ABSTRACT

Osteoporosis (OP) is a chronic, progressive disease of the skeleton characterized by bone fragility due to a reduction in bone mass and possible alterations in bone architecture which lead to a propensity for fractures with minimum trauma. Bone formation is related to osteoblastic proliferation, alkaline phosphatase (ALP) activity, and osteocalcin and collagen synthesis. Bone resorption is associated with osteoclast formation and differentiation, and tartrate-resistant acid phosphatase activity (TRAP). It’s a well established fact that 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 2050-2055. Osteoporosis affects both men and women; however the later are more susceptible targets of this crippling disorder of bone.

Oxidative stress is caused by an imbalance between the generation of reactive oxygen species (ROS) and the activity of antioxidant defense. Severe oxidative stress has been implicated in many chronic and degenerative diseases, including osteoporosis, cancer, ageing, and neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease and amyotrophic lateral sclerosis. Several medications such as calcitonin (CT), calcium products, estrogen, bisphosphonates, ipriflavone and anabolic steroids have been reported to be effective for curing osteoporosis. However, these medications may have serious side effects, may not improve bone quality, or may not reduce susceptibility to fracture. Recently, oriental traditional medicines have been re-evaluated by clinicians because these medicines have fewer side effects and are more suitable for long-term use compared with chemically synthesized medicines. Some natural flavonoids with potent antioxidant activity including scopoletin, resveratrol, and baicalein have found to exert anti-osteoporotic activities through suppressing osteoclast formation and TRAP. Accordingly, natural antioxidants have
become a topic of increasing interest as they could provide a safe, economical and
valuable tool in combating such diseases.

It has been well recognized that infection and inflammation as well as
autoimmune diseases are associated with systemic and local bone loss. Recent studies
show that that T lymphocytes and their product have also been recognized as key
regulators of bone cell formation, lifespan and activity. Numerous proinflammatory
cytokines are reported to be activated in osteoporosis. However, the autocrine
cytokine namely TNF-α is probably the most dominant cytokine, which promotes
osteoclastogenesis by augmenting the production of RANKL, the non-redundant
cytokine responsible for osteoclast development. Such a capability of TNF-α is solely
due to synergistic interactions at the level of NF-κB and activator protein-1 (AP-1)
signaling. In addition to the above, 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, resveratrol, curcumin as well as allicin from
garlic were employed as valuable natural antioxidants / natural tools 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.

In view of reactive oxygen species (ROS) and reactive nitrogen species (RNI)
playing an important role in both estrogens 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. Allicin, resveratrol and curcumin are
one of the most versatile medicinal compounds / natural antioxidants having a wide
Abstract

spectrum of biological activity, where allicin have been reported to have multiple properties some of which include anti-inflammatory, Immunostimulatory, antimalarial, anti-mycobacterial, antipyretic, anti-carcinogenic, anti-oxidant and analgesic activities. In view of the above, thus, we studied the incorporation of such natural antioxidants / compounds in the management for bone loss.

In the first phase of the present study, an attempt was made to carry out a detailed characterization of sera and monocytes of osteoporosis patients in order to assess the activity of predominant antioxidant enzyme, GPx as well as to measure the intramonicyte (GSH) levels. Glutathione (GSH) directly reacts with ROS, and glutathione peroxidase (GPx) catalyses the removal of hydrogen peroxide. Our ELISA results exhibited nearly less than half the activity of enzyme both in sera and monocytes of osteoporosis patients as compared to healthy individuals. Decrease in GPx activity indicates impairment of hydrogen peroxide-neutralizing mechanism. Similarly, suppression in intramonicyte GSH levels was also observed in these patients, which in turn, were indicative of weak antioxidant power. Furthermore, elevated free radicals in osteoporosis patients were also substantiated by MDA determination.

Thereafter, further study showed that the levels of by-product of lipid peroxidation namely malondialdehyde (MDA), to be appreciably high both in sera and monocyte cultures of osteoporosis patients, thereby, further pointing to the augmented / 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, resveratrol and curcumin dose-dependently down-regulated the excess levels of MDA in monocyte cultures of osteoporosis patients.

A wide spectrum of studies show established evidences which suggest that estrogen prevents bone loss by blocking the production of proinflammatory cytokines by bone marrow stromal and bone cells. The most prominent cytokines that are regulated by estrogen are IL-1, IL-6 and TNF α. In the present study also, probe by
ELISA exhibited an appreciable secretion of TNF-α and IL-1β in the sera and monocytes of osteoporosis patients. Infect, 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.

Thereafter, monocytes from osteoporosis patients were also characterized with respect to TNF-α and osteoprotegerin (OPG), wherein monocytes from these patients evaluated by real-time RT-PCR revealed appreciably high basal levels of both TNF-α and OPG mRNA expressions in comparison to healthy controls.

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 the regulation of RANKL by natural antioxidants like allicin, resveratrol and curcumin 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 all the three natural antioxidants namely allicin, resveratrol and curcumin down-regulated the expression of TNF-α mRNA in PBMC’s of osteoporosis patients in a dose-dependent manner. Concentrations of 250 and 500 ngs/ml of allicin and that of 25 µg/ml of resveratrol and curcumin respectively were found to suppress the appreciably high basal levels of TNF 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. However, our laboratory has shown lower doses of allicin (0-500 ng/ml) to be non-toxic and proved to be potent anti-inflammatory agent in other disease conditions such as tuberculosis. From our data, it is evident that whereas low concentrations of allicin, resveratrol and curcumin used in this study appreciably down-regulate the mRNA expression of TNF α, at the same time show no significant effect on the expression of human housekeeping gene R18. Thus, low dose of allicin, resveratrol and curcumin used in the study was non-toxic.
Osteoprotegerin (OPG) is produced in many tissues, but bone derived OPG may be released into circulation, thereby reflecting the situation locally in bone. Our data exhibiting appreciably augmented 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. In the present study, the expression of OPG mRNA correlated positively with that of TNF α mRNA expression. Thus, OPG, a soluble member of TNFR super family of proteins plays an important role in the negative regulation of osteoclastic bone resorption.

In case of osteoporosis patients, sRANKL secretion dose-dependently decreased from 33.16 pg/ml at through 27.54, 20.33, 12.98 and 3.02 pg/ml with 50, 100, 250 and 500 ng allicin respectively. On the contrary, healthy controls exhibited in between 1.33 – 3.12 pg/ml of RANKL. Next, after dose response evaluation, an attempt was also made to re-check the data by co-culturing with the maximum dose of allicin (500 ng/ml) selected in the study, and that, similar results were observed to the one’s observed above in dose response experiments at the maximum dose. Computation of the data exhibited that allicin suppressed the secretion of sRANKL by around 16.94%, 38.69%, 60.85% and 90.89% with 50, 100, 250 and 500 ng allicin respectively. The IC$_{50}$ was computed out to be in between 100 -135 ng/ml.

Thereafter, monocytes of study groups were co-cultured separately with varying concentrations of resveratrol and curcumin (0, 2, 5, 10, 15 20 and 25 µg/ml). In case of osteoporosis patients, sRANKL secretion was found to dose-dependently decrease from 35.16 pg/ml at through 29.11, 24.26, 15.16, 11.33, 9.16 and 8.89 pg/ml with 0, 2, 5, 10, 15, 20 and 25 µg resveratrol respectively. On the contrary, healthy controls exhibited in between 1.3 – 3.2 pg/ml of RANKL.

Next, the effect of varying doses of curcumin on RANKL in 5 days monocyte cultures was also analyzed. Co-culturing of patient monocytes with curcumin exhibited a dose-dependent down-regulation of sRANKL, which was of the order of 32.65, 27.81, 22.45, 17.29, 13.07, 9.44 and 9.23 pg/ml with 0, 2, 5, 10, 15, 20 and 25 µg/ml of curcumin respectively. IC$_{50}$ was computed out to be ~ 9 µg/ml. Next, computational analysis of the data revealed that curcumin down regulated the
secretion of sRANKL by around 15%, 31%, 46%, 60%, 70% and 73% with 2, 5, 10, 15, 20 and 25 µg curcumin respectively.

Bone marker study involving TRAP assay data revealed that healthy control monocyte cultures did not show any multinucleated cells/osteoclasts, whereas those from osteoporosis patient exhibited appreciable number of multinucleated cells/osteoclasts. However, there was no/negligible appearance of osteoclast precursors after 24 hr (1 day) of culture. The number of multinucleated preosteoclasts, arising from PBMCs isolated from the blood of normal healthy individual (data not shown) and osteoporotic patients 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. Interestingly, we observed that co-culturing of PBMCs from osteoporosis patients with allicin, resveratrol and curcumin in osteoclastogenic medium for 3 days resulted in an appreciable amount of reduction in appearance of multinucleated osteoclast precursors. Hence, this reflects the potential of allicin, resveratrol and curcumin to exert regulatory effect in osteoclast generation and differentiation. The above doses of allicin, resveratrol and curcumin were selected after performing dose response experiment, where TRAP assay revealed a linear suppression in the formation of multinucleated cells was observed. The data in the present study shows nearly 20-30% suppression in appearance of multinucleated cells in cultures separately resveratrol (25 µg/ml) or curcumin (25 µg/ml) respectively receiving relative to control devoid of any supplement. Interestingly, around 35-40% suppression in appearance of multinucleated cells was observed in cultures receiving 500 ng/ml of allicin relative to control cultures devoid of any allicin.

Next, the presence of human osteopontin (OPN) was probed both in sera of healthy controls and osteoporosis patients, where healthy controls showed negligible levels of OPN (range: 2.07 ng/ml to 3.24 ng/ml), while sera of osteoporosis patients exhibited appreciable levels of OPN, which was in the range of 15.98 ng/ml to 28.35 ng/ml. Thus, in comparison to healthy controls, around an 8-fold OPN levels were recorded in osteoporosis patients. Also, in the present study also shows no or negligible levels of OPN in 24 hours culture supernatants of monocytes obtained from
Abstract

healthy controls. Around 12 fold levels of OPN was detected in osteoporotic patients in comparison to healthy controls.

Modulation study on monocytes from osteoporosis patients that were cultured for 3 days in osteoclastogenic medium with and without 1 ng/ml of calcitonin (CT), exhibited that those culture wells receiving CT exhibited an augmentation/increase by around 1.4-folds in OPN levels thereby substantiating that CT was a positive modulator of OPN. Next, upon comparison of the data, it was observed that allicin proved to be the most potent suppressor of OPN followed by resveratrol, and in turn, followed by curcumin. Thereafter, the effect of known negative modulator, namely Denosumab (Prolia) of OPN and osteoclasts was probed. The data in the present study show that monocyte cultures of osteoporosis patients receiving denosumab (Prolia) for 3 days in osteoclastogenic medium exhibited remarkably suppressed / down-regulated levels of OPN in comparison to cultures devoid of any supplements. Furthermore, on comparative analysis, it was observed that the degree of suppression/down-regulation of OPN was nearly similar/same with natural antioxidant allicin and monoclonal antibody namely denosumab (Prolia).

Thereafter, the cytokine IL-1β secretions as well as generation of ROS, as reflected by suppressed GPx activity and GSH levels in osteoporosis patients, were mediated through activation of NFκB. This fact was 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. Apart from the above, NAC, a precursor of in vivo antioxidant glutathione, exhibited down-regulation/suppression of IL-1β secretion and exhibited enhancement/amelioration of intramonomocyte GSH levels. Interestingly, allicin and resveratrol showed a higher degree of inhibition of cytokine secretion and enhanced antioxidant effect in comparison to NAC, thereby, proving them as effective natural antagonists of pathogenesis/management of osteoporosis.

Since higher doses of both allicin 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 resveratrol and curcumin (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, resveratrol and curcumin did not non-specifically affect TNF-α transcription in patient monocytes nor cause cellular death.

Furthermore, the present study also revealed all the three natural antioxidants namely, allicin, resveratrol and curcumin augmented / up-regulated / ameliorated the activity of GPx in the monocyte cultures of osteoporosis patients in a dose-dependent manner. Our data indicate an appreciable degree of amelioration in GPx activity at 500 ng/ml allicin and at 20 µg/ml resveratrol and curcumin. It’s an established fact that significant amelioration of GPx activity in osteoporosis patient monocyte cultures indicates reversal of impaired neutralizing mechanisms. Furthermore, allicin, resveratrol and curcumin were also found to appreciably augment / up-regulate the intramonocyte GSH levels in patients with osteoporosis in a dose-dependent manner. Interestingly, in comparison to NAC and SN50, allicin and resveratrol, both exerted more efficient restoration of decreased antioxidant power whereas curcumin exerting somewhat equal restoration of decreased antioxidant power as said above, thereby indicating that these compounds / natural antioxidants 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 and IL-1β expressions at the gene and protein levels and augmentation / up-regulation of GSH levels in the presence of SN50, an inhibitor of NFκB. SN50/M, an inactive analogue of SN50, did not show any such effect. In light of the above, our study demonstrates that this effect involved inhibition of NFκB pathway induced by allicin, resveratrol and curcumin, probably by inhibiting the degradation of IκBα. As a matter of fact, since numerous genes involved in inflammatory responses are regulated by NFκB pathway, thus a
high magnitude suppression / down-regulation of this pathway by allicin, resveratrol and curcumin would predictably minimize / reduce the elaboration of NFκB-mediated OPN, TNF-α, sRANKL, 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 as well as OPN 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 resveratrol, allicin and curcumin which correlates inversely with the down-regulation of OPN, TNF α, sRANKL and IL-1β expressions in monocytes of osteoporotic patients. Thus, allicin, followed by resveratrol followed by curcumin, probably 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.

Therefore, these observations strengthen the idea that these natural antioxidants namely allicin, resveratrol and curcumin can be subjected to in-depth investigation in in vivo models to evaluate their therapeutic potential in the pathogenesis / management of osteoporosis.