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Chapter 2

Bone Substitute Materials in Implant Dentistry

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Additional information is available at the end of the chapter

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1. Introduction

Although bone autografts have been routinely used as "gold standard" for reconstruction/replacement bone defects, because they have osteogenic, osteoinductive, osteoconductive properties, they have a high number of viable cells and are rich in growth factors. However, the use of autograft is limited by several factors, being one of them the insufficient amount of donor tissue. Therefore, bone substitute materials have been extensively studied in order to develop an ideal material for substitution of bone grafts, due to some disadvantages presented by autografts, allografts and xenografts, such as poor bone quality, an inadequate amount of bone and possible immunogenicity for allografts and xenografts, which limit the use of these grafts in specific surgical protocols. These disadvantages have led tissue engineering and biotechnology to develop new materials and promising methods for tissue repair, especially for bone tissue. Thus, bone substitutes, synthetic and/or biotechnologically processed have become potential materials for clinical applications in different areas of health.

An ideal bone substitute (BS) material should provide a variety of shapes and sizes with suitable mechanical properties to be used in sites where there are impact loading; moreover, these materials should be biocompatible, osteoconductive, preferably being resorbable and replaced by new bone formation. In general, resorbable BS materials are preferred, since these materials are expected to preserve the increased bone volume during the reconstruction and simultaneously are gradually replaced by newly formed bone.

Synthetic materials, denominated as alloplastics, may act as scaffolds for bone cells providing tissue growth inside the respective material.

A scaffold must be highly porous with interconnected pores and have adequate mechanical properties. The surface of a scaffold should be similar to extracellular matrix (ECM). These properties enable the scaffold to act as a matrix for tissue regeneration to maintain and improve tissue/organs functions; therefore, it is considered the key element for the success in tissue engineering. Numerous physicochemical features of scaffolds, such as surface chemistry, surface roughness, topography, mechanical properties and interfacial free energy (hydrophobic/hydrophilic balance) are important for cell attachment, proliferation and differentiation. These factors are also critically important to the overall biocompatibility and bioactivity of a particular material [1-3].

Resorption of a biomaterial is related to several factors, such as, particle size, porosity, chemical structure (composition and crystallinity), and pH of body fluids [4, 5]. Particles with nanometric sizes are reabsorbed faster than micrometric particles, because osteoclasts or macrophages act faster on a biomaterial surface. Biomaterial crystallinity also changes the resorption rate, since highly crystalline structures are more resistant to resorption than an amorphous or semi-crystalline structure. Moreover, the chemical composition is also important. Impurities such as calcium carbonate promote faster resorption [6]. The failure or the success of a material for bone fill or replacement may be related to the resorption rate of the material, as well as the regenerative capacity of bone tissue. This process can occur in three forms: 1. insufficient permanence of the material to promote bone apposition and to allow the osteoconductivity; 2. premature destabilization of newly formed bone due to the complete degradation of the material; 3. an exaggerated inflammatory response due to the degradation of the material [7]. Thus, bone substitute materials must have suitable resorption rate in accordance with the rate of tissue formation.

Despite recent advances in the development of new BS for bone tissue engineering, there is still a search for a material or a composite with mechanical properties and physicochemical characteristics similar to autograft and a structure closer to the natural ECM.

2. Ceramic-based bone substitutes

Ceramics are compounds between metallic and nonmetallic elements. Ceramic materials have a several of attractive advantages comparing to other materials. These include high melting points, great hardness, low densities and chemical and environmental stability. However, ceramics are severely affected by lack of toughness; they are extremely brittle, and are highly susceptible to fracture. They are most frequently oxides, nitrides, and carbides, for example, some of the common ceramic materials include aluminum oxide (or alumina, Al_2O_3) and silicon dioxide (or silica, SiO_2), in addition, some traditional ceramics are referred as those composed of clay minerals (*i.e.*, porcelain), as well as cement and glass [8].

The ability of ceramic materials to bond to the bone tissue is a unique property of bioactive ceramics. This property has been led their wide clinical application in both areas as orthopedics and dentistry. The use of ceramics for hard tissues reconstitution has been performed for centuries, but in clinical practice the use of these materials only began in the late eighteenth

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century with the use of porcelain for making dental prostheses. On the other hand, in orthopedics the use of ceramic materials happened in the late nineteenth century with the use of plaster of Paris (calcium sulfate hemihydrate, $CaSO_4$ ½H_2O) for bone defects filling [9].

The term "bioceramic" refers to biocompatible ceramic materials applied to biomedical and clinical use due to certain characteristics such as biocompatibility, excellent tribological properties and high chemical stability, which is superior to metals in different applications, moreover excellent osteoconductive [10].

Among bioceramics, calcium phosphates are ceramics with Ca/P molar ratio ranging from 0.5 to 2.0 and are found in different types [11], in which the best known form is hydroxyapatite (HA), a natural mineral component representing 30 to 70% of the mass of bones and teeth [12]. The chemical structure of biological HA is very complex, because it not presents a totally pure composition (non-stoichiometric), being frequently calcium-deficient hydroxyapatite enriched with carbonate ions forming the carbonate-apatite [13]. Some calcium phosphates of biological relevance are: amorphous calcium phosphate (ACP), dicalcium phosphate dihydrate (DCPD), dicalcium phosphate (DCP), octacalcium phosphate (OCP), tricalcium phosphate (TCP), calcium pyrophosphate (CPP) and hydroxyapatite (HA).

Pure HA, calcium hydroxyapatite specifically, is a stoichiometric composition of $(Ca)_{10}(PO_4)_6(OH)_2$ (Ca/P = 1.67). It is main inorganic component of bone tissue and teeth. For many years, different types of synthesis and applications of these calcium phosphates have been researched for regeneration/reconstruction of bone structures. Synthetic HA has been used for this purpose, because they are bioactive material and can have a Ca/P molar ratio less than 1.67; thus, they are more effective clinically due to its similarities with the composition of bone tissue and their osteoconductive properties [13, 14].

Bioceramics have different rates of *in vitro* solubility, which reflects in the *in vivo* degradation, *i.e*., as greater the Ca/P molar ratio lower is the solubility of bioceramics [15]. However, the rate of dissolution is not only influenced by Ca/P molar ratio, but also may be influenced by other factors such as local pH, chemical composition, crystallinity, particle size and porosity of material [5].

Bioceramics when in contact with body fluids and tissues, in this interface material-tissue, suffer reactions at the molecular scale of type dissolution preferably by the release of Ca^{2+} and PO_4^{3-} ions; however, in this interface there is an increase of local pH promoted by Ca^{2+} ion release. This increase in pH stimulates alkaline phosphatase activity in pre-existing osteoblastic cells and in newly-differentiated active osteoblasts to synthesize more alkaline phosphatase, type I collagen, non-collagen proteins and others. Therefore, pH at the material-tissue interface is gradually reestablished, while occurs the nucleation of crystals of calcium phosphate to the collagen fibers until forming a chemically phase more stable. This event is related to PO_4^{3-} ion release from ATP molecules, pyrophosphate and others, which contain PO_4^{3-} ion from adjacent tissues. Moreover the action of biological buffers containing HCO^{3-} ion, which favor the precipitation of carbonate-apatite as well as the decrease of chemical mediators locally, produced by leukocytes [13]. Table 1 shows the occurrence of calcium phosphates in biological systems.

Apatite phase	Formula	Ca/P
Monocalcium phosphate monohydrate - MCPH	$Ca(H_2PO_4)_2{\cdot}H_2O$	0.5
Monocalcium phosphate anhydrous - MCP	$Ca(H_2PO_4)_2$	0.5
Dicalcium phosphate dihydrate (Brushite) - DCPD	$CaHPO_4{\cdot}2H_2O$	1.0
Dicalcium phosphate anhydrous (Monetite) - DCP	$CaHPO_4$	1.0
Octacalcium phosphate - OCP	$Ca_8H_2(PO_4)6{\cdot}5H_2O$	1.33
Amorphous calcium phosphate - ACP	$Ca_x(PO_4)_y{\cdot}nH_2O$	1.2 - 2.2
α or β-Tricalcium phosphate - TCP	$Ca_3(PO_4)_2$	1.48 - 1.50
Calcium-deficient hydroxyapatite - CDHA	$Ca_9(HPO_4)(PO_4)5(OH)$	1.5
Hydroxyapatite - HA	$Ca_{10}(PO_4)6(OH)_2$	1.67

Table 1. Main calcium phosphate phases. Apatite phase, chemical formula and Ca/P molar ratio.

Bioactive ceramics have been used as bone substitute materials for maxillary sinus lift, alveolar ridge augmentation, inlay bone grafting and as coatings for titanium and their respective alloys. However, bioceramics present a limitation in clinical application due to their low mechanical properties, for instance, low elastic modulus, when compared to other metallic and polymeric biomaterials. Therefore, these ceramic materials cannot be used in sites where there is a high mechanical loading, but can be used for bone fill materials and coatings of metallic surfaces or materials of high mechanical properties [16, 17]. These coatings may accelerate initial stabilization of implants and stimulating bone appositions on the implant surface, promoting a rapid fixation of these devices [18].

The bioceramics may be employed in dense and porous forms. Despite the increase in porosity decrease the mechanical strength of ceramics, the existence of isolated pores with suitable dimensions can favor the ingrowth of tissue through of these pores, promoting a strong entanglement between the material and newly formed tissue [19], moreover this porosity may promote circulation of biological fluids, increases the specific surface area, and thus accelerating the biodegradability.

Bioceramics can be single crystals (sapphire), polycrystalline [alumina, hydroxyapatite (HA), tricalcium phosphate (TCP)] or semi-crystalline structure as glass-ceramics (Ceravital® or A/W glass-ceramic) and composites, which have an amorphous phase and one or more crystalline phases. In addition, bioactive glasses (Bioglass®, PerioGlas®, BioGran®) which are a group of glass-ceramic consist in a structure of amorphous solids.

The initial medical applications of CS were documented in 1961 [20]. This material, plaster of Paris, was used in many bone defects of trauma. In the dental field, one of the first reports of the use of CS was in 1961 by Lebourg and Biou [21]. These authors implanted CS in alveoli after extraction of third molars, even in other bone defects in the mandible and maxilla. After three to four weeks it has been observed that the material had been completely resorbed, and bone healing was accelerated in the treated areas in comparison with the control. The authors concluded that CS was a favorable material for the treatment of bone defects and they justify it by the ability of the material to supply essential inorganic ions for the repair process.

Clinical studies showed positive results regarding the use of CS as material for bone fill and barrier to the preservation of alveolar ridge, post-dental extraction, providing a barrier which

stabilizes the clot, assisting in the healing and bone regeneration of the local to receive the implant. The use of CS hemihydrates (CS) (powder, particulate or cement form) and CS associated with demineralized freeze-dried bone (DFDB) in bone defects, post-extraction dental and periodontal defects, promotes the increase of the quality and quantity of newly formed bone preserving the dimensions of alveolar ridge [22-24]. Moreover, CS or CS associated with DFDB when used to maxillary sinus lift, this bone substitute, favors a good primary stability of dental implants and with relative bone density [25-27]. In addition to these advantages, CS is a BS rapidly resorbable and promotes angiogenesis [27-29]; however, in some clinical situations this rapid absorption *in vivo*, may be a disadvantage, due to its degradation which often occurs before the new bone formation.

Other the bioactive ceramics most commonly investigated as bone substitute materials are HA, β-TCP and bioactive glasses. Synthetic HA, β-TCP and biphasic calcium phosphates (HA:β-TCP) are routinely employed as BS in block or granule forms. Furthermore, cements based on HA and/or β-TCP are excellent bone fill materials, due to their easy manipulation and favor the bone contour, moreover, are clinically used by their similarity to the bone inorganic composition and by osteoconductive property. On the other hand, bioactive glasses are most commonly used in granule forms.

For several years, synthetic HA was used as the main method in the reconstruction of bone defects involving the craniofacial region, oral surgery, orthopedic and implant dentistry [14, 30-32]. HA presents some disadvantages related to its resorption, because it is hardly absorbed, which hampers the remodeling and the new bone formation, and results in poor local stability or permanent stress concentration. Currently, biphasic calcium phosphates, mixtures containing HA/TCP (α-TCP or β-TCP) are preferably used in clinical practice with varied proportions between HA and TCP [33-38] due to their considerably difference in the resorption rate, which HA reabsorbs very slowly compared with TCP. The difference in the resorption rate influences in the osteoconductive property of these materials, TCPs are more osteoconductive than HA, due to their greater biodegradability rate in relation to HA [13, 39, 40]. Clinical and experimental studies have shown that mixture HA/TCP promotes intense activity of bone formation with high osteoconductivity [34, 41-43], whose mixture has demonstrated to be an excellent material for sinus lift [34, 37, 38]. However, even the resorption rate of TCP being faster than HA, clinical studies that used just TCP reported presence of TCP particles after long-term postoperative of maxillary sinus lift and mandible defects [44-49]. Results show that β-TCP is a good material for grafting [44-51], on this account also promotes stability of implants increasing the survival rate [48].

Furthermore, these bioceramics when associated with biopolymers as hyaluronic acid [49] and collagen [52, 53] or other osteoinductive biomolecules (growth factors: bone morphogenetic protein-2 (BMP-2); fibroblast growth factor-2 (FGF-2)) [54-59] have displayed promising results for bone regeneration. These associations have promoted a better quality and quantity of newly formed bone [49, 52, 53, 56, 57, 59], consequently they can improve the primary stability of implants.

Other subgroups of bioceramics quite used as BS material are bioactive glasses and glass-ceramics. Silica glasses are generally classified as a subgroup of ceramics. The glass-ceramics are materials formed by a glass matrix reinforced by ceramic crystals obtained from controlled

crystallization processes [60]. This crystallization process can take place by heat treatment, resulting in a material containing various crystal phases and controlled grain sizes [10]. The glass-ceramic materials have relatively high mechanical strengths, low coefficients of thermal expansion and good biological compatibility. Possibly the most attractive attribute of this class of materials is the ease with which they may be fabricated, in which conventional glass-forming techniques may be used [8]. On the other hand, bioactive glasses present limitations in certain mechanical properties such as low strength and toughness.

In the late 1960s and early 1970s, the several researches for developing implant materials with a better biocompatibility resulted in the new concept of bioceramic materials, which could mimic natural bone tissue. During this period, Hench and coworkers [61] developed a new biocompatible material, silica-based melt-derived glass, for bonding fractured bones, a bioactive glass denominate 45S5 Bioglass®. This denomination was given because the material mimicked normal bone and to stimulate the new bone formation between the fractures [62]. Bioglass® is a commercially available family of bioactive glasses, based on SiO_2, Na_2O, CaO and P_2O_5 in specific proportions, and was one of the first materials completely synthetic with excellent osteoconductive properties, which seamlessly binds to bone [61, 63]. The bioactive glasses, since their discovered, have been widely used in dentistry for bone defects repair/reconstruction, because these glasses exhibit bone bonding, a phenomenon also observed with other bioactive ceramics [64]. Bioglass offers advantages such as control of resorption rate, excellent osteoconductivity, bioactivity, and capacity for delivering cells. This process is a result of the surface reactive silica, calcium, and phosphate groups that are characteristic of these materials. Silica is believed to play a critical role in bioactivity [64].

In the 1970s, Brömer and coworkers [65] developed a glass-ceramic, Ceravital® through reduction of alkali oxides in the composition and the phase precipitation of the glass matrix by heat treatment of Bioglass®. Ceravital® has been used for small bone defects/structure reconstruction as dentistry [66] as other medical applications, *e.g.* tympanoplasties [67].

The use of bioactive glasses as alloplastic bone graft materials for alveolar ridge augmentation [68-71] and maxillary sinus lift [72-74] procedures has received increasing attention in implant dentistry. Besides Bioglass® other commercial types of bioactive glass have been used for bone repair such as PerioGlas® [70, 75, 76] and BioGran® [71-73]. Studies have reported presence of bioactive glass long-term postoperative (1-2 years) [72, 73].

Moreover, several studies have shown that bioactive glasses and glass ceramics stimulates the secretion of angiogenic growth factors on fibroblasts and endothelial cell proliferation [77, 78].

Although they are quite biocompatible and exhibit bone bonding, bioactive glasses are not osteoinductive and are not capable of forming bone in ectopic sites (although they can be used to deliver osteopromotive growth factors) [64].

Another glass-ceramic with potential for application in implant dentistry is apatite/wollastonite (A/W), which was developed by Kokubo et al., in 1982 [79]. This material presents a great capacity for bone bonding and moderate mechanical strength [80], with excellent results in orthopedic applications [81-83]. The resorption rate of this glass-ceramic can be increased when associated with β-TCP [84]. According to Carrodeguas et al. (2008) [85] the report that the new ceramics containing wollastonite did not exhibit toxicity in cell culture with human

fibroblasts. Moreover, they are biocompatible, resorbable and bioactive releasing ions of silica and calcium in the physiological environment, which are capable of stimulating cells to produce bone matrix [86, 87].

Biosilicate®, glass-ceramic developed by Zanotto and coworkers in 2004 [88], which is highly crystalline (~100%), has an elastic modulus value close to cortical bone, and displays high level of bioactivity [89-91]. It is biocompatible and provides efficient new bone formation in sockets preserving alveolar bone ridge height and allowing osseointegration of implants [92].

Table 2 summarizes some bioceramics used in clinical practice.

Material	Application	Results	Ref.
Calcium Sulphate	Sinus lift	Promote new bone formation with new vessels	[27-29, 93]
		High resorption in 1 month	
HA	Bone graft	Promote new bone formation	[30, 31, 94, 95]
	Sinus lift	Produce avoid space for blood clot	
		Increase bone volume in 8 weeks	
		Promote direct mineralized bone-to-implant contact in the augmented area	
β-TCP	Guide bone regeneration	No induce inflammation	[37, 38, 42-49]
	Sinus Lift	Osteoconductive	
	Bone graft	Highly degraded by macrophages and osteoclast	
Biphasic calcium phosphate (HA:β-TCP)	Bone graft	Increase bone volume in 8 weeks	[33-38, 40, 41]
	Sinus lift	Osteoconductive	
		Promote stability of implants	
		Promote new bone formation	
Bioglass®	Bone graft	Increase bone volume	[68, 69, 93]
	Sinus lift	High bioactivity Promote new bone formation	
BONITmatrix®	Bone graft	Stimulate IGF-1 gene expression	[96]
		Enhance Coll-1 expression	
Biocoral®	Bone graft	Promote formation of fibrovascular tissue	[93, 97]
	Sinus lift	New formed bone 39% highly mineralized	
Fisiograft®	Sinus lift	Increase bone volume 33%	[93]
		High absorption	
OSSANOVA	Bone graft	Stimulate IGF-1 expression	[96]

Table 2. Some bioceramics used in clinical practice.

3. Composite and polymer-based bone substitutes

Composite materials are described as those that have at least two components or two phases with distinct physical and chemical properties that are separated by an interface. The purpose of developing composites is to associate different materials to produce a single device with superior properties compared with the isolated components [10]. Separately the constituents of the composite maintains their features, however when mixed they constitute a compound with their own properties inherent to the new composition. Two examples of natural fiber composites are: 1. wood, which is basically formed of cellulose fibers and lignin (amorphous resin which binds the cellulose fibers); 2. bone tissue, which is formed by an inorganic phase, essentially carbonate-apatite, placed in an organic matrix, whose composition is about 95% type I collagen. Therefore, the composites are formed by the matrix, which is the continuous phase ("fiber network") and involves the other phase, the dispersed one. Among the several types of composites, polymer composites exhibit some advantages such as: low weight, corrosion resistance, high temperature resistance and good mechanical properties when compared to conventional engineering materials [98].

However, the current goal of tissue engineering is the development of polymer composites, metal-free, with mechanical properties similar to living tissue, especially bone tissue, for partial or total replacement or reconstruction of the organ or tissue being repaired.

Polymers can be classified as natural or synthetic and degradable or non-degradable. These compounds provide versatility in their structure and can modulate the mechanical properties of other compounds like ceramics. Degradable polymers may be advantageous in certain clinical situations.

Composites produced from a combination of natural polymers (collagen, cellulose, polyhydroxybutyrate), or synthetic [poly (lactic-*co*-glycolic acid) (PLGA), poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL)] associated with bioceramics have been highlighted in academic community [99-106] because they are biocompatible, excellent osteoconductors, bioactives, have satisfactory mechanical properties, and are absorbable, therefore they are potential materials for application in regenerative medicine therapies.

Natural polymers or biopolymers have attractive properties for the construction of 3D scaffolds, such as biocompatibility and biodegradability. Bioactivity of these polymers, if you need to improve, can also be controlled by the addition of chemicals, proteins, peptides, and cells. The most commonly studied natural polymers for the purpose of bone engineering are collagen/gelatin, chitosan, silk, alginate, hyaluronic acid, and peptides [107].

Currently, the most BS, available commercially, for clinical application in implant dentistry based on polymers are barrier membranes for guided bone regeneration (GBR) or collagen sponge/BMPs (INFUSE®) for bone reconstruction.

At the beginning of the use of the GBR the treatment was preferably with non-resorbable membranes based on expanded polytetrafluoroethylene (e-PTFE) [108, 109], because of its inert characteristic and their biological effective and predictable results as a mechanical barrier.

However, resorbable membranes have been widely used to the development of new biomaterials, due to the predictable and similar results compared to the non-resorbable membranes [110-112], moreover resorbable membranes can be used on peri-implant defects, *i. e.*, an advantage in relation to non-resorbable membranes. Among the membranes the most used as resorbable membranes are: collagen, PLA and PLGA [110-113].

The type I collagen is an example of biopolymers quite used to the development of BS. It is a matrix that provides a favorable environment for induction of osteoblasts differentiation *in vitro* and osteogenesis *in vivo* [114]. Type III collagen constitutes the reticular fibers of the tissues and is also widely used in the manufacture of membranes for GBR. The non-mineralized collagen membranes are usually weak (low tensile strength) making their clinical manipulation difficult. The great advantage of them is the excellent cell affinity stimulating the chemotaxis of fibroblasts and acting as support migration of these cells (osteoconduction). Other advantages are: good adaptation to bone surfaces, especially to dental roots and hemostatic effect [113]. When embedded in the bone matrix they are gradually metabolized by the action of collagenase, or can be partially embedded in the bone matrix.

The resorption of collagen occurs parallel to bone formation as well as by the formation of new periodontal tissue such as cementum and periodontal ligament. The resorption time ranges from 06 to 08 weeks depending on the strength of the material, however it can last from 04 to 06 months [115]. In this case, the new bone is protected against the growth of connective tissue within the defect area. Despite prevent cellular infiltration, this membrane is permeable to nutrients, and the degradation occurs through enzymatic reactions without irritating the surrounding tissues. These membranes have adequate mechanical resistance [116]; moreover, they can facilitate the maintenance of the space to be regenerated, similar to the non-resorbable membranes.

The collagen membranes developed in recent years have shown optimal physicochemical characteristics for clinical application [117-121]. According to the literature, the determination of the density of crosslinking reaction (cross-linking) directly influences the physical properties of collagen matrices, *i. e.*, the increased crosslinking of collagen fibrils provides increased tensile strength and enzymatic degradation, and higher thermal stability [118, 119, 121, 122].

The membrane Bio-Gide® has been the most membrane widely used for GBR in the last years [113, 123-126], which is composed of type I and III collagen from porcine. This membrane has a bilayer structure with a compact layer and other porous. The porous layer (inner face) promotes a three-dimensional matrix for bone integration. The natural collagen structure of the Bio-Gide ® is ideal for tissue adhesion, while the newly formed bone is protected against the growth of connective tissue into the defect region; while preventing cellular infiltration this membrane is permeable to nutrients, and the degradation occurs through enzymatic reactions without irritating the surrounding tissues.

Studies with collagen membrane for GBR have reported satisfactory results *in vivo*, for example, the rate of bone regeneration has a similar efficacy to the e-PTFE membranes. This occurred due to the advent of collagen membranes with good mechanical strength. In the past,

it was difficult to obtain such satisfactory and predictable results due to the difficulty of producing collagen membranes with these characteristics [123, 127].

4. New perspectives for bone substitutes

4.1. Bone tissue engineering

In recent years, a new generation of bone substitutes have been developed in an attempt to obtain materials closer to the autograft standard by using biomaterials capable of inducing specific cellular responses at the molecular level, by integrating the bioactivity and biodegradability of these materials [107]. These BS are being based on the concept of bone tissue engineering. Tissue engineering/regenerative medicine has emerged as an interdisciplinary field that includes cell-based therapies and use of porous-bioactive materials for development of functional substitutes for the repair or replacement of damaged tissues or organs [128]. Tissue engineering has achieved great progress in the development of three-dimensional materials (scaffolds) for repair or replacement of damaged tissues or organs, including alloplastic materials such as bioceramics, bioactive glasses and polymers [60, 61, 63, 129, 130] in association to the signaling pathway, molecular and/or biophysical stimulation. Thus, tissue engineering is based on three elements that must be in synergism: matrix (scaffolds), cells and signals (mechanical and/or molecules: proteins, peptides and cytokines) [131, 132]; the absence or dysfunction of one element will halt or delay tissue regeneration. Furthermore, the tissue formation inside the scaffolds is directly influenced by porosity rate and pore size. In the case of bone formation, scaffolds should preferably have pores greater than 300 μM for promoting a good vascularization and a new bone formation, preventing hypoxia and induction of endochondral formation before the osteogenesis [133].

Porous scaffolds have been developed by variety of conventional methods from alloplastic materials, such as particle/salt leaching, chemical/gas foaming, fiber bonding, solvent casting, melting molding, phase separation and freeze drying [134, 135]. However, these methods present some limitations due to their lack of the controlled formation of pores and do not produce interconnected structures to favoring cell growth inside the structure. For overcome these disadvantages, additive manufacturing (AM), also otherwise known as three-dimensional (3D) printing, is a promising option for the production of scaffolds particularly for bone substitutes.

This technique consists in constructing 3D scaffolds by a tool for direct digital fabrication that selectively prints a respective material (layer-by-layer) into/onto a bed, whose shape is given by CAD specifications [136]. A distinctive feature of this layer-by-layer printing process, is the printing of structures with high geometric complexity and well-defined architecture as well as patient-specific implant designs, which are not possible to be constructed by any other manufacturing method (Figure 1).

Some of the commercially available AM techniques are 3DP (ExOne, PA), fused deposition modeling (FDM, Stratasys, MN), selective laser sintering (SLS, 3D Systems, CA), stereolithog-

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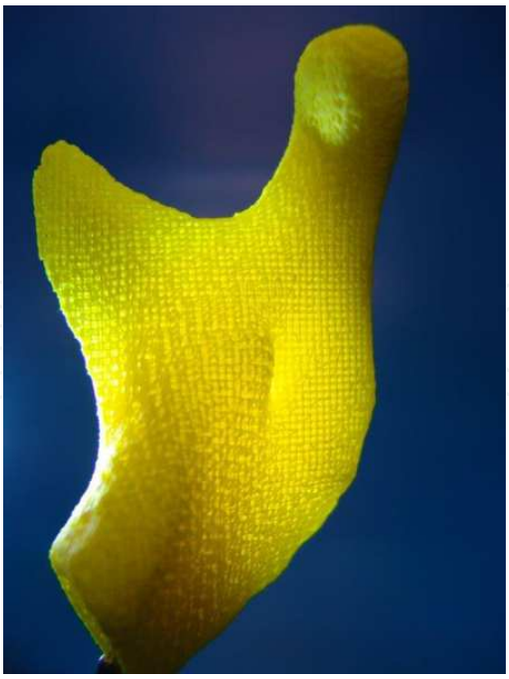

Figure 1. Printed mandibular condyle by Fused Deposition Modeling (FDM) process. *Image provided by Centro de Tecnologia de Informação (CTI) – Renato Archer (Campinas, Brazil).*

raphy (3D Systems, CA), 3D plotting (Fraunhofer Institute for Materials Research and BeamTechnology, Germany), as well as various methods [135]. These AM techniques can be classified as – (a) extrusion (deformation + solidification), (b) polymerization, (c) laser-assisted sintering, and (d) direct writing-based processes [135, 136].

Recently, some researches have performed using 3D printed scaffolds for bone regeneration. Among them we can highlight the use of this 3D printed BS based on bioceramics for vertical bone augmentation as onlay graft. The results shown by these studies are promising and efficient as bone graft compared to autografts [137-139]. Li et al (2011) [140] reported a case report of a 3D printed mandibular condyle implant made of nano-hydroxyapatite/polyamide. The clinical results suggest that this type of 3D printed implant can be a viable alternative to the autografts for maxillofacial defects. 3D printed scaffolds base on PLGA have also demonstrated good results for bone regeneration [141]. So far, as signaling pathway, growth factor and drug delivery, have been reported the use recombinant human BMP-2 (rhBMP-2) [142] and alendronate [143]. PCL/PLGA/gelatin scaffolds containing rhBMP-2 did not induce the osteogenic differentiation of mesenchymal stem cells *in vitro*, however, in the preclinical experiements, PCL/PLGA/collagen/rhBMP-2 showed the best bone healing quality at both weeks (4 and 8 after implantation) without inflammatory response. On the other hand, a large number of macrophages indicated severe inflammation caused by burst release of rhBMP-2 [142]. In addition, a study about 3D-printed bioceramic scaffolds containing alendronate shows that *in vivo* local alendronate delivery from PCL-coated 3DP TCP scaffolds could further induce increased early bone formation [143].

4.2. Growth factors

Osteoprogenitor cells, osteoblasts and osteoclasts are under growth factors activity. The role of growth factors is not only to stimulate cell proliferation through cell cycle regulation by initiating mitosis but also to maintain cell survival and to stimulate the migration, differentiation and apoptosis as well. Osteoblasts proliferate mediated by growth factors released by themselves and by the bone during the resorption process. Among the most important are the TGF-β and the factors released by the bone matrix, such as growth factor similar to insulin (IGF-1 and 2), the fibroblast growth factor (FGF-2) and growth factor derived from platelets (PDGF) [144, 145] which are potent mitogens [146, 147].

Moreover, other factors are secreted during the repair process, such as BMPs and angiogenic factors (vascular endothelial growth factor - VEGF) [147]. TGF-β presents activity in embryonic development, cell differentiation, hormone secretion and immune function, and acts synergistically with TGF-α in the induction of phenotypic transformation [146]. The TGF-β superfamily includes TGF-β1, TGF-β2, TGF-β3 and other important factors, such as BMPs 1-8, which promote several stages of intramembranous and endochondral ossification during bone repair [148].

Among these several growth factors, BMP-2 has received attention from the scientific community due its use in combination with different scaffolds to promote bone repair, especially in tissue engineering. The literature, in the constant search for developing a biomaterial with excellent osteoinductive properties, such as autogenous bone for reconstructive surgery, has recently shown that some polymers and bioceramics can be great carriers for BMPs, especially the collagen [54, 57, 59, 143, 149-152].

The concept of osteoinduction was first described by Urist in 1965 [153] when he observed new bone formation inside the demineralized bone matrices. Since then, these proteins, BMPs, have been reported as factors responsible for bone neoformation [149, 154]. BMPs attract mesenchymal cells to the site of bone formation by chemotaxis, and induces the conversion of these cells to a pre-osteoblastic lineage. BMP-2, 6 and 9 are described as important for the initiation of the differentiation of mesenchymal cells into pre-osteoblasts, while BMP-4 and 7 promote the differentiation of pre-osteoblasts into osteoblasts [155].

Clinical studies with rhBMP-2 using collagen as a carrier for surgical protocol of vertebral column showed similar or better results compared to autografts [150, 156-158]. However, the cost-effectiveness ratio of BMPs is questionable because of the large required amount (12 mg, 1.5 $mg.mL^{-1}$ therapeutic dose of INFUSE®) to obtain an effective bone repair in comparison to conventional surgical techniques [159].

In recent years, the use of synthetic peptides has been highlighted due to the ease of recognition and binding to specific sites of the extracellular matrix proteins increasing the material-cell interaction and for do not promote an immunogenic reaction. In this context, the specific amino acid sequence Arg-Gly-Asp (RGD) of the extracellular matrix proteins, such as fibronectin and osteopontin is recognized by the transmembrane receptors (integrins) [160, 161], and promotes better adhesion, and consequently a greater proliferation of osteoblastic cells. This RGD

sequence has been widely used for functionalization of biomaterials in order to stimulate the initial process of cell adhesion [162-164].

5. Cytototoxic, genotoxic and mutagenic tests of biomaterials

Biomaterials may have low, medium or high potential risk to human safety, depending on the type and extent of the patient contact. Safety assessments of medical biomaterials are guided by the toxicological guidelines recommended by the International Organization of Standardization (ISO 10993-1/EN 30993-1). One of the recommended and appropriate steps for the biological assessment of potential medical biomaterials consists of an *in vitro* evaluation of cytotoxicity and genotoxicity [165].

It is important to consider the possible impact of the composition on processes linked to cell proliferation and survival. It is essential to ensure that the proportional amounts of each component do not impoverish the cytocompatibility of the final composite, due to the release of toxic or irritating components. Therefore, *in vitro* cytotoxicity tests represent critical requirements previous to the clinical application of such materials (ISO 10993-12; [166])The choice of one or more cytotoxic tests depends on the nature of the sample to be evaluated, the potential site of use and the nature of the use (ISO 10993-5).

Cytotoxicity can be evaluated regarding the cell viability. XTT is a soluble variation of the widely employed MTT test, which accounts for mitochondrial activity in the tested material [166, 167]. Dimethyl sulfoxide solubilization of cellular-generated 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) - formazan presents several inherent disadvantages of this assay, including the safety hazard of personnel exposure to large quantities of dimethyl sulfoxide, the deleterious effects of this solvent on laboratory equipment, and the inefficient metabolism of MTT by some human cell lines [167, 168]. Recognition of these limitations prompted development of possible alternative microculture tetrazolium assays utilizing a different tetrazolium reagent, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT), which is metabolically reduced in viable cells to a water-soluble formazan product. This reagent allows direct absorbance readings, therefore eliminating a solubilization step and shortening the microculture growth assay procedure [167]. Therefore, in XTT test mitochondrial dehydrogenase activity is measured by the ability of such enzymes to reduce the reagent XTT to soluble formazan salts, with differing color.

To evaluate cell survival, Neutral Red uptake cytotoxicity test detects membrane intact viable cells by incorporation of the dye in their lysosomes [166, 169]. It is one of the most used cytotoxicity tests with many biomedical and environmental applications and most primary cells and cell lines from diverse origin may be used [169]. The procedure is cheaper and more sensitive than other cytotoxicity tests (tetrazolium salts, enzyme leakage or protein content) [169].

Bone substitute and implant materials have been evaluated regarding cytotoxicity by different assays [166, 170-173].

It is inherent in the provision of safe medical devices that the risk of serious and irreversible effects, such as cancer or second-generation abnormalities, can be minimized to the greatest extent feasible. The assessment of mutagenic, carcinogenic and reproductive hazards is an essential component of the control of these risks (ISO 10993-3). An international standard (ISO 10993) lays down specific requirements for biocompatibility, including the tests based on the nature of the contact and the duration of implantation of the biomaterial. The standard stipulates that all materials that will be in contact with mucous, bone, or dentinal tissue if the contact exceeds 30 days, as well as all implantable devices if the contact exceeds 24h, must undergo genotoxicity testing [174].

A useful approach for assessing genotoxic activity is the single cell gel electrophoresis (SCGE) or Comet assay. Singh et al. (1988) [175] introduced a microgel technique involving electrophoresis under alkaline conditions for detecting DNA damage in single cells which led to a sensitive version of the assay that could assess both double- and single-strand DNA breaks as well as the alkali labile sites expressed as frank strand breaks in the DNA. In this technique, cells are embedded in agarose gel on microscope slides, lysed by detergents and high salt, and then electrophoresed for a short period under alkaline conditions [175]. The assay is called a comet assay because the damaged cells look like a comet under a microscope. Cells with increased DNA damage display increased migration of DNA from the nucleus toward anode [176], so it appears like a comet tail that moves away from the unbroken DNA ("comet head") (Figure 2). Cells with increased DNA damage display increased migration of DNA from the nucleus toward anode [175]. Staining with different fluorescent dyes like ethidium bromide, propidium iodide, SYBR green quantifies the migrating DNA [176]. The most flexible approach for collecting comet data involves the application of image analysis techniques to individual cells, and several software programs are commercially available [176].

Some advantages of the SCGE assay is its sensitivity for detecting low levels of DNA damage, the requirement for small numbers of cells per sample, its flexibility and the short time needed to complete a study [176].

The SCGE assay has the capability to assess an increasing genotoxicity of a biomaterial model, whatever the cause and mechanism of the genotoxicity [174].

The *in vitro* micronucleus assay is well established in the field of toxicology for screening the effects of physical and chemical agents that may damage the DNA of eukaryotic cells [177]. The micronucleus assays have emerged as one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage to be measured reliably [178]. Because of the uncertainty of the fate of micronuclei following more than one nuclear division it is important to identify cells that have completed one nuclear division only [178]. In the cytokinesis-block micronucleus (CBMN) assay the cytokinesis is blocked using cytochalasin-B (Cyt-B). Cyt-B is an inhibitor of actin polymerization required for the formation of the microfilament ring that constricts the cytoplasm between the daughter nuclei during cytokinesis [178].

Micronuclei (MNi) are acentric chromosome fragments or whole chromosomes that are left behind during mitotic cellular division and appear in the cytoplasm of interphase cells as small

http://dx.doi.org/10.5772/59487

additional nuclei [179]. MNi are morphologically identical to nuclei but smaller (Figure 3). The diameter of MNi usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei [180]. The number of micronuclei in 1000 binucleated cells should be scored and the frequency of MN per 1000 binucleated cells calculated [178].

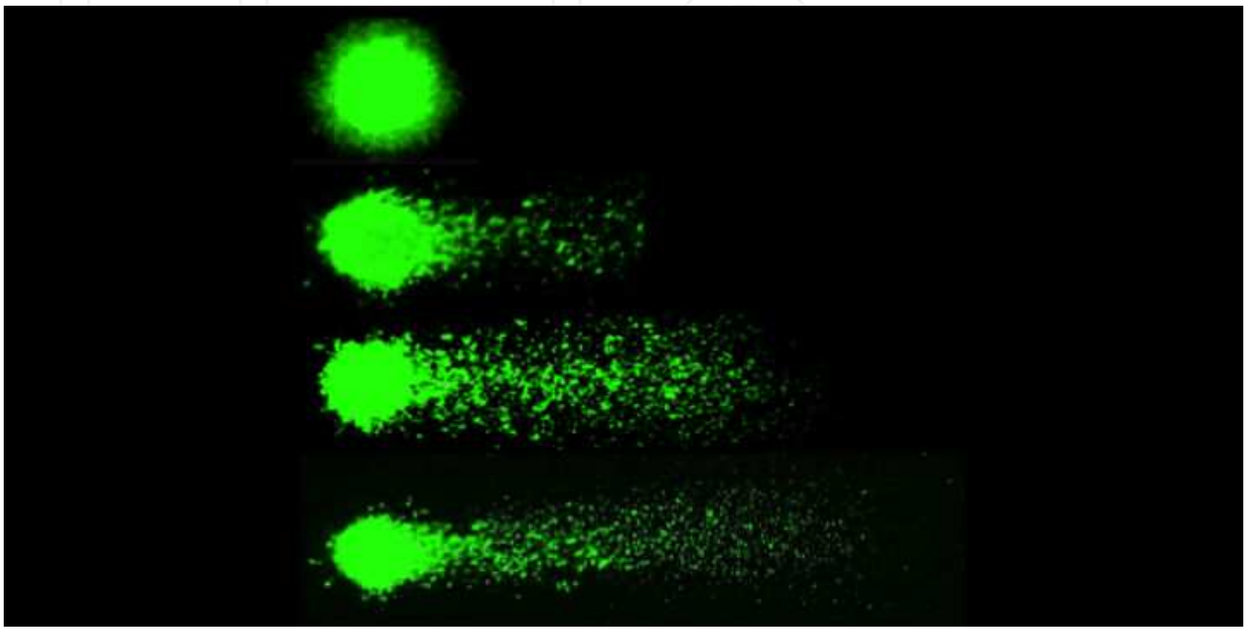

Figure 2. CHO-K1 cells exposed to different treatments. We can observe cells with different quantity of DNA damage obtained from Comet Assay. CHO-K1 cells stained by SYBR green. The cell located more superiorly presents minimal damage (about 5%) and the other cells show higher DNA damage. The longer is the tail of the "comet", the greater is the migration of damaged DNA.

Due to CBMN assay reliability and good reproducibility, it has become one of the standard cytogenetic tests for genetic toxicology tests in human and mammalian cells [180].

The measurement of nucleoplasmic bridges (NPBs), nuclear buds (NBUDs) and MNi of binucleated cells led the development of the concept of the cytokinesis-block micronucleus cytome (CBMN Cyt) assay [180]. The frequency of binucleated cells with MNi, NPBs or NBUDs provides a measure of genome damage and/or chromosomal instability. An NPB is a continuous DNA-containing structure linking the nuclei in a binucleated cell which originates from dicentric chromosomes (resulting from misrepaired DNA breaks or telomere end fusions) in which the centromeres are pulled to opposite poles during anaphase [180]. NBUDs represent the mechanism by which a nucleus eliminates amplified DNA and DNA repair complexes. They are similar to MNi in appearance with the exception that they are connected with the nucleus by a bridge [180]. Figure 3 shows NPB and NBUD in binucleated cells.

Since no single test has proved to be capable of detecting mammalian mutagens and carcinogens with an acceptable level of precision and reproducibility, a battery of tests is needed (ISO 10993-3).

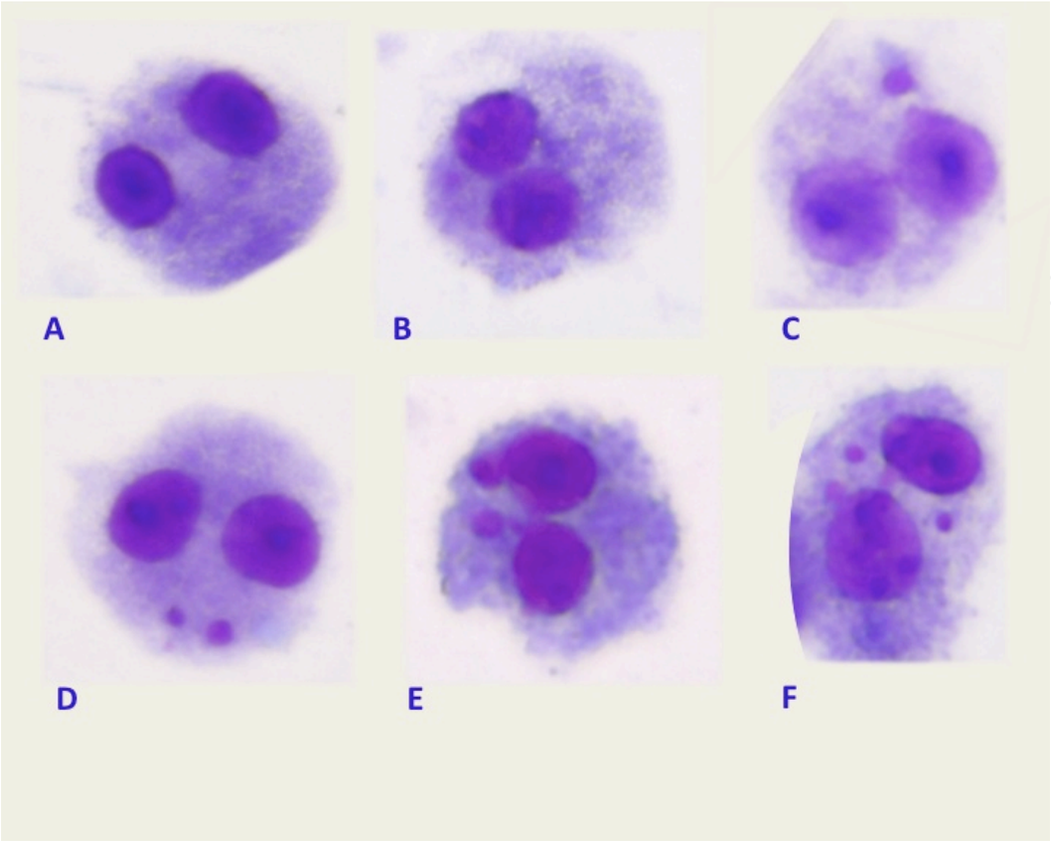

Figure 3. CHO-K1 cells after CBMN assay. We can observe binucleated cells (A, B); a binucleated cell with one micronucleus (C); a binucleated cell with two micronuclei (D); a binucleated cell with NBUDs (E); a binucleated cell with micronuclei and a NPB between the main nuclei.

5.1. Some biomaterial studies — Cytotoxic, genotoxic, mutagenic assays

Because of the low biodegradation rates of hydroxyatatite (HA), beta-tricalcium phosphate was added to HA, generating a biphasic calcium phosphate (BCP) composite, which may play an important role during assisted bone regeneration [166]. The authors [166] evaluated the cytocompatibility of dense HA, porous HA, dense BCP and porous BCP by three different cell viability parameters (XTT, Crystal Violet Dye Elution, Neutral Red assay) on human mesenchymal cells. No significant differences on mitochondrial activity (XTT) or cell density (Crystal Violet Dye Elution) were observed among groups. Dense materials induced lower levels of total viable cells by Neutral Red assay. It was concluded that porous BCP has shown better results than dense materials and these ceramics are suited for further studies [166].

Authors [165] evaluated cytotoxic, genotoxic and mutagenic effects of fluor- hydroxyapatite (FHA) and fluorapatite (FA) eluates on Chinese hamster V79 cells and compared them with the effects of hydroxyapatite (HA) eluate. The results showed that the highest test concentrations of the biomaterials (100% and 75% eluates) induced very weak inhibition of colony growth (about 10%). On the other hand, the reduction of cell number per colony induced by these concentrations was in the range from 43% to 31%. The comet assay showed that biomaterials induced DNA breaks, which increased with increasing test concentrations in the order

HA < FHA < FA. None of the biomaterials induced mutagenic effects compared with the positive control; and DNA breakage was probably the reason for the inhibition of cell division in V79 cell colonies.

Calcium phosphate cements are an important class of bone repair materials. Dicalcium phosphate dihydrate (DCPD) cements were prepared using monocalcium phosphate monohydrate (MCPM) and hydroxyapatite (HA) [170]. Degradation properties and cytocompatibility of this cement were analyzed and compared with β-tricalcium phosphate (β-TCP). The percent of viable cells as well as the percent of necrotic and apoptotic ones were evaluated by flow cytometry-based cell viability/apoptosis assay. According to the results, although conversion to HA has been noted in DCPD cements prepared with β -TCP, the conversion occurred rapidly when HA was used as the base component. HA during cement preparation seemed to accelerate the process and led to a rapid pH drop, extensive mass loss, a complete loss of mechanical integrity, and reduced cytocompatibility [170].

Authors [173] evaluated poloxamines, i.e., X-shaped poly(ethylene oxide)-poly(propylene oxide) block copolymers with an ethylenediamine core (Tetronic®), as an active osteogenic component and as a vehicle for rhBMP-2 injectable implants [173]. After cytotoxicity screening of various poloxamine varieties, Tetronic® 304, 901, 904, 908, 1107, 1301, 1307 and 150R1 and poloxamer Pluronic® F127 were analyzed. Tetronic® 908, 1107, 1301 and 1307 solutions were the most cytocompatible and it was concluded that the intrinsic osteogenic activity of poloxamines offers novel perspectives for bone regeneration using minimally invasive procedures (i.e., injectable scaffolds) and overcoming the safety and the cost/effectiveness concerns associated with large scale clinical use of recombinant growth factors [173].

Recombinant human bone morphogenetic protein 2 (rhBMP-2) has been widely employed for the induction of bone growth in animal models and in clinical trials [177]. Authors [177] prepared their own rhBMP-2 and the micronucleus assay was used to evaluate the genotoxic effect of it. It was concluded that author's preparations of recombinant human BMP-2 prepared in E. coli do not promote DNA damage in the concentration range tested.

A fully crystallized bioactive glass–ceramic material (Biosilicate®) for bone repair was developed and the biocompatibility was evaluated by means of histopathological (after subcutaneous test), cytotoxic (MTT) and genotoxic analysis (Comet assay). Neonatal murine calvarial osteoblastic (OSTEO-1) and murine fibroblasts (L929) were employed in this study. The results indicated that Biosilicate® scaffolds was biocompatible and noncytotoxic and did not induce DNA strand breaks at any evaluated period [172].

Polymethyl methacrylate (PMMA) is an acrylic resin which is widely used as a biomaterial due to its excellent biocompatibility and haemocompatibility [181]. *In vitro* micronucleus (MN) induction by PMMA bone cement was analyzed in cultured human lymphocyte [181]. The results showed a highly significant increase in MN frequency in human lymphocytes treated with PMMA and consequently a genotoxic effect of this substance or of the aphorised residual ingredients, which continue to be released in small amounts from the polymer. According to the authors, after the polymerization process, small quantities of ingredients usually present

in self-curing methacrylate bone cements are released and their rate of diffusion depends on storage conditions.

Titanium has been one of the most clinically applicable metals in bone tissue to serve as fracture fixation devices and also as endosseous implants for the rehabilitation of various parts of human body, especially in the oral maxillofacial region [182]. Piozzi et al. (2009) [182] evaluated whether liver, kidney, and lung of rats were particularly sensitive organs for DNA damaging (Comet assay) and cytotoxicity (histopathological changes) following implantation of internal fixture materials composed by titanium alloy in rats. No histopathological changes in cells of lung, kidney or liver were observed in the negative control group and in the experimental groups. The liver, lung and kidney cells did not show any genotoxic effects along the time course experiment. In the same way, no cytotoxic effects were present since neither tissue alterations nor signals of metals deposition were evidenced in these organs, even after 180 days of titanium exposure [182].

Metallic implants can release not only biocompatible ions but also some particles from mechanical wear or degradation. After corrosion or mechanical wear, these metal biomaterials release toxic elements such as ions or particles to the environment. Biodegradable metals seem to be the suitable material for orthopedic applications. Screws and plates made of magnesium alloys may work as stable biodegradable implants, which avoids the instance of a second operation. However, despite their use in novel technology, there is no available information about the possible toxic effects of magnesium particles (MP) from wear debris on human health [171]. Authors [171] used Mg powder to simulate the presence of MP wear debris within a cell culture and cytotoxic and genotoxic effects (comet assay and micronucleus induction) were analyzed. Neutral red (NR) incorporation and acridine orange/ethidium bromide (AO/EB) staining techniques were used to analyze the cytotoxic effects at 25–1000 μg/mL concentration range. Changes in lysosome activity were observed after 24 h only at 1000 μg/mL. Accordingly, AO/EB staining showed a significant decrease in the number of living cells at 500 μg/mL. A significant dose-dependent increase in MN frequencies was observed at 25–100 μg/mL range (nontoxic range). DNA damage induction was observed by comet assay only at 500 μg/mL. Therefore, authors verified a dose-dependent cytotoxic and genotoxic effects of MP on UMR106 cells with different threshold values of MP concentration.

6. Summary

This chapter approaches the most current bone substitute materials used in implant dentistry, as in research as in clinical application, for alveolar ridge augmentation, maxillary sinus lift and guided bone regeneration, such as: alloplastic materials (bioceramics, bioactive glasses, glass-ceramics, polymers and composites) and bioactive molecules (peptides and growth factors). In addition, concepts of tissue engineering used for the development of the new materials and techniques for implant dentistry were approached. Moreover, this chapter approached some cytotoxic, genotoxic and mutagenic assays used to evaluate the safety of biomaterials. Some studies that evaluated cytotoxicity, genotoxicity and/or mutagenicity of biomaterials were presented.

Thus, the use of bone substitutes continues to increase along with the availability of new technologies. Many alternatives for the replacement of autografts, allografts and xenografts are emerging. Rigorous preclinical and clinical studies are necessary to confirm the cost-effectiveness of these approaches over traditional bone grafts methods with benefits of technological advancement exceeding risks to the patient and costs of implantation.

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