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Mini Review

# FoxOs, Wnts and oxidative stress-induced bone loss: new players in the periodontitis arena?

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*Background and Objective:* Chronic periodontitis is a widespread disease affecting tooth-supporting structures that can lead to extensive loss of periodontal ligament and bone, ultimately resulting in tooth loss. Extensive evidence has demonstrated a strong association between age, metabolic disorders such as type II diabetes, oxidative stress and alveolar bone loss. The molecular players controlling bone maintenance and underlying age-related bone loss and its links to the general metabolism are currently the object of intense research.

*Material and Methods:* Recent findings are summarized to elucidate the molecular mechanisms linking oxidative stress, bone loss and metabolic factors.

*Results:* It is well known that reactive oxygen species are an inevitable consequence of cellular respiration and that organisms have developed an efficient array of defenses against them. The core of this complex defense line is a family of transcription factors, known as FoxOs, which can bind to β-catenin and initiate a transcriptional programme regulating cell apoptosis, DNA repair and degradation of reactive oxygen species. An increase in reactive oxygen species due, for example, to age or insulin resistance, generates a situation in which bone formation is impaired by activation of FoxO, and a decrease in Wnt signaling and bone resorption are promoted.

*Conclusion:* The balance between FoxO and the Wnt pathway is finely tuned by systemic and local factors, creating a far-reaching mechanism that dictates the fate of mesenchymal progenitors and regulates the homeostasis of bone, providing a rationale for the impairment of systemic and alveolar bone maintenance clinically observed with age and metabolic diseases.

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Periodontis is an inflammatory disease that affects tooth-supporting structures and can lead to loss of periodontal ligament and alveolar bone, with increased tooth mobility and eventually tooth loss. Its pathogenesis is multifactorial and involves the periodontal microbiota, the host's immune responses and behavioral or concomitant medical conditions. There appears to be an epidemiological association between periodontitis and metabolic diseases, including diabetes, and an association between periodontitis and aging has long been suggested, although not conclusively proved (1–13). Importantly, the main factor that seems to be linking all these medical conditions is increased oxidative stress (3,14–16). Humans live in an oxygen-rich environment, and their survival depends on a subtle mitochondrial electric current that generates ATP, the common energy source for all cells. Electrons, however, can escape from the mitochondrial transport chain and generate reactive oxygen species

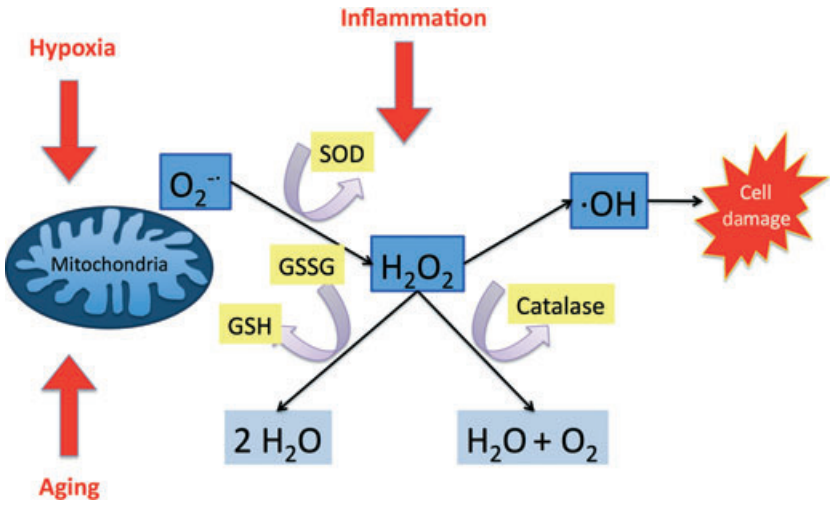


*Fig. 1.* Diagram showing generation of reactive oxygen species (ROS) in the mitochondria and antioxidant cellular defenses. Hypoxia, aging and inflammation can increase the formation of highly reactive oxygen species, such as superoxide radicals ($O_2^-$). These can be converted to $H_2O_2$ by the enzyme superoxide dismutase (SOD). Hydrogen peroxide can then be converted to $H_2O$ by glutathione or the enzyme catalase. Accumulation of ROS in the cells can lead to cellular damage. GSSG, oxidized glutathione; GSH, reduced glutathione.

(ROS), such as hydrogen peroxide ($H_2O_2$), oxygen superoxide ($O_2^-$) or the hydroxyl radical ($OH^-$; Fig. 1; 17–19). These are highly reactive oxygen radicals that are responsible for most oxidative stress in cells (20–22). Although low levels of ROS are, in fact, necessary for several cellular signaling pathways, such as ERKs, p38 MAPK and tyrosine phosphatases (23,24), their continuous production within cellular organelles causes cellular microdamage that accumulates over time (25–28). Broadly speaking, oxidative stress can cause cellular damage and death in many tissues, and it has been inversely correlated with lifespan in nematodes, flies and mammals (29), to the point of being considered the primary force driving aging. Increased oxidative stress is a hallmark of inflammation, because it can be produced by neutrophils as a defense against invading microorganisms or by the microorganisms themselves, and this is well known to contribute to tissue damage (30–34). Importantly, several pathologic conditions, such as wound healing, ischemia, or diabetes-related changes in the microvasculature, are associated with hypoxia, a shortage of oxygen supply (35,36). Although apparently in contrast, hypoxia and generation of ROS are coupled processes. During hypoxia, ROS can be released into the cytoplasm as a consequence of mitochondrial failure. Reactive oxygen species have also been demonstrated to impair the immune responses to microorganisms, and several reports directly suggest that oxidative stress is an important factor in the pathogenesis of periodontitis (30,37–39). Moreover, recent clinical studies have demonstrated a higher concentration of oxygen metabolites in the serum of periodontal patients (40,41), a reduction in the serum levels of oxidized low-density lipoproteins (42) or an increase in total antioxidant capacity after periodontal treatment (43–45). Some studies showed a relation between serum (46,47) or salivary antioxidants (48,49) as predictors of the insurgence of periodontitis or peri-implantitis.

As aging and a wealth of metabolic disorders have been associated with both systemic and alveolar bone loss, and the same conditions are also characterized by a marked increase in oxidative stress, the idea that oxidative stress is the culprit for bone loss is becoming increasingly attractive (3,50). To support this hypothesis, some recent studies have shown that bone formation in young mice is decreased by inhibiting the antioxidant glutathione, that increased lipid oxidation may reduce pro-osteogenic stimuli in the skeleton (51) and that administration of the antioxidant *N*-acetylcysteine reverses bone loss in a murine model of estrogen deficiency (52–55). Interestingly, Toker *et al.* (31) showed that the same antioxidant, *N*-acetylcysteine, can reduce alveolar bone loss in a rat periodontitis model, while Tomofuji *et al.* (56) demonstrated that a cocoa-enriched diet protected rats from periodontitis-induced alteration in serum antioxidant levels and inhibited alveolar bone loss.

The molecular mechanisms that link oxidative stress and bone loss are complex and still not fully understood. However, understanding them can provide a critical key to hamper or prevent bone loss in clinical conditions of increased oxidative stress. Recent exciting discoveries about the signal pathways controlling the balance between cell fate and the cellular defenses against ROS can provide a rationale for many important clinical observations and help identify potential therapeutic targets.

An association between periodontal disease and metabolic syndrome has been established. These medical conditions are characterized by increased oxidative stress. It has been shown that Reactive oxygen species can induce bone loss, thus providing a rationale for tissue destruction in periodontitis.

## The FoxO family of transcription factors

Since oxidative stress is an inevitable and, within certain limits, not undesired side-effect of cell respiration, cells have developed several antioxidant mechanisms to contain ROS-mediated damage, while allowing them to function in cell signaling. Some of these defenses rely on thiol-containing peptides, such as glutathione and thioredoxine, which can reduce ROS into harmless alcohols (57). Cells can, however, also resort to more sophisticated transcriptional programmes, such as the ones controlled by FoxOs.

FoxOs are members of the O ('other') class of the Forkhead superfamily (58), originally called FKHRs

(forkhead in rhabdomyosarcomas). Four members of this class are known at present, FoxO1, FoxO3, FoxO4 and FoxO6, of which FoxO1–3 are broadly expressed, whereas FoxO6 is restricted to the developing brain (60). They are all characterized by a 100 amino-acid helix–loop–helix DNA binding domain, called the Forkhead domain (61,62), which is recognized by a consensus sequence (G/T)(T/A)AA (C/T)AA, called the FoxO-recognized element (FRE; 63–65). FoxOs can move from the nucleus, where they act as transcription factors, to the cytoplasm, where they are inactive, depending on their phosphorylation state (62). In the absence of growth factors or insulin or in the presence of stress stimuli, FoxOs reside in the nucleus and actively transcribe several target genes. Some of them are involved in apoptosis signaling, like the tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL), Fas ligand and Bim (66,67). Interestingly, it has been shown that the expression of a phosphorylation-resistant form of FoxO3 induces cell apoptosis (61). FoxOs can also induce cell cycle arrest (68–70) by up-regulating the expression of the cyclin-dependent kinase inhibitor, $p27^{KIP1}$ (71), of the retinoblastoma protein p130 (72) and down-regulating cyclin D1 and D2 (73,74), a critical function, because an inactivation of FoxO activity can be found in 60–80% of prostate cancers (75). FoxOs antagonize oxidative stress through the transcription of manganese superoxide dismutase (MnSOD), which catalyzes the transformation of $O_2^-$ into $H_2O_2$, catalase, which neutralizes $H_2O_2$, and Growth Arrest and DNA Damage 45, which repairs DNA damage (68,76,77). By promoting cell cycle arrest and quiescence, FoxOs help cells to survive and counteract the action of ROS (67,78). The balance between the pro-apoptotic and the prosurvival actions of FoxOs depends on mechanisms not yet completely clear. However, it is known that the acetylation state of FoxO plays a role in the preferential expression of a subset of genes (79). Sirtuins (SIRTs) have been shown to deacetylate FoxOs in response to oxidative stress, facilitating the expression of genes involved in defense against ROS and cell cycle arrest (80–82).

The pivotal importance of FoxOs for the organism is highlighted by the dramatic consequences of FoxO deletion in mice. FoxO1 null mice die during embryonic development because of defects in angiogenesis (83,84), whereas FoxO3 null mice present with impaired fertility and lymphoproliferative disorders (85,86). Most interestingly, conditional FoxO1,2,3 triple knockout mice show hemopoietic defects, because of increased ROS levels in the hemopoietic stem cells. Reactive oxygen species, in turn, deplete the stem cell reservoir in the bone marrow, by driving hemopoietic stem cells into the cell cycle and differentiation, which is reversed by administration of *N*-acetylcysteine (NAC), a potent antioxidant (78).

Two very recent works have greatly elucidated the importance of FoxO for bone maintenance. Rached *et al.* (87) showed that α1(I) *Collagen-Cre*-mediated conditional deletion of FoxO1 from osteoblasts decreased bone mineral density in the spine and femur. Reduced osteoblast number, bone formation rate and bone volume were observed at histology. Moreover, $FoxO1^{-/-}$ bone phenotype did not progress with age, so these mice lacked age-related bone changes. At the cellular level, the authors showed a decrease in osteoblast proliferation and in antioxidant defense responses and, noticeably, FoxO1 overexpression or NAC administration reversed the bone phenotype. Ambrogini *et al.* (88) conditionally deleted FoxO1,3,4 using the interferon-inducible transgene *Mx-Cre*, and demonstrated a reduction in bone mineral density, osteoblast number, bone formation rate and an increase in osteoblast apoptosis 5 wk after deletion. Conversely, FoxO3 overexpression increased bone mass and reduced oxidative-stress-induced apoptosis (88).

FoxO transcription factors induce the expression of genes controlling defenses against oxidative stress and cell survival. Genetic ablation of FoxOs decreases bone mass and osteoblast numbers. Treatment with antioxidants can reverse this phenotype.

## FoxO control by Akt: the metabolic link

FoxOs possess evolutionarily conserved phosphorylation sites for the survival kinase Akt near the Forkhead domain, on threonine 24, serine 256 and serine 319 (62,68). Akt, also known as protein kinase B, is a serine/threonine kinase activated by a number of receptor tyrosine kinases and G protein-coupled receptors (89,90) through phosphatidyl inositol 3-kinase (PI3K) (91) and its product, phosphatidylinositol 3,4,5-triphosphate ($PIP_3$). $PIP_3$ recruits Akt to the cell membrane, where it phosphorylates FoxO, which is bound by the 14-3-3 chaperone proteins and is thus retained in the cytoplasm. Akt activation is antagonized by the protein Klotho and by phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which removes the 3′ phosphate from $PIP_3$ and thereby attenuates PI3K signaling (92). In the presence of ROS, PTEN is activated, and as a consequence Akt is down-regulated, removing its inhibition on FoxO, which is then free to initiate the transcription of defense factors against oxidative stress. Akt, in contrast, can also increase ROS generation by controlling cell metabolism and oxygen consumption. Akt1 and 2 double knockout mouse embryonic fibroblasts (MEFs) had significantly lower intracellular ROS levels than wild-type MEFs, whereas cells expressing activated Akt or MEFs from $PTEN^{-/-}$ mice showed increased ROS (93). Moreover, activation of Akt provides protection from apoptosis, leads to uncontrolled cell replication and hyperplastic lesions in SCID mice (94). Not unexpectedly then, it also sensitizes cells to oxidative damage (93,95).

The Akt–FoxO axis is of critical importance in the regulation of cell metabolism. Insulin, glucagon-like peptide 1 or insulin-like growth factor induce Akt activation, which in turns retains FoxOs outside the nucleus. The removal of FoxO-mediated inhibition of cyclins allows β-cells in the pancreas to proliferate actively, to supply more insulin when needed, but at the same time weakens cellular defenses against

ROS. Reactive oxygen species, however, can also activate FoxO through c-Jun Kinase (JNK)-mediated phosphorylation, regardless of Akt-mediated insulin signaling, thus decreasing the cell responses to insulin, and therefore insulin sensitivity (Fig. 2). Notably, JNK deletion, FoxO1 (96) or PTEN haploinsufficiency (97), as well as NAC administration (98,99) or overexpression of ROS scavengers (100,101) improved insulin sensitivity in mice. Conversely, FoxO1 overexpression or the expression of a constitutively active FoxO1 impaired glucose metabolism and induced diabetes (102). To make things worse, hyperglycemia and insulin resistance create a situation in which the increased metabolism, due to increased presence of glucose, and proinflammatory cytokines generate more ROS. This requires higher levels of FoxO transcriptional activity to oppose cellular damage, at the expense of the response to insulin.

Metabolic stimuli, such as insulin or insulin-like growth factor, can activate Akt, an inhibitor of FoxOs, thus allowing pancreatic β-cells to proliferate and supply the required amount of insulin, but at the same time reducing defenses against oxidative stress. Reactive oxygen species, however, can activate FoxOs via JNK, independently of Akt, thus reducing insulin sensitivity. This might help explain why periodontal disease worsens diabetes control and vice versa.

## FoxO and T-cell factor (TCF) signaling: β-catenin at the helm

To better understand the consequences of FoxO activation for bone, it must be remembered that FoxO transcriptional activity requires β-catenin, a protein of the Armadillo family and a normal constituent of cell-to-cell junctions. β-Catenin is an essential mediator of several pathways that control the cell fate. One of the best known is the Wnt canonical pathway (103–105). The canonical or Wnt/β-catenin pathway is activated upon binding of some secreted glycoproteins, called Wnt proteins, to Frizzled (Fz) and LRP5/6 receptor (106,107). This induces the activation of dishevelled (Dvl; 108), which releases β-catenin from a multimolecular complex it forms with glycogen synthase kinase 3 and casein kinase 1α, and two scaffold proteins, axin and adenomatous polyposis coli (109), which target β-catenin for proteosomal degradation (110,111). Once released, β-catenin can shuttle to the nucleus, where it binds to a member of the T-cell factor/lymphoid enhancer factor (TCF/Lef1) transcription factor family (112), thus promoting the expression of several target genes (113).

The β-catenin-mediated Wnt signaling is a pivotal pathway in the development of the embryo (114,115) and has been shown to control stem cell proliferation and differentiation (116). The canonical Wnt signaling is also required for osteoblast differentiation (117) and bone formation (118). Patients suffering with van Buchem disease present with dramatic osteopetrosis due to unopposed Wnt canonical signaling in bone owing to the lack of an osteocyte-derived β-catenin inhibitor, sclerostin (119–121). Furthermore, β-catenin is a co-mediator of the action of parathyroid hormone on bone formation (122), and it regulates bone remodeling by increasing the expression of osteoprotegerin (123), which acts as an antagonist of RANKL, the main inducer of osteoclastogenesis, and thus bone resorption. Akt can promote Wnt canonical signaling by phosphorylating glycogen synthase kinase 3 (124) and thus releasing β-catenin, and it has been demonstrated that Akt can exert a broad function in controlling skeletal development in the embryo by tuning the activation of the Akt–glycogen synthase kinase 3 or the Akt–FoxO axis (125).

β-Catenin is then at the crossroad between two alternative pathways with juxtaposed effects, the former, mediated by TCF, which promotes cells proliferation and commitment, and the latter, mediated by FoxOs, that induces quiescence, preservation of stemness and defenses against ROS (53,126).

To activate cellular defenses against ROS, therefore, FoxOs must compete with TCF/Lef1 to bind β-catenin, and it has been shown that induction of oxidative stress by ROS antagonizes Wnt canonical signaling, by diverting the pool of free cytosolic β-catenin away from TCF (52,127) to bind FoxO

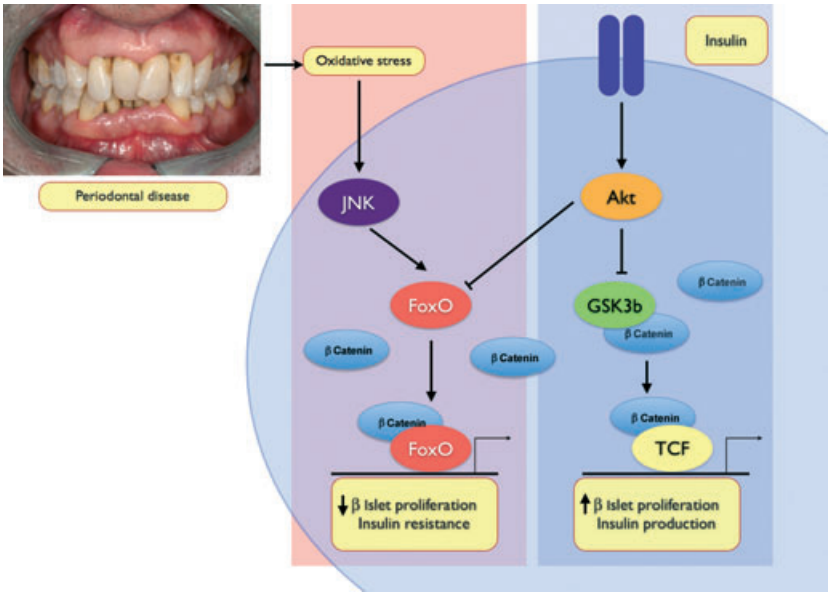


*Fig. 2.* Insulin binding to membrane receptors in the pancreatic islets leads to Akt activation through PIP3. This in turn phosphorylates FoxO transcription factors, excluding them from the nucleus and promoting the activation of alternative pathways that induce cell proliferation, to produce more insulin. The presence of ROS, as a consequence of periodontitis, however, can activate FoxOs independently of Akt, leading to insulin resistance. PIP3, Phosphatidylinositol (3,4,5)-trisphosphate; JNK, c-Jun N-terminal Kinase; TCF, T-cell factor.

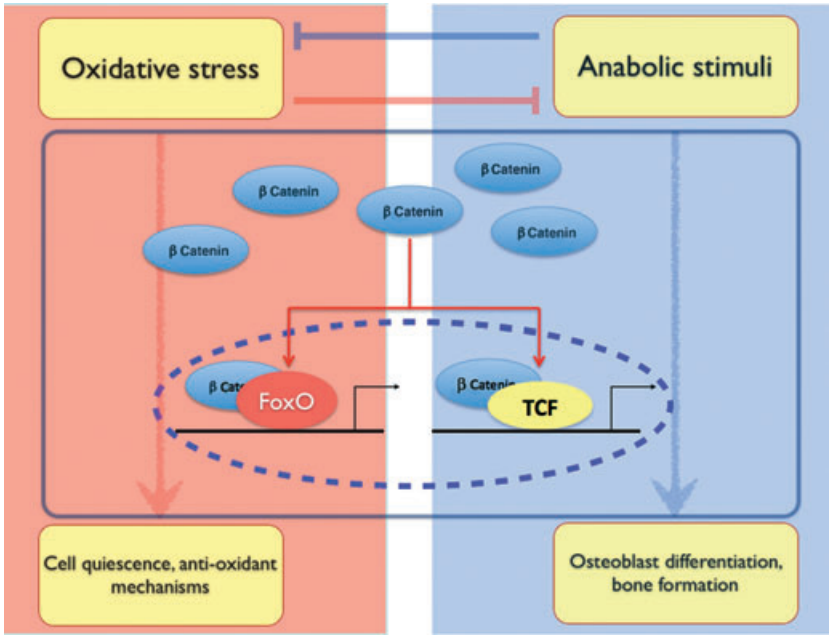


*Fig. 3.* FoxOs and TCF compete for the co-transcription factor β-catenin. β-Catenin can thus initiate two alternative pathways with opposite effects, leading to cell quiescence, defenses against ROS and apoptosis or cell proliferation and differentiation. External stimuli, such as the presence of ROS, hormones and growth factors, can affect the balance between these two pathways.

(Fig. 3). The balance between the two pathways is regulated by the presence of stress factors; ROS activate JNK both independently of and via Dvl, and JNK in turn also phosphorylates FoxO, increasing its activity at the expense of β-catenin/TCF (128). JNK could also directly inactivate TCF by phosphorylating it, much like a similar kinase, Nemo-like kinase (129).

This may provide a rationale for the impaired bone formation and increased osteoblast apoptosis in diabetic rodents and for alveolar bone loss in diabetic rodents (1,130–132).

FoxOs and TCF compete for binding β-catenin. This co-transcription can thus participate in the activation of transcription programmes for defenses against oxidative stress or alternatively for osteoblast differentiation. Continuous stimulation of FoxO/β-catenin by ROS results, therefore, in reduced bone formation.

## Reactive oxygen species, lipid oxidation and bone resorption

An increase in ROS can oppose osteoblastogenesis and bone formation by another mechanism. Reactive oxygen species have been proved to enhance the activity of the lipoxygenase Alox-15 (51), converting polyunsaturated fatty acids, such as linoleic acid, to oxidized fatty acids, including 9-hydroxy-10,12-octadecadienoic acid., a high affinity ligand of the adipogenic transcription factor Peroxisome proliferator-activated receptor gamma (PPAR-γ) (133). A vicious cycle may ensue, because these oxidized compounds can also break into unsaturated aldehydes, such as 4-hydroxynonenal, which can deplete glutathione and thus further increase ROS (134). Sheikhi *et al.* (33) hypothesized that lipid peroxidation by activated neutrophils could be responsible for tissue damage in periodontal disease. As a matter of fact, pharmacologic inhibition of Alox15 has been shown to improve bone mass in mice (135). Adipogenesis and osteoblastogenesis are balanced and almost antagonistic processes in the skeleton. It has been demonstrated that PPAR-γ haploinsufficiency in adipocytes increases bone mass and osteoblastogenesis (136), whereas PPAR-γ stimulation by rosiglitazone or oxidized fatty acids induces bone loss in humans and rodents (137–139). PPAR-γ can bind to β-catenin and degrade it, while at the same time the increase in oxidative stress promotes FoxO/β-catenin transcriptional activity, diverting β-catenin from binding TCF.

Moreover, ROS play an important role in the formation, survival and resorbing activity of osteoclasts by up-regulating RANKL and tumor necrosis factor-α expression through ERK and nuclear factor-κB activation (140–142). Inhibition of ROS by the antioxidant NAC prevents osteoclastogenesis (143). Furthermore, recent reports have shown that RANKL induces osteoclast formation by generating ROS in osteoclast precursors, and mice lacking the antioxidant gene peroxiredoxin II have reduced bone mass (143,144). Likewise, it has been reported that overexpression of chloroplastic glutathione peroxidase, an enzyme responsible for $H_2O_2$ degradation in osteoclast precursors, impairs osteoclast formation and RANKL signaling (142). Consistent with these results, Srinivasan *et al.* (145) demonstrated that hypoxia promoted osteoclast formation by increasing ROS and that homocysteine enhanced bone resorption through induction of ROS (146). Notably, there is important evidence that the effects on bone mass of estrogens and androgens, hormones that have been long associated with maintenance of bone homeostasis, are mediated by antioxidant effects, and that their effect in preventing ovariectomy-induced bone loss can be recapitulated by NAC (50,147,148). A recent report by Jilka *et al.* (149) also showed that the effects of intermittent parathyroid hormone on bone anabolism are more marked in aged bone compared with the young skeleton because of its antioxidant activity.

It is therefore possible to envisage a scenario where, in the presence of periodontitis, a localized and systemic state of oxidative stress is created (39,40), which activates a cascade of oxidation products and stimulates bone resorption while inhibiting bone formation. The cellular mechanisms that antagonize ROS in the presence of

*Table 1.* List of studies investigating the effects of antioxidants on periodontitis, the model used and their effect

| Study | Year | Model | Antioxidant | Effect |
|---|---|---|---|---|
| Abou Sulaiman *et al.* (43) | 2010 | Periodontitis, human | Vitamin C | n.s. |
| Maruyama *et al.* (150) | 2011 | Periodontitis, rat | Green tea catechins | + |
| Govindaraj *et al.* (151) | 2010 | Periodontitis, rat | Proanthocyanidin | + |
| Toker *et al.* (31) | 2009 | Periodontitis, rat | *N*-Acetylcysteine | + |
| Tomofuji *et al.* (152) | 2009 | Periodontitis, rat | Vitamin C | + (*) |
| Tomofuji *et al.* (56) | 2009 | Periodontitis, rat | Cocoa | + |
| Hirasawa *et al.* (154) | 2002 | Periodontitis, human | Green Tea catechins | + |
| Khmelevskii *et al.* (153) | 1985 | Periodontitis, human | Vitamin A, E, K | + |

n.s., no significant effect observed; +, significant difference between treatment in the presence or in the absence of antioxidant; *, only oxidative parameters considered, no clinical outcome.

sustained oxidative stress are detrimental to bone formation and facilitate progressive damage to alveolar and periodontal structures.

Oxidative stress can exert a detrimental effect on bone by generating oxidized fatty acids, which stimulate adipogenesis and inhibit osteoblastogenesis, and by directly stimulating osteoclast formation and activity. Taken together, these mechanisms provide a framework in which periodontal and bone damage ensues as a consequence of a periodontitis-related highly oxidative state.

## What next?

Attempts to counteract oxidative stress to improve the outcome of treatment of periodontitis with local or systemic factors have been reported (31,43,56, 150–154), and most of them appeared successfully to hamper periodontal destruction, at least in rodent models (Table 1). The available antioxidants have some limitations, however. *N*-Acetylcysteine, though effective in rodents, has been shown to inhibit Wnt canonical signaling, which might decrease its overall benefits (149). Improved antioxidants should be developed to avoid undesired effects, and novel therapeutic approaches should take advantage of known molecular pathways underlying cell defenses against ROS.

## Conclusions

Oxidative stress is a central event for the fate of cells. Its ubiquitous presence has led to the development of a complex genetic network, co-ordinated by FoxOs and β-catenin, aiming to protect cells and balance cell activities to best oppose ROS. An increase in ROS, which may result from age or insulin resistance, generates a situation in which bone formation is impaired and bone resorption is strongly promoted. Understanding the mechanisms underlying ROS-mediated bone loss is the key to developing new therapeutic approaches to systemic and localized bone disorders, but also to periodontitis and possibly peri-implantitis.

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