Calcium Metabolism and Oxidative Stress in Bone Fractures: Role of Antioxidants

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Abstract: Calcium ion is an essential structural component of the skeleton. There is growing evidence for the importance of nutrition in the maintenance of bones and joints health. Nutritional imbalance combined with endocrine abnormalities may be involved in osteoporosis. For example, essential fatty acids and their metabolites were reported to have beneficial action in osteoporosis. The mechanism by which fatty acids prevent osteoporosis may involve inhibition of pro-inflammatory cytokines, which are known to have a major role in osteoporosis through induction of oxidative stress which had adverse effects on the skeleton. Other risk factors for osteoporosis, such as smoking, hypertension and diabetes mellitus are also associated with increased oxidative stress and free radicals levels.

When bone fracture occurs, a remarkable yield of free radicals is generated by the damaged tissues. However, controlled production of free radicals by normally functioning osteoclasts could accelerate destruction of calcified tissues and assist bone remodeling. Enhanced osteoclastic activity observed in bone disorders may have been responsible for increased production of reactive oxygen species [ROS] in the form of superoxide, which is evident by increased levels of serum malondialdehyde [MDA] levels. One of the most damaging effects of ROS is lipid peroxidation, the end product of which is MDA which also served as a measure of osteoclastic activity. Inhibition of the antioxidant enzymes activities, such as superoxide dismutase and glutathione peroxidase, was found to increase superoxide production by the osteoclasts which represented by increased levels of MDA. Therefore, oxidative stress is an important mediator of bone loss since deficiency of antioxidant vitamins has been found to be more common in the elderly osteoporotic patients. It is concluded from this review that increased free radical production overwhelms the natural antioxidants defense mechanisms, subjecting individuals to hyperoxidant stress and thus leading to osteoporosis. In addition, administration of antioxidants might protect bones from osteoporosis and also might help in the acceleration of healing of fractured bones.

1. INTRODUCTION

Osteoporosis, which is characterized by low bone mass and microarchitectural deterioration of bone tissue leading to increased bone fragility, can result in an increased risk of fractures. Osteoporosis in men is now recognized as an increasingly important public health issue [1]. One out of five osteoporosis patients is male and 30% of hip fractures occur in men [2]. The pathogenesis of osteoporosis in men is poorly understood. Although aging and genetic factors are important, many factors such as smoking, immobilization, inefficient calcium intake, thyroid and parathyroid function disorders, gastrointestinal and kidney diseases cause osteoporosis and 30-60% of cases are associated with one or more of the secondary risk factors in male osteoporosis [3]. It has been reported that approximately one third of osteoporotic men have an idiopathic disease [4]. On the other hand, free oxygen radicals or reactive oxygen species [ROS] including hydroxyl radicals [OH·], superoxide anion radical [$O\cdot_2^-$], hydrogen peroxide [H_2O_2] and nitric oxide [NO] lead to specific oxidation of some enzymes, protein oxidation and degradation [5, 6]. Their effects are eliminated by enzymatic antioxidant mechanisms such as superoxide dismutase [SOD], glutathione peroxidase [GPx] and catalase [7]. Oxidative stress is an imbalance between the free radicals and antioxidant mechanism in biological systems and damages cellular macromolecules and functions. It is responsible for the pathophysiology of the aging process and many diseases such as atherosclerosis, carcinogenesis, myocardial infarction and muscle diseases [8]. The role of free radicals and oxidative stress in osteoporosis is unknown. However, there is evidence that NO modulates bone remodeling and bone loss *in vitro* and *in vivo* [9]. Several studies have demonstrated that bone cells can produce NO and express NOS [nitric oxide synthase] enzymes. Also, NO plays an important role as the paracrine and autocrine mediator of bone cells in response to diverse stimuli such as proinflammatory cytokines [10], mechanical strains [11] and sex hormones. However, there is not enough evidence about the role of MDA [malondialdehyde] and antioxidant enzymes [SOD, GPx] on bone tissue and the effect of oxidative stress regarding the development of osteoporosis. The aim of this review is to throw more light on the role of the different factors including pro- and antioxidants which play a significant role in the integrity of bones in health and diseases.

2. CALCIUM HOMEOSTASIS

Calcium ion plays a key role in many fundamental biologic processes including muscle contraction, blood coagulation, enzyme activity, neural excitability, hormone release, and membrane permeability, in addition to being an essential structural component of the skeleton. Therefore, the precise control of calcium ion in extracellular fluids is vital to the health of humans. To maintain a constant concentration of blood calcium, despite marked variations in intake and excretion, endocrine control mechanisms have evolved. Although the direct roles of parathyroid hormone [PTH], calcitonin [CT], and vitamin D frequently are emphasized in the control of blood calcium [3, 12], other hormones such as adrenal corticosteroids, estrogens, thyroxine, somatotropin, and glucagon may contribute to the maintenance of calcium homeostasis under certain conditions. If the blood calcium level is elevated by the intravenous infusion of calcium, there is a rapid and pronounced reduction in circulating levels of immunoreactive parathyroid hormone [iPTH]. Conversely, if the blood calcium level is lowered by ethylenediaminetetra-acetic acid EDTA, there is a brisk and substantial increase in iPTH levels [13]. The concentration of blood phosphorus has no direct regulatory influence on the synthesis and secretion of PTH; however, certain disease conditions characterized by hyperphosphatemia in both animals and humans are associated clinically with hyperparathyroidism. An elevated blood phosphorus level may lead indirectly to parathyroid stimulation by virtue of its ability to lower the blood calcium level [14]. Vitamin E deficiency impaired bone calcium homeostasis with subsequent secondary hyperparathyroidism and vertebral bone loss [15,16].

If the blood phosphorus level is elevated significantly by an infusion of phosphate and calcium administered simultaneously in amounts to prevent the accompanying reduction of the blood calcium level, plasma iPTH levels remain within the normal range. In

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1389-2002/07 $50.00+.00

addition, hyperphosphatemia suppresses the rate of formation of the biologically active, hormonal form of vitamin D [1,25-dihydroxycholecalciferol 1,25-diOH-CC] in the kidney, which further contributes to the development of hypocalcemia and parathyroid stimulation. A number of investigations have shown that the hormonal regulation of calcium metabolism exhibits a certain degree of seasonal variation. Both, serum levels of 25-hydroxyvitamin D3 (25OHD3) [17,18] and urinary calcium excretion [19] are elevated in late summer and decreased in winter, whereas PTH levels tend to increase during winter [20-22]. Less attention, however, has been paid to the annual variations in biochemical parameters of bone turnover. It has been shown that bone turnover, as assessed by specific biochemical markers, is accelerated during winter, with more pronounced findings in females. Furthermore, the seasonal increase in bone metabolism appears to coincide with a significant reduction in serum values of 25-OHD3 in females. Also, analysis of life style habits revealed that regular alcohol consumption (in both sexes) and current smoking (in females only) are associated with reduced levels of bone turnover. In contrast, physical activity in both sexes seems to be associated with higher levels of bone formation markers and reduced levels of bone resorption markers. However, for most of the biomarkers studied, seasonal variations accounted for more of the observed variability than any of the other factors except age.

Magnesium ion has an effect on PTH secretory rate similar to that of calcium but not equipotent [23]. The more potent effects of calcium ion in the control of PTH secretion together with its preponderance over magnesium in the extracellular fluid suggest a secondary role for magnesium in parathyroid control.

3. NITRIC OXIDE AND BONE METABOLISM

Nitric Oxide is generated by the nitric oxide synthase enzymes [NOS] from molecular oxygen and the terminal guanidino nitrogen of the amino acid L-arginine, yielding L-citrulline as a coproduct [24]. This reaction can be inhibited by substituted arginine analogues such as L-NG-monomethyl arginine [L-NMMA] and L-nitro-arginine-methyl ester [L-NAME]. NO can also be generated nonenzymatically from nitrite in the acid environment of the stomach and pharmacologically by compounds such as organic nitrates [e.g. nitro-glycerine] and sodium nitroprusside, which are used clinically as vasodilators [24].

Nitric oxide is a free radical involved in the regulation of many physiological processes, such as vascular relaxation, neurotransmission, platelet aggregation and in immune regulation [25]. During the last decade, it has become apparent that NO has important effects on bone cell function. The endothelial isoform of nitric oxide synthase [eNOS] is widely expressed in bone on a constitutive basis, whereas inducible NOS is only expressed in response to inflammatory stimuli.

Accumulating evidence suggest that the iNOS pathway plays an important role in cytokine and inflammation induced bone loss however, Inflammation induced osteoporosis has been shown to be mediated in part by activation of the iNOS pathway [26] and other studies have shown that activation of the iNOS pathway is essential for IL-1-stimulated bone resorption, both *in vivo* and *in vitro* [27]. The mechanism by which iNOS promotes IL-1 induced bone resorption has been investigated in cocultures of normal osteoblasts with iNOS-KO osteoclast precursors. These studies have shown that IL-1 primarily acts on osteoblasts to increase NO synthesis by activation of the iNOS pathway and that this in turn promotes nuclear translocation of the transcription factor NFkB in osteoclast progenitors. Whilst osteoclast progenitors from iNOS deficient animals also show NFkB activation in response to IL-1, the response is transient, implying that NO has a key role to play in sustaining NFkB activation in osteoclast precursors [27].

On the other hand, nitric oxide appears to have biphasic effects on osteoblast activity. Studies *in vitro* have indicated that the small amounts of NO which are produced constitutively by osteoblasts may act as an autocrine stimulator of osteoblast growth and cytokine production [28]. Whilst some investigators have shown that slow release NO donors stimulate osteoblast growth and differentiation *in vitro* [29]. Other workers reported that NO donors and NOS inhibitors had little effect on osteoblast growth or differentiation, except at high concentrations where inhibitory effects were observed [30].

4. ESTROGEN AND BONE INTEGRITY

Estrogen has immunomodulatory effects and anti-inflammatory actions. In healthy premenopausal women who underwent oopherectomy, increases in GM-CSF activity was observed as early as 1 week after surgery, whereas elevations in IL-1 and TNF-α were detectable 2 weeks after surgery [3]. In those who did not receive estrogen replacement therapy, IL-1, TNF-α, and GM-CSF reached the highest levels 8 weeks after oopherectomy, and these changes in the cytokine profile were found to be associated with indices of bone resorption [31]. On the contrary, those who received estrogen replacement therapy within 4 weeks after oopherectomy showed reduction in the secretion of GMCSF, IL-1, and TNF-α. The cytokine profile did not change in the female controls that underwent simple hysterectomy. This suggests that estrogen suppresses the production of these three cytokines [31] and, thus, prevents osteoporosis [32]. It is also known that estrogen enhances transforming growth factor-[TGF- β] production [33,34]. TGF- β has antioxidant and anti-TNF actions, it suppresses free radical generation, and it shows anti-inflammatory actions as well [34,35]. TGF-β plays an important role in bone formation, induction, or repair [33,36]. Injection of TGF- β over frontal or parietal bones in neonatal mice or rats and over femur in newborn rats stimulates bone formation [33]. TGF-β can regulate cell proliferation and phenotypic expression in the fracture callus *in vitro* and it can enhance chondrogenesis and osteogenesis *in vivo* [33]. When used at physiological concentrations, 17β-estradiol stimulated the production of TGF- β *in vitro* [37]. It was also observed that estrogen modulates IL-1 actions on human osteoclasts [38]. Isolated human osteoclasts and primary bone marrow derived osteoclast-like cells expressed both the signaling [IL-1RI] and decoy [IL-1RII] IL-1 receptors, whereas only IL-1RI was detected in osteoblasts [38]. IL-1RII/IL-1RI mRNA ratios and release of soluble IL-1RII [sIL-1RII] were found to be lower in osteoclast-like cells derived from women in the late postmenopausal period compared with younger women. Estrogen directly reduced *in vitro* osteoclast- like cell IL-1RI mRNA levels, while it increased IL- 1RII mRNA levels and sIL-1RII release [38]. Estrogen pretreatment significantly inhibited two IL-1 responses, suppressing IL-1-mediated IL-8 mRNA induction and IL-1-promoted osteoclast survival. It is known that IL-8 is released at high levels by human osteoclasts; osteoclast-derived IL-8 inhibits multiple osteoblast bone formative functions [39]; IL-8 stimulates osteoclast migration; and IL-8 promotes osteoclast recruitment, development, and bone-resorptive activity [40]. Thus, one mechanism by which estrogen exerts bone-protective effects may include a selective modulation of IL-1R isoform levels in osteoclasts, thereby reducing their IL-1 responsiveness and cell survival. Lin *et al*. 1997, reported increased expression of the IL-6 receptor in cells of the bone marrow stromal/osteoblastic lineage after loss of sex steroids [41]. This is consistent with the observation that increased IL-6 levels can be detected in bone marrow supernatants from oopherectomizedcompared with sham-operated animals [32,42], and in postmenopausal compared with premenopausal women [31]. Thus, estrogen suppresses the production of proinflammatory cytokines [IL-1, IL-6, and TNF-α] and their actions, and augments the production of TGF-β, events that inhibit bone resorption and prevent osteoporosis. Paradoxically, glucocorticoids, which suppress the production of TNF and other pro-inflammatory cytokines [43], do not prevent osteoporosis. In fact, they induce osteoporosis by suppression of the osteoblast. This indicates that there could be a

second messenger involved in osteoporosis through which the actions of cytokines and corticosteroids on bone remodeling are brought about. Post-menopausal reduction in skeletal mass appears to be associated with excessive osteoclastic activity. Direct stimulatory effect of estrogen on osteoblasts is possible due to estrogen receptors on osteoblasts [44]. Enhanced osteoclastic and depressed osteoblastic activity is attributable to primary estrogen deficiency characteristic of post-menopausal osteoporosis [45].

5. NUTRICEUTICALS AND BONE DISEASES

There is growing recognition of the importance of nutritional factors in the maintenance of bone and joint health, and that nutritional imbalance combined with endocrine abnormalities may be involved in the pathogenesis of osteoarthrosis [OA] and osteochondritis dissecans [OCD] [46-48]. Despite this, dietary programs have played a secondary role in the management of these connective tissue disorders [49]. Articular cartilage is critically dependent upon the regular provision of nutrients [glucose and amino acids], vitamins [particularly vitamin C], and essential trace elements [zinc, magnesium, and copper] [50,51]. Therefore, dietary supplementation programs and nutriceuticals used in conjunction with non-steroidal, anti-inflammatory drugs [NSAIDs] may offer significant benefits to patients with joint disorders, such as OA and OCD [52].

Essential fatty acids [EFAs] and their metabolites such as γ-linolenic acid [GLA], eicosapentaenoic acid [EPA], and docosahexaenoic acid [DHA] were also reported to have beneficial action in osteoporosis [35, 53]. Fish oil can prevent nephrocalcinosis in animals by reducing urinary calcium excretion. Wohl *et al*., demonstrated that a diet rich in saturated fat interfered with calcium absorption and decreased cancellous bone strength [54]. It has been suggested that beneficial effects of long-term fish oil feeding in maintaining higher bone mineral density [BMD] and lower synovitis in mouse models. These beneficial effects may be due, in part, to increased activity of antioxidant enzymes, decreased expression of RANKL, and increased expression of OPG in fish oil [FO] fed mice thereby altering the RANKL/OPG ratio. These significant beneficial effects on BMD suggest that FO may serve as an effective dietary supplement to prevent BMD loss in patients with RA [55].

EFA-deficient animals develop severe osteoporosis and increased renal and arterial calcification [35], a finding that is similar to the osteoporosis and calcification of arteries and kidneys seen in elderly people. A combination of GLA and EPA decreased calcium excretion, enhanced intestinal calcium absorption, and increased bone calcium content [35]. One mechanism by which these fatty acids prevent osteoporosis may involve inhibition of pro-inflammatory cytokines IL-1, IL-2, and TNF-α [35], which are known to have a major role in osteoporosis. Similar to statins, EFAs and their metabolites can lower cholesterol, triglyceride, and low-density lipoprotein levels, and they can block HMG-CoA reductase activity [56; 35]. Animals with EFA deficiency showed an increase in HMGCoA reductase activity, which reverted to normally following topical application of linoleic acid [LA] [35,56]. Further, both ω-3 and ω-6 fatty acids showed inhibitory action on HMG-CoA reductase activity [35]. Hence, similar to statins, EFAs and their metabolites also may enhance the expression of BMPs. This could be yet another mechanism by which EFAs are useful in osteoporosis [35]. It may be noted here that even though EFAs and their metabolites are thought to be useful in osteoporosis, more definitive studies need to be performed in humans. It is also possible that EFAs have to be given for long periods of time to obtain their possible benefit in osteoporosis.

6. REACTIVE OXYGEN SPECIES AND BONE INTEG-RITY

It has previously been shown *in vitro* and in rodents that free radicals are involved in osteoclastogenesis and in bone resorption [57]. Osteopetrosis has been found in mice lacking NF-kB [58], the discovery of receptor activator of NF-kB [RANK], the RANK ligand, and the decoy receptor osteoprotegerin, all demonstrated the great importance of NF-kB in osteoclastogenesis. NF-kB is an oxidative stress-responsive transcription factor [59]. Thus, free radicals may increase bone resorption through activation of NF-kB. Several risk factors for osteoporosis, such as smoking [60], hypertension [61] and diabetes mellitus [62,63] are associated with increased oxidative stress. Smoking increases the concentrations of free radicals, which have been suggested to be involved in bone resorption [64]. It has been shown that oxidant stress had adverse effects on the skeleton of smokers, and that an insufficient dietary intake of vitamin E and C may substantially increase the risk of hip fracture in current smokers, whereas a more adequate intake seems to be protective [64]. It has been demonstrated that an insufficient dietary intake of antioxidants substantially increases the risk of hip fracture in current smokers, whereas current smokers with a more adequate intake seem to have a fracture risk that is similar to that seen among never smokers [64], supporting this hypothesis that oxidative stress has important effects on bone in man. A major problem associated with the assessment of free radical-induced oxidative stress in various diseases has been the limitation in available assay methods for *in vivo* measurement of free radical generation or end products of free radical catalyzed oxidation of lipids. The role of reactive oxygen species [ROS] in bone metabolism is unique and dual considering their effect under physiological and pathological conditions [65,66]. Under physiological conditions, the production of ROS by osteoclasts assists in accelerating destruction of calcified tissue and hence assists in bone remode-ling [67,68]. Moreover, osteoclasts contain a NADPH oxidase [69], an enzyme that is capable of cytokine-regulated generation of ROS. ROS are also potent inducers in many cells of TNF-α and other cytokines strongly implicated in the bone loss of estrogen deficiency. Thus, if estrogen increases oxidant defenses in bone, estrogen deficiency might directly or indirectly stimulate osteoclastic bone resorption [69]. The major determinants of the reductive state of soluble proteins are glutathione and thiored-oxin. Upon oxidation, their oxidized forms are reverted to the reduced state by glutathione reductase and thioredoxin reductase, respectively, substantial decreases in glutathione and thioredoxin and in the enzymes that regenerate their reduced forms has been found in rodent bone marrow. These decreases were reversed by estrogen administration. It was also found that administration of antioxidants completely prevented bone loss in oopherectomized mice, while L-buthionine-[S,R]-sulphoximine [BSO], a specific inhibitor of glutathione synthesis, depleted glutathione and caused substantial osteolysis in ovary-intact mice [69].

7. BIOCHEMICAL EVENTS DURING BONE FRACTURES

Intact osteocalcin and bone-specific alkaline phosphatase (ALP) activity are also markers of osteoblastic activity. Both osteocalcin and ALP levels increased after fracture, although the time to reach the first peak differed. Consistently increasing levels of ALP and intact osteocalcin in serum after fracture were also reported by another investigator [70]. It has been reported an assay of the serological indicators of collagen synthesis, suggesting that serological measurements may reflect events occurring at the fracture site.

7.1. Role of Calcitonin

There are numerous experimental studies in the literature investigating the action of calcitonin on fracture healing [71]. All these studies have been done in rats or rabbits where a fracture of the peripheral skeleton was performed. The dose administered corresponds to 2-5 IU of salmon calcitonin depending on the type, age and weight of the animal. The parameters examined are the histological study of callus, calcium content, radioactive isotope retention, and bone enzyme and biochemical indices of callus, as well as of serum and urine and finally, the mechanical strength of the callus. Of the above studies, they examined the effect of calcitonin upon secondary (osteocartilaginous) callus formation. In some of

these studies, it was not possible to detect any beneficial effect of calcitonin on fracture healing between the animals [72,73]. In other studies calcitonin was found to have an adverse effect by way of decreased collagen formation and mineralization as well as decreased callus strength [73,74]. However, in the other studies [75, 76] a positive effect on fracture healing was found, which is shown from the histological appearance of callus, biochemical and immunohistochemical findings and radiological appearance. The conclusion in the majority of these studies was that calcitonin stimulates endochondral ossification during fracture healing, causing an increase in cartilaginous callus and faster maturation. The differences found may be caused by inadequate or very high doses of calcitonin, resulting in secondary hyperparathyroidism due to hypocalcaemia [74]. In cases of primary fracture healing without the process of endochondral ossification, calcitonin has no effect, although its administration inhibits regional osteoporosis under the materials of fixation [74,77]. The action of calcitonin on fracture healing in humans has not been extensively studied. In some clinical studies [78], it has been established that there is a clinical and radiological improvement in patients with recent fractures of the peripheral skeleton, an acceleration in the formation of radiologically visible callus, and clinical improvement in Paget's disease patients with multiple fractures [79]. Calcitonin has also been used in clinical settings of patients with injuries of the musculoskeletal system, such as incorporation of bone grafts after local injections of calcitonin [80], restoration of bone cysts after dental extractions and an improvement in delayed fracture healing after local administration of calcitonin in patients with neglected fractures [81]. Finally, the analgesic effect of calcitonin in patients with recent osteoporotic vertebral fractures is of great importance [82].

7.2. Role of Growth Factors

Fracture healing is a physical recovery process not comparable to any other type of tissue regeneration leading to bone restoration of original quality and function. The healing process reflects embryonic development and bone growth. During the last decade, research concerning bone regeneration has been enormously intensified. Through immunohistochemical deter-mination and analysis of gene expression, the necessity of various biochemical factors for fracture healing was confirmed [83]. Gerstenfeld *et al*. described more than 50 cytokines, morphogens, proteases, and angiogenetic factors which demonstrably play a role in fracture healing [84]. Most of the current knowledge on growth factors involved in fracture healing is derived from animal experiments or *in vitro* studies [85]. In a large prospective multi-center clinical trial, Friedlaender *et al*. could show that implantation of OP-1 (BMP-7) with a type I collagen carrier and bone autograft performed equally in therapy of tibial nonunions in patients [86]. Further, Einhorn *et al*. demonstrated acceleration of fracture healing after a single percutaneous injection of BMP-2 in rats [87]. Beside the direct stimulation of osteoblasts, chondroclasts, and chondro-cytes, TGF-h intensifies the effect of other bone growth factors like BMP and IGF [88]. The stimulating effect of TGF-h on fracture healing could be demonstrated in animal experiments. However, these effects were usually reached by unphysiologically high doses of TGF-h and showed controversial effects [89].

Various authors suggested that not only local release of growth factors at the fracture site but also a systemic reaction is necessary to trigger local effects [90]. An insufficient systemic supply of growth factors leads to a loss of bone substance and to reduced differentiation of osteoblasts [91]. This hypothesis is supported by a study of Gazit *et al*. which showed that systemic application of TGF-h increased bone density in osteopenic mice [92]. Repeated surgical explorations of fracture sites on patients are not justifiable for ethical reasons. However, further studies on elective osteotomies in animal experiments showed simultaneous local and systemic increase of certain growth factors [93]. From that it is assumable that local concentrations of growth factors are mirrored in the circulation, rendering systemic measurements of growth factors an elegant and promising way to trace the process of normal and pathological fracture healing on human beings *in vivo*. Drawing and analysis of peripheral venous blood is considered a standard method for the documentation of serum concentration of various growth factors after trauma [93]. A published analysis showed that gene expression of TGF-h in older rats with delayed healing of femoral fractures followed the same pattern than the one in younger rats showing normal fracture healing [94]. The authors concluded that failure of older rats to heal fractures timely was not due to lack of expression of a single growth factor or cytokine, but inability of older rats to sustain high levels of gene expression of osteoinductive factors until achievement of bony consolidation.

Fibrous growth factors (FGFs) are related to bone formation during the fracture healing process [95]. Insulin-like growth factors (IGFs) also stimulate osteoblastic cell proliferation and are expressed in the cells of healing fracture callus [96]. On the other hand, IGF-binding proteins (IGF-BPs), which are produced by several kinds of cells including bone cells, modulate IGF activities. Six IGFBPs, IGFBP-1 through IGFBP6, have been identified, and more than 95% of IGF-I are bound to IGFBp-3 in serum, which prolongs the half-life of IGFs in the circulation and may serve as a potential reservoir [70].

7.3. Role of Reactive Oxygen Species in Bone Fractures

When bone fractures occur, a remarkably high yield of radicals is generated. It is suggested that as a break occurs, the minimal crystallites separate at grain boundaries with no major chemical changes, but the tightly bound collagen strands running through the mineral phase are forced to break homiletically. Some react with oxygen and yield oxygen radical metabolites [97]. However, though enhanced osteoclastic activity and increased production of ROS are linked in many skeletal pathologies, it remains to be studied whether increased ROS production overwhelms the antioxidant defenses, subjecting the individual to hyperoxidant stress. Osteoclasts destroy calcified tissue by complex developmental steps. In particular, controlled production of free radicals by normally functioning osteoclasts could accelerate destruction of calcified tissue and assist in bone remodelling [68]. Enhanced osteoclastic activity observed in bone disorders may have been responsible for increased production of ROS in form of superoxide, which is evident by increased levels of serum MDA levels. One of the most damaging effects of ROS is lipid peroxidation, the end product of which is MDA [98]. MDA in addition to serving as an index of lipid peroxidation has also served as a measure of osteoclastic activity. Depressed acti-vities of the antioxidant enzymes, SOD and GSH-PX illustrated a defense mechanism that may have been overwhelmed in mitigating the increased superoxide production by the osteoclasts represented by increased levels of MDA in the serum.

The generation of free radicals has been implicated in the causation of several diseases, and compounds that can scavenge free radicals have great potential in ameliorating these disease processes. Thioredoxin (TRX) is an oxidative stress-inducible biological antioxidant that is highly expressed in the synoviocytes of rheumatoid arthritis (RA) patients [99]. There is much evidence that oxidative stress plays a key role in the inflammation and destruction of RA joints [99]. The inflammatory and joint-damaging processes of RA using a murine model in which arthritis was induced by administering a mixture of anti-type II collagen monoclonal antibodies (mAb) and lipopolysaccharide (LPS) were studied. In Wt mice mAb/LPS injection induced neutrophil infiltration, cartilage destruction, and chondrocyte apoptosis within the joints, all of which were dramatically suppressed in TRX transgenic (TRX-Tg) mice [99]. Another previous study aimed to investigate the possible antioxidant potential of Ligularia fischeri leaves on collagen type II induced arthritis (CIA) in DBA/1J mice [100]. The induction of arthritis significantly increased malondialdehyde (MDA), oxidized

proteins such as protein carbonyl (PCO), advanced glycation end-products (AGE), anti-collagen antibody, rheumatoid factor, interleukin (IL)-1beta, IL-6, and low density lipoprotein in the serum of patients [101]. The antiarthritic and anti-inflammatory efficacy of N-acetyl-L-cysteine (NAC) was tested in male DBA/1 hybrid mice suffering from type II collagen-induced arthritis [102]. After treatment with 100 mg/kg of NAC from day 42 after immunization over a period of six weeks, the ROS production was reduced to levels occurring in whole blood of healthy animals. It has been found that low-molecular-weight antioxidants such as NAC may be adequate for controlling oxidative stress-derived damage in rheumatic diseases by modulation of ROS-dependent signal transduction pathways [102]. Amelioration of collagen-induced arthritis (CIA) in mice by alpha-lipoic acid was associated with reduction in oxidative stress, as well as inhibition of inflammatory cytokine activation and NF-kappaB DNA binding activity [103]. Moreover, alpha-lipoic acid inhibited bone destruction *in vivo* and osteoclastogenesis *in vitro* [103]. Oxidative stress induced by reactive oxygen species (ROS) is associated with the risk of osteoporosis, and can be reduced by certain dietary antioxidants. Lycopene is an antioxidant known to decrease the risk of age-related chronic diseases [104]. It has been suggested that the dietary antioxidant lycopene reduces oxidative stress and the levels of bone turnover markers in postmenopausal women, and may be beneficial in reducing the risk of osteoporosis [104]. Levels of oxidative stress markers reduced (GSH) and oxidized (GSSG) gluthatione and malondialdehyde (MDA) were assayed in loose and stable hips revised for high rate of wear and osteolysis [105]. Collagen in the periprosthetic tissues was measured as hydroxyproline content [105]. MDA and both GSH and GSSG levels were correlated significantly with hydroxyproline level [105]. This study could provide an evidence on the role of malondialdehyde in the destruction of collagen through releasing of hydroxyproline [105].

7.4. Role of Antioxidants

Experimental studies on animals or cultured human cell lines support a role of polyphenols in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases, diabetes, or osteoporosis [106]. 2,6-Diisopropylphenol is an intravenous anesthetic agent used for induction and maintenance of anesthesia. Since it is similar to alpha-tocopherol, 2,6-diisopropylphenol may have antioxidant effects [107]. Osteob-lasts play important roles in bone remodeling [107]. 2,6-Diisopropylphenol significantly reduced hydrogen peroxide -induced oxidative stress. Exposure of osteoblasts to SNP and HP decreased cell viability time-dependently. 2,6-Diisopropylphenol protected osteoblasts from sodium nitroprusside - and hydrogen peroxide -induced cell damage [107]. Ascorbic acid [AA] plays a key role in the regulation of differentiation and activation of osteoclast [108]. It has been demonstrated that AA inhibits RANKL-induced differentiation of OCL precursor cells into mature OCL and reduces the formation of bone resorption pits *in vitro* [108]. It has been shown that AA induces embryonic stem cells to differentiate into osteoblasts. The mechanism by which AA sustains pre-osteoblast proliferation and commitment is mediated through the synthesis of collagen type I, interaction with alpha2- and beta1-integrin, activation of the mitogen-activated protein kinase pathway, and phosphorylation of osteoblast-specific transcription factors [109]. By using DNA micro-arrays containing 15,000 genes, it has been identified several genes in MC3T3-E1 cultured with AA for 24h whose expression was significantly up or downregulated. The differentially expressed genes covered a broad range of functional activities, [1] cell growth; [2] metabolism; [3] morphogenesis; [4] cell death; [5] cell communication. The data reported are the first genetic portrait of early stage stimulation of pre-osteoblasts by AA, and may be relevant to better understand the molecular mechanism of pre-osteoblast proliferation and commitment. Elucidation of the molecular mechanism has important clinical implications because it may facilitate the correct use of AA to accelerate bone regeneration [109]. Oxidative stress are an important mediator of bone loss. TNF-alpha, which plays a critical role in the bone loss after menopause, has been shown to increase intracellular oxidative stress [109]. TNF-alpha is an important mediator of bone loss. In the HS-5 hBMSC, TNF-alpha and H2O2 increased intracellular ROS levels and induced cell apoptosis through activation of caspases, JNK and NF-kappaB. alpha-Lipoic acid prevented these changes induced by TNF-alpha and H2O2, suggesting its potential therapeutic applications in attenuating bone loss [109, 110].

Subclinical vitamins deficiency is common in the elderly, especially in osteoporotic patients [111]. Improving these vitamins status may help to treat and prevent osteoporosis in elderly people [112]. Higher vitamin D intake is recognized to be needed to keep not only bone health but also muscle strength. More sun exposure might be needed for improved bone health in the elderly. Deficiency of Vitamin K, C, or B [113] may be also important modifiable risk factors for osteoporosis and bone fracture [49]. Excessive retinal supplementation may become associated with higher bone loss. Thus such diet rich in fruit and vegetables together with fish and meat could fulfill a balance among these vitamins and should be recommended for prevention or treatment of osteoporosis [49].

Administration of antioxidant vitamins or radical scavengers has an effect on the oxidative stress in various experimental models [114-116]. Furthermore, it has been found that in the case of an individual with a head injury, melatonin can enhance osteogenesis. Osteoblastic activity rises with the increase of melatonin. Healing of a fracture of long or large bone can often be accelerated in patients with severe traumatic brain injury. However, a melatonin which could perhaps induce enhanced osteogenesis has not yet been identified. Melatonin might cause early bone healing and hypertrophic callus [116]. Flavonols, in contrast to soybean isoflavones, are the most abundant phytoestrogens in western diets, being present in onions, beans, fruits, red wine, and tea. They may protect against atherosclerosis, inhibit certain cancer cell types, and reduce bone resorption. The most widely distributed flavonol is quercetin, which occurs mainly as its glycoside, rutin, but data are very scarce regarding the precise mechanism of action of these compounds on bone-resorbing cells at concentrations similar to those detected in human plasma [117]. Osteoclasts and osteoclast progenitors contained estrogen receptor alpha ERalpha, ERbeta, and RANK proteins. Both flavonols increased nuclear ERbeta protein and decreased ERalpha protein of osteoclast progenitors. Moreover, rutin reduced RANK protein, whereas 17beta-oestradiol and quercetin promoted apoptosis by cleavage of caspase-8 and caspase-3. The anti-resorbing properties of flavonols are mainly mediated by ER proteins through the inhibition of RANK protein or the activation of caspases [118].

In our previous studies, we had found that antioxidants could protect organs against the toxicity of toxic compounds and also improved male infertility [119-121]. It is concluded from this review that increased free radical production overwhelms the natural antioxidants defense mechanisms, subjecting individuals to hyperoxidant stress and thus leading to osteoporosis. In addition, administration of antioxidants might protect bones from osteoporosis and also might help in the acceleration of healing of fractured bones.

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Received: March 12, 2007 **Revised: April 9, 2007** **Accepted: April 11, 2007**