Chapter – 4

DISCUSSION
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Osteoporosis 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 [Kang et al., 2014]. Bone formation is related to osteoblastic proliferation, ALP activity, and osteocalcin and collage synthesis. Bone resorption is associated with osteoclast formation and differentiation, and TRAP [Kang et al., 2014]. 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 [Kang et al., 2014]. Several medications such as CT, calcium products, estrogen, bisphosphonates, ipriflavone and anabolic steroids have been reported to be effective for curing osteoporosis [Kang et al., 2014 and references within]. However, these medications may have serious side effects, may not improve bone quality, or may not reduce susceptibility to fracture [Kang et al., 2014 and references within]. 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 [Kang et al., 2014 and references within]. 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 [Kang et al., 2014]. Accordingly, natural antioxidants have become a topic of increasing interest as they could provide a safe, economical and valuable tool in combating such diseases.

Low bone mass and micro architectural deterioration of bone tissue, increasing susceptibility to fracture is one of the main characteristics of osteoporosis [Heinemann, 2000]. Although osteoporosis is often described as a silent disease because it is typically asymptomatic until a fracture occurs, the disease negatively and significantly impacts morbidity and mortality as it can lead to severe pain, deformity, disability, and death [Heinemann, 2000; Salkeld et. al., 2000]. Osteoporosis is an
under recognized disorder in men whereas a highly recognized and predominant phenomenon in post menopausal women [Ebeling, 2008; Adler, 2011; Burge et al., 2007]. When compared to females, reports show that males often develop fractures around a decade later in life [Adler, 2011] and that, are less likely to survive with a high mortality rate after hip fracture [Bass et al., 2007; Jiang et al., 2005]. Besides from bone fractures because osteoporosis, a variety of bone parameters across different types of hip osteoarthritis and their relationship to osteoporotic fracture risk have also been widely studied [Castaño-Betancourt et al., 2013].

TNF-α - which belongs to class of inflammatory autocrine cytokine, have been shown to inhibit osteogenic differentiation of MSCs and bone formation in estrogen deficiency-induced osteoporosis. Unfortunately, till date, the precise or exact mechanism responsible for it remains poorly understood. Furthermore, reports indicate that microRNAs (miRNAs) have been shown to regulate MSC differentiation [Yang et al., 2013. Next, in an another other experiment carried out recently by Yang et al., 2013, has identified a novel mechanism whereby TNF-α, suppressing the functional axis of a key miRNA (miR-21) contributes to estrogen deficiency-induced osteoporosis. Moreover, in the same study, workers have reportedly screened differentially expressed miRNAs in MSCs derived from estrogen deficiency-induced osteoporosis and found miR-21 was significantly down regulated. miR-21 was suppressed by TNF-α during the osteogenesis of MSCs. Furthermore, miR-21 was confirmed to promote the osteoblast differentiation of MSCs by repressing Spry1, which can negatively regulate the osteogenic differentiation of MSCs. Up regulating miR-21 partially rescued TNF-α-impaired osteogenesis of MSCs. Whereas, it was observed that blocking of TNF-α ameliorated the inflammatory environment and significantly enhanced bone formation with increased miR-21 expression and suppressed Spry1 expression in ovariectomized (OVX) mice. Also, their results revealed a novel function for miR-21 and suggested that suppressed miR-21 may contribute to impaired bone formation by elevated TNF-α in estrogen deficiency-induced osteoporosis [Yang et al., 2013]. A wide spectrum of studies have reported increased production of TNF by cultures of mononuclear cells derived from osteoporosis patients, an effect reversed by estrogen replacement [Shevde et al., 2000].
ROS may play a role in bone loss in osteoporosis patients by generating a more oxidized bone microenvironment [Almeida and O’Brien, 2013]. 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 OPN and CT 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, orthopedic implant loosening, etc. Thus, if ROS production in vivo is regulated by natural antioxidants, then the above-mentioned bone markers associated with chronic bone conditions may be easily regulated.

Therefore, in view of the above, we probed here the management and/or regulation of ROS and TNF-α activation in osteoporosis patient monocytes by employing various natural antioxidants like allicin from garlic, resveratrol, curcumin and EGCG from green tea. The mechanisms of cellular activation as well as TNF-α and ROS enhancement would definitely help in better understanding the pathogenesis of osteoporosis.

It has been well established that the autocrine cytokine TNF-α is implicated in the pathophysiology of bone metabolism, where 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 women as well as osteoporosis patient is well documented [Pacifici, 2010]. Interestingly, ROS are also involved in the etiopathology and progression of osteoporosis as reported earlier, and that, radicals generated in cells of osteoporosis patient stimulate TNF-α, causing accelerated bone loss.

In the present study, we employed various natural antioxidants like allicin from garlic as well as resveratrol and curcumin and also to a lesser extent, EGCG from green tea, whose exact mechanism underlying their antioxidant activity still remains poorly understood. Allicin-induced enhancement of GPx activity has been
To the best of our understanding, we show for the first time that allicin, resveratrol and curcumin exerts potent anti-inflammatory and free radicals scavenging effects on osteoporosis patient mononuclear cells.

Doses of allicin higher than 100 µM and those of EGCG higher than 23 µg/ml have proven to be toxic [Ankri et al., 1997; Hasan et al., 2006; Kawai et al., 2005], thus, doses below the above mentioned doses were selected in the present study. Therefore, prior to all investigations, cell viability and potential cytotoxicity were determined for the doses employed in this study using trypan blue and MTT assay where viability of ~98–99% was observed with the lower doses selected in the present study. Moreover, our laboratory has previously demonstrated that the doses employed in the present study for allicin or EGCG had no effect on human housekeeping genes namely R18, thereby demonstrating that the effect of allicin or EGCG, was not mediated by cellular death, but rather by specific inhibition of expression and secretion of pro-inflammatory molecules [Hasan et al., 2006; Singh et al., 2002; Fatima et al., 2012]. Hence, data has not been shown here. Similarly, after performing dose response effects of resveratrol and curcumin, it was found that cells remained viable to the extent of ~98-99% at 20-25 µg/ml of resveratrol and curcumin respectively as revealed by trypan blue and MTT assay, and that both these natural antioxidants had no adverse effect on human housekeeping gene R18.

The real time RT-PCR results indicate an appreciable / high degree of down-regulation in endogenous TNF-α mRNA expression by allicin, resveratrol and curcumin in osteoporosis patient monocytes cultured under osteoclastogenic medium. Our results regarding TNF-α mRNA and OPG mRNA expressions in osteoporosis patients as well as regulation by the above antioxidants are in accordance to similar pattern observed by other workers with different antioxidants than ours [Nazrun et al., 2012]. Furthermore, TNF-α generation in monocytes is regulated at multiple intracellular levels, beginning with transcription [Raabe et al., 1998]. Elevated expression of TNF-α mRNA and activation of a relevant transcription factor, NF-κB, have been reported in monocytic cells derived from osteoporosis patient.
accordance to earlier finding, in the present study, we also report here the up regulation of TNF-α mRNA expression as well as activation of NF-κB in human monocytes derived from osteoporosis patient patients. The induction of TNF-α expression was observed to be mediated through activation of NF-κB, as evidenced by the suppression of TNF-α mRNA in the presence of SN50, an inhibitor of NF-κB. On the contrary, the control SN50/M, an inactive analogue of SN50, failed to show any such effect. It has been well established that TNF-α induced nuclear translocation of NF-κB was inhibited by SN50 peptide as demonstrated in EMSA [Lin et al., 1995].

Therefore, in light of the above view, the data in the present study demonstrated that this effect involved inhibition of the NF-κB pathway induced by various natural antioxidants namely allicin, resveratrol and curcumin, probably by inhibiting the degradation of IκBα. The NFκB heterodimer is retained in the cytoplasm in an inactive form through association with one of the IκBs inhibitory proteins. As a consequence of stimulation by TNF-α, the IκBα gets phosphorylated by a specific kinase complex (IKK) leading to its ubiquitination, and subsequent proteolysis by the 28S proteosome [Lang et al., 2004; Li and Verma, 2002]. The degradation of IκB releases active NF-κB, which translocates to the nucleus and regulates gene expression by binding to κB binding sites or by interacting with other transcriptional factors [Brown et al., 1995]. Since a number of genes involved in inflammatory responses are regulated by NF-κB pathway, thus a high magnitude downregulation of the NF-κB pathway by allicin and or resveratrol and curcumin would predictably reduce the elaboration of NF-κB-mediated TNF-α mRNA expression.

Next, it’s a well established fact that glutathione directly reacts with ROS, and GPx catalyzes the removal of hydrogen peroxide [Mesiter and Anderson, 1983]. Down-regulation or decrease in GPx activity indicates impairment of hydrogen peroxide-neutralizing mechanisms [Rukmini et al., 2004]. Here, in the present study, it was observed that in osteoporosis patient monocytes that were untreated with allicin or resveratrol or curcumin, there was an appreciably suppressed GPx activity, thereby in turn, correlating with earlier reports that substantial amounts of ROS are being
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generated in bone cells of osteoporosis patient due to lowering of antioxidant defences in such type of cells. Augmentation / enhancement of GPx activity in osteoporosis patient monocyte cultures after addition of NAC, a precursor of in vivo antioxidant glutathione, indicates reversal of impaired neutralizing mechanisms. Interestingly, here slightly augmented GPx activity was observed when allicin or resveratrol or curcumin was co-cultured instead of NAC, indicating allicin as well as resveratrol and curcumin to be an effective natural antioxidant combating ROS, generated as a consequence of cellular activation in osteoporosis patient monocytes. Thus, our data exhibited amelioration in GPx activity by allicin, resveratrol and curcumin, which in turn, correlated inversely with the down regulation of TNF-α mRNA expression and ROS in monocytes of osteoporosis patient.

Next, in our study, peripheral blood mononuclear cells (PBMCs) were used directly for the generation of osteoclast precursors under osteoclastogenic medium. The multinucleated osteoclast precursors were observed to appear on day 3 and the number increased after 5 days of culture, as revealed by Tartrate Resistant Acid Phosphatase (TRAP) staining. However, after 24 h (1 day) of culture, appearance of osteoclast precursors was negligible. 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, where individual variation in osteoclast generation from different donors were observed.

Interestingly, we observed that co-culturing of PBMCs with resveratrol (25 µg/ml) or curcumin (25 µg/ml) or allicin (500 ng/ml) in osteoclastogenic medium for 3 and 5 days resulted in an appreciable amount of reduction in appearance of multinucleated osteoclast precursors, thereby reflecting the potential of these natural antioxidants to exert regulatory effect in osteoclast generation and differentiation. Nearly 30% suppression in appearance of multinucleated cells was observed in cultures receiving 20 µg/ml of resveratrol or curcumin relative to control cultures devoid of any supplements as said above. Interestingly, around 40% suppression in appearance of multinucleated cells was observed in cultures receiving 500 ng/ml of allicin relative to control cultures devoid of any supplement i.e. allicin.
Our data shows around 12-fold augmented OPN levels in culture supernatants of monocytes from osteoporotic patients in comparison to healthy controls. To the best of our knowledge, we show for the first time the natural antioxidants (allicin, resveratrol and curcumin) – induced down-regulation / suppressed levels of OPN, and that too, to an appreciable magnitude. Even, modulation with positive modulator namely CT failed to minimize the negative modulatory effects of allicin, resveratrol or curcumin on OPN. Out of these three natural antioxidants, allicin proved to be the most potent suppressor / down-regulator of OPN followed by resveratrol followed by curcumin.

One of the most striking findings was that allicin followed by resveratrol was equally good as Denosumab (Prolia), which has recently been employed in the treatment of osteoporosis, in suppressing OPN levels. Denosumab (Prolia®) [Rizzoli et al., 2010] offers a new approach in the treatment of osteoporosis [Dubois et al., 2011]. At present, it is being clinically used for the treatment of osteoporosis and cancer-related bone disorders in Japan, Europe, United States and many other countries [Yasuda, 2013]. It decreases bone resorption by inhibiting osteoclast formation, function and survival [Dubois et al., 2011]. Denosumab is a fully human monoclonal IgG2 antibody that binds RANKL with high affinity and specificity, preventing interaction with RANK on the osteoclast membrane [Yasuda, 2013]. In other words we can say that it is a fully human anti- RANKL monoclonal antibody or a RANKL neutralizing antibody [Yasuda, 2013]. It is a strong inhibitor of RANKL. It blocks the interaction of RANKL with RANK [Schmiedel et al., 2012]. Thus, it mimics the endogenous effects of osteoprotegerin (OPG) [Yasuda, 2013]. By attaching to and blocking RANKL, denosumab inhibits osteoclast differentiation, activation and survival [Dubois et al., 2011]. This favors bone formation over bone resorption, increasing bone mass and reducing the risk of fractures [Cummings et al., 2009]. It also reduces the release of RANKL-induced immunomodulatory factors by acute myeloid leukemia (AML) cells and their immunomodulatory effects [Schmiedel et al., 2012]. In addition, Denosumab prevents inhibitory RANK signaling into NK cells, which results in enhanced NK cell antitumor reactivity [Schmiedel et al., 2012]. It has been developed and shown to be effective in treatment of non-malignant and
malignant osteolysis [Lacey et al., 2012]. It has also been demonstrated to prevent or delay skeletal-related events (SREs) in patients with solid tumors that have metastasized to bone [Dougall et al., 2014]. Denosumab is administered once every 6 months as a 60-mg subcutaneous injection [Dubois et al., 2011]. It is very stable in the blood stream for several months after single subcutaneous injection [Yasuda, 2013]. During treatment, calcium and vitamin D supplementation is important [Dubois et al., 2011]. Adverse Effects of Denosumab (Prolia®) have also been reported, where patients with hypocalcaemia and/or chronic kidney disease may develop symptomatic hypocalcaemia upon treatment with denosumab [Dubois et al., 2011]. Therefore, hypocalcaemia should be corrected before therapy and serum calcium concentration monitored [Dubois et al., 2011]. Because RANKL also has a function in the immune system, denosumab could adversely influence infections of the urinary and upper respiratory tracts [Dubois et al., 2011].

Thus, in the present study, the suppressed levels of OPN which was induced by allicin as well as resveratrol and curcumin respectively may probably be by blocking RANKL by these natural antioxidants just like denosumab, and in turn, inhibits osteoclast differentiation, activation and survival, and consequently, in turn, favors bone formation over bone resorption, increasing bone mass and reducing the risk of fractures. Recent report shows the effect of resveratrol in rats that improves bone repair by modulation of bone morphogenetic proteins and OPN gene expression [Casarin et al., 2014].

Next, our data revealed that allicin, resveratrol and curcumin appreciably down regulated / suppressed the secretion of sRANKL. With allicin, it was observed that sRANKL dose-dependently decreased from 33.16 pg/ml at 0 ng/ml by around 16.94%, 38.69%, 60.85% and 90.89% with 50, 100, 250 and 500 ng allicin respectively, where the IC_{50} was computed out to be in between 100-135 ng/ml. Similarly, analysis of the data exhibited that resveratrol down regulated / suppressed the secretion of sRANKL 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. The IC_{50} was computed out to be around 8 µg/ml of Resveratrol. Furthermore,
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, and that, the IC50 was found to be around 9 µg/ml of Curcumin. Therefore, the present study provides for the first time evidences that natural antioxidants namely allicin from garlic as well as resveratrol and curcumin inhibited RANKL mediated signaling events that lead to osteoclast differentiation and function in monocyte cultures. Incubation of osteoclast progenitor cells with allicin or resveratrol or curcumin inhibited TRAP activity and TNF-alpha mRNA expression in a dose dependent manner.

The findings in the present study are novel and contribute to define a mechanism for the altered bone turnover in osteoporosis patients that, which in turn, may protect patients from early bone loss or lead to increased bone mass. Bone mass is tightly regulated by osteoclastic and osteoblastic bone remodeling. The contribution of natural antioxidants like allicin from garlic as well as resveratrol and curcumin to bone remodeling in vivo is poorly understood. The findings of the present study are indicative/suggestive that allicin, resveratrol and curcumin are inhibiting osteoclastogenesis, likely by preventing the formation of pre-osteoclast cells capable of fusing into multinucleated osteoclasts. ROS including superoxide anion and hydrogen peroxide (H$_2$O$_2$) have been recognized as major intermediaries in the formation and activation of osteoclasts in vitro and in vivo [Wittrant et al., 2008; Suda et al., 1993; Garrett et al., 1990; Lee et al., 2005]. RANKL-mediated ROS production serves to regulate RANKL signaling pathways required for osteoclast differentiation [Koh et al., 2006; Ha et al., 2004; Wittrant et al., 2008]. Furthermore, in RAW264.7 and BMM precursor cells, RANKL increases ROS, whereas expression of catalase in BMM cells blocks RANKL-induced ROS production and inhibits the formation of TRACP+osteoclasts [Wittrant et al., 2008; Lee et al., 2005], and that, such effects were reported to be correlated with the decreased formation of multinucleated osteoclast-like cells in the TRACP assays and likely contributed to the inability of these cells to differentiate in response to RANKL.
Therefore, in summary, our data regarding regulation of TNF-α, sRANKL, OPN, GPx activities, osteoclast formation and differentiation, etc by allicin from garlic as well as resveratrol and curcumin could provide a valuable tool in probing the control molecular mechanism of osteoporosis, and in turn, such natural antioxidants may serve as adjuncts in the management of osteoporosis.