporosis as described by us previously (Hasan *et al.*, 2006; Hasan *et al.*, 2007; Islam *et al.*, 2004).

2.7. Quantitative real-time RT-PCR and expression of sTNF-alpha in culture supernatants

Quantitative real-time RT-PCR with internal fluorescent hybridization probes was employed to quantify host TNF- α gene transcription as described by us previously (Hasan *et al.*, 2006; Hasan *et al.*, 2007; Islam *et al.*, 2004). This technique affords a sensitive and specific quantification of individual RNA transcripts (Islam *et al.*, 2004). Human R18 housekeeping gene was employed to normalize gene expression. TaqMan PCR primers and probes as well as target-specific RT primer for each assay were designed as described by us elsewhere (Hasan *et al.*, 2006; Hasan *et al.*, 2007; Islam *et al.*, 2004). The primer and probe sequences used have been previously reported by us as well as elsewhere (Islam *et al.*, 2004; Holland *et al.*, 1991). To assure lack of DNA contamination in the RNA samples, in some experiments, a duplicate tube of sample with no RT enzyme was included as control. DNA contamination remained negligible. In each sample, host 18S ribosomal RNA was used as internal control. Expression of TNF- α mRNA was corrected to internal control (host 18S rRNA) in the same sample and was expressed as copies of TNF- α in 10^{10} copies of R18 (equivalent to 1×10^6 monocytes).

The expression of sTNF-alpha in culture supernatants of PBMC's from patients with osteoporosis that were co-cultured with and without EGCG (20 μ g/ml) as described above was

evaluated as described by us earlier (Hasan *et al.*, 2006; Hasan *et al.*, 2007; Islam *et al.*, 2004).

2.8. Glutathione peroxidase assay

The activity of glutathione peroxidase (GPx) was measured as described elsewhere (Holland *et al.*, 1991). Briefly, culture cells from patient with osteoporosis as described above were co-cultured with or without EGCG (20 µg/ml) for 5 days in osteoclastogenic medium. Thereafter, cells were scrapped and centrifuged as described earlier (Hasan *et al.*, 2006), and the supernatants were subjected to GPx activity determination. The GPx activity was quantified in 100 µl of each sample, with continuous photometric monitoring of oxidized glutathione (GSSG) at 37°C. The conversion of NADPH to NADP was evaluated using UV absorbance at 340 nm (Flohe and Gunzler, 1984). GPx activity was calculated after subtraction of the blank value, as µmol of NADPH oxidized/min/mg protein (U/mg protein).

2.9. Statistical analysis

Results were analyzed by use of paired t test and expressed as means ± S.E. of ten experiments. P < 0.05 was considered statistically significant

3. Results:

3.1. Generation of Human Osteoclast Precursors from Peripheral Blood Mononuclear Cells (PBMCs) and Suppressive Effect of Epigallocatechin gallate (EGCG) on the Generation of Human Osteoclasts

Peripheral blood mononuclear cells (PBMCs) were used directly for the generation of osteoclast precursors after centrifugation with Ficoll-Hypaque. Multinucleated osteoclast precursors were observed to appear after the 3 day culture duration in

osteoclastogenic medium (α -MEM culture medium supplemented with 10% FCS, 100U/ml penicillin, 100 μ g/ml streptomycin, 50ng/ml M-CSF and 25ng/ml RANKL) (Fig. 1). Interestingly, in comparison to the data of 3 days culture, the tartarate resistant acid phosphatase (TRAP) staining exhibited the number of multinucleated cells to increase appreciably after 5 days of culture (Figs. 2). However, the first two days of culture showed no detectable appearance of osteoclast precursors (data not shown). The number of multinucleated preosteoclasts, arising from PBMCs isolated from the blood of normal healthy individual (data not shown) and osteoporotic patients (n=30) (Fig. 1 and 2) were counted by TRAP staining. Individual variation in osteoclast generation from different donors was observed (individual data not shown and instead mean values are shown). Next, co-culturing of PBMCs with EGCG (20 μ g/ml) in osteoclastogenic medium for 5 and 3 days exhibited an appreciable suppression in the range of around 56% to 37% in appearance of multinucleated osteoclast precursors (Figs. 1 and 2). Hence, this shows the potential of EGCG to exert regulatory effect in osteoclast generation and differentiation.

3.2. Dose Response Effect of Epigallocatechin gallate (EGCG) on the Generation of Human Osteoclasts

The above dose of EGCG was selected after performing dose response experiment, where TRAP assay revealed a linear suppression in the formation of multinucleated cells was observed (Fig. 3). Nearly 20-25% suppression in appearance of multinucleated cells was observed in cultures receiving 20 and 25 μ g/ml of EGCG relative to control cultures devoid of any EGCG (Fig. 3).

3.3. Effect of EGCG on TNF- α gene expression

The expression of TNF- α was determined at both the protein and mRNA levels. PBMC's from normal healthy and patients with osteoporosis (n=20) were co-cultured for 24 h with 20 μ g/ml EGCG. Cultures devoid of EGCG served as controls. Culture supernatants were subjected to evaluation of sTNF- α expression at the protein level by ELISA. As evident from Fig. 4A, EGCG (20 μ g/ml) down regulated the expression of sTNF- α by around 66.97% (P<0.001) relative to cultures devoid of any EGCG. Similar observations were recorded at the mRNA level. The suppressive effect of EGCG (20 μ g/ml) on the amplification of TNF- α gene was found to be of appreciable magnitude, as was revealed by real-time RT-PCR, where in comparison to control, a down regulation of TNF- α mRNA copy numbers by 4.1~logs (P < 0.001) was recorded with 20 μ g/ml of EGCG (Fig. 4B). Our previous study on cultures from normal healthy donors with or without EGCG did not show any significant TNF- α or any effect of EGCG (30). Thus, EGCG potently inhibits the pro-inflammatory cytokine TNF- α in PBMC cultures of patients with osteoporosis.

3.4. Glutathione peroxidase activity

GPx activity was determined in PBMC's culture of patient with osteoporosis, which were co-cultured for 5 days with or without 20 μ g/ml of EGCG. Normal healthy cells devoid of EGCG served as controls. As evident from Fig. 4C, GPx activity was found to be suppressed by ~ 52.61 % (P < 0.001; n=20) in cultures of patient's cells relative to normal healthy cells. Interestingly, in comparison to patient's cells, co-culturing with EGCG (20 μ g/ml) revealed amelioration in the GPx activity by ~ 40.3% (P < 0.001) (Fig. 5). Thus, EGCG proved to be a potent enhancer of GPx activity in culture cells of patient with osteoporosis.

4. Discussion:

Globally, osteoporosis is known since the origin of human civilization, but complete understanding about its management still remains poorly understood till date. Augmented generation of ROS in vivo due to a wide spectrum of in-vivo-related reasons, leads to the activation and up-regulation of bone markers like pro-inflammatory cytokine TNF- α and its super family member OPG as well as osteopontin and calcitonin at both the protein and gene i. e. mRNA levels. This in turn results in accelerated osteoclast differentiation, thereby resulting to loss of bone mass, including osteoporosis, arthritis, orthopaedic implant loosening, etc.

Thus, if ROS production in vivo is regulated by natural antioxidants like EGCG from green tea, neem extract, turmeric, allicin from garlic, etc., then the above-mentioned bone markers associated with chronic bone conditions may probably be easily regulated. We have previously shown the effect of EGCG in other diseases (Islam *et al.*, 2000; Yang *et al.*, 2014). Several studies have reported increased production of TNF by cultures of mononuclear cells derived from osteoporotic and postmenopausal women, an effect reversed by estrogen replacement (Alldredge *et al.*, 2009; Islam *et al.*, 2004), and that, ROS may play a role in bone loss by generating a more oxidized bone microenvironment (Buckley *et al.*, 2005; Holland *et al.*, 1991).

We probed here the management and/or regulation of ROS and TNF- α activation in PBMC's from patients with osteoporosis that were cultured under osteoclastogenic medium. Probing the adjuncts for down regulation of augmented TNF- α and ROS in patients with osteoporosis would definitely help in better understanding the pathogenesis of osteoporosis.

TNF- α is implicated in the pathophysiology of bone metabolism. The presence of elevated levels of TNF in the bone

marrow of ovx animals and in the conditioned media of peripheral blood cells of postmenopausal and osteoporosis subjects is well documented (Wein *et al.*, 2005; Alldredge *et al.*, 2009; WHO, 1994). Moreover, ROS are also involved in the etiopathology and progression of osteoporosis. Radicals generated in cells of postmenopausal and osteoporosis patients stimulate TNF- α , causing accelerated bone loss.

EGCG- a green tea polyphenol as well as a natural antioxidant was employed in the present study whose exact mechanism underlying its antioxidant activity still remains poorly understood. EGCG-induced enhancement of GPx activity has been reported

Augmented expression of TNF- α mRNA has been reported in monocytic cells derived from patients with osteoporosis (Raisz, 2005). Consistent with these finding, we also report the up regulation of TNF- α mRNA expression in PBMC's from patients with osteoporosis that were cultured under osteoclastogenic medium.

The most striking finding of the present study shows that EGCG exerts potent anti-inflammatory, antioxidant and anti-osteoclast effects on PBMC's from patients with osteoporosis that were cultured under osteoclastogenic medium. Since higher doses of EGCG (>23 μ M) have proven toxic, thus lower doses (0-20 μ M) were employed in the present study. The results indicate an appreciable suppression in endogenous TNF- α mRNA expression by \sim 4.1 logs with 20 μ g/ml of EGCG in mononuclear cells of patients with osteoporosis.

Glutathione directly reacts with ROS, and GPx catalyzes the removal of hydrogen peroxide (Flohe and Gunzler, 1984). Lowering in GPx activity infers impairment of hydrogen peroxide-neutralizing mechanisms (Flohe and Gunzler, 1984). Here, we observed a suppression in GPx activity in PBMC's from patients with osteoporosis that were cultured under osteoclastogenic medium, thereby correlating with earlier

reports that substantial amounts of ROS are being generated in bone cells due to lowering of antioxidant defenses. Amelioration of GPx activity was observed in PBMC's from patients with osteoporosis that were co-cultured with EGCG under osteoclastogenic medium. This indicates EGCG-induced reversal of impaired neutralizing mechanisms in PBMC's from patients with osteoporosis that were cultured under osteoclastogenic medium. Thus, our study shows ameliorated GPx activity by EGCG, which correlated inversely with the down regulation of TNF- α mRNA expression and ROS in culture cells of patients with osteoporosis.

In summary, dietary supplementation in anti-bone resorptive therapy is relevant as a safe and economic option, where EGCG may act as an effective herbal therapeutic, as it is a strong anti-oxidant, anti-inflammatory and anti-osteoclast agent having beneficial effects on bone metabolism. EGCG, a green tea polyphenol has been reported to exert potent anti-oxidant and anti-inflammatory effects by inhibiting signaling and gene expression (Yang *et al.*, 2014). Thus, it is hoped that the present study may help in the better understanding of the management of osteoporosis.

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Figures:

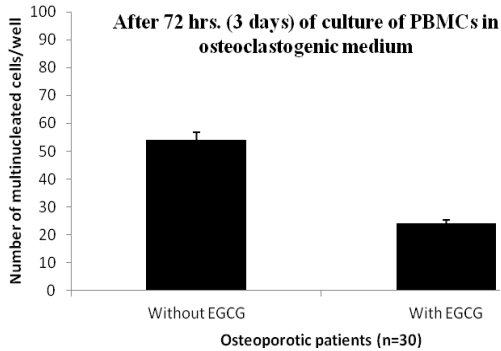


Fig. 1. 72 hrs cultures: Human osteoclasts were generated from peripheral blood mononuclear cells (PBMCs) in 72 hrs cultures as described in methods. The results from 30 patient donors (mean value) are presented as quantification of TRAP-positive multinucleated cell number. The effect of EGCG (20 μ g/ml) is shown in black bars. ($P < 0.001$ in all cases)

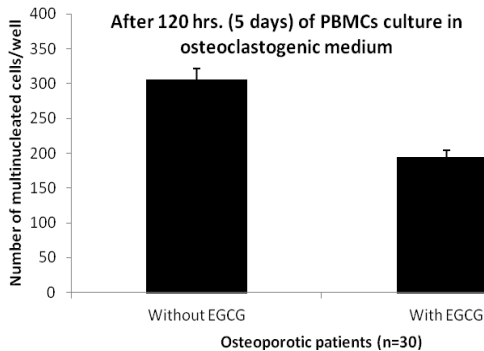


Fig. 2. 120 hrs cultures: Human osteoclasts were generated from peripheral blood mononuclear cells (PBMCs) in 120 hrs cultures as described in methods. The results from 30 patient donors (mean value) are presented as quantification of TRAP-positive multinucleated cell number. The effect of EGCG (20 μ g/ml) is shown in black bars. ($P < 0.001$ in all cases)

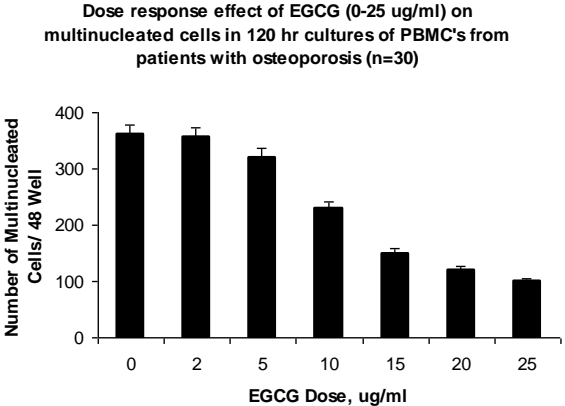


Fig. 3. Dose response effect of EGCG (0-25 $\mu\text{g/ml}$) on multinucleated cells in 120 hr cultures of PBMC's from patients with osteoporosis (n=30). The cells were cultured under osteoclastogenic medium as described in methods (P<0.001).

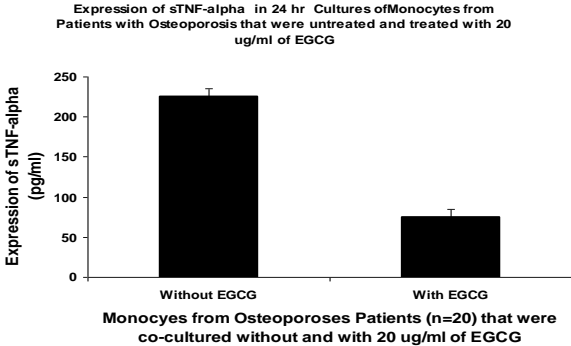


Fig. 4A. Expression of soluble TNF- α by ELISA in 24 hr cultures of PBMC's from patients with osteoporosis (n=20) that were untreated and treated with 20 $\mu\text{g/ml}$ of EGCG under osteoclastogenic medium (P<0.001).

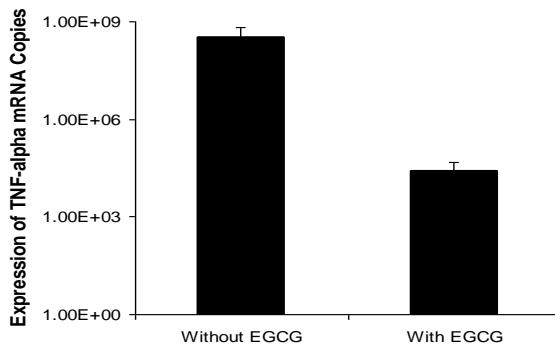


Fig. 4B. Modulation of TNF- α mRNA expression by Real Time RT-PCR in 24 hr cultures of PBMC's isolated from patients with osteoporosis (n=6; P<0.001). The cells were cultured under osteoclastogenic medium as described in methods.

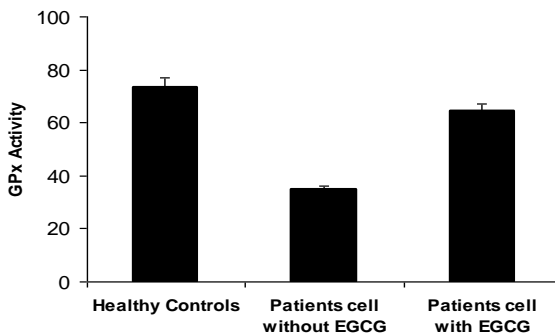


Fig. 4C. Amelioration of GPx activity by EGCG (20 μ g/ml) in 120 hr cultures of PBMC's isolated from patients with osteoporosis (n=20; P<0.001). The cells were cultured under osteoclastogenic medium as described in methods. GPx activity is expressed as U/mg protein.