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Review

Chitosan stabilizes platelet growth factors and modulates stem cell differentiation toward tissue regeneration

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ABSTRACT

The idea of using chitosan as a functional delivery aid to support simultaneously PRP, stem cells and growth factors (GF) is associated with the intention to use morphogenic biomaterials to modulate the natural healing sequence in bone and other tissues. For example, chitosan–chondroitin sulfate loaded with platelet lysate was included in a poly(D,L-lactate) foam that was then seeded with human adipose-derived stem cells and cultured *in vitro* under osteogenic stimulus: the platelet lysate provided to the bone tissue the most suitable assortment of GF which induces the osteogenic differentiation of the mesenchymal stem cells. PDGF, FGF, IGF and TGF-β were protagonists in the repair of callus fractures. The release of GF from the composites of chitosan–PRP and either nano-hydroxyapatite or tricalcium phosphate was highly beneficial for enhancing MSC proliferation and differentiation, thus qualifying chitosan as an excellent vehicle. A number of biochemical characteristics of chitosan exert synergism with stem cells in the regeneration of soft tissues.

Contents

Abbreviations: ADSC, adipose derived stem cell; AFM, atomic force microscopy; ALP, alkaline phosphatase; BMP, bone morphogenetic protein; BMSC, bone marrow stem cells; $Ca_3P_2O_8$, tricalcium orthophosphate; DMEM, Dulbecco modified Eagle's medium; ECM, extracellular matrix; ESC, embryonic stem cells; FGF, fibroblast growth factor; GAG, glycosaminoglycans; GF, growth factors; GFP, green fluorescent protein; H&E, hematoxylin and eosin; HPC, hematopoietic progenitor cells; HSC, hematopoietic stem cells; IGF, insulin-like growth factor; NMI-chitosan, N-methylimidazole chitosan; MSC, mesenchymal stem cell; PDGF, platelet derived growth factor; PDLSC, periodontal ligament stem cells; PRP, platelet-rich plasma; RGD, Arg-Gly-Asp tripeptide; SA-chitosan, salicylate-modified chitosan; SOD, superoxide dismutase; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; TXA_2, thromboxane A_2.

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1. Introduction

The present article reviews the associations of chitosans (a family of cationic partly acetylated and optionally modified (1-4)-2-amino-2-deoxy-β-D-glucans) with growth factors, either from platelets or stem cells, mainly intended for the regeneration of bone and soft tissues.

Chitosans and platelets were described for the first time by C. Rouget (1859) and G. Bizzozero (1881), respectively (Coller, 1984; Gravela, 1989; Muzzarelli, 1977; Muzzarelli, Boudrant, et al., 2012; Paweletz, 2001; Ribatti & Crivellato, 2007). Nearly one century had to elapse before platelets were studied for the development of wound dressing endowed with exceptionally high hemostatic activity, manufactured from chitosans from marine diatoms and crustaceans.

In particular the diatom chitin/chitosan fibers were found to have superior hemostatic activity compared to the other chitosans (Fischer, Bode, Demcheva, & Vournakis, 2007). Examples include Syvek-Patch® whose chitin fibers are obtained from aseptic culture of the diatom *Thalassiosira fluviatilis*, and hydrogels consisting of either partially or fully deacetylated chitosans. Chitin/chitosan fibers tightly bind major plasma proteins and a specific sub-set of platelet surface proteins, resulting in the acceleration of fibrin gel formation when platelet integrins contact plasma protein-saturated chitin/chitosan fibers (Fischer et al., 2005). The essential reasons for the exceptional performances reside in the chemical nature and tertiary/quaternary structure of the chitin/chitosan whose surface is recognized by the platelets: *via* the staining of the activation marker P-selectin, it was possible to reveal the sudden activation of the platelets whose pseudopodia make a robust contact immediately upon activation.

In a time scale of nano- to micro-seconds, the consequences are the start of the coagulation cascade, the red blood cell agglutination, thrombin generation and fibrin mesh synthesis in the microenvironment of the polysaccharide. The mechanical constriction is then augmented by the vasoconstriction generated by the platelets through the clot retraction.

A number of manufacturers are today producing chitosan-based hemostatic bandages approved by FDA and certified by CE and NATO (see Table 2 in Muzzarelli, 2009), currently used in emergency and under extreme conditions in consideration of their exceptional performances (Devlin, Kircher, Kozen, Littlejohn, & Johnson, 2011; Klokkevold, Fukayama, Sung, & Bertolami, 1999; Pozza & Millner, 2011; Pusateri et al., 2003; Sugamori, Iwase, Maeda, Inoue, & Kurosawa, 2000; Weiner, Fischer, & Waxman, 2003; Whang, Kirsch, Zhu, Yang, & Hudson, 2005). This applied research area developed at a very high pace, while the basic knowledge on chitosan combined with platelet-rich plasma or single GF for tissue regeneration progressed slowly.

Many GF have well established roles in embryonic development and skeletal homeostasis. Angiogenic factors are important in consideration of the transport of oxygen, nutrients and circulating cells: thus, angiogenesis is regulated by soluble compounds such as VEGF, PDGF, FGF and IGF. Among osteogenic factors, the members of the highly conserved TGF-β superfamily play an important role in embryonic development, tissue morphogenesis, cell proliferation and cell differentiation; TGF-β1 and TGF-β2 inhibit bone resorption and osteoclast activity, and trigger rapid maturation of collagen in early wounds. Because fracture healing is characterized by inflammation, renewal and remodeling phases, the control of inflammation involves the manipulation of pro-inflammatory cytokines and growth factors temporally and spatially released following bone injury. It was shown that inflammatory compounds including TNF-α, interleukins, interferon-γ and prostaglandins stimulate the migration and differentiation of osteoblasts and osteoclasts. Several authors discussed the role of various factors in bone regeneration, and examined the requirements for protein, cell and gene therapy for bone regeneration, with emphasis on the materials and methods for controlled delivery (Panuganti, Schlinker, Lindholm, Papoutsakis, & Miller, 2013; Takayama & Eto, 2012; Vo, Kasper, & Mikos, 2012).

Pessimistic forecasts on the application of GF in the management of chronic ulcers were based on the rationale that GF are inactivated by high levels of MMP in the ulcers, have scarce bioavailability and high manufacturing cost (Sweeney, Miraftab, & Collyer, 2012). Therefore, their use would be justified only in the presence of a functional delivery aid or scaffold capable to offset said drawbacks, or even enhance the activity of the GF: the covalent immobilization of BMP-2 onto chitosan nanofibers imparts higher bioactivity, and greater cell proliferation, when compared with plain absorption (Zustiak, Wei, & Leach, 2013). Moreover, chitosan is an inhibitor of MMP (Kim, Ahn, Kong, & Kim, 2012).

Besides the inhibitory activity against MMP, characteristic properties of chitosan appreciated for tissue engineering in regenerative medicine are the following: atoxicity; biodegradability; availability in various physical forms; chemical and enzymatic versatility; mucoadhesivity; suitability for controlled release of cytokines, extracellular matrix components and antibiotics and for retention of the normal cell morphology; entrapment of GF to accelerate the healing; stimulation of integrin-mediated cell motility and increased *in vitro* angiogenesis; integrin-dependent regulation of the pro-angiogenic transcription factor Ets1; promotion of the attachment, proliferation and viability of seeded stem cells.

Basic information on chitins and chitosans is amply available in the bibliographies of most recent review articles (Muzzarelli, Boudrant, et al., 2012; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012; Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). In particular, the following characteristics that qualify chitosans in wound healing can be recalled: chemoattraction and activation of macrophages and neutrophils to initiate the healing process; promotion of granulation tissue and re-epithelialization; limitation of scar formation and retraction; analgesic and hemostatic activities; activation of immunocytes; release of glucosamine and N-acetylglucosamine monomers and oligomers that stimulate cellular activities besides being used as building blocks in the synthesis of the ECM; intrinsic antimicrobial activity.

Platelet-rich plasma has been proposed for therapeutic purposes and for tissue engineering as well (Borzini & Mazzucco, 2005; Crovetti, Martinelli, & Issi, 2004; Ehrenfest, Rasmusson, & Albrektsson, 2009; Gigante et al., 2010, 2012; Marx, 2004; Mazzucco, Balbo, Cattana, Guaschino, & Borzini, 2009; Okamoto et al. (2003); Rodriguez-Merchan, 2012; Whitaker, Quirk, Howdle, & Shakesheff, 2001). PRP is obtained easily on the day of surgery from autologous whole blood (Kutlu, Aydin, Akman, Gumusderelioglu, & Nohutcu, 2013; Weibrich, Kleis, & Hafner, 2002), thus the autologous origin excludes the risk of transmission of diseases (Soyer, Cakmak, Aslan, Senyucel, & Kisa, 2013).

GF tend to be released quickly from PRP, and as a consequence, they soon lose their activity, and their clinical efficacy. Although centrifugation, freeze-thawing and thermal cycles are simple operations (Leytin, Mody, Semple, Garvey, & Freedman, 2000; Picker, 2011; Zaky, Ottonello, Strada, Cancedda, & Mastrogiacomo, 2008) the handling requires precautions, because the platelet density values in PRP may change according to the preparation technique, ranging from 2- to 7-fold above physiological levels (Santo, Duarte, et al., 2012; Santo, Gomes, et al., 2012). Therefore, a major concern comes from the fact that hyper-concentration of platelets induces further platelet activation and leads to decrease or loss of platelet functionality (Gollehon, King, & Craig, 1998). For these reasons, scaffolds suitable for the stabilization of platelets and GF are sought.

Stem cells are extraordinary insofar as they self-renew and differentiate to mature somatic cells *in vitro* and *in vivo*. They offer unlimited and consistent supply of physiologically relevant cells from pathogen-free sources for cell replacement therapies, and other applications. The emerging area of induced pluripotent stem cells (iPS) has stimulated the interest in stem cell technology because somatic cells can be reprogrammed to a pluripotent state. The microenvironment in which cells are cultured is to be optimized because the spatial configuration of stem cells and the ECM substrate has great consequences on their fate. Moreover, the pharmaceutical industry is realizing the potential of stem cells as an important alternative to recombinant cell lines.

In general, human primary hematopoietic stem cells derive from cord blood, bone marrow, and peripheral blood; human pluripotent stem cells have been proposed as an alternative source (Panuganti et al., 2013; Takayama & Eto, 2012) because they can proliferate infinitely *in vitro*, thereby providing samples with reproducible performances. Platelets are generated from human embryonic stem cells and induced pluripotent stem cells *in vitro* mainly for transfusion therapy and genetic studies, that guarantee the availability of a permanent supply of GF for experiments in tissue regeneration.

Cells capable of multi-lineage differentiation release specific GF when in contact with the suitable milieu (Rehman, Traktuev, & Li, 2004). An elegant demonstration was provided with the aid of green fluorescent protein transfection for *in vivo* tracking of stem cells delivered to an injured site: engrafted GFP cells were identified throughout the layers of the dermis and the cutaneous appendages after surgery. Engrafted ADSC were identified on the basis of the GFP signal, staining positive for the nuclear marker of proliferation Ki67, indicating ongoing proliferation of ADSC. The latter displayed a fibroblastic phenotype, on the basis of GFP/HSP-47 co-staining. In fact, ADSC produce significant amounts of IGF-1 and VEGF (Altman et al., 2009; Sadat, Gehmert, & Song, 2007; Traktuev, Merfeld-Clauss, & Li, 2008).

Adipose tissue obtained from liposuction contains a large quantity of mesenchymal stem cells that can be used clinically to compensate for said deficiency (Gimble, Katz, & Bunnell, 2007). The multilineage differentiation of these cells has been demonstrated. Otherwise said, when seeding MSC, in particular ADSC, into a wounded tissue, suitable GF are produced by ADSC and do not need to be supplied artificially.

Protocols for rapid and optimal expansion and maintenance of undifferentiated hMSC have been developed (Gjelstrup & Stelzer, 2012). hMSC are candidates for clinical use because they lend themselves to expansion in culture while preserving their prerogative to differentiate at a later stage (*e.g.* incorporation into a scaffold) into the osteogenic, adipogenic or chondrogenic lineages. The proliferation and differentiation of stem cells on chitosan hydrogels has been discussed (Debnath et al., 2013): oligomeric chitosan helps the mice marrow cells to proliferate and induces CD34+ cells into megakaryocyte progenitor cells. The primitive HSC can differentiate to two groups: one is the myeloid HSC, which differentiates to erythrocyte, granulocyte, monocyte, and blood platelet; the other one is the lymphoid stem cell (Wei, Chen, Mao, & Wang, 2013).

The reader is referred to the book chapters and reviews by Croisier and Jerome (2013), Jain et al. (2013), Jiang, Deng, Abdel-Fattah, and Laurencin (2012), Liu, Ma, Mao, and Gao (2011), Muzzarelli (2009), Muzzarelli (2011), Sahoo, Pan, Li, and He (2013) and St.Denis, Dai, Huang, and Hamblin (2012) that are good sources of basic information. On the other hand, the present article testifies a sudden upsurge of interest in the title topic in the early 2013 bibliography. Thus the primary scope of this review article is the identification and presentation of a novel research trend that identifies chitosan as the best functional and stabilizing carrier intended for (i) the delivery of platelet-rich plasma and platelet lysates as well as stem cells to wounded tissues and (ii) the proliferation of the host cells as well as the differentiation of artificially seeded stem cells. The appreciation of the synergy of the unique chemical performances of the chitosan multiform wound dressings with the biochemical actions of platelets and stem cells in the regeneration of lost and wounded tissues is a further scope of this article.

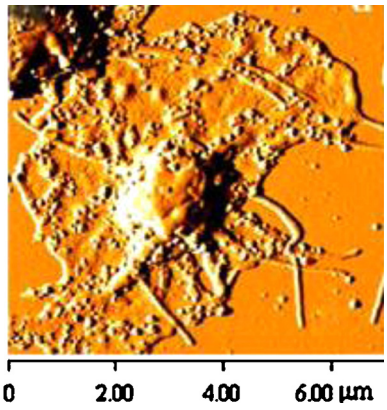

Fig. 1. AFM image of platelets adhered to a hyaluronan–collagen/chitosan film. Many granules are noticeable on the whole body of the platelets. Alpha granules excrete a number of growth factors; dense granules release calcium, serotonin, and ADP or ATP.
From Wu et al. (2008).

2. Compatibility of platelets, growth factors and stem cells with chitosan

2.1. Platelets

During the growth of 3T3 fibroblasts on the surface of a hyaluronan–collagen–chitosan film, the spreading and aggregating behavior of platelets made visible by AFM indicated that the great majority of them exhibited pseudopodia (Hayashi, Yamada, Guchi, Koyama, & Ikeda, 2012; Wu, Hu, Cai, Ma, & Wang, 2008). Most important, there were many 60–100 nm granules on the surface of the spreading platelets (Fig. 1). One of the conclusions of the work was that the hyaluronan–collagen/chitosan film, even after partial crosslinking with glutaraldehyde, is safe for fibroblast growth, and enables the platelets to have a regular behavior (Wu et al., 2008). Films made of 5 variously carboxymethylated chitosans and 2 oligomeric chitosans exhibited platelet aggregates and clumps on the surface of the membrane layer with 70–80% coverage. All samples qualified as hemostatic agents, and enhanced platelet adhesion and aggregation *in vitro* (Periayah et al., 2013). Likewise, chitosan-coated polycaprolactone electrospun nanofibers exhibited pharmacological effects including increased platelet aggregation, and anti-bacterial, anti-adhesive and anti-inflammatory activities. The enhancements of the platelet aggregation were further promoted by calcium ions incorporated in the chitosan overlayer (Bai, Chou, Tsai, & Yang, 2013). Therefore, what already mentioned above for chitosan isolated from diatoms holds true for marine crustacean chitosan (Lord, Cheng, McCarthy, Jung, & Whitelock, 2011) as well as for carboxymethyl chitosans.

Two methods can be used to prepare PRP-loaded chitosan scaffolds: (i) PRP was added to chitosan gel before freeze-drying and (ii) PRP was embedded into freeze-dried chitosan scaffolds. SEM demonstrated that in group (i), there is no deterioration of scaffold porosity and interconnections. Sustained release of GF was achieved in group (i) while a sharp burst release was observed for group (ii). This study demonstrated that chitosan scaffold (i) could be an appropriate carrier for PRP applications by providing sustained release of GF (Kutlu et al., 2013).

2.2. Growth factors

Of course there are many studies about synergic activities of selected GF: for example, a dual growth factor release system with PDGF and basic fibroblast growth factor (bFGF) was developed where GF were encapsulated within chitosan nanoparticles embedded in bacterial gellan and xanthan gels as well as directly seeded within the latter. The said gels become highly viscous at body temperature upon injection at the injury site. The good differentiation of ADSC and the production of GAG took place in the course of 7 days; the viability of both L929 fibroblasts and ADSC was over 90% (Dyondi, Chandra, Bhonde, & Banerjee, 2012). Likewise, but without using PRP, highly porous chitosan alone and in combination with IGF-1 and BMP-2 was assayed in bone regeneration. Histological results pointed out good osteoblastic proliferation along with proliferating fibroblasts, vascularization and reticular networks. High adsorption (90%) for both IGF-1 and BMP-2 imparted narrower distribution of macropores, and better mechanical strength. *In vivo*, the controlled release of IGF-1 and BMP-2, accompanied by the enzymatic depolymerization of chitosan and presence of chitosan oligomers, significantly promoted bone regeneration (Nandi, Kundu, & Basu, 2013). Coaxial electrospinning techniques were adopted to encapsulate VEGF and PDGF, with the purpose of regulating proliferation of vascular endothelial cells and vascular smooth muscle cells. Four weeks of *in vivo* replacement of rabbit carotid artery demonstrated that VECs and VSMCs developed on the lumen and exterior side of grafts, respectively, without thrombus formation. Dual-delivery of VEGF and PDGF by the electrospun membranes facilitated revascularization (Zhang, Jia, et al., 2013).

PRP lends itself to hydrogel formation, into which seeded cells can grow and differentiate. For example, PRP releases endogenous GF, that aid BMSC and ADSC seeded therein to differentiate into chondrocytes for cartilage repair (Xie et al., 2012). Fig. 2 shows the xerogels as such (A) and with seeded MSC (B).

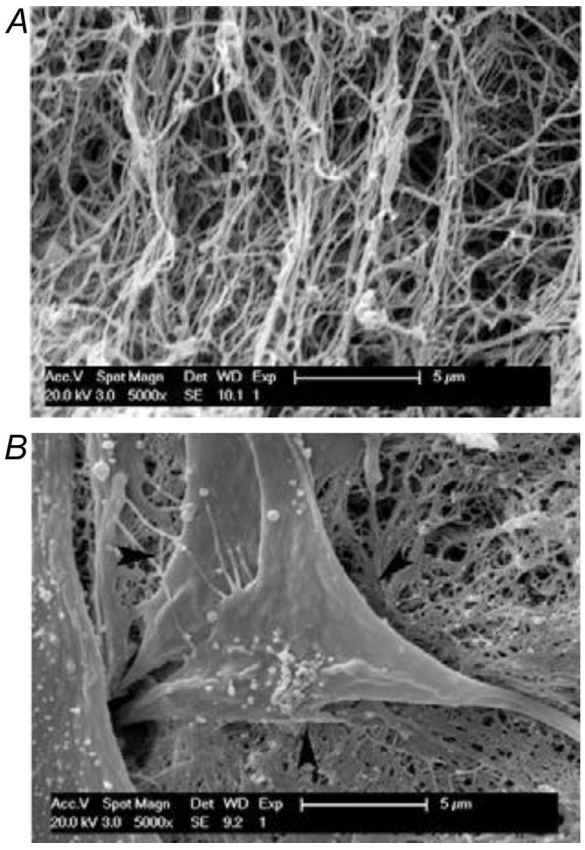

Fig. 2. Components and structure of the platelet-rich plasma. (A) SEM of the PRP scaffold as a xerogel and (B) SEM of the PRP seeded with mesenchymal stem cells, as a xerogel. Bars, 5 μm.
From Xie et al. (2012).

2.3. Stem cells

Rabbit autologous ADSC were expanded *in vitro*, seeded on the polyglutamate chitosan polyelectrolyte complex (PLG–chitosan) and chondrogenically induced with growth medium supplemented with TGF-β1, IGF-1, dexamethasone and transferin; after 24 h incubation the cell-scaffold constructs were cultured *in vitro* for 5 weeks to be later implanted to heal articular cartilage defects (Zhang, Zhang, et al., 2013). The long-term outcomes were followed by evaluation of the hyaline cartilage regeneration. The amount of GAG increased impressively because the PLG–chitosan scaffolds were suitable for the chondrogenic differentiation of ADSC, representing a micro-environment similar to the natural one. The ADSC grew along the pore rims and after 2 weeks they filled most pores to be embedded in dense ECM; they exhibited minimal cell mortality before implantation. Deposition of GAG and collagen-II increased significantly since the beginning; chondrogenic markers, including collagen-II, aggrecan, and SOX9 were evidently expressed during *in vitro* induction for as long as 5 weeks, indicating that PLG–chitosan scaffolds with pore size of 150–200 μm were suitable for ASCs chondrogenic differentiation. The compressive modulus of the engineered cartilage progressively increased to nearly 78% after 12 weeks. In summary, the scaffolds seeded with induced ADSC achieved hyaline cartilage regeneration, thus repairing a full thickness articular cartilage defect. An injectable preparation included rhBMP-2 and dexamethasone to support proliferation and osteoblastic differentiation (Gao, Cai, Kong, Han, & Yao, 2013).

This example permits to appreciate a particularly important step forward from the standpoint of this article: the GF seeded or released by chitosan-supported platelets and by chitosan-supported stem cells enable the latter to proliferate and differentiate in the most desirable way, thus leading to rapid and regular regeneration of lost/wounded tissues.

3. Bone healing

The idea of using chitosan as a functional delivery aid to support simultaneously PRP, stem cells and GF has been developed in the course of the last decade in consideration of the respective characteristic properties (Lee et al., 2000; Mooney & Vandenburgh, 2008), and was associated with the intention of using morphogenic biomaterials to mimic/modulate the natural healing sequence in bone (Mehta, Schmidt-Bleek, Duda, & Mooney, 2012). A crucial point to be considered preliminarily, however, is the porosity of the chitosan-based scaffold.

In fact, the clinical success of bone regeneration would be impeded if the vascularization obtainable in a scaffold is inadequate. The lack of vascular networks leads to insufficient supply of oxygen and nutrients, and jeopardizes the survival of implanted cells and their overall performance. To ensure the viability of seeded cells, less than 200 μm is required between cells and blood vessel (Kaully, Kaufman-Francis, Lesman, & Levenberg, 2009). However, as far as the clinical applications are concerned, 200 μm is such a small distance that the inner cells will have little chance to participate in the regeneration process. Therefore, a scaffold seeded with appropriate pluripotent cells, providing a favorable micro-environment and sufficient nutrient for prolonged viability, and with angiogenic and osteogenic potential, could be a reasonable starting point.

The speed and degree of vascularization play a critical role in the efficiency and integration of the engineered bone. The main

mechanism involved is angiogenesis, which demands the participation of endothelial cells and pericytes affected by multiple GF functioning in different stages of the process, such as the VEGF, angiopoietin, FGF, PDGF, and the family of TGF (Kanczler & Oreffo, 2008).

The mild gelling conditions of chitosan preserve the viability of incorporated cells. Chitosan is being favored by researchers for several other properties, such as good biocompatibility, high porosity and interconnected porous structure that favor nutrients and oxygen diffusion. Consequently, ADSC and a mixture of GF contained in PRP could confer osteogenic and angiogenic potential to the polysaccharidic carrier. For example, an *in vitro* study based on Ca alginate microspheres showed that PRP promoted migration and osteogenic differentiation of encapsulated ADSC, and sustained their viability. *In vivo* data demonstrated that the injectable microspheres loaded with 10–15% PRP-ADSC induced vascularization and mineralization (Man et al., 2012). The PRP-ADSC-loaded alginate microspheres may be applied in the micro invasive approach, and used also in the form of genipin-crosslinked alginate as well (Paul et al., 2013) once safety and efficacy are duly tested.

Bone engineering applications of clinical interest include the use of GF, BMP-2 and BMP-7, and PDGF (Lieberman, Daluiski, & Einhorn, 2002). Platelet-rich plasma has also been widely explored (Calori et al., 2008; Griffin, Smith, & Costa, 2009; Sarkar et al., 2006). Centeno, Busse, Kisiday, Keohan, and Freeman (2008) increased the knee cartilage volume in degenerative joint disease by using percutaneously implanted, autologous mesenchymal stem cells together with platelet lysate.

Although no significant improvement was seen after the addition of PRP in bone formation in non-critical size defects in a rabbit cranial model, autogenous bone (plain or with added PRP) showed a histo-morphometric tendency toward increased bone formation during the first month (Aghaloo, Moy, & Freymiller, 2002). The effect of chitosan sponge and PRP gel alone as well as their combination was studied on bone regeneration in rabbit cranial defects (4.5 mm diam.) (Oktay et al., 2010). Higher bone formation was observed in the PRP group when compared with the other groups at weeks 4 and 8. All parts of the defects were filled with thick trabecular new bone in the PRP group. Application of PRP showed histologically a tendency toward increased bone formation.

Chitosan–chondroitin sulfate nanoparticles loaded with platelet lysate were included in a poly(D,L-lactate) foam produced by supercritical fluid foaming. The foams were then seeded with human ADSC and cultured *in vitro* under osteogenic stimulus. The osteogenic differentiation of the seeded ADSC takes place with the lysate-loaded constructs, as revealed by the earlier detection of alkaline phosphatase and calcium. The release of platelet lysate from chitosan–chondroitin sulfate nanoparticles enhanced *in vitro* the osteogenic differentiation of human ADSC, as shown by the increased levels of mineralization, providing evidence not only of the role of the delivery system but also of the role of platelet lysate as an osteogenic supplement. The experimental data indicate the synergistic effect of ADSC and platelet lysate and throw light on the capacity of the latter to increase the functionality of the material (Santo, Gomes, et al., 2012). In fact the platelet lysate provides the bone tissue with the most suitable assortment of GF which induce the osteogenic differentiation of the mesenchymal stem cells: PDGF, FGF, IGF and TGF-β were protagonists in the repair of callus fractures (Bourque, Gross, & Hall, 1993; Xia et al., 2011).

Other *in vivo* studies show that platelet concentration required for a positive effect on bone regeneration spans a limited range of 2–8-fold above the physiological levels while lower concentrations have sub-optimal effects, and higher concentrations might have a paradoxically inhibitory effect. The 5-fold increase in the platelet concentration was the optimal one to induce a positive effect on osteogenic differentiation: of course, the standardization of the isolation procedure is highly recommended (Santo, Duarte, et al., 2012; Zhang, Madhu, Dighe, Irvine, & Cui, 2012).

Chitosan composites with inorganic compounds such as tricalcium orthophosphate are of paramount importance in the regeneration of bone, particularly so when they are amenable to injectable hydrogels. Chitosan solution and $Ca_3P_2O_8$ powder were mixed at the ratio of 10:6 (ml:g), then 1 ml of PRP was added to the mixture. As soon as PRP was activated by bovine thrombin and calcium chloride (100 IU thrombin and 1 ml 10% $CaCl_2$ solution per ml PRP), the final composite (PRP–$Ca_3P_2O_8$–chitosan) was injected into circular molds to make cylinders and disks for mechanical testing and cell culture. To make the chitosan citrate solution three components were mixed at a weight ratio of 2:25:20 (chitosan/citric acid/glucose) in distilled water. The final mass fraction of chitosan was 2%, the degree of deacetylation was 94%, and the molecular weight was 680 kDa (Bi, Cheng, Fan, & Pei, 2010). The presence of PRP in the $Ca_3P_2O_8$–chitosan composite had no adverse effect on the mechanical strength and there was no diverse compressive strength in PRP–$Ca_3P_2O_8$–chitosan and plain $Ca_3P_2O_8$–chitosan. Instead, cell count and ALP activity of PRP–$Ca_3P_2O_8$–chitosan were higher than those of plain $Ca_3P_2O_8$–chitosan. By reverse transcriptase polymerase chain reaction, the high levels of Cbfa1 and TGF-β were detected early in MSC induced by PRP–$Ca_3P_2O_8$–chitosan. Bone formation following expression of collagen-I, osteocalcin, osteonectin and calcium nodules was also observed in MSC induced by PRP–$Ca_3P_2O_8$–chitosan. Moreover, this composite was injected into the tibial bone defect in a goat model, for the confirmation of its ability to induce bone regeneration (Bi et al., 2010).

The same authors observed rapid proliferation of MSC in the early culture stages due to the biological effect of TGF-β1. Given the fact that Cbfa1 is essential for osteoblastic differentiation and bone formation (Street & Lenehan, 2009), the early detection of Cbfa1 in MSC induced by PRP–$Ca_3P_2O_8$–chitosan indicates that PRP enhances osteoblastic differentiation and bone formation. Both collagen-I and osteocalcin were detected in MSC induced by PRP–$Ca_3P_2O_8$–chitosan: the former accounts for 90% of bone matrix proteins, while osteocalcin is a major non-collagenous protein component of the bone ECM. The dynamic interactions of MSC with hydroxyapatite in a composite with chitosan and gelatin promoted osteogenic differentiation, denoting the capacity of hydroxyapatite to exert synergic action (Hunter & Ma, 2013).

The hESCd-MSC proliferation and osteogenic differentiation on $Ca_3P_2O_8$ admixed with chitosan covalently linked to RGD led to high percentage of live cells and high cell density (Chen et al., 2013). SEM examination showed hESCd-MSCs with a healthy spreading morphology. hESCd-MSCs expressed high levels of osteogenic markers, including alkaline phosphatase, osteocalcin, collagen-I, and Runx2. RGD-chitosan improved the strength and toughness of $Ca_3P_2O_8$, and greatly enhanced the incorporation of the latter into bone, as well as the hESCd-MSC attachment and proliferation. Similar conclusions were reached in a simultaneous work with RGD-chitosan (Tsai, Chen, & Liu, 2013). The osteogenic differentiation of MSC was also studied with an injectable scaffold made of chitosan, β-glycerophosphate and collagen, that supported neovascularization and differentiation of MSCs toward osteogenic lineage (Ding, Zhang, Yang, & Xu, 2013).

Woven bone and lamellar bone were identified in most of the specimens, and the defects were completely repaired within 16 weeks with little residual material observed in group PRP–$Ca_3P_2O_8$–chitosan (Fig. 3 upper frame). By contrast, in the control group, defects were filled with abundant non-degraded material: newly formed bone in the center and edge areas (Fig. 3 lower frame). The release of GF from the PRP–$Ca_3P_2O_8$–chitosan was found to be highly beneficial for enhancing MSC proliferation and differentiation, thus qualifying chitosan as an excellent vehicle;

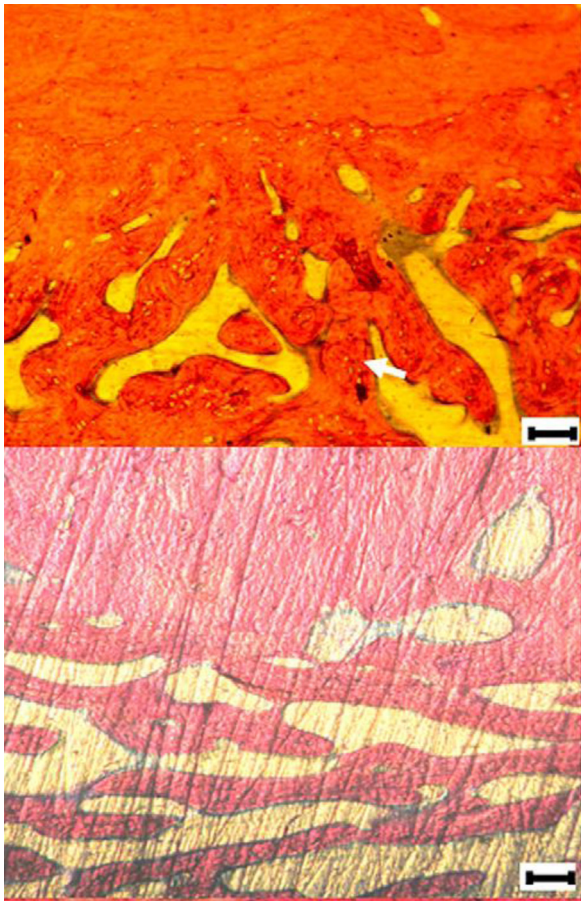

Fig. 3. Abundant woven bone and lamellar bone (white arrow in upper frame) were identified in most of the specimens in group PRP–$Ca_3P_2O_8$–chitosan, at 16 weeks. In group $Ca_3P_2O_8$–chitosan, the defects were filled with abundant non-degraded material, and only limited newly formed bone could be detected (lower frame). In the blank control group, the soft tissue in the center of the defects became dense and fibrotic with no evidence of newly formed bone [bars = 100 μm].
From Bi et al. (2010).

this material is injectable and can take the shape of the cavity into which it is placed (Bi et al., 2010). Likewise, $Ca_3P_2O_8$ was deemed to be a resorbable material endowed with biocompatibility and osteoconductivity (Dong, Uemura, Shirasaki, & Tateishi, 2002).

The behavior of human periodontal ligament stem cells seeded in a genipin-chitosan scaffold coated with nano-hydroxyapatite was evaluated in terms of the effects on seeded PDLSC in the course of bone repair. The PDLSC, that had the capacity to undergo osteogenic and adipogenic differentiation, exhibited great viability, alkaline phosphatase activity, and upregulated the bone-related markers, namely bone sialoprotein, osteocalcin and osteopontin to a great extent: the use of PDLSC-seeded scaffolds promoted calvarial bone repair in a rat calvarial defect model. This study demonstrated the remarkable role of nano-hydroxyapatite in the composite scaffold, and the potential of the stem cells combined with the said hybrid scaffold as a promising tool for bone regeneration (Ge et al., 2012).

It is worth mentioning that nano-hydroxyapatite was also studied in novel hybrid hydrogels prepared by introducing it into chitin solutions with the aid of NaOH/urea aqueous media at low temperature, and then cross-linking with epichlorohydrin. The hydroxyapatite nano-particles were uniformly dispersed in the crosslinked chitin hydrogel networks, a macroscopic consequence being that the latter exhibited about 10 times higher mechanical properties than the control chitin hydrogel. Importantly, cell culture experiments proved that cells could adhere and proliferate well on the hybrid hydrogels, indicative of good biocompatibility and suitability for tissue engineering, and absence of deleterious effects exerted by the nanoparticles on the cells (Chang et al., 2013).

3.1. Potentially enhanced performances of ad hoc modified chitosans

Exceedingly large osseous defects, deprived of the capacity to repair spontaneously, have been treated with chitosans that elicited the production of newly formed bone tissue to fill the defects completely. A modified chitosan carrying covalently linked imidazole groups was used to stimulate bone formation. N-Methylimidazole chitosan (NMI-chitosan) was prepared with the isomers imidazole-2-carboxaldehyde and imidazole-4-carboxaldehyde, *via* Schiff reaction followed by hydrogenation with sodium borohydride at pH 5: its identity was assessed *via* FTIR and HNMR (Fig. 4). The NMI-chitosan was in the form of isolated films, microspheres and freeze-dried scaffolds; its nitrogen content was 6.8%, and the pK_a 5.4 (instead of 7.89 and 6.1 for the parent chitosan, respectively). The preparation of N-imidazolemethyl chitosan *via* reductive Schiff reaction with said aldehydes demonstrated the possibility to avoid the use of carbodiimide and ancillary chemicals proposed by other authors. While the amide function is known to contribute negatively to the solubility of chitosans, the preservation of the primary amino group of chitosan in the form of a secondary amino group would instead be an additional contribution to the desirable cationicity of the imidazole-bearing polysaccharide for a number of applications among which, *in primis*, the regeneration of bone (Muzzarelli et al., 1994).

Lesions (7 mm diameter) were surgically made in the femoral condyle of sheep and treated with NMI-chitosan. Within 40 d after surgery, the neoformed tissue occluded the surgical hole and assumed a trabecular structure in the peripheral area of the lesion, while looking like a mineralization nodule in the central part in association with a fibrous component. In the control, no sign of osteoinduction or reparative process was observed and bone marrow was rich in adipocytes (Muzzarelli et al., 1994). NMI-chitosan appears to be much more effective in bone healing than chitosan itself and other modified chitosans so far tested, including the methyl pyrrolidinone chitosan used in an animal model and in the medication of the surgical lesion consequent to wisdom tooth avulsion (Muzzarelli et al., 1993). A most similar chitosan modified with imidazole groups for high-efficiency gene delivery was developed recently, having characteristics of interest in bone healing such as high solubility at physiological pH, low cytotoxicity, good biodegradability, besides high (70%) cell transfection (Shi, Zhang, Shen, Bi, & Dai, 2013).

4. Healing of soft tissues

4.1. Dermal wounds, ulcers and burns

A chitosan gel matrix can be seen as an acellular scaffold capable of enhancing by itself the tissue regeneration. Besides the ample information supporting this view, and the numerous articles containing data on the associations of chitosan with collagen (Hayashi, Yamada, Guchi, Koyama, & Ikeda, 2012), specific data on chitosan–atelocollagen 1:1, an easily bioresorbable hydrogel (Judith, Nithya, Rose, & Mandal, 2012), deserve to be recalled here. Topical application of chitosan–atelocollagen to the wounds was protracted for 10 days and the PDGF and nerve GF were determined in the epithelialized tissue in order to assess their over-secretion. In fact, as expected, the treated wound tissue showed higher concentrations of PDGF, and PDGF-mediated

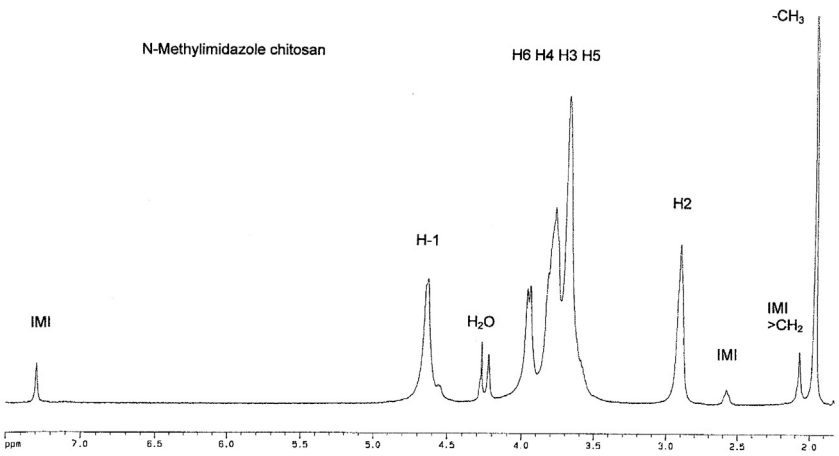

Fig. 4. The ^{1}H NMR spectrum of NMI-chitosan reveals the chemical identity of a carbohydrate polymer particularly suitable for bone regeneration in large bone defects. The new signal at 2.08 ppm is assigned to the methylene of the substituent, in analogy with the signal at 2.35 for 2-methyl imidazole. Moreover, the signal at 7.28 ppm corresponds to that at 6.90 for authentic 2-methyl imidazole and 7.10 ppm for authentic imidazole. NMI-chitosan is potentially suitable for the preparation of hydrogels including PRP. Courtesy of: Muzzarelli, RAA, original unpublished results.

NGF production. Therefore, the topical application of a gel comprising depolymerized atelocollagen and easily soluble chitosan triggers the healing process by inducing the extracellular matrix to facilitate cellular activities, and thus it promotes the various phases of repair. The protein level seemed to be higher in animals treated with chitosan + collagen compared to other groups (Judith et al., 2012). In their previous work, the same authors had in fact evaluated the potential of the collagen-chitosan gel containing PDGF to enhance healing and reduce the time of wound closure (Judith, Nithya, Rose, & Mandal, 2010). PDGF (0.2 μg) was dissolved in 1 ml of 10 mM acetic acid. The PDGF-containing collagen–chitosan gel was prepared by mixing100 mg collagen and 100 mg chitosan with 5 μl of 10 mM acetic acid that contained 1.25 ng PDGF. Compared to the control, the tissue samples from the group treated with the collagen–chitosan–PDGF gel had remarkable increase in antioxidants and lipid peroxide: the concentration of glutathione, ascorbic acid, catalase, SOD and GPX was increased in each of the treated groups and was greatest in albino rats treated with collagen–chitosan–PDGF (Table 1). Additionally, the histology and ultrastructure of re-epithelialized tissues at the wound site revealed that the collagen–chitosan gel loaded with PDGF exerted biomimetic and chemotactic effects and promoted wound healing. In addition to enhancing the cellular activities *via* PDGF treatment, the wound bed preparation using the said gel improved wound healing. Owing to the incorporation of PDGF, the chitosan gel used here optimized healthy tissue granulation and improved the therapeutic efficacy; the time to wound closure was reduced (Judith et al., 2010).

The mechanism of action of PRP being the molecular and cellular induction of normal wound healing responses, the therapy has clinical significance regardless of the wound etiology. Hence, PRP applications have gained considerable popularity in tissue regeneration (Han et al., 2007). Significant clinical outcomes indicated that many previously non responsive wounds began healing actively in the course of the platelet-rich plasma therapy (Carter et al., 2011). Patients with chronic ulcers underwent intralesional injection of PRP: 10 of them healed within a mean period of 7 weeks. In the other 16 cases, PRP prepared the wound bed for the final and simpler reconstructive procedure (Dionyssiou et al., 2012). Histological evidence testified effective therapeutic action on chronic ulcers. The platelet gel was mostly effective within the first 2 weeks of treatment while a prolonged treatment did not provide additional advantages when compared to the best current protocols (Scevola et al., 2010). The efficacy of platelet release on the healing of chronic diabetic foot ulcers was studied in comparison with platelet-poor plasma on 24 patients: the results showed that healing of the PRP group patients was significantly faster (Saad Setta, Elshahat, Elsherbiny, Massoud, & Safe, 2011). Likewise, analogous observations on decubitus ulcers in the horse have been made (Iacono et al., 2012).

Therefore it is convenient to use PRP with carriers that provide controlled release in order to increase the bioavailability of GF. Chitosans are efficient vehicles for the simultaneous release of various GF, and the rationale behind their use resides in the good results so far obtained with other polysaccharides, as well as in the superior biochemical significance of chitosans among the latter. Moreover, the gentle gelling conditions of polysaccharides offer the advantage

Table 1
Concentration of antioxidants and enzymes in the skin of control and experimental groups of female albino rats treated with chitosan.

Antioxidants	Normal skin	Wounded skin
Antioxidant concentration (μg per mg protein)		
Glutathione		
Control	2.62 ± 0.18	2.68 ± 0.09
Chitosan–PDGF	4.84 ± 0.39***	10.46 ± 1.23***
Collagen–chitosan–PDGF	6.56 ± 0.59***	11.21 ± 1.62***
Ascorbic acid		
Control	2.01 ± 0.07	2.12 ± 0.09
Chitosan–PDGF	2.57 ± 2.57**	2.62 ± 0.23**
Collagen–chitosan–PDGF	3.06 ± 0.13***	3.49 ± 0.36***
Catalase		
Control	2.02 ± 0.03	2.37 ± 0.04
Chitosan–PDGF	2.42 ± 0.22*	2.58 ± 0.31**
Collagen–chitosan–PDGF	3.13 ± 0.26***	3.61 ± 0.43***
SOD		
Control	1.46 ± 0.04	1.51 ± 0.03
Chitosan–PDGF	2.03 ± 0.11***	2.29 ± 0.18***
Collagen–chitosan–PDG	1.83 ± 0.10***	2.52 ± 0.13***
GPX		
Control	1.54 ± 0.26	2.96 ± 0.27
Chitosan–PDGF	6.83 ± 0.89***	9.26 ± 1.34***
Collagen–chitosan–PDG	9.43 ± 1.92***	11.43 ± 2.18***

From Judith et al. (2010).
Values are expressed as the mean ± SD for six animals. A Student's *t*-test was performed to compare the mean values of the various parameters between the experimental and control groups.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$

of preserving viability of incorporated cells and proteins. For example, patients with wound dehiscence and tendon exposure showed complete healing of the wounds 30 days after surgery by virtue of a combination of platelet-rich plasma and hyaluronan (De Angelis et al., 2012).

Chitosans and modified chitosans are deemed to be suitable for the delivery of platelet-rich plasma or platelet lysate to the bed of wounds, burns and ulcers (Crovetti et al., 2004; Degim, 2008; Rossi et al., 2012). In fact, chitosan dressings maintain the platelet GF in unaltered active form (as assessed by dosing PDGF, the representative of the entire pool present in the lysate, with the aid of the ELISA test).

Although chitosan polyglutamate scaffolds were found to support cell attachment and growth (Yan et al., 2013; Zhang, Zhang, et al., 2013), the choice of glutamate as a stoichiometric counter ion in the polyelectrolyte complex remains questionable owing to the high MW (128 Da) of glutamate that becomes an important mass fraction as high as 80% of the chitosan mass in the complex. This situation might prevent the reproduction of the results in case a smaller anion is selected such as glycolate (MW 87 Da) with a more reasonable mass fraction of 54% of the stoichiometrically corresponding chitosan. Moreover, neurotoxicity of glutamate has long been claimed (Muzzarelli, 2010; Olney, 1969; Wang, Wang, Kong, Man, & Qu, 2012).

In general, deep second-degree burns heal with extensive areas of scarring. Platelet-rich plasma obtained by centrifugation and addition of thrombin and calcium, results in a gel containing abundant GF. The objective of a study was to monitor the healing of said burns that involve all epidermis layers, including the basal laminae, and to characterize the PRP antibacterial effect in horses' burns (Maciel et al., 2012). All groups had bacterial contamination but no infection; PRP accelerated the repair, induced fibrosis and probably provided antibacterial activity in horses with burns. Previous data (Kellouche et al., 2007) concerned a dermal substitute (DS) for the definitive coverage of full-thickness burns. A dermal substitute composed of a collagen–GAG–chitosan dermal matrix (DM) bearing foreskin fibroblasts (FF) was described. FF-DS were compared to the DM seeded with adult dermal fibroblasts (DF). FF-DS expressed more fibrillin and tropoelastin than that with DF-DS. In the presence of platelet lysate both FF-DS and DF-DS produced cutaneous wound healing mediators in a dose-dependent manner. After freeze-thawing, the FF-DS were recovered in culture and retained their ability to produce VEGF. Grafting of dermal substitute into nude rats achieved complete healing of a dermal–epidermal lesion.

A chitosan gel, containing 10 μg EGF per ml, healed second degree burn wounds in rats in 14 days. There were significant increases in cell proliferation in the EGF gel group, and the epithelialization rate in the chitosan-EGF group was higher compared to the EGF solution group: thus it was concluded that chitosan promoted better and faster epithelialization (Alemdaroglu et al., 2006). Similarly, a chitosan gel formulation containing liposomes loaded with EGF was evaluated for the healing of second-degree burn wounds in rats. Liposomes containing 10 μg/ml EGF in 2% chitosan gel, EGF-chitosan gel, and EGF-loaded liposome formulations were applied daily to the burn wounds. Based on biopsies evaluated immuno-histochemically, significant increase in cell proliferation was reported for the EGF-liposome group, which also exhibited the highest epithelialization rate (Degim et al., 2011).

Finally, it is interesting to mention a study making use of ADSC for the purpose of illustrating the present trend in dermal tissue regeneration (Altman et al., 2009). A 75:25 silk fibroin–chitosan blend scaffold served as a delivery aid for human ADSC in a soft tissue injury murine model. Said delivery not only is feasible but confers the benefits of accelerated and physiologically valid wound closure, in agreement with the observation of enhanced wound closure with stem cell therapy. After being transplanted in their new microenvironment, ADSCs engraft, proliferate, and differentiate into fibroblastic, vascular, and epithelial phenotypes (Fig. 5).

4.2. Healing of corneal lesions

Fibroblasts have a crucial role in corneal healing: after a wound occurs, within the stroma activated fibroblasts appear in response to injury. Moreover, fibroblasts migrate to the wound area, secreting collagenases, proteases and extracellular matrix components, which contribute to the reconstruction of the damaged stroma (Geremicca, Fonte, & Vecchio, 2010). A review article on the ocular applications of chitosan has been published (Basaran & Yazan, 2012), while data on polysaccharides have been reviewed (Pahuja, Arora, & Pawar, 2012).

It was also possible to develop formulations suitable for prolonging the contact of platelet lysate with the damaged cornea, for the time necessary to exert a therapeutic effect. Polyacrylate and chitosan glutamate were autoclaved and loaded with platelet lysate and the resultant formulations were characterized in terms of rheology, mucoadhesion, vehicle compatibility and stability. The proliferation effect was tested rabbit corneal epithelial cells and fibroblasts; an *in vitro* wound-healing test was performed on fibroblasts (Sandri et al., 2011). Both formulations, compared with platelet lysate diluted with saline at the same concentration, were found to maintain the rheological and mucoadhesive properties of the vehicles, and the proliferative activity of the platelet lysate. Chitosan significantly enhanced epithelial cell growth even after storage for up to 2 weeks (in-use conditions), while the polyacrylate was less efficient. The *in vitro* wound-healing test performed on fibroblasts confirmed the differences between the two carriers. The effect induced by the chitosan formulated with platelet lysate was faster than that of the polyacrylate with lysate; complete *in vitro* wound repair was achieved within 48 h.

4.3. Neuronal tissue regeneration

The beneficial effects of PRP on wounds in persons with spinal cord injury are known (Rappl, 2011). As an alternative, somatic cells are currently reprogrammed *via* genetic modification with defined factors to induce pluripotent stem cells (iPS), which resemble ESC (Takahashi & Yamanaka, 2006). These innovated iPS cells can be a crucial solution to overcome the surgical rejection and the ethic issue for medical purposes (Jaenisch & Young, 2008). Owing to the stimulation by the micro-environment (such as the chitin/chitosan gels) and the induction by neuron growth factor (NGF), the iPS cells differentiate into neurons, improve axonal growth, and promote neurite regeneration for treating spinal cord injury (Salewski, Eftekharpour, & Fehlings, 2010; Tsuji et al., 2010). The iPS cells could maintain their phenotype and differentiate into neurons under induction by N-cadherin (Haque, Yue, Motazedian, Tagawa, & Akaike, 2012).

A mixture of chitin, chitosan, and gelatin gels was shortly treated with 1% (w/v) genipin to covalently crosslink them reciprocally. The volume ratio of chitin:chitosan:gelatin was typically 2:1:1. The resulting chitin–chitosan–gelatin gel was infiltrated into a sintered crystal template of colloidal polystyrene arrays and then the resulting composite was immersed in tetrahydrofuran overnight to dissolve the polystyrene microspherical beads. The neuronal differentiation of iPS cells in the ordered empty rooms left by the polystyrene was faster than that in the control. Based on this fact, the controlled geometry in chitin–chitosan–gelatin crossliked scaffolds can accelerate the production of neuron-lineage cells from iPS cells. The combination of genipin-crosslinked chitin–chitosan–gelatin scaffolds and iPS cells was used to design specialized biomedical devices for nerve regeneration. Similarly, alginate–chitosan–gelatin scaffolds crosslinked with genipin and

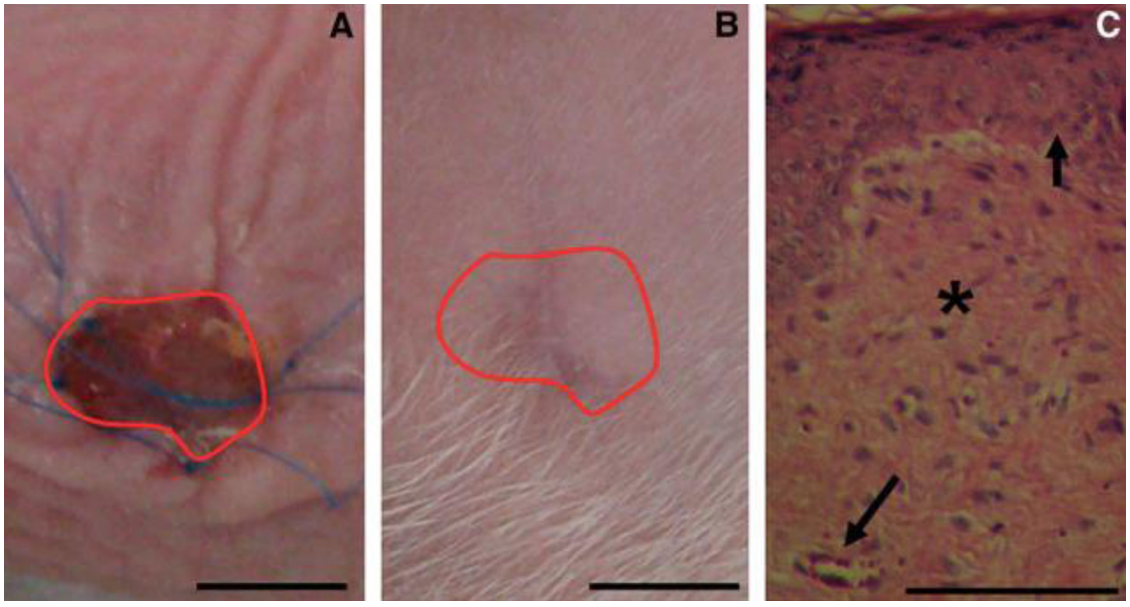

Fig. 5. Immediate post-operative and 2-week images of human ADSC-seeded chitosan–silk fibroin scaffold engrafted into a 6-mm-diameter, full-thickness cutaneous wound in athymic male mice, photomicrograph from region of regenerated wound bed tissue, and demonstration of significantly enhanced microvessel density in ADSC–chitosan–silk fibroin engraftment biopsies at 2 weeks after surgery. (A) Graft immediately after surgery. Scale bar 5 mm. (B) Healed wound bed at 2 weeks after surgery. Scale bar 5 mm. (C) Image from healed wound bed region: long arrow indicates vascular structure with residual erythrocytes; * indicates papillary dermis; short arrow indicates intact epidermal epithelium. Scale bar 100 μm.
From Altman et al. (2009).

holding NGF on their surface were found able to modulate the differentiation of iPS cells for the purpose of regenerating neurons (Daley, Peters, & Larsen, 2008; Hanna, Saha, & Jaenisch, 2010; Kuo & Lin, 2013; Kuo & Wang, 2013).

4.4. Potentially enhanced performances of an ad hoc prepared chitosan

Taurine, 2-aminoethane sulfonic acid, an ubiquitous strongly acidic aminoacid, plays various important physiological roles in almost all mammalian tissues (Huxtable, Azuma, Kuriyama, Nakagawa, & Baba, 1996; Ripps & Shen, 2012). It exhibits antioxidant effects and it influences cell proliferation, inflammation and collagenogenesis. In mice, the taurine-chitosan gel releases taurine slowly. A plain chitosan solution (1.5%) and the same with added taurine (50 mM) were applied to full thickness skin wounds of mice once a day for 7 days and then malondialdehyde and hydroxyproline levels along with the tensile strength of wound tissues were measured. Locally administered chitosan taurine salt increased wound tensile strength by decreasing malondialdehyde and increasing hydroxyproline levels, as supported by histological findings (Degim et al., 2002). Thus the taurine gel may be effective in wound healing because it would act synergistically with chitosan, and it is compatible with PRP and lysate: it should be underlined that the taurine concentration is 400-fold higher in platelets than in plasma and that it dissolves chitosan beads yielding neutral chitosan taurine salt solutions (Ahtee, Boullin, & Passonen, 1974; Frendo, Koj, & Zgliczynski, 1959).

5. Conclusions

Chitosan wound dressing and bandages have been approved and are now commercially available for the control of massive traumatic hemorrhage in emergency and battlefields, and in surgery on certain organs, owing to their unmatched performances. The latter are based on the recognition of chitin/chitosan fibrils surface by blood platelets, and consequent acceleration of the blood clot formation by a redundant biochemical mechanism.

On the other hand, chitosan-based dressings for the usual wound care do not have the prominence that they would deserve, evidently because the market is controlled by a number of manufacturers who have no interest in promoting new materials. Nevertheless, chitosan has been fully assessed not only in the human care field but also in veterinary medicine, in consideration of its most appreciable characteristics such as the capacity to avoid scar formation, and its antimicrobial activity.

When combining the knowledge on the recognition of the chitosan surface by platelets and on the efficacy of plain and modified chitosans to heal surgical and traumatic wounds, it is possible to envisage a novel kind of applications of chitin/chitosan for the regeneration of lost or wounded tissues. A number of recent articles underline the inherent favorable qualities of chitosan when used as a delivery aid of platelet-rich plasma and stem cells, optionally accompanied by inorganics such as $Ca_3P_2O_8$ and nano-hydroxyapatite.

While a chitosan gel matrix can be seen as an acellular scaffold capable to enhance by itself the tissue regeneration, in view of its peculiar characteristics listed above, this article identifies some advantages offered by chitosan as a GF delivery aid, and a support for seeded cells, namely: chitosan enhances the release of GF from the aggregated platelets; it provides a favorable microenvironment for prolonged viability of cells owing to well known angiogenic and osteogenic capacities; the gentle gelling conditions of chitosan preserve the viability of incorporated cells and host cells such as fibroblasts; chitosan dressings maintain the platelet GF in active form; chitosan prolongs the contact of platelet lysate with the damaged tissue for the time necessary to exert a therapeutic effect; chitosan significantly promotes better and faster epithelialization compared to polyacrylate; chitosan is suitable for the modulated differentiation of ADSC, the latter appearing to be more accessible than BMSC and ESC cells for surgical and ethical reasons, respectively; within chitosan ADSC differentiate into fibroblastic, vascular and epithelial phenotypes; MSC undergo osteogenic

differentiation in the scaffold made of hydroxyapatite-chitosan-gelatin, the favorite combination for bone formation; chitosan improves the strength of inorganics and enhances their incorporation into bone as much as the ESC-MSC proliferation; pluripotent stem cells most similar to ESC, in a chitin/chitosan milieu might offer a chance to overcome both surgical and ethical difficulties; in the wound site, a chemically tuned chitosan could become an unsurpassed scaffold for the modulation of the platelet activity, as well as for the consequent proliferation and differentiation of the seeded stem cells.

High throughput technologies to investigate stem cells and finding out improved culture protocols are expected to potentiate the efforts being made for their biopharmaceutical applications; the same can be said for platelets. The novel trend indicates that chitosan will supersede a number of natural and synthetic polymers. Of course advanced analytical data on the materials, well-designed protocols and adequately powered clinical trials are needed.

Conflict of interests

No conflict of interests exists.

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