Study on the Role of Natural Antioxidants in the Regulation of Osteoporosis

ABSTRACT

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Osteoporosis is as old as the origin of human civilization yet complete understanding about it remains to be achieved till date. Osteoporosis and associated fractures constitute a major public health issue together representing an important cause of mortality and morbidity with annual incidence of fracture rates exceeding the combined incidence of breast cancer, stroke and heart attacks in postmenopausal women (PMW). Emergence of osteoporosis as a global bone health concern is reflected in the occurrence of 1.6 million hip fractures annually worldwide and the number projected to go up four times by 2050. Though osteoporosis affects both men and women, the later are more susceptible targets of this crippling disorder of bone.

Osteoporosis has been recognized as that infection, inflammation, and autoimmune disorders are associated with systemic and local bone loss. However, it is only recent that T lymphocytes and their product have been recognized as key regulators of bone cell formation, lifespan and activity. Of many proinflammatory cytokines activated in osteoporosis, TNF-α is probably the dominant cytokine, which promotes osteoclastogenesis by augmenting the production of RANKL, the non-redundant cytokine responsible for osteoclast development. This ability of TNF-α is due to synergistic interactions at the level of NF-κB and activator protein-1 (AP-1) signaling. Furthermore, TNF stimulates osteoclast (OC) activity and inhibits osteoblastogenesis thus further driving an imbalance between bone formation and bone resorption. With ever increasing number of elderly people in the population, improved survival and an increase in the age-specific fracture rates, there is an immediate need to develop new cost-effective therapeutic strategies against osteoporosis. Thus, in the present study allicin from garlic and EGCG from green tea were employed in order to investigate the above.

Allicin, from garlic is the major biologically active thiosulfinate compound of freshly crushed garlic. It has been reported to possess potent antibacterial properties besides showing broad range antimicrobial and antifungal activities. Besides, allicin has radical scavenging properties in activated granulocytes and may also inhibit iNOS expression in activated macrophages. EGCG-a green tea polyphenol, is one of the most versatile medicinal plants having a wide spectrum of biological activity. Both allicin and EGCG have been reported to have multiple properties some of which
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include anti-inflammatory, immunostimulatory, anti-malarial, anti-mycobacterial, antipyretic, anticarcinogenic, anti-oxidant and analgesic activities.

In view of reactive oxygen species (ROS) and reactive nitrogen species (RNS) playing an important role in both estrogen related and unrelated osteoporosis, thus, greater emphasis now being laid on development of compounds from natural sources having antioxidant and anti-inflammatory properties in combating osteoporosis. Earlier work in our laboratory has proved the antioxidant and anti-inflammatory effects of allicin from garlic in other diseases. Thus, we studied the incorporation of such compounds like allicin from garlic, and EGCG- a green tea polyphenol as well as a natural antioxidant in the treatment for bone loss.

The initial phase of the present study undertook a detailed characterization of sera and monocytes of osteoporosis patients to assess the activity of predominant antioxidant enzyme, GPx and to measure the intramonoocyte (GSH) levels. Glutathione (GSH) directly reacts with ROS and glutathione peroxidase (GPx) catalyses the removal of hydrogen peroxide. ELISA results showed almost less than half the activity of enzyme both in sera and monocytes of osteoporosis patients as compared to healthy individuals. Decrease in GPx activity therefore, indicates impairment of hydrogen peroxide-neutralizing mechanism. A similar suppression in intramonoocyte GSH levels was observed in these patients indicative of weak antioxidant power. The presence of elevated free radicals in osteoporosis patients was also substantiated by MDA determination.

Next, the levels of malondialdehyde (MDA), a by-product of lipid peroxidation were observed to be appreciably high both in sera and monocyte cultures of osteoporosis patients further pointing to the increased oxidant stress in such patients. Subsequent increase in hydrogen peroxide levels due to depressed GSH levels and GPx activity in osteoporosis patients might have induced the peroxidation of polyunsaturated fatty acids, thereby leading to the formation of high MDA levels. Moreover, due to high reactivity of MDA towards amino groups may also lead to deactivation of enzymes. Allicin and EGCG dose-dependently down-regulated the excess levels of MDA in monocyte cultures of osteoporosis patients.
Multiple evidences suggest that estrogen prevents bone loss by blocking the production of proinflammatory cytokines by bone marrow stromal and bone cells. Cytokines regulated by estrogen are IL-1, IL-6 and TNF α. In our study also, probe by ELISA showed an appreciable secretion of TNF-α and IL-1β in the sera and monocytes of osteoporosis patients. Infact, IL-1 is a potent stimulator of bone resorption together with TNF, and both are well-recognized inhibitors of bone formation. IL-1 and TNF are also powerful inducers of other cytokines such as IL-6, M-CSF and GM-CSF, which potentiate the effect of IL-1 on osteoclastogenesis.

Next, an attempt was also made to characterize monocytes from osteoporosis patients with respect to TNF-α and osteoprotegrin (OPG). Monocytes from these patients were subjected to TNF-α and OPG mRNA evaluation by real-time RT-PCR revealing appreciably high basal levels of both mRNA expressions.

The role of OPG in the pathogenesis of osteoporosis has not been clear since OPG levels are not consistently altered. In humans, OPG levels increasing with age are understood as a homeostatic response to limit the bone loss that occurs with an increase in other bone resorbing factors. Thus, in the present study, we attempted to probe the expression level of OPG and RANKL together with TNF α and their regulation by natural antioxidants like allicin from garlic and EGCG from green tea in osteoporosis.

In order to probe the above, in the present study, a real-time RT-PCR was carried out, where the data exhibited that both allicin and EGCG down-regulated the expression of TNF-α and OPG mRNA in PBMC's of osteoporosis patients in a dose-dependent manner. Concentrations of 250 and 500 ng/ml of allicin and that of 15 μg/ml and 20 μg/ml of EGCG respectively were found to suppress the appreciably high basal levels of TNF and OPG mRNA's by an appreciable degree. It is to be pointed out that previous reports indicated higher doses of allicin were toxic to human cells. Similarly, previous reports show that concentrations of EGCG above 23 μg/ml induced apoptosis in mononuclear cells. However, our laboratory has shown lower doses of allicin (0-500 ng/ml) as well as EGCG (0-20 μg/ml) to be non-toxic and proved to be potent anti-inflammatory agent in other disease conditions such as tuberculosis.
our data, it is evident that whereas low concentrations of allicin and EGCG used in this study appreciably down-regulate the mRNA expression of TNF α and OPG, at the same time show no significant effect on the expression of human housekeeping gene R18. Thus, low dose of allicin and EGCG used in the study was non-toxic.

Although OPG is produced in many tissues, bone derived OPG may be released into circulation, thereby reflecting the situation locally in bone. Our results showing increased OPG mRNA expression in the osteoporosis patient PBMC’s therefore may indicate a compensatory response to increased osteoclastic bone resorption and the resultant bone loss caused by estrogen deficiency; this considering the fact that OPG has bone sparing activity. The expression of OPG mRNA correlated positively with that of TNF α mRNA expression. Therefore, OPG, a soluble member of TNFR superfamily of proteins plays an important role in the negative regulation of osteoclastic bone resorption.

Next, as RANKL is necessary for osteoclast differentiation, thus the levels of RANKL in monocyte cultures of osteoporosis patients in comparison to healthy controls was also probed. Our results showed that in comparison to healthy controls, the patient’s cultures exhibited around 9-fold augmented levels of RANKL. Thereafter, dose response effects of allicin and EGCG showed interesting results. In case of osteoporosis patients, sRANKL secretion dose-dependently decreased from 30.68 pg/ml at through 25.36, 18.12, 9.56 and 4.01 pg/ml with 50, 100, 250 and 500 ng/ml allicin respectively (P<0.001). Computation of the data revealed that allicin down regulated / suppressed the secretion of sRANKL by around 17.34%, 40.93%, 68.83% and 86.92% with 50, 100, 250 and 500 ng/ml allicin respectively. The IC₅₀ was computed out to be in between 100-125 ng/ml. Similarly, in case of osteoporosis patients, sRANKL secretion was found to dose-dependently decrease from 32.65 pg/ml at through 27.81, 22.45, 17.29, 13.07 and 9.44 pg/ml with 2, 5, 10, 15 and 20 μg/ml EGCG respectively. Computational analysis of the data revealed that EGCG down regulated / suppressed the secretion of sRANKL by around 14.82%, 31.24%, 47.04%, 59.96% and 71.08% with 2, 5, 10, 15 and 20 μg/ml EGCG respectively. The IC₅₀ was computed out to be in between 7.5 – 10 μg/ml of EGCG.
TRAP assay data revealed healthy control monocyte cultures did not show any multinucleated cells/osteoclasts, whereas those from osteoporosis patient exhibited appreciable number of multinucleated cells/osteoclasts. Interestingly, monocyte cultures of 5 days for osteoporosis patient that were co-cultured with 2 ng/ml of TNF-alpha exhibited an enhanced number of multinucleated cells/osteoclasts, when compared to cultures devoid of any TNF-alpha. Furthermore, in monocyte cultures of osteoporosis patient, when co-cultured with 500 ng/ml of allicin and 20 μg/ml of EGCG respectively for 5 days, the TRAP assay data showed that both 500 ng/ml allicin as well as 20 μg/ml EGCG, inhibited / suppressed / down-regulated the formation of multinucleated cells/osteoclasts. The suppression was clearly more in cultures receiving allicin in comparison to EGCG.

Next, secretion of cytokine IL-1β and generation of ROS, as reflected by suppressed GPx activity and GSH levels in osteoporosis patients, were mediated through activation of NFκB as evidenced by their suppression in monocyte cultures in the presence of SN50, an inhibitor of NFκB while SN50/M, an inactive analogue of SN50, failed to show any such effect. Furthermore, NAC, a precursor of in vivo antioxidant glutathione, caused suppression of IL-1β secretion and exhibited enhancement / amelioration of intramonoocyte GSH levels. Interestingly, allicin and EGCG showed a higher degree of inhibition of cytokine secretion and enhanced antioxidant effect in comparison to NAC, thereby, proving them as effective natural herbal antagonists of pathogenesis of osteoporosis.

Since higher doses of both allicin and EGCG have previously proven to be toxic by various investigators, our present study employed lower concentrations (0-500 ng/ml) of Allicin as well as that of EGCG (0-20 μg/ml), both of which in their respective range of doses, failed to show any toxic effect on human monocytes as revealed by MTT assay. Also no effect was observed on human housekeeping gene R18 as revealed by real-time RT-PCR, thereby indicating that allicin and EGCG did not non-specifically affect TNF-α and OPG transcription in patient monocytes nor cause cellular death.

Apart from the above, the present study also revealed both allicin and EGCG up-regulated the activity of GPx in the monocyte cultures of osteoporosis patients in a
dose-dependent manner. The results indicate an appreciable degree of amelioration in GPx activity at 500 ng/ml allicin and at 20 μg/ml EGCG. Significant amelioration of GPx activity in osteoporosis patient monocyte cultures indicates reversal of impaired neutralizing mechanisms. Similarly, allicin and EGCG were also found to appreciably up-regulate the intramonocyte GSH levels in these patients in a dose-dependent manner. Interestingly, in comparison to NAC and SN50, allicin and EGCG, both exerted more efficient restoration of decreased antioxidant power indicating these compounds to be effective herbal antioxidants combating ROS, generated as a consequence of excess cellular activation in monocytes of osteoporosis patients.

Next, it’s well established that the Intracellular signaling pathways, especially NFκB, are known to be ROS sensitive. Our results indicate that the increased secretion of TNF-α, sRANKL and IL-1β at protein levels as well as TNF-α and OPG at the gene i.e. mRNA levels, and excess ROS generation as mirrored by decreased GPx activity and GSH levels, are interconnected and involve cellular activation of NFκB. This fact is evidenced by the suppression of TNF-α, OPG and IL-1β expressions at the protein and gene levels and up-regulation of GSH levels in the presence of SN50, an inhibitor of NFκB. SN50/M, an inactive analogue of SN50, failed to show any such effect. In view of it, our data demonstrate that this effect involved inhibition of NFκB pathway induced by allicin and EGCG probably by inhibiting the degradation of IκBα. Since a number of genes involved in inflammatory responses are regulated by NFκB pathway, thus a high magnitude down-regulation of this pathway by allicin and EGCG would predictably reduce the elaboration of NFκB-mediated TNF-α, sRANKL, OPG and IL-1β expressions and generation of ROS.

In conclusion, the presence of high levels of proinflammatory cytokines such as IL-1β, TNF α, sRANKL and a related TNFR super family member OPG together with antioxidant deficiency in terms of both low GPx activity and GSH levels and a simultaneous increase in MDA levels add to the severity of estrogen deficiency bone loss. Our study shows appreciable reversal of impaired neutralizing mechanisms by allicin and EGCG which correlates inversely with the down-regulation of TNF α, sRANKL, IL-1β, and OPG expressions in monocytes of osteoporotic patients. Thus, allicin and EGCG may prove to be valuable natural antioxidant and anti-inflammatory
agents in the management of osteoporosis and, therefore, be useful adjuncts in the treatment of bone loss. These observations strengthen the idea that allicin and EGCG should be tested in in vivo models to evaluate their therapeutic potential in the pathogenesis of osteoporosis.