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Insights into oxidative stress in bone tissue and novel challenges for biomaterials

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Insights into oxidative stress in bone tissue and novel challenges for biomaterials

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Highlights**:**

- High levels of oxygen free radicals (ROS) can affect bone tissue promoting its catabolism;

- Aging, fractures, biomaterial implantation and body response are sources of ROS;

- The modulation of ROS levels can improve bone biomaterial integration;

- Emerging strategies for modulating ROS levels and support bone regeneration.

Abstract

The presence of Reactive Oxygen Species (ROS) in bone can influence resident cells behaviour as well as the extra-cellular matrix composition and the tissue architecture. Aging, in addition to excessive overloads, unbalanced diet, smoking, predisposing genetic factors, lead to an increase of ROS and, if it is accompanied with an inappropriate production of scavengers, promotes the generation of oxidative stress that encourages bone catabolism. Furthermore, bone injuries can be triggered by numerous events such as road and sports accidents or tumour resection. Although bone tissue possesses a well-known repair and regeneration capacity, these mechanisms are inefficient in repairing large size defects and bone grafts are often necessary. ROS play a fundamental role in response after the implant introduction and can influence its success. This review provides insights on the mechanisms of oxidative stress generated by an implant *in vivo* and suitable ways for its modulation. The local delivery of active molecules, such as polyphenols, enhanced bone biomaterial integration evidencing that the management of the oxidative stress is a target for the effectiveness of an implant. Polyphenols have been widely used in medicine for cardiovascular, neurodegenerative, bone disorders and cancer, thanks to their antioxidant and anti-inflammatory properties. In addition, the perspective of new smart biomaterials and molecular medicine for the oxidative stress modulation in a programmable way, by the use of ROS responsive materials or by the targeting of selective molecular pathways involved in ROS generation, will be analysed and discussed critically.

Graphical abstract
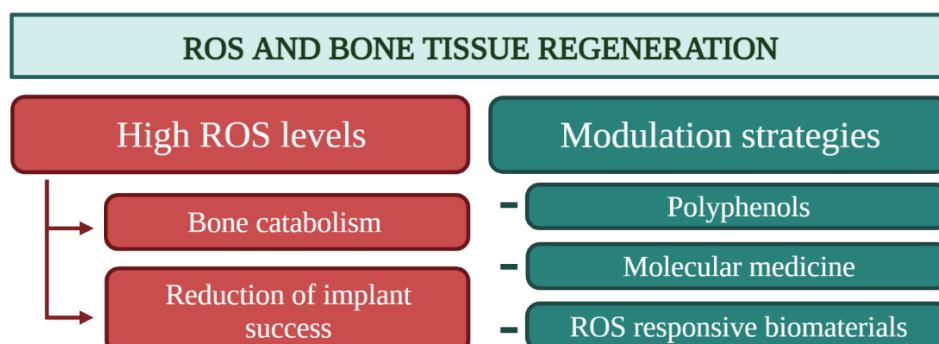
Keywords:

Bone, ROS, oxidative stress, Biomaterial, Polyphenol, molecular medicine

Abbreviations:

ECM, Extracellular Matrix; OBs, Osteoblasts; OCs Osteoclasts; OCYs, Osteocytes; CT, Calcitonin; PTH, Parathyroid Hormone; BMPs, Bone Morphogenetic Proteins; TGF-β, Transforming Growth Factor-β; FGF, Fibroblast Growth Factor; IGF-1, Insulin Growth Factor-1; RANKL, Receptor Activator of Nuclear factor-κB Ligand; OPG, Osteoprotegerin; ROS, Reactive Oxygen Species; O_2^-, superoxide anion; H_2O_2, Hydrogen Peroxyde; OH, hydroxyl radicals; ATP, Adenine Triphosphate; NADPH, Nicotinamide Adenine Dinucleotide Phosphate; SOD, Superoxide Dismutase; GSH, Reduced Glutathione; GSSG, Oxidized Glutathione; SIRT, Sirtuins; MMPs, Metelloproteinasis; TRAP, Tartrate-Resistant Acid Phosphatase; TCL/LEF, T-cell factor/lymphoid enhancer factor; Runx-2, Runt-Related Transcription Factor-2; FoxOs, Forkhead box O transcription factors; ERK1/2, extracellular signal-regulated kinases; JNK, c-Jun-N terminal kinase; MAPK, Mitogen activated protein kinases; MSCs, Mesenchymal Stromal Cells; FBR, Foreign Body Material Response; FBGCs, Foreign Body Giant Cells; ILs, Interleukins; PDGF, Platelet-Derived Growth Factor; DAMPs, Damage-Associated Signals; M1, Pro-Inflammatory Macrophages; MCP-1, Monocyte Chemotactic Protein 1; MIP-1, Macrophage Inflammatory Protein 1; M2, Regulatory Macrophages; PI3K, phosphatidylinositol-4,5-bisphosphate kinase 3-kinase; Akt, Protein Kinase B; PKC, Protein Kinase C; ERs, Estrogen Receptors; OPCs, Oligomeric Proanthocyanidins; siRNAs, Short Interfering RNAs; miRNAs, microRNAs; RNAi, RNA interfering; mRNA, messenger RNA; DKK-1, dickkopf-1; hBMSCs, Human Bone Marrow Mesenchymal Stromal Cells; NOG, Noggin; PEI, polyethyleneimine.

1. *Introduction*

Bone is a dynamic tissue characterised by cells within a highly organized organic and inorganic extracellular matrix (ECM). Its main organic component is collagen followed by non-collagenous proteins such as Transforming Growth Factor-β, Insulin-like Growth Factor-1, Decorin, Osteonectin, Osteopontin, Bone Sialoprotein, and Osteocalcin [1]; while, hydroxyapatite, made of calcium and phosphate, represents the major inorganic component [2].

Bone is constantly undergoing a physiological remodelling that adjusts its architecture in response to mechanical needs and helps the repair of micro-damages. Moreover, bone remodelling plays an important role in maintaining plasma calcium homeostasis [3]. The effectors of this process are osteoblasts (OBs) and osteoclasts (OCs): OBs are qualified to synthesize new ECM, while OCs are responsible for bone resorption. Imbalances in the coupling between OBs and OCs can increase the activity of one cell population to the detriment of the other one and can cause damages to the bone ECM [4]. Bone homeostasis, the result of OBs and OCs coupled activity, is finely controlled by both mechanical and biological mechanisms [5]. Bone cells, mainly Osteocytes (OCYs), are able to translate mechanical stimulations in biological signals [6] via signalling pathways that are still unclear. A mechanotransducer that plays a critical role in bone formation is Piezo 1 and its lack in osteoblasts lineage cells determines mechanical-load-independent bone formation with the disruption of the tissue architecture [7]. Furthermore, systemic factors like Calcitonin (CT), parathyroid hormone (PTH), vitamin D3 [$1,25(OH)_2$ vitamin D_3] control bone remodel activity and play a pivotal role in blood calcium levels [8]. Also, estrogens, androgens, thyroid hormone, and glucocorticoids affect bone homeostasis [8]. Recently, the role of growth factors attracted considerable interest. Bone morphogenetic proteins (BMPs) [9], transforming growth factor (TGF)-β, epidermal growth factor receptor (EGFR), Fibroblast growth factors (FGF) and Insulin-like growth factor-1 (IGF-1) binding to bone cell receptors stimulate different molecular pathways that drive cells toward bone synthesis or resorptions [9–12]. Mechanotransduction and biological

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molecules activating cascades that control the production of Receptor Activator of Nuclear factor-κB Ligand (RANKL) and Osteoprotegerin (OPG) [13] will be discussed in chapter 4.

Reactive oxygen species (ROS) are essential in bone remodelling machinery as they help the degradation of the mineralized matrix by OCs, and an impairment in their regulation can affect the behaviour of all the cells involved in this process as well as the precursor cells [14,15]. The control of ROS release could be an interesting approach suitable for bone tissue regeneration and this review aims to provide an overview of the role of ROS on bone ECM and resident cells, as well as an analysis of the sources of production during biomaterials implantation and degradation. In addition, herein, we discussed future perspectives for ROS modulation. In this regard, we suggest several strategies: (i) polyphenols and their transport, (ii) biomaterial with ROS-smart activity, and (iii) technique based on molecular medicine and its delivery.
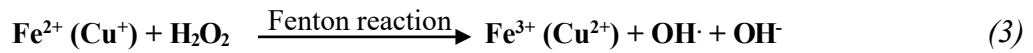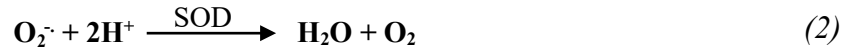
2. *Reactive oxygen species*

The three most relevant molecules of the ROS family are the superoxide anion ($O_2^{-\cdot}$), the hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH) that take part in the redox signalling, an important process involved in cell proliferation, inflammation and aging [16].

ROS are chemical products of oxygen metabolism identified as molecules with unstable bonds (not radicals) or species with one or more unpaired electrons on the external orbital (radicals). These conditions provide instability causing exceptional reactive behaviours [17]. To reach the equilibrium, ROS need another molecule to steal an electron from. This molecule, which lost an electron, oxidizes and, in turn, looks for another molecule able to provide a new electron [18].

ROS generation can take place via both endogenous and exogenous causes [19] via reactions indicated in equations 1-4 [20]. The endogenous production can be accidental or induced: for instance, ROS can be accidentally generated during biochemical reaction such as the adenine triphosphate (ATP) production process in the mitochondria, during the respiratory process [21], or their synthesis can be induced during phenomena such as phagocytosis through the activation of the

NADPH oxidase (Nox) systems [22]. Otherwise, the exogenous production of ROS can occur physically by exposure to pathogens, chemicals and ultraviolet radiations [19].

$$O_2 \xrightarrow{\ e\ } O_2^{\cdot -} \xrightarrow{e+2H^+} H_2O_2 \xrightarrow{e+2H^+} OH^{\cdot} \xrightarrow{e+2H^+} H_2O \qquad (1)$$

$$O_2^{\cdot -} + 2H^+ \xrightarrow{\ SOD\ } H_2O + O_2 \qquad (2)$$

$$Fe^{2+} (Cu^+) + H_2O_2 \xrightarrow{Fenton\ reaction} Fe^{3+} (Cu^{2+}) + OH^{\cdot} + OH^- \qquad (3)$$

$$O_2^{\cdot -} + {}^1O_2 \longrightarrow O_2 + O_2^- \qquad (4)$$
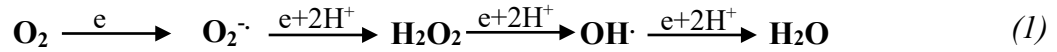
Basal ROS levels contribute to normal cell homeostasis and functions and, the variation in ROS amount influences cell behaviour: ROS reduction induces the cell cycle arrest, while their increase activates the defence response [23–25]. Finally, excessive ROS levels lead to cell death, inducing pro-apoptotic cascade, and in extreme cases, cellular necrosis [26].

ROS perform their physiological functions under strictly controlled conditions maintained by "scavengers", which are enzymatic or not enzymatic molecules capable to shut down the oxidative chain reactions [16]. In enzymatic defences, the Superoxide Dismutase (SOD) that converts the superoxide in hydrogen peroxide (H_2O_2), is of primary importance for cell survival. Hydrogen peroxide could be removed by catalase and glutathione peroxidase, producing water and oxygen [27] as indicated in equations 5 and 6.

$$2H_2O_2 \xrightarrow{\ Catalase\ } H_2O + O_2^{\cdot -} \qquad (5)$$

$$H_2O_2 + 2GSH \xrightarrow{Glutathione\ peroxidase} GSSG + 2H_2O \qquad (6)$$
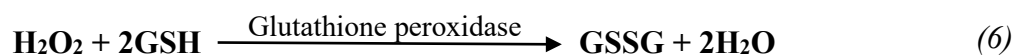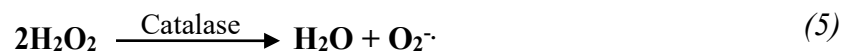
Non- enzymatic antioxidant compounds react directly with different oxidant agents, which can be hydrophilic such as vitamin C (ascorbic acid), vitamin E (α-tocopherol), and Glutathione (GSH) or hydrophobic such as carotenoids (β-carotene) and ubiquinone [28].

When ROS production increases, the balance between their amount and antioxidant defences is altered and the oxidative stress occurs [29]. Excessive intracellular ROS can greedily react with several molecules like proteins, fats, carbohydrates, and nucleic acids whilst, extracellular ones

oxidise lipoproteins and activate the collagen-degrading matrix metalloproteinases [30]. ROS products produce several chain-propagating reactions with the formation of new radicals, more harmful than the first one, which lead to the spread of the damage within the bone tissue [17].

3. *Oxidative stress and bone health*

A healthy skeleton must control properties such as stiffness (resistance to deformation) and strength (maximum stress to failure) that are required to support loads, and toughness, or ductility, which is essential for the energy absorption from impact loads [31]. Bone strength is determined by the mineral amount and its distribution along with the applied forces [32].

Since aging is a process affecting all the body tissues, the skeleton is involved. In this regard, bone mechanical behaviours and morphological structure undergo several changes, during aging, correlated to ECM variations. These modifications are the result of both extrinsic and intrinsic mechanisms. The former group includes sex- hormone deficiency, decreased physical activity, nutritional deficit, glucocorticoid excess, and alcohol or smoking consumption. The latter includes cell senescence and alterations in the mechanisms of cell damage repair [33]. Despite the reduction of sex hormones has been considered the first extrinsic cause of bone loss during aging, recent evidences revealed that bone failure begins before the decrease of sex steroids both in men and women [34]. In support of this, Almeida et al. [35] investigated that sex-steroid sufficient mice suffer of a decrease in bone mass under oxidative stress conditions. They demonstrated that high ROS level impairs osteoblast lifespan and ECM deposition, suggesting that oxidative stress could potentially be the main inductor of bone decay effect, accelerated by the loss of oestrogens or androgens. The resulting decrease in bone mineral density can reduce bone mass and encourage the onset of osteoporosis[36].

Furthermore, the repetition of stressful loading events (such as walking, running, or climbing stairs) can promote the development of sub-micron fractures, that, if enlarged without being repaired through the remodelling process, can lead to bone fractures [37]. Damaged tissues generate a

conspicuous amount of free radicals following the ischemia-reperfusion process [38,39]. It was showed through blood analysis that the increase in ROS lipids products appears after a 3-day period of fracture healing, during the callus formation [40,41]. In this time, the formation of new capillaries increases the vascularisation of the site and promotes the influx of inflammatory cells with the consequent increase in ROS [42].

Bone health is governed by numerous factors such as genetic heritage, hormones, mechanical stress as well as the equilibrium between ROS and antioxidant levels. The oxidative stress, that is generated by an increase of ROS production and /or a decrease of antioxidants, affects this tissue and leads to bone loss, influencing the behaviour of bone cells (detailed in following chapter) as well as of cartilage in the surrounding area [43].

4. *How oxidative stress influences bone cells?*

Osteocytes (OCYs) derive from the osteoblasts (OBs) remained buried in their own ECM during its deposition process. During the differentiation, these cells modify their protein expression and change morphology with the formation of numerous dendritic-type extensions which extend into the bone canaliculi [44–48]. OCYs compose the 90-95% of all bone cells [49] and mediate the bone adaptation to mechanical loading by controlling OBs matrix mineralization [50]. Although OCYs are not the final effectors of the remodelling process, they can act as coordinators by directing the homing of OCs to the site of interest and producing factors that influence OBs and OCs differentiation and lifespan [51]. Moreover, OCYs can manage the production of RANKL and OPG by OBs and bone lining cells, influencing the activation of OCs precursors [52]. OCs differentiation, resorptive activity and survival are mediated by RANKL/RANK binding [53] while them are inhibited by OPG, a soluble mediator that acts as a decoy receptor for RANKL, blocking the receptor recognition [54].

The role of ROS in OBs differentiation is still not well understood. It seems that OBs perform their function under a stringent elimination of intracellular ROS [55,56]. OBs progenitors intensify the ATP production with an increase of mitochondrial biogenesis and oxygen consumption to satisfy

the new bioenergetic needs due to an enhancement of the protein production [57,58]. Despite the intensification of respiratory burst, the level of ROS remains low during the differentiation due to the mitochondrial stress regulator SIRT3/SOD2, which plays a vital role in OBs differentiation and bone formation [59].

In spite of this, ROS are positive modulators of OCs differentiation and maturation, taking part in RANKL/RANK signal pathway as second messengers [14] and allowing actin ring formation [60]. Moreover, it was also reported that active OCs produce a massive amount of ROS via NADPH oxidase [61], that cooperate with proteinases, metalloproteinases (MMPs) and tartrate-resistant acid phosphatase (TRAP) to degrade bone extracellular matrix both extracellularly and intracellularly [14,62].

OPG production [63], as well as osteogenesis[64], bone matrix formation/mineralization [65] and bone cell apoptosis [66] are regulated by the Wnt/βcatenin that appears the main pathway suffering the increase of ROS levels in OBs [67,68]. β-catenin is a scaffold protein constantly produced by cells and removed by a degradation complex. Wnt signal induces the release of β-catenin from the complex that, once accumulated in the cytoplasm, migrates into the nucleus where β-catenin binds to TCF/LEF transcription factors [69]. This binding is crucial for runt-related transcription factor-2 (Runx-2) activation, the key mediator of osteogenic commitment [70]. The Forkhead box O transcription factors (FoxOs) are important regulators of the oxidative stress response and promote the activation of the antioxidant defences [71]. In the presence of ROS, FoxOs utilise β-catenin to transcribe their targets limiting its availability and, therefore, hampering OPG production, OBs differentiation and mineralisation potential [15].

Moreover, oxidative stress stimulates the expression of RANKL via ERKs and HSF2 pathways in human OBs [72] and encourage OCYs apoptosis by different mitogen-activated protein kinase (MAPK) pathways such as extracellular signal-regulated kinases (ERK1/2), c-Jun-N terminal kinase (JNK) and p38 Mitogen activated protein kinases (p38 MAPK) [73]. Apoptotic OCYs can, in turn, stimulate OBs and bone lining cells to produce RANKL [74].

Figure 1 summarises how oxidative stress influences the bone cells behaviour shifting the balance between resorption and deposition. Elevated ROS levels promote osteoclast activity by increasing RANKL production and decreasing OPG. Furthermore, they push OBs to reduce their activity of extracellular matrix synthesis and mineralization leading to the start of the catabolic process and, therefore, bone loss. In Table 1 it is summarised the role of ROS on bone cells, with a focus on the effect of ROS level on each cell type, the specific pathways involved, and the respective cells or murine models reported in the literature.
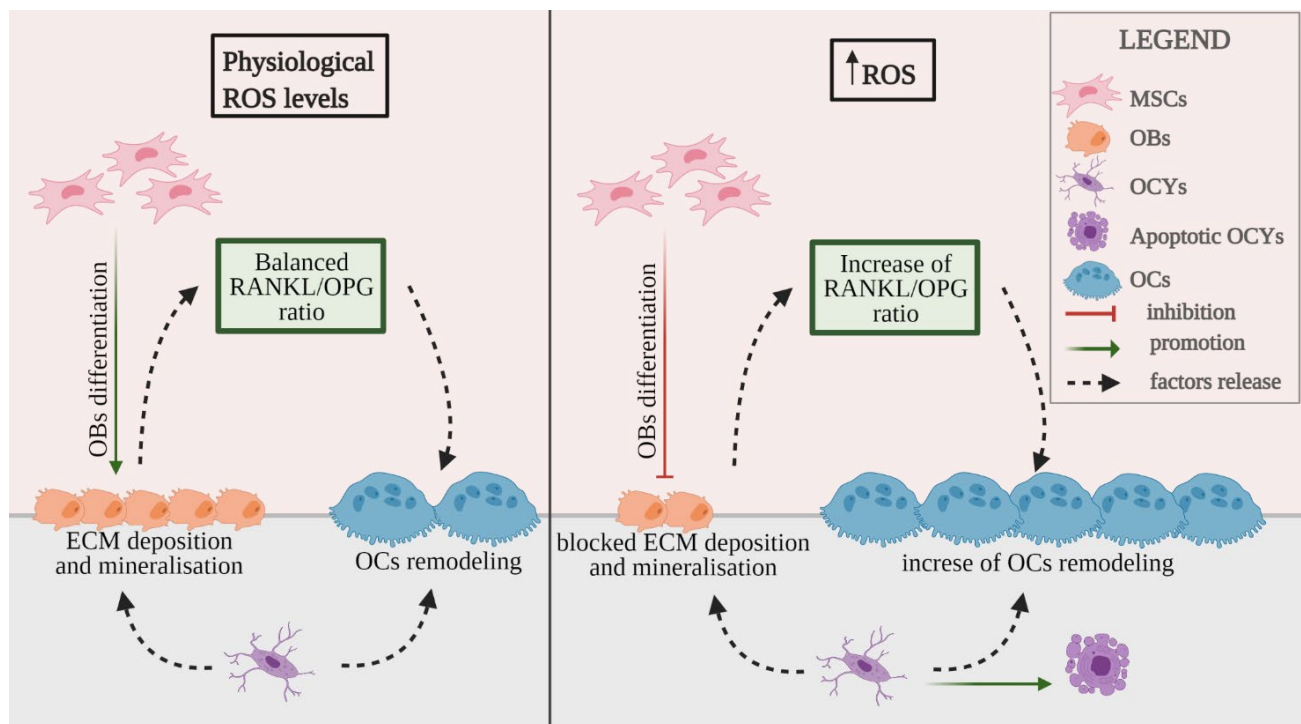
Figure 1: ROS influence on bone cells. In the left panel the physiological behaviour of bone resident cells in normal ROS conditions: MSCs differentiate in OBs, able to synthetise and mineralise bone ECM; also, they produce RANKL and OPG to control OCs formation and activity. OCYs regulate OBs and OCs homing and activity. In the right panel the modification induced by ROS increase are showed: MSCs differentiation into OBs is reduced and the mature ones are unable to synthetise and mineralise bone ECM; the increase of RANKL and OPG ratio promote OCs differentiation and activity. High ROS induce OCYs to send catabolic signals to OBs and OCs as well as lead to OCYs apoptosis.

Table 1: ROS and bone cells

Cells	Effects of the increase of ROS levels	Pathways involved	Cells or models used	References
Osteoblasts	Inhibition of differentiation	Wnt/βcatenin	OB-6	[15]
			FOXO 1, -3, -4 f/f; Osx1-Cre mice	[75]
			Ctnnb1loxP/loxP; Dmp1-Cre mice	[64]
	Increase of antioxidants production	Nrf2	MC3T3	[76]
	Low expression of specific markers	Nrf2/Runx-2	MC3T3	[77]
	Stimulation of RANKL expression	ERKs and HSF2	MG63	[72]
Osteoclasts	Stimulation of differentiation	NADPH-oxidase b558 subunits	HD-11EM	[78]
		NF-kB	RAW 264.7, mouse BMMs	[79]
	Differentiation and activity	Akt, NF-kB, ERK	mouse BMMs	[60]
Osteocytes	Apoptosis	JNK and ERK1/2	MLO-Y4	[80]
		JNK/p38 MAPK	MLO-Y4	[81]
	RANKL production	unclear	MLO-Y4	[82]
		JAK2	MLO-Y4	[83]

List of the main mechanisms affected by high levels of ROS, involved pathways and in vitro or murine models.

5. *Exogenous causes of bone oxidative stress*

Although bone has a repairing ability, the chances of success of large bone defects healing are extremely low and, in those cases, the introduction of grafts/biomaterials is necessary. Autologous graft is considered the "gold standard" technique but the disadvantages deriving from this practice are numerous such as secondary damage, high donor site morbidity, limitations in shape, insufficient tissue [84].

To address these limitations that could obstacle clinical practice, biomaterials are largely used in orthopaedic [85]. The implant of a biomaterial within the body originates a sequence of responses that need to be correctly addressed to avoid device rejection.

5.1. ROS production by Foreign Body Response

The implant of biomaterial determines the inflammation onset in terms of foreign body material response (FBR), which is a mechanism activated by the human body to repair the damaged tissue and prevent further injuries [86]. ROS play important roles during the initiation and the progression of inflammation. Specifically, the H_2O_2 produced in the sites of the foreign material insertion acts as a chemo-attractor for the recruitment of immune cells, [87] that produces $O2^{-\bullet}$ acting as a neutralizing factor for the foreign body [22]. Bone FBR could be divided into acute and chronic responses and ends successfully with osseointegration [88]. The initial response (acute) is intended for the degradation of the foreign body and the repair of the damages given by the biomaterial implantation, whilst the secondary (chronic) response is characterised by the formation of foreign body giant cells (FBGCs), the biomaterial isolation via the formation of a fibrous capsule around the biomaterial, and the subsequent production of ROS and enzymes [89]. When the FBR completes its tasks of cleaning up the foreign bodies, the healing phase begins. Figure 2 shows the consecutive phases of body response to an exogenous graft until the achievement of osseointegration. The acute response is represented in the left, the chronic response in the middle, the final stage of chronic response (with the formation of fibrous capsule) in the panel and the osseointegration in the right.
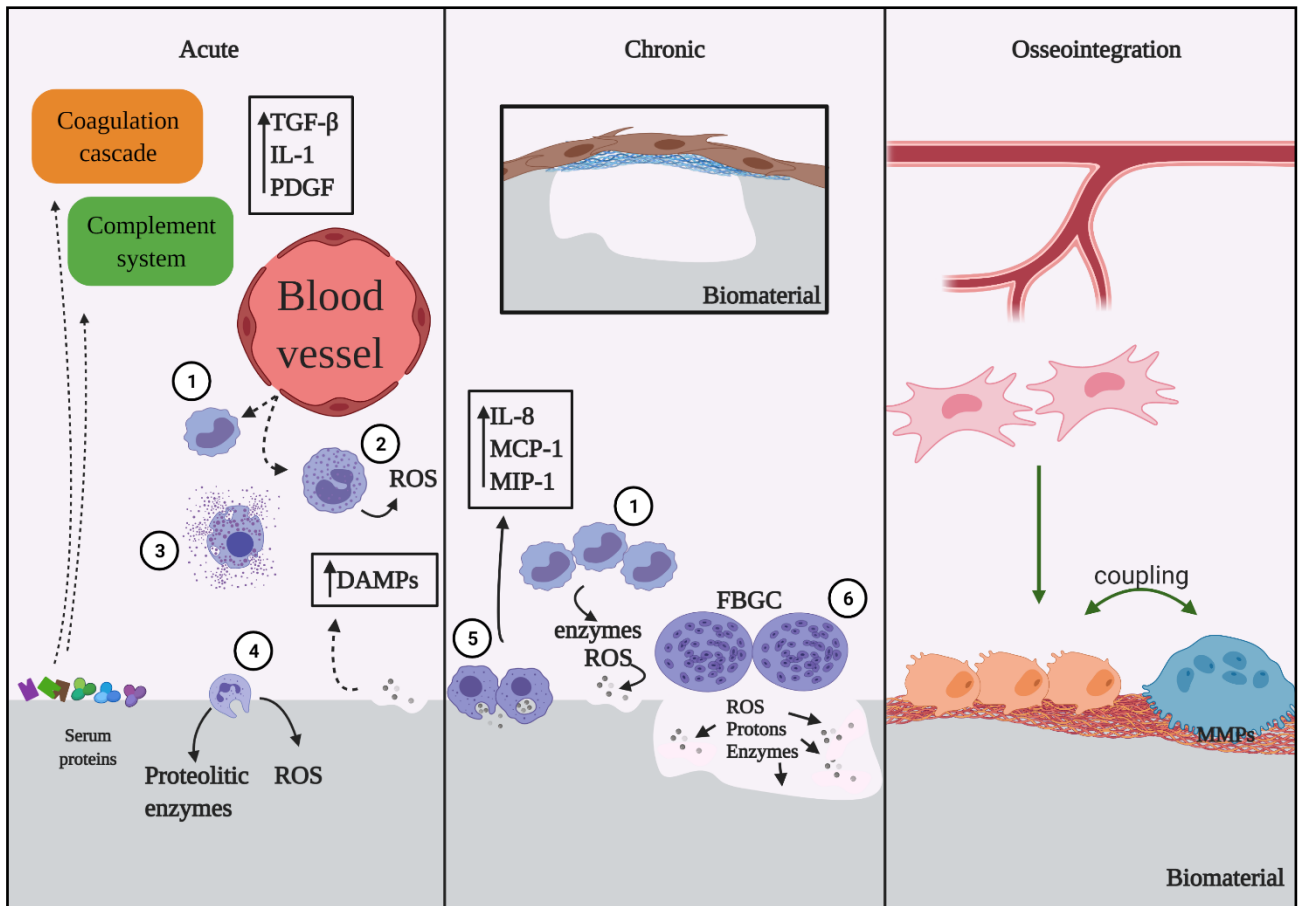
Figure 2: Scheme of Foreign Body material Response (FBR) phases. *Cells actively involved in acute and chronic responses and derived from hematopoietic compartment are in violet: (1) monocyte, (2) granulocytes, (3) activated granulocytes, (4) neutrophils, (5) macrophages and (6) foreign body giant cells. In the insert: fibroblasts (brown) produce a fibrous capsule (in blue) isolating the biomaterial. Osseointegration and remodelling of ECM are performed by MSCs (pink), OBs (orange) and OCs (blue) crosstalk.*

Immediately after the grafting, blood proteins, such as albumin, fibrinogen, fibronectin, vitronectin, complement proteins, globulins and other immunomodulatory proteins derived from serum, are absorbed within the biomaterial surface according to the Vroman effect. These proteins provide biochemical signals to attract immune cells to the site of implantation and play a critical role in cell recognition and adhesion to the material [89,90]. Furthermore, some of these proteins modulate the cascade of coagulation and activate the complement system. The latter is triggered by classical or alternative pathways and, in turn, induces the degranulation of mast cells, increases vascular

permeability, and attracts granulocytes and monocytes. Granulocytes release ROS and contribute to the inflammation response. The coagulation cascade and the complement system closely interact, producing chemoattracts and chemokines, such as transforming growth factor (TGF-β), interleukin-1 (IL-1), and platelet-derived growth factor (PDGF), which are released from activated platelets and injured cells around the biomaterial [90,91]. Moreover, the production of damage-associated signals (DAMPs), which are components deriving from dying cells or the breakdown of the ECM, stimulates further recruitment of mast cells into the connective tissue surrounding the implantation site [92]. Neutrophils begin to release destructive agents such as proteolytic enzymes and ROS (by oxidative burst and NoX enzymes), which are also involved in pathogen removal, and whose unbalance could lead to infections [93]. Leukocyte-released factors attract monocytes, macrophages, immature dendritic cells, and concomitantly suppress their infiltration. At the end of the acute response, the leukocytes are surrounded by macrophages and should disappear after a few days from implantation.

The chronic response is characterised by the established presence of macrophages, monocytes, lymphocytes, and the formation of connective tissue and new blood vessels [94]. Macrophages spread on the biomaterial surface with other activated cells, such as granulocytes and monocytes, and may induce endocytosis of parts of the implant to destroy it. Alternatively, the inflammatory cells secrete ROS and proteolytic enzymes into spaces between cells and implant to degrade the material into nano- and micro- particles that can be then phagocytised [86].

The differentiation of macrophages can derive from multiple pathways, each of them leading to the obtainment of cells with different behaviour. During the early phase of inflammation, the infiltrated macrophages are pro-inflammatory (M1), the principal mediators of chronic response. To clean the biomaterial, M1 macrophages secrete several inflammatory mediators such as IL-8, the monocyte chemotactic protein 1 (MCP-1), and the macrophage inflammatory protein 1 (MIP-1) to continuously attract inflammatory cells. Afterward, they fuse under IL-14 and IL-13 stimuli to form FBGCs [95] and increase their capability to phagocytose particles larger than 5μm [96]. The

macrophages fusion and adhesion are also sustained by a high amount of vitronectin. Therefore, FBGCs form a sealed compartment where they release ROS, protons, and enzymes to clean the biomaterial. In the later phase, the most abundant cells are regulatory macrophages (M2), capable of dampening the inflammation process. If this mechanism is finely tuned, fibroblasts form a capsule around the implant to prevent the damage of the surrounding tissues [89] but, if the inflammatory process prevails over the modulatory one, it appears an increase in the capsule's thickness, compromising the device's functionality and a rejection could occur [97].

The proportion of M1 and M2 macrophages appears to be crucial for the tissue remodelling process [98] and ROS are both critical for M1 activation and functions, and necessary for M2 differentiation [99]. The development of biomaterials capable to efficiently modulate the oxidative stress at the implant site affecting macrophages response will improve new bone tissue deposition: the release of osteogenic cytokines appears when the shift from M1 to M2 macrophages it is timely balanced [100].

The osseointegration phase, that occurs when the immune-mediated FBR reaches the balance [101], is characterised by provisional ECM production and the formation of new blood vessels. ROS can influence cell adhesion, spreading, proliferation and differentiation; also, redox variation can influence MSCs viability, plasticity, and osteoblast lineage commitment [102]. The interim ECM undergoes a remodelling process whereby it is replaced by a mature ECM, and the cell density decreases [103]. During tissue remodelling the oxidative stress can influence the production of matrix metalloproteases (MMPs) that regulate ECM degradation. The ECM breakdowns release the DAMPs that may lead to macrophages activation causing further oxidative signals. Inflammation and remodelling, until the biomaterial completely integration, can generally last for months or years. In every phase of this progression, oxidative stress plays a determinant role, affecting the rapid regeneration of the tissue. The possibility to modulate oxidative stress with a targeted method could improve and speed up the bone tissue regeneration [104].

5.2. Capability of different materials to promote ROS formation.

Biomaterials for bone regeneration should preferably be *osteoinductive* (capable of encouraging the differentiation of progenitor cells into an osteoblastic lineage), *osteoconductive* (support bone growth and boost the ingrowth of the surrounding bone), and capable of inducing *osseointegration* (direct structural and functional connection between living bone and the surface of a load-carrying implant [105]) [85]. While providing several advantages suitable for orthopaedic and maxillofacial implants (i.e. strength, ductility, tenacity, hardness, fracture toughness, formability and biocompatibility), metals can undergo degradation following the corrosion and wear [106,107]. Particularly, all metallic materials undertake electrochemical corrosion that affects their structural integrity and determines the release of degradation products. The galvanic effect of a metal exposed to biological fluids is based on the release of high concentration of metallic ions such as Fe^{2+}, Cu^{2+}, Cr^{6+} and Co^{2+} on the anodic side, which undergo further redox-cycling reactions and may contribute to the OH formation; meanwhile, on the cathodic side, oxygen reduction occurs with the formation of ROS as intermediate products [108]. The reduction of this effect could make metals more attractive for clinical applications and, amongst them, titanium and tantalum express highly corrosion-resistant property that, in addition to their durable and not biodegradable capabilities, makes them an intriguing source for bone substitutes [109–111].

Moreover, an additional source of oxidative stress after the implant seems related to the release of fragments in the surrounding environment because of wear, mechanical stress, and body fluids. Metal fragments are in the range of 0.01-0.03 μm while ceramic particles in 0.1–7 μm [99]; once internalised by cells, both particles can contribute to the ROS generation [112–114]. Simultaneously, tissues that surround the implant reduce the production of antioxidant enzymes and display high lipid peroxidation [115].

The extremely attractive polymers are deeply exploited for tissue regenerative approaches, due to their easy chemical tailoring and tuneable properties. In this regard, it is of remarkable interest their use in composite materials especially in combination with bioactive glasses [116–118]. The main

reason that justifies the use of bioactive glasses is related to their capability to accelerate bone regeneration through the release of ions such as Zn^{2+}, Sr^{2+}, $Co^{2+,}$ or Cu^{2+} that can be substituted or doped into silica or phosphate- based glasses and glass-ceramics [119–121], but might also sustain FBR oxidative stress that participates in the polymer chain scission, determining its degradation [122].

Biomaterial degradation can contribute to ROS generation depending on their nature. The generated oxidative stress attacks the tissues around the implant influencing its success. The awareness of the oxidative stress induction for a specific material, could be the target for the improvement of its integrative properties.

6. ROS production modulation: future perspectives

Biomaterials, by their nature and by FBR they trigger, are massive producers of ROS that can affect bone cells behaviour, favouring bone tissue catabolism. Moreover, the increase of oxidative stress affects the surrounding tissues and contributes to scaffold damage and the slowing-down of the implant integration [123].

These observations highlight that ROS modulation could be a hypothetical target to improve implant success, as well as the speed-up of its integration. The use of polyphenols and smart ROS-responsive materials were already exploited, but further improvements are still necessary. In addition, the development of new recent technologies has made possible the breakdown of barriers until now unattainable, also combining multisectoral skills. The connection between engineering and molecular biology competences could be a strategy of success. For this purpose, we will introduce the RNA*interference* strategy for the modulation of ROS in bone tissue.

6.1. Polyphenols

Polyphenols are derived from plants and have high antioxidant power due to their ROS scavenger properties [124]. They can be classified in phenolic acids, flavonoids, stilbenes, tannins, coumarins and lignans, according to the number of phenolic rings and on the different radicals [125].

Polyphenols are extensively used in bone regeneration [126]: these molecules can (i) exert anti-inflammatory activities interacting with intracellular signalling cascades such as phosphatidylinositol-4,5-bisphosphate kinase 3-kinase (PI3K) [127], protein kinase B (Akt) [128], protein kinase C (PKC) [129] and mitogen-activated protein kinases (MAPKs) [130]; (ii) down-regulate inflammatory mediators, like cytokines, implicated in OCs maintenance [131]; (iii) exert a bone anabolic effect stimulating multiple pathways such as Wnt/βcatenin [132], BMPs [133], Runx-2 [134], and Osterix [135]; (iv) work as hormone analogues binding to Estrogen Receptors (ERs) thanks to the structural similarities that they share with estrogens [136]. These molecules can protect the bone tissue by stimulating bone mass production and slowing down bone turnover [131]. Polyphenols are also used in vascular grafts for their anti-calcification proprieties. They prevent the adhesion of inflamed cells and the subsequent release of MMPs and TNF-α [137]. So, polyphenols enhance the mineralization in bone tissue, while they inhibit calcification in the vascular system.

The therapeutic potential of these compounds is still limited by the low molecule bioavailability due to poor solubility and/or stability, which makes it difficult to provide a relevant antioxidant effect. To overcome these drawbacks, several delivery systems have been proposed in literature, such as encapsulation [138], chemical modification [139], design of colloidal systems [140], use of nanoparticles [141], and implant surface modifications [142]. Amongst them, the encapsulation technology seems the most promising strategy. Micro- and nano- encapsulated methods allow to maintain the structural integrity of the polyphenol until the administration, increase its water solubility and bioavailability, and convey it precisely towards physiological targets [138].

Polyphenols have also been used to modify the mechanical, biological and degradation properties of polymers intended for bone tissue regeneration. Usually, different crosslinking (physical, chemical, enzymatic and non-enzymatic) strategies are used to improve bone scaffold mechanical properties [143], but, unfortunately, with the augmentation of mechanical proprieties also the cytotoxicity increases [144]. Zhao et al. [145] compared the crosslinking capability on gelatin of genipin and a polyphenol extract of *Fructus Chebulae*. Their study demonstrated that the polyphenol conferred

the highest gel strength, the most compact surface and greater temperature stability; however, no toxicity studies have been reported. In addition, for tuning the degradation rate, oligomeric proanthocyanidins (OPCs), a class of polyphenols found in a variety of plants such as blueberry, have been characterised by highly hydroxylated structures that can form insoluble complexes with carbohydrates and proteins [146]. OPCs can stabilise the collagen matrix, reducing the rate of degradation both *in vivo* and *in vitro* [147]. Kim et al [148] demonstrated the absence of cytotoxicity of OPCs-crosslinked chitosan/gelatin films. OPCs can also inhibit the activity of some enzymes such as MMP-2,-8, -9 and cathepsin B and K [149], slowing down the blend degradation.

6.2. Smart biomaterials

Since the presence of ROS is required during the early phase of FBR for their antimicrobial activity [150] and an inadequate ROS suppression could hinder the healing, then the careful maintenance of redox balance is required for tissue regeneration. Hubbel and co-workers were the first using ROS-responsive biomaterials technology for drug delivery [151], which quickly spread and find applications in different pathologies [152].

This smart technique is based on the capability of systems to change their conformation in an oxidative microenvironment [153,154]. Thus, the biomaterial can be loaded with antioxidants or selected molecular compounds, which can be released only in presence of oxidation and in a proportional manner. This capability is conferred by the presence of functional groups that, if activated, can change the solubility or the chemical bonds, leading to the polymer degradation [152]. This approach would provide a tool that can improve polyphenols applicability, managing their release and concentration. Figure 3 concisely shows the mechanism of release of ROS-responsible biomaterials, where the material is loaded with one or more drugs (which can be antioxidants such as polyphenols) and, in presence of oxidative environment, it can modify its structure and allow drug release.
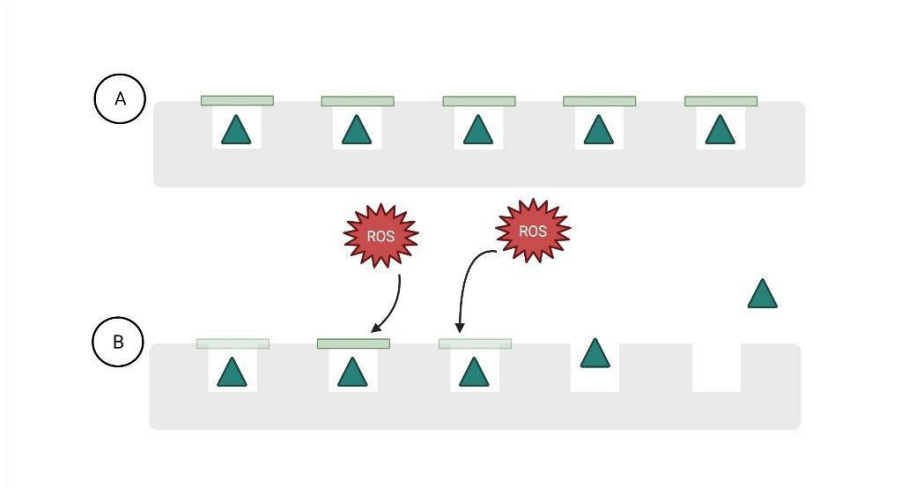
Figure 3: Illustration of ROS-responsible biomaterial mechanism of action. A) The drug, that can be an antioxidant or a target molecule, is loaded in an isolated compartment of the biomaterial; B) when the concentration of ROS in the environment is high, the biomaterial conformation change allowing the drug release.

6.3. Molecular medicine

Recently, the new understanding of the gene regulation role of short interfering RNAs (siRNAs) and microRNAs (miRNAs) have attracted increasing attention in plenty of fields, as well as in bone tissue regeneration. These "non-coding RNAs" can act on "non-druggable" targets [155] and can be designed to virtually affect any gene of interest.

SiRNAs therapeutic approaches induce an RNAi that inhibits the expression of a specific messenger RNA (mRNA), obtaining a gene-silencing effect [156]; on the other side, miRNAs can be used for both inhibition and replacement approaches. In the first case, inhibitory miRNAs (also known as antagomirs or anti-miRs) are applied to block endogenous miRNAs favouring the protein synthesis, whilst with the replacement strategies, synthetic miRNAs (miRNAs mimics) are used to mimic the roles of endogenous miRNAs [157].

The differences between these RNAs are complementary to their targets and the inhibition mechanism. SiRNAs are totally complementary to a specific mRNA and induce its selective cleavage, whilst miRNAs, for their not full matching property, can influence a pool of mRNAs and take part in the regulation of protein expression, by translation repression or degradation by

deadenylation, decapping, or exonuclease action [158]. Because of the different mechanisms of action of siRNAs and miRNAs, their therapeutic applications are different: siRNAs are extremely useful to target single gene disorders [159–161] while miRNAs are applied for multigenic disorders thanks to their capability to modulate multiple pathways [162].

RNAi is also applied in bone tissue regeneration to stimulate osteogenic commitment and increase the power of the success of devices [163]. BMP and Wnt/βcatenin pathways control several genes involved in osteogenesis and their direct or indirect (antagonists) targeting by miRNAs affect Runx-2 expression and its downstream genes. For example, miRNA433-3p promotes OBs differentiation by targeting dickkopf-1 (DKK-1) [164], an antagonist of Wnt signalling pathway, while anti-miR34 targets Notch signalling [165], a pathway involved in the switch of the commitment of hBMSCs into osteogenic lineage rather than the adipogenic fate [166]. For what concerns silencing, the main osteogenic suppressors in hMSCs have been revealed, making available useful targets for the modulation of cell differentiation [167]. The silencing technique was applied to drive MSCs to an osteogenic commitment by stimulating BMP expression through the insertion of a siRNA against the NOG gene [168].

The main problem that compromises the therapeutic applicability of RNAi is their cell delivery. The most efficient methods include the use of viral vectors with serious limitations, including high immunogenicity [169], the risk of insertional mutagenesis [170], the low packaging capacity [171] and the high production cost [172]. Therefore, non-viral vectors are becoming attractive alternatives, although their lower transfection capability.

The reduced bone perfusion makes the local delivery more promising than the systemic one, and this could be advantageous for the use of scaffold. Between several carrier materials investigated, hydrogel-based scaffolds [173–176] represent the most relevant platform able to encapsulate RNAi molecules and guarantee a sustained availability to the nearest cells [177]. Strategies, such as inclusion within nanoliposomes or ionic complexation with cationic polymers such as polyethyleneimine (PEI), have been successfully applied [178,179].

The RNAi technique is applicable to modulate the expression of plenty of genes and pathways including the genes involved in the oxidative stress generation and response. Somasuntharam et al [180] demonstrated, both *in vitro* and *in vitro,* the efficacy of delivery particles loaded with a siRNA inhibiting the NADPH oxidase complex 2 to reduce the oxidative stress in the myocardium. As stated before, the reduction of ROS favours bone anabolism and, although RNA interference is not still applied for ROS modulation in the bone field, it could open new perspectives to ameliorate the tissue regeneration. The selective targeting of ROS production pathways can improve, not only the osteogenic commitment, but also the reduction of the inflammation that the material can sustain in its surrounding. The simple shift of RNAi technology to ROS pathways, instead of the differentiation one, could be a more effective upstream target to drive bone cells to promote biomaterial integration.

Conclusions

Bone is an extraordinarily organized tissue where the finely balanced action of OBs and OCs preserve its healthy and functional state. Here, we underlined the role of ROS in bone tissue, the way they interfere with bone metabolism and the role of biomaterials in influencing ROS *in vivo* production. As high and sustained ROS levels around bone implant affect its integration this review focuses on strategies for oxidative stress modulation were described. It is important to note that the complete eradication of the redox state may represent an adverse effect since oxidative stress is an important mediator of inflammation and it is required for pathogens removal.

The first evidence confirming that oxidative stress modulation could be a target for the improving of bone biomaterial integration was achieved by using polyphenols. Plenty approaches of bone biomaterials delivering polyphenols are continuously emerging, indicating the easy feasibility of this system.

The capability of biomaterials to modulate their antioxidant properties by smart techniques could be a further step to ameliorate bone integration. Here, two innovative strategies are explored, not still

applied for bone regeneration. They include the selective silencing of molecular pathways involved in oxidative stress generation by miRNA or siRNA, and the use of "smart" materials able to modify their conformations in response to high levels of ROS and release their cargo. This latter strategy (*i.e.* oxidative-induced cargo release) could represent an interesting approach to modulate polyphenols activity and/or provide an implementation of miRNA and siRNA selective targeting. Several studies are still necessary for the use of these innovative stratagems for bone tissue regeneration but their feasibility in other recovering attempts is encouraging.

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