Role of oxidative stress in osteoporosis

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The maintenance of bone mass is influenced by genetic race, hormonal, mechanical and nutritional factors which modulate the local and systemic mechanisms regulating bone turnover. One of the factors significantly influencing bone mass is oxidative stress. This paper aims to provide a review of the state of the science of oxidative stress and osteoporosis. To reach this objective, a search of the literature using Medline/Index Medicus, EMBASE/Excerpta Medica and Chemical Abstracts were performed, and most of relevant citations were studied and summarized. It is concluded that oxidative stress by itself and by influencing the regulatory cytokines such as tumor necrosis factor and interlukins are involved in osteoporosis. Use of antioxidants can be helpful in the management of osteoporosis.

Keywords: antioxidants, osteoblast, osteoclast, osteoporosis, oxidative stress, reactive oxygen species

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Osteoporosis has been defined as a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enlarged bone fragility and a resulting increase in fracture risk [1]. It is one of the most common disorders in the world. Risk factors of osteoporosis are seen in **Box 1** and the causal factors are illustrated in **Box 2** [2].

Risk factors, such as thalessemia, are most important in some countries [3], but some, like vitamin D deficiency, seem to be a worldwide problem reported from 4 to 80% in different parts of the world [4,5]. Some studies also investigated relations between other factors, such as diastolic blood pressure, tea consumption, soy intake and zinc level with bone markers and found some relations [6–9].

Osteoporosis, the chronic disease of bone, characterized by deterioration of bone tissue, loss of bone mass and risk of fracture, is due to a change in the balance between activities of osteoblasts and osteoclasts. These specialized cells are responsible for bone formation and resorption. Negative balance between osteoblastic and osteoclastic activity would ultimately lead to osteoporosis [10]. The activities of the bone cells can be influenced by a variety of nutritional and cellular factors, including the supply of oxygen, nutrients, endocrines, cytokines, growth factors and free radicals. The differentiation of osteoblasts and osteoclasts are believed to be very important in the pathogenesis of osteoporosis [11,12]. Osteoblasts are derived from osteoprogenitors that reside in the bone marrow. Although evidence exists that many transcription factors and signaling pathways may be involved in osteoblast differentiation, the signaling mechanisms that decrease osteoblastic differentiation in osteoporosis are not well known [13,14].

Free radical products of oxygen metabolism, such as superoxide and hydrogen peroxide, are generated and released under environmental stimuli (e.g., cytokines, ultra violet radiation), and pathologic circumstances. These reactive oxygen species (ROS) are neutralized by the antioxidant system in the body. This system consists of agents such as vitamins E and C, reduced glutathione (GSH), glutathione peroxidase and superoxide dismutase (SOD). Oxidative stress occurs when there is an imbalance between free radical production and antioxidant capacity implicating in the pathogenesis of various chronic diseases including atherosclerosis, diabetes, neurodegenerative disorders, toxic exposure and in aging itself [15,16]. At the cellular level, oxidative injury expresses a wide spectrum of responses, ranging from proliferation to growth or differentiation arrests, senescence and cell death by activating numerous major signaling pathways, such as nuclear factor (NF)-κB, mitogen activated protein kinases (MAPKs), P 53 and heat shock factor (HSF) in a variety of cultured mammalian cell types. The severity of effect depends on the cell type and the concentrations of free radicals [17,18].

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 [19]. Osteoblasts produce antioxidants such as glutathione

Box 1. Risk factors for osteoporosis.

Risk factors that cannot be changed
- Race
- Sex and age
- Genetics
- Previous fractures

Risk factors that can be changed
- Chronic inactivity
- Microgravity
- Excessive sports activity
- Low body weight
- Low lifetime calcium intake
- Hormones
- Medication

Oxidative stress-related factors
- Smoking
- Low antioxidant status
- Excessive lipid intake
- Nutrition deficiency

peroxidase (GPX) to protect against ROS [20]. Several factors affect osteoclasts and, thereby, enhance ROS production. These factors are shown in **Figure 1** [19]. Under these conditions, the imbalance between ROS production and antioxidant mechanisms may be disturbing, resulting in oxidative stress which affects the bone and may causes osteoporosis. In fact, some investigators have suggested that osteoporosis is associated with biochemical markers of oxidative stress, such as urinary excretion of isoprostanes [21] and plasma antioxidants [22]. Basu and colleagues used the urinary excretion of the F2-isoprostane as a marker of *in vivo* oxidative stress and found that the formation and urinary excretion of this compound was negatively related to bone mineral density in individuals who were carefully characterized for other variables that could influence *in vivo* lipid peroxidation [21].

However, it has not been explained by which mechanism ROS assists in accelerating destruction of calcified tissue and bone resorption. In this paper, we attempted to describe the effects of oxidative stress and various factors on different bone cells and materials.

Oxidative stress & osteoporosis

A summary of information in this topic is provided in **Table 1**.

Osteoblasts

Reduction in skeletal mass appears to be associated with increased osteoclastic and decreased osteoblastic activity [23]. Oxidative stress has an inhibitory effect on osteoblasts. Mody and colleagues have demonstrated that oxidative stress inhibits differentiation of M2-10B4, a marrow stromal cell line and MC3T3-E1, a preosteoblastic cell line, by measuring alkaline phosphatase (ALP) as an early differentiation marker. Furthermore, oxidative stress inhibits mineralization in these cell lines. These effects of ROS were counteracted by the antioxidants trolox and pyrrolidine dithiocarbamate [24]. More recent data from rabbit bone marrow stromal cells (BMSCs) and calvarial osteoblasts demonstrated that a much lower dose of H_2O_2 (0.1 mM) inhibits expression of osteoblastic differentiation markers, while a higher dose of H_2O_2 (1 mM) induces cell death [25]. Necrosis of MC3T3-E1 cells has been induced by free radicals at high doses [26]. Liu and colleagues demonstrated that using exogenous metallothionein (MT) or inducing endogenous MT expression could protect BMSCs against H_2O_2 induced inhibition of osteoblastic differentiation, demonstrated by release of an H_2O_2 inhibited osteo-blastic differentiation marker (ALP). MT has free radical scavengering properties and is known to act in a similar fashion to GSH. The ability of MT to scavenge hydroxyl and superoxide radicals and function like superoxide dismutase in microorganisms has been proved [27]. Recently, it has been shown that MT inhibits H_2O_2-activated NF-κB signaling during osteoblastic differentiation of BMSCs and implied NF-κB in MT protection against H_2O_2-induced inhibition of osteoblastic differentiation [28].

Osteoclasts

ROS, such as superoxide and hydrogen peroxide, have been implicated as regulatory factors in the control of osteoclastic bone resorption. Generated superoxide from osteoclasts directly contributes to bone degradation. The presence of superoxide at the osteoclast–bone interface proposes a direct effect of superoxide in osteoclastic bone resorption [29,30]. In addition, inhibition of osteoclastic superoxide availability causes a reduction in bone resorption [31,32]. Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)-oxidase is the enzyme involved in the production of ROS by osteoclasts [33]. Treatment with interferon (IFN)-γ, a stimulator of NADPH oxidase activity, improves defective osteoclastic function in osteoporotic, microphtalmic mice *in vivo* and in calvaria

Box 2. Causes of osteoporosis.

Medication
- Chronic use of glucocorticoids (e.g., prednisone at 5 mg/day for more than 6 months)
- Excessive use of thyroxin
- Long-term use of certain anticonvulsants (e.g., phenytoin)
- Anticoagulant (e.g., heparin, warfarin)
- Cytotoxic agents
- Gonadotropin-releasing hormones agonists or analogues
- Intramuscular medroxyprogesterone contraceptive
- Immunosuppressives (e.g., cyclosporin)

Genetic disorders
- Hemophilia
- Thalessemia
- Hypophosphatasia
- Hemochromatosis

Disorders of calcium balance
- Hypercalciuria
- Vitamin D deficiency

Endocrinopathies
- Cortisol excess
- Cushing's syndrome
- Gonadal insufficiency (primary and secondary)
- Hyperthyroidism
- Type I diabetes mellitus
- Primary hyperparathyroidism

Gastrointestinal diseases
- Chronic liver disease (e.g., primary billiary cirrhosis)
- Malabsorption syndromes (e.g., celiac disease, Crohn's disease)
- Total gastrectomy
- Billroth I gastroenterostomy

Other disorders and conditions
- Multiple myeloma
- Lymphoma and leukemia
- Systemic mastocytosis
- Nutritional disorders
- Rheumatoid arthritis
- Chronic renal disease

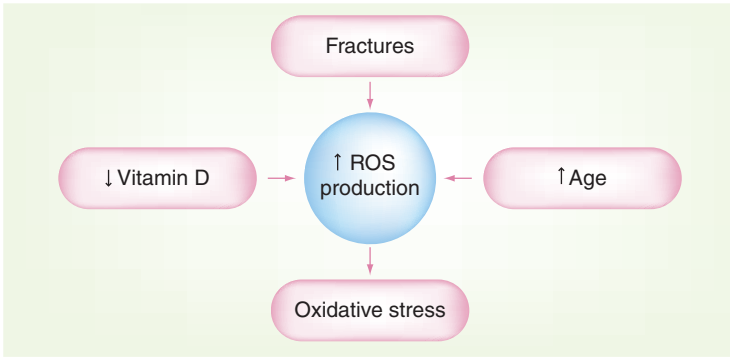
cultured from these animals [34]. The enzymatic generation of superoxide by xanthine and xanthine oxidase was accompanied by increased bone resorption in mouse calvarial organ cultures and injection of these agents over the calvarial bone of mice *in vivo* also caused a local increase in osteoclastic bone resorption. In contrast, there is evidence that H_2O_2 does not increase bone resorption [35]. Other experiments suggested that H_2O_2 may act directly to increase bone resorption by isolated osteoclasts and to increase osteoclast formation in marrow cultures [36,37]. Furthermore, the effects of H_2O_2 and superoxide on bone resorption in mouse calvarial organ cultures was investigated. H_2O_2 stimulated bone resorption in a concentration-dependent manner in calvarial organ cultures. In contrast, the combination of the xanthine–xanthine oxidase system, which generates superoxide anions, failed to stimulate bone resorption. Analysis of calvarial bones which were exposed to H_2O_2 showed a significant increase in osteoclast numbers suggesting that H_2O_2 may stimulate osteoclast formation in addition to enhancing activity of mature osteoclasts. H_2O_2 also exerted a direct stimulating effect on the generation of resorption pits by mature rat osteoclasts [38]. NF-κB is an oxidative stress-responsive transcription factor that is incorporated in gene activation and plays a critical role in various types of cellular activities associated with the regulation of cell growth, differentiation, death and development. In bone, its major role was marked by the phenotype of NF-κB knockout mice showing osteoporosis, chiefly due to impairment in osteoclastogenesis and osteoclastic function [39]. At the same time, several repots focused on the negative regulation of osteoblast differentiation by NF-κB in mouse osteoblastic (MC3T3) cells [40] and the human osteosarcoma cell line Saos-2 [41].

Several potential mechanisms might support the relationship between oxidative stress and bone loss. For example, NF-κB, which is known to mediate some of the important functions of tumor necrosis factor (TNF)-α, a cytokine produced in the bone microenvironment, on osteoclastogenesis, is activated in osteoblast-like cells by mitogens and cytokines through the production of ROS [39,42,43]. In other words, intercellular free radical production might signify the final common mechanism of NF-κB activation by various factors [44]. If this mechanism-of-action of osteclastogenic cytokines adequately confirms most models of bone loss, it is reasonable that low intracellular and probably interstitial levels of antioxidants are a signal, that is, a result of increased osteoclastogenic activity and bone turnover. Alternatively, it is also possible that low levels of intracellular antioxidants will exaggerate osteoclastogenesis through uncontrolled availability of excess ROS.

Extracellular matrix of bone

Oxidative stress also damages fibronectin, one of the major components of the extracellular matrix of bone. This glycoprotein acts as a substratum of the osteoblast and is involved in various cellular activities such as adhesion, proliferation, migration, cell shape and differentiation. Since

Figure 1. Conditions enhancing production of ROS in osteoclasts.



ROS: reactive oxygen species

the metabolic turnover of fibronectin is generally much slower than that of other cellular components, it will be affected by various nonenzymatic modifications, including the formation of oxygen-free radicals during the aging process. ROS causes partial degradation and modification of fibronectin molecules. These damaged fibronectin molecules lose their function in bone nodule formation [45].

Mitochondrial DNA deletions & osteoporosis

The mitochondrial genome of 15 men with symptomatic vertebral fractures was screened for the presence of mitochondrial DNA (mtDNA) deletions. Various deletions in mtDNA of these patients were found which span part of the gene coding and lead to inefficient oxidative phosphorylation, defective transport of electrons in the electron transport chain and an increase in oxygen-free radical production. Such defects would be expected to be associated with oxidative stress. mtDNA mutations are either maternally inherited or may be acquired through the accumulation of somatic alteration in the mitochondrial genome [46]. In this way, direct effects of oxidative stress on bone have been discussed but indirect effects remain to be reviewed after further study.

Cytokines & osteoporosis

Cytokine are important regulators of bone cell activity. For example, interleukin (IL)-1 is one of the most potent stimulators of bone resorption at high concentrations by increasing the number of osteoclasts by precursor fusion and inhibiting collagen synthesis. IL-6 appears to be a potent osteotropic factor that may play an important role in disease characterized with increased bone resorption and promotion of osteoclast fusion. TNF-α, as IL-1, enhances bone resorption by stimulating the development of osteoclast progenitors and increasing the activity of mature cells [47–49]. It has been demonstrated that oxidative stress is a powerful stimulant for the increased expression of pro-inflammatory cytokines in heart [50], inflammatory bowel disease [51], and pancreas [52]. It is suggested that oxidative stress has similar effects on bone and increases cytokine levels, thus inducing osteoporosis.

Antioxidants & osteoporosis

An informative table regarding the effects of different antioxidants in osteoporosis is provided (**Table 2**). Oxidative stress, defined as an imbalance between antioxidants and pro-oxidants, in consideration of the former, potentially causing damage, generally indicates that antioxidants are low and markers of oxidative stress are increased [53]. Lipid peroxidation is one of the most harmful effects of ROS, of which MDA is the end product [54] and it was seen that serum levels of MDA increased in bone disorders. Decreased activities of antioxidant enzymes superoxidase dismutase (SOD)

Table 1. Effects of ROS on different bone cells and materials.

Bone agents	Activity of ROS
Osteoblasts	Inhibition of differentiation
	Inhibition of mineralization
	Induction of necrosis
Osteoclasts	Enhancement of activity
	Stimulation of fomation
	Generation of resorption
Fibrinonectin	Partial degradation and modification
	Inhibition of bone nodule formation
Pro-inflamatory cytokines	Increasing expression

Table 2. The antioxidants and their mechanism of action on bone mass.

Antioxidant	Effect
Vitamin E	Vitamin E deficiency impairs calcium transport via the intestine and reducs bone density, free radical scavenger
Vitamin C	Enhances collagenogenesis, possible effect on bone formation, the antioxidant effect on osteoporosis is under investigation
Vitamin A	Excess vitamin A intake has been associated with accelerated bone loss but this requires further investigation
Genistein	Free radicals scavenger, inhibitor of cell-derived hydrogen peroxide formation
Ginkgo biloba	Free radical scavenger

and Glutathione peroxidase (GSH-PX) explain a defense mechanism that may have been overcome in alleviating increased superoxide production by osteoclasts displayed by increased levels of MDA in the serum. Antioxidants are generally believed to be protective against oxidative stress by several mechanisms (**Figures 2&3**) [55,56]. Indeed, it would appear to play a role in inhibition of osteoporosis caused by oxidative stress and despite experimental, and epidemiological evidence regarding the relationship between antioxidant vitamins and osteoporosis, there are no data on special antioxidant plasma levels in osteoporotic subjects.

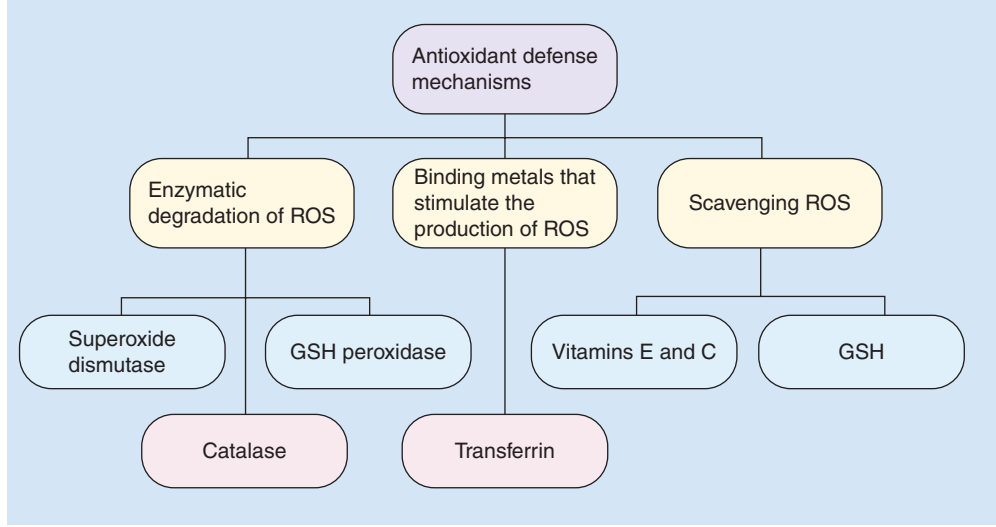
Vitamin E

Vitamin E is an important antioxidant and has been shown to offer protection against free-radical-associated diseases, such as atherosclerosis and cancer [57]. It has been shown that vitamin E deficiency impairs calcium transport via the intestines and reduces bone calcium content. This was thought to be due to impaired conversion of vitamin D3 to its active metabolites [58]. Not enough evidence was found to prove the benefits of vitamin E or β-carotene in postmenopausal femoral neck bone mineral density, but further studies on other bone sites such as the trabecular bone of the spine have been suggested [59].

Estrogen

Estrogen is an important hormone influencing bone mineral density. In addition to its hormonal effects on bone, its phenolic structure means that it shares similarities with the structure of well-known lipophilic antioxidants such as α-tocopherol. Therefore, it allows the molecule to detoxify accumulated ROS [60].

Figure 2. Mechanisms by which antioxidants counter the action of ROS.



GSH: Glutathione, ROS: Reactive oxygen species.

Estrogen deficiency, after menopause leads to bone loss through increased osteoclastic function and subsequently demonstrates the major pathologic determinant responsible for postmenopausal bone loss, as a result of osteoclast stimulation by ROS [10,60].

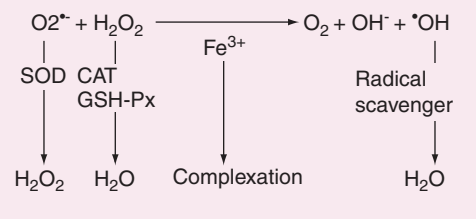
Genistein

The effects of genistein, an isoflavone, on the viability of MC3T3-E1 cells under oxidative stress induced by free radicals during cell proliferation, have been investigated. It has been shown that genistein is the most potent antioxidant among the isoflavones in both the aqueous and lipophilic phases of cells. Genistein is not only an effective scavenger of free radicals *in vitro,* but also a strong inhibitor of cell-derived H_2O_2 formation *in vivo*. Genistein was found very effective in decreasing the effects of oxidative agents at physiologic concentrations. In addition, the antioxidative properties of genistein at physiologic concentrations (10^{-7}–10^{-10}M) were more effective than the actions of vitamin C and E in protecting against cellular damage of free radicals [17].

Ginkgo biloba

An extract of *Ginkgo biloba,* which contains several antioxidative polyphenolics, may prevent bone loss and osteoporosis-related bone fractures. The effects of this herbal product (EGb-761) on the proliferation of MC3T3-E1 cells, an osteoblast-like cell line, have been examined. A significant increase by 3.72-fold in MC3T3-E1 cell proliferation was found with treatment of EGb-761 at 1000 μg/ml compared with the control (vehicle) group and 3.86-fold with treatment of EGb-761 at 2000 μg/ml compared with control. Two mechanisms have been suggested for this effect. Firstly, EGb-761 may act by facilitating glucose uptake and pyruvate concentrations by these bone cells which leads to increased cellular energy, and hence, proliferation. Secondly, the complex ring structure of one or more of the molecules in EGb-761 appears to scavenge the free radicals effectively by successfully accepting free electrons which eliminates the damaging properties of the free radicals [26].

Figure 3. Targets of antioxidants.



CAT: Catalase; GSH-Px: Glutathione peroxidase; SOD: Superoxide dismutase.

Vitamin C

Vitamin C is a key antioxidant vitamin, as well as being an essential co-factor in the formation of stable collagen, both in growing and in mature connective tissue [61,62]. Scorbut, a condition of vitamin C deficiency, establishes a nonorganized and thinner growth plate, thinner trabecular network, very low collagen synthesis and decreased differentiation of osteoblasts from mesenchymal cells. In preosteoblastic and osteoblast-like cell lines, vitamin C enhances collagenogenesis, vitamin D-stimulated expression of ALP and on the whole mineralization [63–67]. It seems that vitamin C is the antioxidant with the most significant evidence for a possible effect on bone formation/bone loss. It has also been assumed that TGF-β may act through vitamin C to stimulate osteoblast differentiation and then bone formation [68]. Moreover, guinea-pig studies have demonstrated that low vitamin C intake in the growing animal is associated with higher-than-normal bone turnover [69]. Several epidemiologic studies have shown an association between dietary vitamin C intake and bone mineral density (BMD) in postmenopausal women. This evidence is reasonable for early postmenopausal subjects who have a daily intake of at least 500 mg calcium [70–72]. Feasible indications of a correlation between antioxidants and bone health derive from studies of isoflavones, weak bone-sparing agents that have been shown to display significant antioxidant properties in studies conducted *in vitro* on sperm and lymphocytes and *in vivo* on low-density lipoprotein (LDL) oxidation in humans [73–76]. Despite the experimental and epidemiological evidence relation between antioxidant vitamins to osteoporosis and fracture risk, there are no data on vitamin C or other antioxidant plasma levels in osteoporotic subjects. A negative role for antioxidant deficit in age-related bone loss has been reported [22]. Data from various observational studies, although not in conformity, seem to show a positive role for vitamin C in contrasting age-related bone loss of women in their early and midpostmenoposaul years, especially if they were calcium, but not estrogen repleted [71–73].

In addition, dietary intake of vitamin C and E has demonstrated protective against hip fracture in a selective subset of female smokers chosen from a large population of women followed prospectively for up to 5 years [77].

Vitamin A

In contrast, excess dietary retinol (vitamin A) intake has been recently recognized as a risk factor for hip fracture and has been associated with accelerated bone loss [78–81]. According to some reports, this nutrient could provide a reasonable explanation for the higher incidence of osteoporotic fractures in Sweden and Norway, where the diet is particularly rich not only in calcium and vitamin D compared with the rest of Europe, but also in vitamin A, contained in cod liver oil, dietary products and milk usually fortified with vitamin A and D [78]. Nevertheless, some data seem to contrast with this view, which shows lower levels of vitamin A in osteoporotics [22]. Furthermore, vitamin A was the dietary antioxidant most strongly and positively correlated with bone mass in the osteoporotic population. The investigators conclude by indicating at the presence of an elegant balance between confirming adequate vitamin A intake and exaggerated age-related bone loss due to excessive retinol supplementation. For any condition or disease in which a decrease in antioxidant defence is established, the question has to be answered whether this is principally due to an increased production of free radicals or to a poor dietary antioxidant intake. When free radicals are generated in greater quantities than the body can neutralize them, a state of oxidative stress takes place.

Expert commentary & outlook

Bone metabolism depends on osteoblastic and osteoclastic activity. These two types of cells are derived from progenitors. The development of osteoclasts from their progenitors depends on stromal-osteoblastic cells that is an important source of critical cytokines in osteoclastogenesis, such as IL6 and IL-11. The production of these cytokines and the response of bone marrow cells to them are regulated by sex hormones. Decreasing gonadal function, followed by increasing age, results in increasing the formation of osteoclasts and osteoblasts in the bone marrow, which is mediated, not only by hyperproduction of cytokines, but also hyper-responsiveness of progenitor cells to IL-6 and the other cytokines.

Conversely, it has been demonstrated that free radicals are involved in osteoclastogenesis and bone resorption. NF-κB is an oxidative stress responsive transcription factor and recently, some of the studies found that a lack of NF-κB in mice can result in osteoporosis. Thus, free radicals may increase bone resorption through activation of NF-κB. Several risk factors for osteoporosis, such as hypertension, smoking, diabetes mellitus and aging are associated with oxidative stress or decreased natural antioxidants [36,41].

There is some evidence demonstrating the role of unknown environmental factors, such as diet, on the development of osteoporosis. Although studies on vitamin C and its relation to BMD are conflicting, a positive association has been established. Recently it has been shown that vitamin C supplementation has a beneficial effect on BMD in postmenopausal women [82].

Accordingly, further studies are needed to elucidate the important roles of pro- and antioxidants in osteoporosis.

Highlights

- Reactive oxygen species (ROS) enhance resorption by isolated osteoclasts and increases osteoclast formation in marrow culture.
- Oxidative stress inhibits differentiation and mineralization and induces necrosis of osteoblasts.
- ROS causes partial degradation and modification of fibronectin molecules. These damaged fibronectin molecules lose their function in bone nodule formation.
- Oxidative stress may increase expression of cytokines in the bone and thus induce osteoporosis.
- Antioxidants may have a role in the inhibition of the effects of free radicals on the bone and hence in the prevention of osteoporosis.

Bibliography

1. Conference report: Consensus development conference: prophylaxis and treatment of osteoporosis. *Am. J. Med.* 90, 107–110 (1991).
2. Rao LG. Will tomatoes prevent osteoporosis? *Endocr. Rounds* 5(2), (2005).
3. Shamshirsaz A, bekheinia MR, Larijani B *et al.* Metabolic and endocrinological complication in β-thalessemia major: A multicenter study in Tehran. *BMC Endocr. Disord.* 3(1), 4 (2003).
4. Hashemipour S, Larijani B, Adibi H *et al.* Vitamin D deficiency and causative factors in the population of Tehran. *BMC Public Health* 4(1), 38 (2004).
5. Mckenna MJ, Differences in vitamin D status between countries in young adults and elderly. *Am. J. Med.*. 93(1), 69–77 (1992).

6. Larijani B, Bekheirnia MR, Soltani A, Khalilifar A, Adibi H, Jalili RB. Bone mineral density is related to blood pressure in men. *Am. J. Hum. Biol.* 16(2), 168–171 (2004).
7. Hosseinnezhad A, Soltani A, Rahimi I, Shafaie A, Maghbooli Z, Larijani B. Relation between tea drinking and bone mineral density. *Tabib-Shargh* 2(1), 29–38 (2003).
8. Haghighian A, Tahbaz F, Larijani B *et al*. Effect of soy protein on bone metabolism biomarkers in postmenopausal women with osteopenia *J. Nutr.*. (2005) (In press).
9. Bekhaiia MR, Shamshirsaz A, Larijani B *et al*. Serum zinc and its relation to bone mineral density in β-thalassemic adolescent. *Biol. Trace Elem. Res.* 97(3), 215–224 (2004).
10. Monologas SC, Jilka RL. Bone marrow, cytokines and remodeling: Emerging insights into the pathophysiology of osteoporosis. *N. Engl. J Med.* 322, 305–311 (1995).
11. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature* 423, 337–342 (2003).
12. Monolagas SC. Birth and death of bone cells. basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr. Rev.* 21, 115–117 (2000).
13. Ducy P, Zhang R, Geoffory V, Ridal AL, Darsenty G. Osf2/Runx2: a transcriptional activator of osteoblast differentiation. *Cell* 89, 747–754 (1997).
14. Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. *Nature* 423, 349–355 (2003).
15. Abdollahi M, Ranjbar A, Shadnia S, Nikfar S, Rezaiee A. Pesticides and oxidative stress: a review. *Med. Sci Monit.* 10(6), RA144–RA147 (2004).
16. Finkel T, Holbrook NJ. Oxidants, oxidative stress and biology of aging. *Nature* 408, 239–247 (2000).
17. Lee YS, Chen X, Anderson JJB. Physiological concentrations of genistein stimulate the proliferation and protect against free radical-induced oxidative damage of MC3T3-E1 osteoblast-like cells. *Nutr. Res.* 21, 1287–1298 (2001).
18. Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suiside and survival. *J. Cell. Physiol.* 192, 1–15 (2002) (2001).
19. Sontakke AN, Tare RS. A duality in the roles of reactive species with respect to bone metabolism. *Clin. Chim. Acta* 318, 145–148 (2002).
20. Dreher I, Schuetz N, Baur A *et al*. Selenoproteins are expressed in fetal human osteoblast-like cells. *Biochem. Biophys. Res. Commun.* 245, 101–107 (1998).
21. Basu S, Michaelsson K, Olofsson H *et al*. Association between oxidative stress and bone mineral density. *Biochem. Biophys. Res. Commun.* 288, 275–279 (2001).
22. Maggio D, Barabani M, Pierandrei M *et al*. Marked decrease in plasma anti-oxidants in aged osteoporotic women: results of a cross-sectional study. *J. Clin. Endocrinol. Metab.* 88, 1523–1527 (2003).
23. Heaney RP, Rehecher RR *et al*. Menopousal changes in bone remodeling. *J. Lab. Clin. Med.* 92(6), 953–963
24. Mody M, Parhami F, Sarafian TA, Demer LL. Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. *Free Radical Bio. Med.*. 31(4), 509–519 (2001) (1978).
25. Bai XC, Lu D, Bai J *et al*. Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-κB. *Biochem. Biophys. Res. Commun.* 314, 197–207 (2004).
26. Brayboy JR, Chen XW, Lee YS, Anderson JJB. The protective effects of Ginkgo biloba extract (EGb 761) against free radical damage to osteoblast-like bone cells (MC3T3-E1) and the proliferative effects of EGb 761 on these cells. *Nutr. Res.* 21, 1275–1285 (2001).
27. Thornalley PJ, Vasak M. Possible role for metallothionein in protection against radiation induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochim. Biophys. Acta* 827, 36–44 (1985).
28. Liu A, Zhang Z, Zhu B, Liao Z, Liu Z. Metallothionein protects bone marrow stromal cells against hydrogen-peroxide induced inhibition of osteoblastic differentiation. *Cell. Biol. Intern.* 28, 905–911 (2004).
29. Key LL, Ries WL, Taylor RG *et al*. Oxygen-derived free radicals in osteoclasts: the specificity and location of the nitroblue tetrazolium reaction. *Bone* 11, 115–119 (1990).
30. Key LL, Wolf WC, Guadberg CM *et al*. Superoxide and bone resorption. *Bone* 5, 431–436 (1994).
31. Darden AG, Ries WL, Wolf WC *et al*. Osteoclastic superoxide production and bone resorption: stimulation and inhibition by modulators of NADPH oxidase. *J. Bone Miner. Res.* 11, 671–675 (1996).
32. Ries WL, Key LL, Rodriguiz R. Nitroblue tetrazolium reduction and bone resorption by osteoclasts *in vitro* inhibited by a manganese-based superoxide dismutase mimic. *J. Bone Miner. Res.* 7, 931–939 (1992).
33. Steinbeck MJ, Appel WH, Verhoeven AJ, Karnovsky MJ. NADPH-Oxidase expression and in situ production of superoxide byosteoclasts actively resorbing bone. *J. Cell. Biol.* 126(3), 765–772 (1994).
34. Rodriguiz RM, Key LL, Ries WL. Combination macrophage colony-stimulating factor and interferon-γ administration ameliorates the osteopetrotic condition in microphthalmic (mi/mi) mice. *Pediatr. Res.* 33, 382–389 (1993).
35. Garrett IR, Boyce BF, Oreffo ROC, Bonewald L, Poster J, Mundy GR. Oxygen-derived free radicals stimulates osteoclastic bone resorption in rodent bone *in vitro* and *in vivo*. *J. Clin. Invest.* 85, 632–639
36. Bax BE, Alam ASTM, Banerji B *et al*. Stimulation of osteoblastic bone resorption by hydrogen peroxide (H2O2). *Biochem. Biophys. Res. Commun.* 183, 1153–1158 (1992) (1990).
37. Suda N, Morita I, Kuroda T, Murota S. Participation of oxidative stress in the process of osteoclast differentiation. *Biochim. Biophys. Acta.* 1157, 318–323 (1997).
38. Fraser JHE, Helfrich MH, Wallace HM, Ralston SH. Hydrogen peroxide, but not superoxide, stimulates bone resorption in mouse calvariae. *Bone* 19(3), 223–226 (1996).
39. Iotsova V, Caamano J, Loy J. Osteoporosis in mice lacking NF-κB1 and NF-κB2. *Nature Med.* 3, 1285–1289 (1997).
40. Deyama Y, Takeyama S, Suzuki K *et al*. Inactivation of NF-κB involved in osteoblast development through IL-6. *Biochem. Biophys. Res. Commun.* 282, 1080–1084 (2001).
41. Andela VB, Sheu TJ, Puzas EJ *et al*. Malignant revesion of a human osteosarcoma cell line, Saos-2, by inhibition of NF-κB. *Biochem. Biophysic. Res. Commun.* 297, 237–241 (2002).
42. Schreck R, Rieber P, Baeuerle P A. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO J.* 10, 2247–2258 (1991).
43. Sen CK, Packer I. Anti-oxidant and redox regulation of gene transcription. *FASEB J.* 10, 709–720 (1996).

44. Kurokouchi K, Kambe F, Yasukawa K *et al.* TNF-α increases expression of IL-6 and ICAM-1 gene through activation of NF-κB in osteoblast like ROS17/2.8 cells. *J. Bone Miner. Res.* 13, 1290–1299 (1998).
45. Suzuki H, Hayakawa M, Kobayashi K, Takiguchi H, Abiko Y. H_2O_2-derived free radicals treated fibrinectin substratum reduces the bone nodule formation of rat calvarial osteoblast. *Mech. Ageing Dev.* 98, 113–125 (1997).
46. Varanasi SS, Francis RM, Berger CEM, Papiha SS, Datta HK. Mitochondrial DNA Deletion Associated Oxidative Stress and Severe Male Osteoporosis. *Osteoporos. Int.* 10, 143–149 (1999).
47. Zheng SX, Vrindts Y, Lopez M *et al.* Increase in cytokine production (IL-1 β, IL-6, TNF-α but not IFN-γ, GM-CSF or LIF) by stimulated whole blood cells in postmenopausal osteoporosis. *Maturitas* 26, 63–71 (1997).
48. Baker PJ. The role of immune responses in bone loss during periodontal disease. *Microbes Infect.* 2, 1181–1192 (2000).
49. Ralston SH, Grabowski PS. Mechanisms of cytokine induced bone resorption: role of nitric oxide, cyclic guanosine monophosphate, and prostaglandins. *Bone* 19(1), 29–33 (1996).
50. Sia YT, Parker TG, Liu P, Tsoporis JN, Adam A, Rouleau JL. Improved post-myocardial infarction srvival with pobucol in rats: effects on left ventricular function, morphology, cardiac oxidative stress and cytokine expression. *J. Am. Coll. Cardiol.* 39(1), 148–156 (2002).
51. Afshari M, Larijani B, Abdollahi M *et al.* Ineffectiveness of allopurinol in reduction of oxidative stress in diabetic patients; A randomized, double-blind placebo-controlled clinical trial. *Biomed. Pharmacother.* 58, 546–550 (2004).
52. Astaneie F, Larijani B, Abdollahi M *et al.* Alterations in anti-oxidant power and levels of epidermal growth factor and nitric oxide in blood and saliva of diabetic Type 1 patients. *Arch. Med. Res.* 36, 376–381 (2005).
53. Polidori MC, Stahl W, Eichler O *et al.* Profiles of anti-oxidants in human plasma. *Free Radic. Biol Med.* 30, 456–462 (2001).
54. Eberhardt MK. *Reactive oxygen metabolites: Chemistry and medical consequences.* CRC Press, FL, USA, 264 (2001).
55. Penckofer S, Schwertz D, Flerczak K. Oxidative stress and cardiovascular disease in Type 2 diabetes: The role of anti-oxidants and pro-oxidants. *J. Cardiovasc. Nurs.* 16(2), 68–85 (2002).
56. Korachich GB, Mishra OP. Lipid peroxidation in rat brain cortical slices as measured by TBA test. *J. Neurochem.* 35, 1449–52 (1980).
57. Ozer NK, Sirikci O, Taha S *et al.* Effect of vitamin E and probucol on dietary cholesterol-induced atherosclerosis in rabbits. *Free Radic. Biol. Med.* 24, 226–233 (1998).
58. Ima-Nirwana S, Suhaniza S. Effects of tocopherols and tocotrienols on body composition and bone calcium content in adrenalectomized rats replaced with dexamethazone. *J. Med. Food* 7(1), 45–51 (2004).
59. Behl C. Estrogen can protect neurons: modes of action. *J. Steroids Biochem. Mol. Biol.* 83, 195–197 (2003).
60. Oeffinger KC, Scurvy. more than historical relevance. *Am. Fam. Physician.* 48, 609–613 (1993).
61. Peterkofsky B. Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. *Am. J. Clin. Nutr.* 54, 1135S-1140S (1991).
62. Sugimoto T, Nakada M, Fukase M *et al.* Effects of ascorbic acid on alkaline phosphatase activity and hormone responsiveness in the osteoblastic osteosarcoma cell line UMR-106. *Calcif. Tissue Int.* 39, 171–174 (1986).
63. Aronow MA, Gerstenfeld LC, Owen TA *et al.* Factors that promote progressive development of osteoblast phenotype in cultured fetal rat calvarial cells. *J. Cell. Physiol.* 143, 213–221 (1990).
64. Owen TA, Aronow MA, Shalhoub V *et al.* Progressive development of the rat osteoblast phenotype *in vitro*: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the one extracellular matrix. *J. Cell. Physiol.* 143, 420–430 (1990).
65. Franceschi RT, Lyer BS . Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *J. Bone Miner. Res.* 7, 235–246 (1992).
66. Franceschi RT, Young J. Regulation of alkaline phosphatase by 1,25-dihydroxyvitamin D3 and ascorbic acid in bone-derived cells. *J. Bone Miner. Res.* 5, 1157–1167 (1990).
67. Dixon SJ, Wilson JX. Transforming growth factor-β stimulates ascorbate transport activity in osteoblastic cells. *Endocrinology* 130, 484–489 (1992).
68. Kipp DE, Grey CE, Mcelvian ME *et al.* Long-term low ascorbic acid intake reduces bone mass in guinea-pig. *J. Nutr.* 126, 2044–2049 (1996).
69. Hall Sl, Greendale CE. The relation of dietry vitamin C intake to bone mineral density: results from the PEPI study. *Calcif. Tissue Int.* 63, 183–189 (1998).
70. Leveille SG, Lacroix AZ, Koepsell TD *et al.* Dietary vitamin C and bone mineral density in postmenopausal women in Washington state, USA. *J Epidemiol. Commun. Health* 51, 479–485 (1997).
71. Wang MC, Luz Villa M, Marcus R *et al.* Associations of vitamin C, calcium, and protein with bone mass in postmenopausal Mexican American women. *Osteoporo. Int.* 7, 533–538 (1997).
72. Viereck V, Grundker C, Blaschke S *et al.* Phytoestrogen genistein stimulates the production of osteoprotegrin by human trabecular osteoblasts. *J. Cell. Biochem.* 84, 725–735 (2002).
73. Sierens J, Hartley JA., Campbell MJ *et al.* *In vitro* isoflavone supplementation reduceshydrogen peroxide-induced DNA damage in sperm. *Teratog. Carcinog. Mutagen.* 22, 227–234 (2002).
74. Sierens J, Hartley JA, Campbell MJ *et al.* Effect of phytoestrogen and anti-oxidant supplementation on oxidative DNA damage assessed using the comet assay. *Mutat. Res.* 485, 169–176 (2001).
75. Wiseman H, Reilly JD, Adlercreutz H *et al.* Isoflavone phytoestrogens consumed in soy decrease F2 isoprostane concentrations and increase resistance of low-density lipoprotein to oxidation in humans. *Am. J. Clin. Nutr.* 72, 395–400 (2000).
76. Melhus H, Michaelsson K, Holmberg L *et al.* Smoking anti-oxidant vitamins, and the risk of hip fracture. *J. Bone Miner. Res.* 14, 129–35 (1999).
77. Melhus H, Michaelsson K, Kindmark A *et al.* Excessive dietary intake of vitamin A is associated with reduced bone mineral density and increased risk for hip fractures. *Ann. Intern. Med.* 129, 770–8 (1998).
78. Whiting SJ, Lemke B. Excess retinol intake may explain the high incidence of osteoporosis in northern Europe. *Nutr. Rev.* 57, 192–198 (1999).
79. Feskanich D, Singh V, Willet WC *et al.* Vitamin A intake and hip fractures among postmenopausal women. *JAMA* 287, 47–54 (2002).

80. Promislow JHE, Goodman-Gruen D, Slymen DJ *et al*. Retinol intake and bone mineral density in the elderly. The Rancho Bernardo Study. *J. Bone Miner. Res.* 17, 1349–1358 (2002).
81. Anderson JJB. Oversupplementation of vitamin A and osteoporotic fractures in the elderly: to supplement or not to supplement with vitamin A. *J. Bone Miner. Res.* 17, 1359–1362 (2002).
82. Morton DJ, Barrett-Connor EL, Schneider DL. Vitamin C supplement use and bone mineral density in postmenopausal women. *J. Bone Miner. Res.* 16, 135–140 (2001).

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