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Review article

Repair of bone defects with gelatin-based composites: A review

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ABSTRACT

Numerous biomaterials are used in bone replacement therapy to repair defects caused by trauma, inflammation, tumor resection, or skeletal abnormalities. Ideally, the replacement material must be biocompatible and must be able to be reabsorbed or to dissolve naturally as the bone grows, yielding a newly remodeled bone. Gelatin, a partially denatured derivative of collagen, is biodegradable, exhibits good biocompatibility, and is less antigenic than collagen. Gelatin-based composites, therefore, provide an excellent scaffold for bone replacement. This paper provides a review of the work of the past decade in our laboratory on the development of gelatin-based composites that are suitable for repairing bone defects.

1. Introduction

Bioactive ceramics, such as hydroxyapatite, tricalcium phosphate (TCP), and bioglass are widely applied in clinical settings to strengthen the biocompatibility and osteoconductive characteristics of bone replacements [1–3]. Granular TCP ($Ca_3(PO_4)_2$) is a biodegradable bone replacement material composed of calcium and phosphate ions that is commonly employed to repair bone defects. Although TCP can be easily packed into the bone defect without prior molding or shaping, granular TCP is difficult to maintain within the defect site and lacks structural stability.

Gelatin binds well to TCP, resulting in a composite material that has good biocompatibility, mechanical strength, and plasticity [4]. However, *in vivo* studies have shown that gelatin-TCP-composite materials are readily resorbed primarily due to dissolution and enzymatically catalyzed hydrolysis of the gelatin component of that composite material [5]. Various synthetic crosslinkers, such as formaldehyde, glutaraldehyde, polyepoxy compounds, tannic acid, dimethylsuberimidate, carbodiimides, and acyl azide have been used to crosslink gelatin to prolong the absorption of the gelatin in living tissue and improve the mechanical properties of the composites [6–8]. These synthetic crosslinkers are, however, highly cytotoxic, thereby reducing the biocompatibility of gelatin-based synthetically crosslinked implants. In our previous study, we used glutaraldehyde to fix a gelatin-TCP mixture (GTG) [9]. The *in vitro* cytotoxicity evaluation revealed that the concentration of glutaraldehyde solution used as the crosslinking agent in GTG composites should be lower than 8%. Additionally, GTG should be soaked in distilled water for at least 4 days to decrease its toxicity before clinical application.

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Bone tissue engineering has the potential to regenerate bone with natural form and function. Successful bone tissue engineering depends on a suitable source of cells, appropriate culture conditions, and a biocompatible scaffold with a highly porous and interconnected pore structure. Recent advances in regenerative medicine have shown that stem cells can play an important role in the repair of skeletal defects. Bone marrow stromal cells (BMSCs) are a promising cell source for the restoration of bone defects because of their relative ease of procurement, great ability to self-renew, and their favorable osteogenic differentiation capabilities [10]. Several researchers have shown that BMSCs are effective at repairing bone defects in various animal models [11–13]. In addition, many researchers have studied the combination of BMSCs with gelatin-based scaffolds for bone tissue engineering [14–16]. In this review, we summarize the results of 10 years of research conducted in our laboratory on the effectiveness of gelatin-based composites as viable material for repair of bone defects.

2. Gelatin crosslinked with natural crosslinking reagents

Genipin is a natural crosslinking agent that is extracted from the fruits of *Gardenia jasminoides* Ellis. In our laboratory, we developed a material composed of genipin-crosslinked gelatin combined with TCP ceramic particles (GGT) that could be used as a scaffold for filling bone defects. We found that the degree of crosslinking and the rate of *in vitro* degradation of genipin-crosslinked gelatin could be controlled by varying the concentration of genipin [17]. The concentration of genipin for more complete crosslinking reaction in the GGT composite was 0.5 wt% [17,18]. However, the concentration of genipin must be greater than 0.5 wt% to eliminate the risk of cytotoxicity [19]. In fact, we found that a concentration of genipin that exceeds 80 ppm in the culture medium was cytotoxic to osteoblasts [18]. Experiments on the subcutaneous implantation of the GGT composite in rats demonstrated that the foreign body capsule of the composite that had been crosslinked with 1.00 wt% genipin was much thicker than that of composites that had been crosslinked with 0.05, 0.10 or 0.50 wt% genipin. In addition, the composite had a low degree of porosity (68.0% ± 2.5%) after adding genipin. Therefore, low-toxicity crosslinkers are required to enable implants to form stable and biocompatible crosslinked materials without inducing a cytotoxic effect.

Oligomeric proanthocyanidins (OPCs) are naturally occurring, minimally cytotoxic crosslinkers that are present in many fruits, vegetables, nuts, seeds, and flowers as well as in the bark of many plant species. OPCs belong to a subgroup of flavonoid-condensed tannins, which are highly hydroxylated structures that can form insoluble complexes with carbohydrates and proteins [20]. These proanthocyanidins have been successfully used to fix biologic tissues and biomaterials, including porcine valves, collagen matrices, and chitosan-gelatin films and have been shown to be minimally cytotoxic [21–24].

We prepared a biodegradable GTP composite comprising an OPC-crosslinked gelatin mixed with TCP for use as a bone substitute [25,26]. When 5.0 wt% of OPC was added, the crosslinking reaction between gelatin and OPCs proceeded as close to completion as possible. The result indicated that the ability to crosslink gelatin molecules was higher in genipin than in OPCs. However, adding 5 wt% of OPCs to composites was effective at providing resistance to degradation *in vitro* and *in vivo*. Moreover, OPCs at a concentration of less than 100 ppm were able to promote the proliferation of MG-63 cells, indicating that genipin has higher toxicity than OPCs. Additionally, evaluation of cytotoxicity demonstrated that OPCs, gelatin, and calcium ion gradually released from the GTP composite facilitated the growth of MG-63 cells *in vitro*. The results of the *in vivo* evaluation of the subcutaneous implantation of the composite material in rats revealed a fairly uniform layer of surrounding fibrous tissue. There was no variation in the thickness of foreign body capsules of the GTP composites crosslinked with OPCs at a wt% concentration of 5.0, 7.5 or 10.0 wt%. Furthermore, experiments on the biological response of the GTP composite using a rabbit calvarial defect model demonstrated that the GTP composite did not cause any deleterious effects on the underlying brain tissues. Moreover, there was more evidence of new bone formation in the group that received a GTP composite comprising 5.0 wt% of OPCs than in the group that received deproteinized bovine bone over all implantation periods. Progressive replacement of the GTP composite by new bone was preceded by a combination of osteoconduction and biodegradation.

3. BMSCs loaded onto a porous gelatin-based composite scaffold

The excellent biocompatibility and osteoconduction of the GTP composites make them promising materials for the clinical repair of bone defects. However, GTP composites have a dense morphology after the addition of OPCs. The dense morphology will impede the supply of oxygen and nutrients to the attached cells inside the scaffold and will detrimentally affect cell ingrowth. On the contrary, high porosity, large pores, and a three-dimensionally interconnected pore structure in the scaffold will provide sufficient space for the ingrowth of both new bone tissue and blood vessels following implantation into the host tissue.

We prepared a macroporous GTP composite containing TCP and OPCs crosslinked with gelatin using a salt-leaching method and found that the amount of salt added strongly influenced the morphology of the GTP composite. The GTP composite had a relatively homogeneous pore structure and a high porosity (~73%) when the weight ratio of salt particulates to composite was 4:1. The macropore sizes ranged from 400 μm to 550 μm. The cytotoxicity assay demonstrated that the extract from the porous GTP composite enhanced the proliferation of rat BMSCs and that the enhanced proliferation was attributable to the release of gelatin, OPCs, and calcium. In another experiment, we combined BMSCs with a porous GTP composite as a scaffold for bone tissue engineering. Rat BMSCs were seeded onto the porous GTP composite and cultured in a spinner flask to improve nutrient supply and metabolite removal. After 2 weeks of dynamic culturing, we found that the cells penetrated the pores and proliferated on

the scaffolds, indicating that the porous GTP scaffold is an appropriate material for the ingrowth of cells. We also tested whether the GTP scaffold could promote the ingrowth of new blood vessels from neighboring host tissues. The defect cavity in rats was filled with a BMSC-seeded GTP scaffold. We found that numerous erythrocytes were present in the BMSC-seeded scaffold at Week 4, suggesting that the interconnected macropores in the GTP scaffold promoted the ingrowth of new blood vessels from neighboring host tissues. We also found that there were numerous cells around the pores in the BMSC-seeded GTP scaffold. The cells that differentiated into bone-forming osteoblats were probably derived from the seeded BMSCs. These results indicate that the seeded BMSCs, the postrepair vascularization, and the release of gelatin, calcium, and OPCs from the scaffold are possible causes of the abundant proliferation of the cells at the cranial bone defect. These regenerating cells might modulate further development of bone tissue. After 8 weeks of implantation, the BMSC-seeded scaffold promoted more new bone formation than the acellular scaffold, suggesting that the use of BMSCs reduces the time required for the cells to invade the defect site.

4. Gelatin-based composites containing traditional Chinese herbal medicine

Many traditional Chinese herbal medicines are commonly used to treat orthopedic disorders and have been proven to be effective for bone regeneration [27]. We fabricated a composite composed of gelatin, TCP, genipin, and *Chi-Li-Saan*, a Chinese medicinal remedy, as a bone substitute to fill a large defect in the calvarial bone in rabbits [28]. The results revealed that animals that received the *Chi-Li-Saan* composite demonstrated a significantly greater amount of new bone growth than animals with bone defects that were not subjected to reconstruction therapy. In another study, we evaluated the activity of rat bone cells in animals that had been exposed to five different Chinese herbal drugs [29]. The results of bone cell culture experiments revealed that *Cuscuta chinensis* Lam. enhanced the proliferation and differentiation of osteoblasts but inhibited the activity of osteoclasts. *Loranthus parasiticus* Merr. and *Achyranthes bidentata* Bl. produced opposite results. *Eucommia ulmoides* Oliv. and *Dipsacus asper* Wall. promoted the proliferation and differentiation of osteoblasts but did not affect the activities of osteoclasts. The results of neonatal rat calvarias organ culture that had been exposed to a mixture of GGT composite and traditional Chinese medicine, such as *Cuscuta chinensis* Lam., *Eucommia ulmoides* Oliv. and *Dipsacus asper* Wall., demonstrated that these Chinese medicinal herbs effectively promoted the regeneration of defective bone tissue.

The dried rhizome of perennial pteridophyte *Drynaria fortunei* (Kunze) J. Sm., also known as *Gu-Sui-Bu* (GSB), is widely used in Asia for the treatment of bone-related diseases, including bone fracture, osteoporosis, and arthritis, and has been shown to have therapeutic effects on bone healing [27]. Naringin, a polymethoxylated flavonoid, is reportedly the main effective component of GSB, and has been shown to increase the amount of bone morphogenetic protein in osteoblasts. Several studies have demonstrated that GSB can enhance the proliferation and differentiation of osteoblasts as well as bone cell activities *in vitro*, and that it can inhibit the formation of osteoclasts [30–32]. We have found that GSB at a concentration of 100 μg/mL leads to a significant increase in osteoblast numbers, intracellular alkaline phosphatase levels, and nodule numbers, without influencing osteoclast activity [33]. Moreover, we have found that addition of GSB to GGT composites accelerates the regeneration of defective bone tissue.

We hypothesized that a combination of osteoinductive agents and BMSCs would promote bone healing. We, therefore, prepared a macroporous GGT composite using a salt-leaching method to carry GSB (GGT-GSB). The composite had a homogeneous pore structure with pore sizes ranging from 280 μm to 430 μm and a high porosity (~80%). Rabbit BMSCs were then seeded onto the porous composites. After a week of culture in a spinner flask, the cells effectively entered the scaffold. They were then autotransplanted into critical size calvarial defects in rabbits. After 8 weeks of implantation, new blood vessels formed and many erythrocytes were present in the BMSC-seeded GGT-GSB scaffold, indicating that blood vessels from the neighboring host tissues had successfully invaded the scaffold. There were also many regenerating osteoblasts in the peripheral and central areas of the autologous BMSC-seeded scaffold. These cells were probably derived from the seeded autologous BMSCs. Furthermore, the autologous BMSC-loaded GGT-GSB scaffold promoted more new bone formation at the defect site than the BMSC-seeded GGT and acellular scaffolds. In addition, new bone replaced a significant amount of GGT-GSB scaffold, revealing that the autologous BMSCs were responsible for bone formation at their locations. Moreover, GSB was gradually released from the biodegradable scaffold, which was most likely due to the effect of bone regeneration. Therefore, GSB can induce the formation of new bone by providing an effective biodegradable delivery system. Accordingly, a porous GGT scaffold composed of a GSB-based and autologous BMSC-based composite is an ideal biomaterial for the generation of new bone.

5. Conclusion

Bone tissue engineering is effective at repairing damaged or diseased skeletal tissue. A combination of gelatin with TCP can be used to create a biocompatible scaffold with osteoconduction characteristics. The addition of genipin and OPCs, naturally occurring low-cytotoxic crosslinkers, effectively reduces the degradation rate of the gelatin-TCP mixture. Moreover, incorporating BMSCs, GSB, and Chinese medicinal extracts into a porous gelatin-TCP scaffold can accelerate bone regeneration.

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