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RESEARCH PAPER

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Epigallocatechin-3-gallate (EGCG), A Polyphenol and Natural Anti-Oxidant, Down Regulates Multinucleated Osteoclasts

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ABSTRACT

Osteoporosis has been shown to be associated with reactive oxygen species (ROS) which in turn is actively related to production of bone markers promoting osteoclast differentiation thereby leading to osteoporosis. Most of the in vitro modulation/regulation of osteoclast differention is on cell lines or dentine slices but there are only fewer studies on modulation/regulation of osteoclast differentiation in in vitro cultures of human PBMC's from patients with osteoporosis. We report that epigallocatechin-3-gallate (EGCG), one of the most potent polyphenol, an active ingredient of green tea (Camellia sinensis), as well as an natural antioxidant coupled with strong immunomodulatory activity appreciably suppresses osteoclasts differentiation in cultures of human PBMC's from osteoporosis patients. Therefore, EGCG may have the potential to be used in the treatment/prevention of osteoporosis by suppressing/preventing the generation of multinucleated osteoclasts.

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

INTRODUCTION

Bone is a vital, dynamic and complex connective tissue having multiple functions in vertebrates, including protection of vital organs and the environment and niches required for haematopoiesis, mechanical and structural support for muscles and joints, and a mineral reservoir that is essential for calcium homeostasis, and of growth factors stored in the matrix [Lacey *et al.*, 2012; Rouhi, 2012; Boyce and Xing, 2008; Marcus *et al.*, 2008].

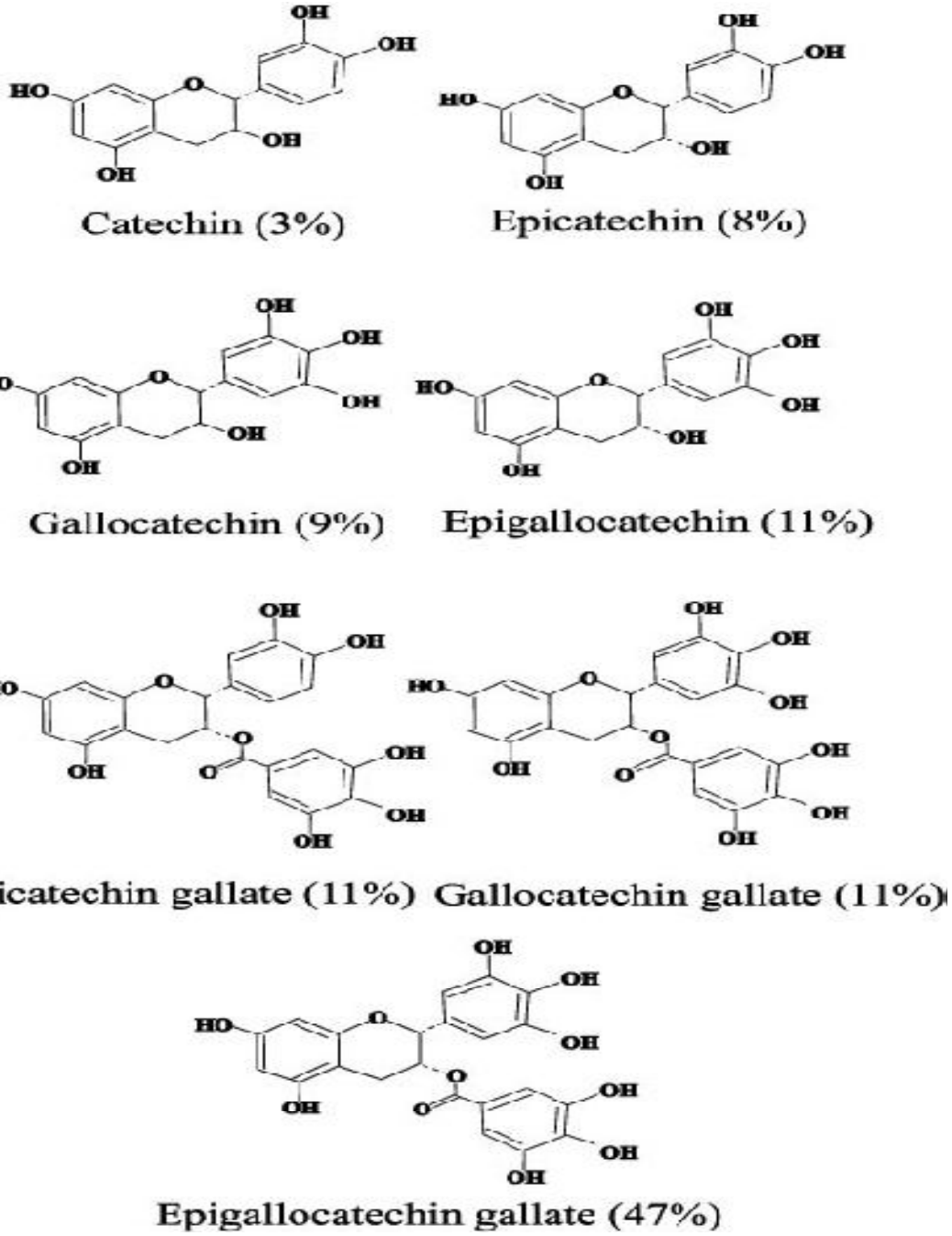
The structure-function relationships observed in bone under normal physiological conditions, coupled with its role in maintaining mineral homeostasis, strongly suggest that it is an organ of optimum structural design [Rouhi, 2012]. In maintaining the structure–function relationship, bone tissue is constantly being broken down and rebuilt in a process called remodeling [Rucci, 2008]. Bone has the potential to adapt its architecture, shape, and mechanical properties via a continuous process termed adaptation in response to altered loading conditions [Burr *et al.*, 2002; Forwood and Turner, 1995; Hsieh and Turner, 2001]. The regulation of bone mass in mammals is governed by a complex interplay between bone-forming cells termed osteoblasts (OBs) and bone-resorbing cells termed osteoclasts (OCs), and is guided physiologically by a diverse set of hormones, cytokines and growth factors. The balance between these processes changes over time, causing an elevated risk of fractures with age [Lacey *et al.*, 2012]. With age, remodeling tends to result in a negative bone balance due to decrease in the number of osteoblasts, in that at each remodeling site slightly less bone is deposited than is resorbed. This negative balance leads to osteopenia and osteoporosis (OP), thus predisposing the bone to fracture during even minimal trauma [D'ippolito *et al.*, 1999; Marcus *et al.*, 2008].

Tea is the most consumed beverage worldwide next to water. Tea is a flavonoid-rich beverage and contributes substantially to the intake of dietary catechins. Tea also contains some flavonols, particularly quercetin and kaempferol [McKay and Blumberg, 2007]. About 20% of the consumed tea is green tea, which is primarily consumed in China and Japan and contains mostly nonoxidized Polyphenols, more particularly catechins [Graham, 1992; Mukhtar and Ahmad, 2000]. After harvesting, the leaves of the bush are steamed and dried to produce green tea leaves. In green tea leaves, the following polyphenolic compounds are found: (-)-epigallocatechin-3-gallate (EGCG), a major component; (-)-epicatechin (EC); (-)-epigallocatechin (EGC) and (-)-epicatechin-3-gallate (ECG).

Epigallocatechin gallate (EGCG) is also known as epigallocatechin-3-gallate. It is the ester of Epigallocatechin and Gallic acid, and is a type of catechin. EGCG is the most abundant catechin in tea and is a potent antioxidant that may have therapeutic applications in the treatment of many disorders. EGCG accounts for more than 50% of the total of catechins [Nagal *et al.*, 2006]. 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].

EGCG has the physiological role in the induction of osteoclast cell death [Nakagawa *et al.*, 2002; Yun *et al.*, 2007] and mineralization of osteoblasts [Takita *et al.*, 2002; Vali *et al.*, 2007]. EGCG is found to reduce the generation of TRAP positive multinucleated cells, bone resorption activity, and osteoclast-specific gene expression without affecting cell viability [Morinobu *et al.*, 2008]. EGCG down-regulated expression of nuclear factor of activated T cells c1 (NF-ATc1), but not of NF-κB, c-Fos, and c-Jun, suggesting that down regulation of NF-ATc1 is one of the molecular bases of EGCG action [Morinobu *et al.*, 2008]. EGCG is found to suppress osteoclast differentiation and ameliorated experimental arthritis in mice over the short term [Morinobu *et al.*, 2008]. The inhibitory effect of EGCG to osteoclastogenesis has been reported to be associated with a down regulation of

RANKL/RANK signal, and increased apoptosis of preosteoclasts [Zhao *et al*., 2014]. Mah *et al* has reported 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].



Molecular structures of *C. sinenesis* polyphenols. Green tea extract contains 85% polyphenols by weight. Composition of polyphenols in green tea extract used is shown as % total polyphenols (Zhong *et al*., 2003).

Most of the osteoclast differentiation studies have been done mostly on cell lines or dentine slices and less on human PBMCs from osteoporosis patients. Thus, we have carried out preliminary study to check whether natural antioxidant EGCG causes osteoclast differentiation or not in *in vitro* cultures of human PBMCs from osteoporosis patients.

MATERIAL AND METHODS

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)**.**

Culture Conditions for Osteoclastogenesis

The isolated PBMCs were centrifuged at 400g for 30 minutes at 21°C. Thereafter, the buffy layer was removed and washed twice in PBS. These isolated mononuclear cells were plated at a density of $2x10^6$ cells/cm^2 in 10 cm Petri dishes in α-MEM culture medium supplemented with 10% FCS, 100U/ml penicillin, 100 µg/ml streptomycin, 50ng/ml M-CSF and 25ng/ml RANKL (Osteoclastogenic medium) at 37°C in a humidified 5% CO_2 atmosphere. The cells were then cultured for 24 hr, and the non-adherent cell fraction was subsequntly 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 5 days and the cells were subjected to phenotypic characterization by TRAP staining and were identified as committed multinucleated preosteoclasts (pOCs) [Susa *et al.*, 2004].

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

TRAP staining of adherent cultures was done with a kit from Sigma on a 96-well culture plate, exactly according to manufacturer's instruction. The stained cells developed red color of different intensity. The number of TRAP-positive multinucleated (>2 nuclei per cell) preosteoclast 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.

Effect of Epigallocatechin gallate (EGCG) on the generation of Osteoclasts from PBMCs

To investigate the effect of natural antioxidants like epigallocatechin-3-gallate (EGCG) on osteoclast generation from PBMCs, 15 µg/ml of EGCG was added to the PBMC cultures seeded at a cell density of $2x10^5$ cells/cm^2 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% CO_2. Thereafter, the cells were analysed with TRAP staining.

RESULTS

Generation of Human Osteoclast Precursors from Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were used directly for the generation of osteoclast precursors after centrifugation with Ficoll-Hypaque. The

results show that after the 3 day culture duration in osteoclastogenic medium (α-MEM culture medium supplemented with 10% FCS, 100U/ml penicillin, 100 µg/ml streptomycin, 50ng/ml M-CSF and 25ng/ml RANKL), multinucleated osteoclast precursors begin to appear and the number increased after the 5 day of culture which were characterized by Tartrate Resistant Acid Phosphatase (TRAP) staining (Fig. 1, 2, 3, 4, 5, 6). It may be pointed out that there was no appearance of osteoclast precursors after 24 h (1 day) of culture (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 (shown in Fig. 1, 2, 3, 4, 5, 6), were counted by TRAP staining. Interestingly, we observed an individual variation in osteoclast generation from different donors as depicted by different number of multinucleated cells in Fig 1,2, 3.

Effect of Natural Antioxidant, Epigallocatechin gallate (EGCG), on the Generation of Human Osteoclasts

Interestingly, we observed that co-culturing of PBMCs with EGCG (15 µg/ml) in osteoclastogenic medium for culture duration of 5 days leads to appreciable amount of reduction in appearance of multinucleated osteoclast precursors (Fig. 1, 2, 3, 4, 5, 6). Hence, this data gives an idea of potential of EGCG to exert regulatory effect in osteoclast generation and differentiation. More deep studies are underway in our laboratory and would be communicated elsewhere.

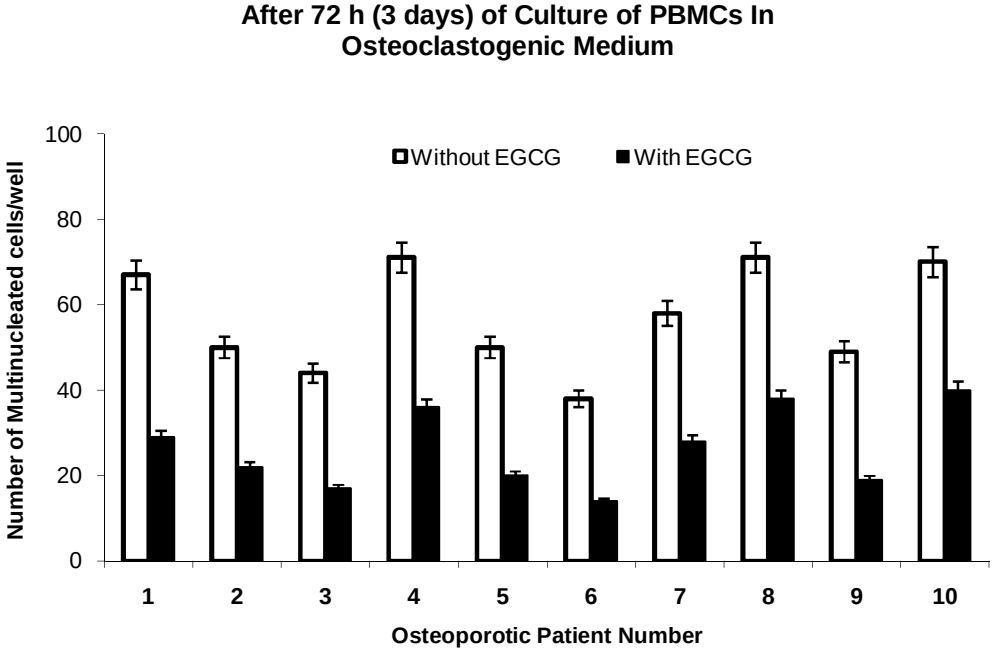


Fig. 1 Patient No. 1 to 10: Individual variation in osteoclast generation from different donors. Human osteoclasts were generated from peripheral blood mononuclear cells (PBMCs) as described under Methodology. The results from 10 patient donors are presented as quantification of TRAP-positive multinucleated cell number. The effect of EGCG (15 µg/ml) is shown in black bars. The results are shown as means ± SEM.

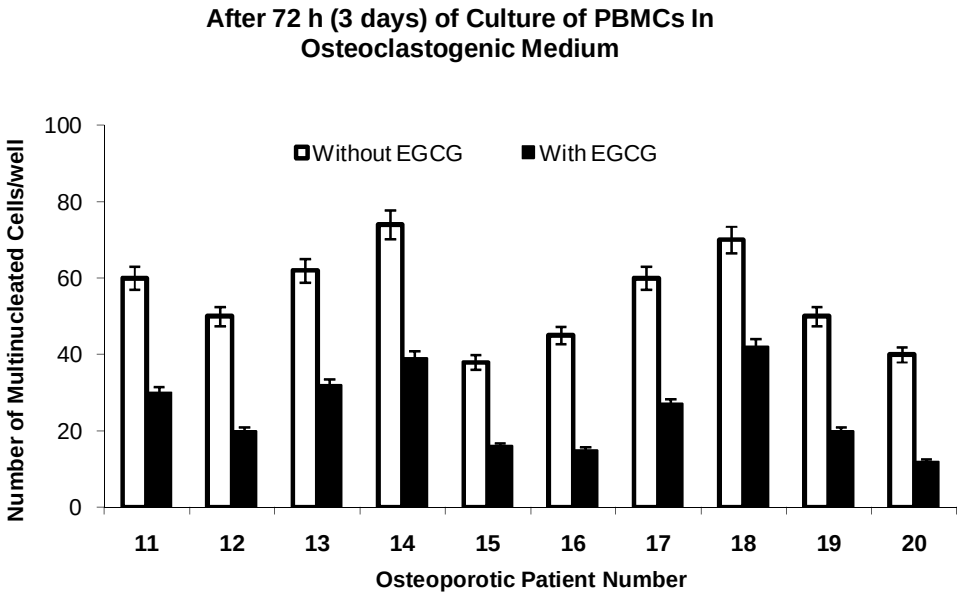


Fig. 2 Patient No. 11 to 20: Individual variation in osteoclast generation from different donors. Human osteoclasts were generated from peripheral blood mononuclear cells (PBMCs) as described under Methodology. The results from 10 patient donors are presented as quantification of TRAP-positive multinucleated cell number. The effect of EGCG (15 μg/ml) is shown in black bars. The results are shown as means ± SEM.

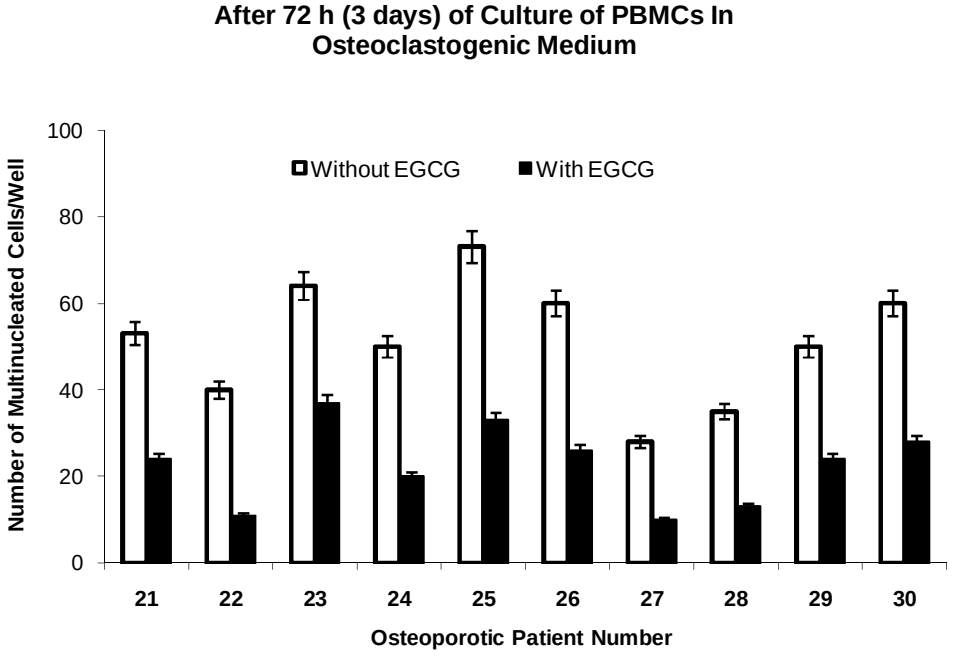


Fig. 3 Patient No. 21 to 30: Individual variation in osteoclast generation from different donors. Human osteoclasts were generated from peripheral blood mononuclear cells (PBMCs) as described under Methodology. The results from 10 patient donors are presented as quantification of TRAP-positive multinucleated cell number. The effect of EGCG (15 μg/ml) is shown in black bars. The results are shown as means ± SEM.

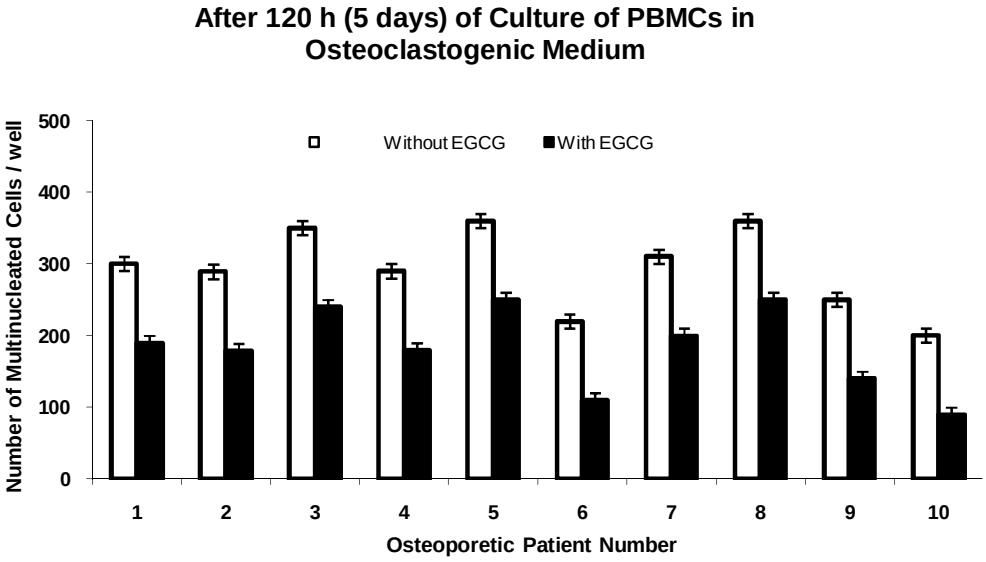


Fig. 4 Patient No. 1 to 10: Individual variation in osteoclast generation from different donors. Human osteoclasts were generated from peripheral blood mononuclear cells (PBMCs) as described under Methodology. The results from 10 patient donors are presented as quantification of TRAP-positive multinucleated cell number. The effect of EGCG (15 μg/ml) is shown in black bars. The results are shown as means ± SEM.

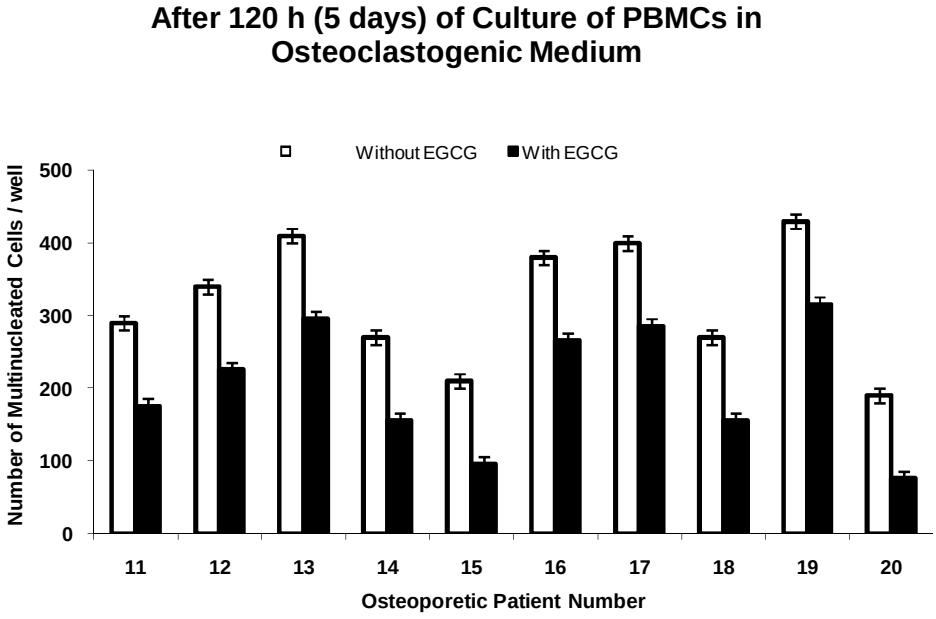


Fig. 5 Patient No. 11 to 20: Individual variation in osteoclast generation from different donors. Human osteoclasts were generated from peripheral blood mononuclear cells (PBMCs) as described under Methodology. The results from 10 patient donors are presented as quantification of TRAP-positive multinucleated cell number. The effect of EGCG (15 μg/ml) is shown in black bars. The results are shown as means ± SEM.

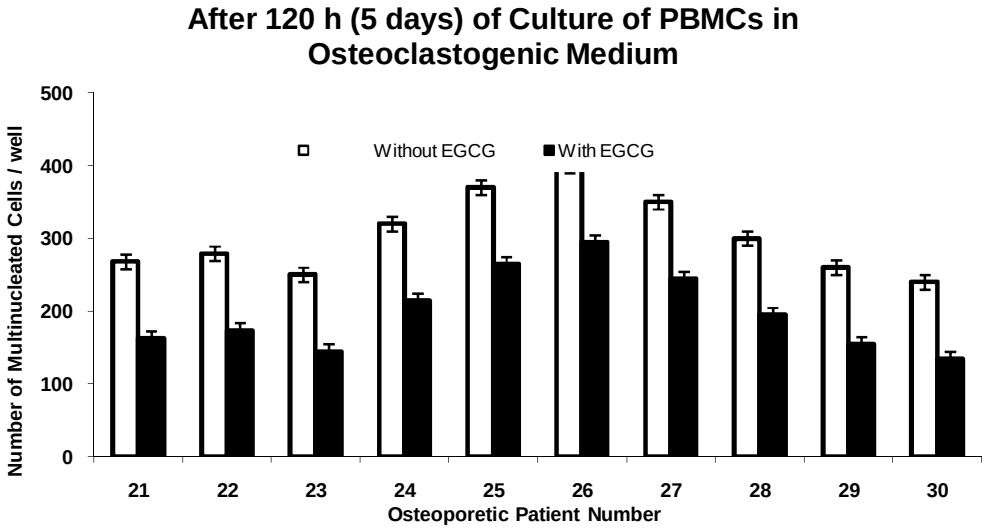


Fig. 6 Patient No. 21 to 30: Individual variation in osteoclast generation from different donors. Human osteoclasts were generated from peripheral blood mononuclear cells (PBMCs) as described under Methodology. The results from 10 patient donors are presented as quantification of TRAP-positive multinucleated cell number. The effect of EGCG (15 μg/ml) is shown in black bars. The results are shown as means ± SEM.

DISCUSSION

The global prevalence of osteoporosis is probably as old as the origin of human civilization, but unfortunately till date, complete understanding about the management of osteoporosis in humans still remains poorly understood. Accelerated human osteoclast differentiation is responsible for most chronic conditions that lead to loss of bone mass, including osteoporosis and arthritis etc.

It is well recognized that hormonal deficiency (estrogen) is a major contributory factor to postmenopausal osteoporosis. However, in non-postmenopausal osteoporosis, various intrinsic factors and not hormonal deficiency contribute to osteoporosis. Bone molecular biology and physiology research, which leads to improved bone health of men and women, will reduce the risk of osteoporosis and other bone disorders later in life. 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 leading to accelerated osteoclast differentiation, thereby resulting to loss of bone mass/ osteoporosis.

Thus, if ROS production in vivo is regulated by natural antioxidants like EGCG from green tea then the bone markers associated with chronic bone conditions may probably be easily regulated. We have previously shown the effect of EGCG in other diseases [Fatima *et al,* 2012; Islam *et al*, 2000].

Several studies have reported increased production of TNF-α 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 [Faust *et al*, 1999; Buckley *et al*, 2005].

In this preliminary study, we have successfully proved here the EGCG-induced suppression of multinucleated osteoclasts in PBMC's from patients with osteoporosis that were cultured under

osteoclastogenic medium. Probing the adjuncts for down regulation of augmented osteoclasts probably due to ROS in patients with osteoporosis would definitely help in better understanding the pathogenesis of osteoporosis.

Thus, it is hoped that the present study could be of immense help in the better understanding of osteoporosis/ postmenopausal osteoporosis management.

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