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The use of physical hydrogels of chitosan for skin regeneration following third-degree burns

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Abstract

Skin repair is an important field of the tissue engineering, especially in the case of extended third-degree burns, where the current treatments are still insufficient in promoting satisfying skin regeneration. Bio-inspired bi-layered physical hydrogels only constituted of chitosan and water were processed and applied to the treatment of full-thickness burn injuries. The aim of the study was at assessing whether this material was totally accepted by the host organism and allowed *in vivo* skin reconstruction of limited area third-degree burns. A first layer constituted of a rigid protective gel ensured good mechanical properties and gas exchanges. A second soft and flexible layer allowed the material to follow the geometry of the wound and ensured a good superficial contact. To compare, highly viscous solutions of chitosan were also considered. Veterinary experiments were performed on pig's skins and biopsies at days 9, 17, 22, 100 and 293, were analysed by histology and immuno-histochemistry. Only one chitosan material was used for each time. All the results showed that chitosan materials were well tolerated and promoted a good tissue regeneration. They induced inflammatory cells migration and angiogenetic activity favouring a high vascularisation of the neo-tissue. At day 22, type I and IV collagens were synthesised under the granulation tissue and the formation of the dermal–epidermal junction was observed. After 100 days, the new tissue was quite similar to a native skin, especially by its aesthetic aspect and its great flexibility.

Keywords: Chitosan; Physical hydrogel; Skin regeneration; Third-degree burns

1. Introduction

Every year in the world, hundred thousands patients require hospitalisation for burn injury [1]. The major lesions require a rapid resection of necrotic tissue, and an immediate cover recuperates the most important skin function (physical barrier). The most conventional treatment uses skin grafts. These grafts can compensate the tissue loss at multiple levels acting as occlusive dressings, providing both skin replacement and stimuli for healing [2]. Problems associated with grafts have prompted the research toward an alternative that would be more widely available with properties close to those of a natural skin. Research in this field resulted in the use of biosynthetic materials and tissue engineered living skin replacement. Three types of skin substitutes can be considered: those consisting only in epidermal equivalents, those encompassing dermal components from processed skin and those possessing distinct dermal and epidermal components, referred to as composite skins [3]. Most of skin substitutes consist in matrix seeded cell cultures. Because of Rheinwald and Green's works [4] cultured epidermal grafts were developed. They proved that a combination of growth factors and irradiated murine 3T3 fibroblasts was likely to support the proliferation of human keratinocytes on synthetic polymer surfaces. Keratinocyte sheets were formed, deposited on gauze and grafted on wounds.

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Different supports for these sheets have been tested such as polyurethane [5] and derivatives of hyaluronic acid [6]. These systems did not yield satisfactory results due to their mechanical instability. They were brittle and then, a blistering of the epidermis occurred. The grafted skin was also reported to be more prone to shrinking yielding poor aesthetic results [7].

Although numerous studies on dermal equivalents used as grafts were reported, acellular matrices are still proposed. We may mention the case of: Alloderm™, obtained from cadaver skin [8], Integra™, constituted of glycosaminoglycans and bovine collagen, covered with a silicon sheet [9]. Cellular dermis equivalents like Dermagraft™ consisting in a culture of neonatal fibroblasts onto a bioresorbable synthetic matrix are also considered [10]. Composites with both dermal and epidermal components were first introduced by Bell et al. but were not fully satisfying for the treatment of severely burned patients [11]. Nevertheless, they allowed the development of new strategies and new materials such as Apligraft® [12] constituted of a fibroblast/collagen matrix on which a fine layer of stratified human epithelium was cultivated. Biodegradable polymeric three-dimensional matrices are gaining attention for cell culture such as sponges of collagen/polycaprolactone [13]. Damour et al. [14] developed a sponge constituted of bovine type I collagen/chondroitin-4-6 sulphate/chitosan for *in vivo* skin reconstruction.

In this paper, we propose a new acellular biomaterial based on physical hydrogels of chitosan to cover burn areas. The major goal is to achieve a permanent skin regeneration with both dermal and epidermal tissues, good functional and aesthetic characteristics. Chitosan, fully absent in mammals corresponds to an interesting series of natural glycosaminoglycans possessing the rare property of bioactivity [15]. Chitin with chitosan constitute the series of the linear copolymers of (1→4)-2-amino-2-deoxy-β-D-glucan (GLcN) and (1→4)-2-acetamido-2-deoxy-β-D-glucan (GlcNAc). DA, the degree of acetylation refers to the molar fraction of *N*-acetyl units present in the polymer chains. When the distribution of the two constitutive residues is random, chitosan corresponds to DAs below 70% [15], and is soluble in dilute acidic solutions. Chitosan-based matrices have been widely used in the biomedical field: for cells encapsulation [16], drug delivery [17], cell culture [18], hyaline cartilage repair [19] and bone reconstruction [20]. In the case of wound healing, Ueno et al. [21] demonstrated that chitosan in the form of a chitosan-cotton blend was an accelerator of wound healing by the activation and infiltration of polymorphonuclear cells at the wound site. Recently, Mizuno et al. also concluded that chitosan was a good wound healing material and that incorporation of basic fibroblast growth factors in the chitosan material accelerated the rate of healing [22]. Park et al. [23] studied *in vitro* the effect of carboxymethylchitosan on the proliferation of normal human skin and keloid fibroblasts. *In vitro* studies also showed that chitosan accelerated the proliferation of keratinocytes [24].

Pure physical hydrogels of chitosan were recently obtained [25–27]. These materials only use intermolecular physical cross-links of low energy (⩽ kT): hydrogen bonding and hydrophobic interactions to elaborate a three-dimensional network of polymer chains, without any use of external chemical agent. Encouraging results were obtained with chondrocyte cultures [19]. In this work, we prepared a bi-layer physical hydrogel, only constituted of chitosan and water. This material was tested *in vivo* for the skin reconstruction after third-degree burns on pig back skins. A kinetics study of the healing showed the full skin reconstruction. The present data also allowed us to confirm the first results obtained with these gels by Montembault et al. [19] for *in vitro* chondrocyte cultures inducing the production of a cartilaginous matrix.

2. Materials and methods

2.1. Purification of chitosan

We used a chitosan produced from squid pens, purchased by Mahtani Chitosan (batch number 114, 15/11/02). For purification, the polymer was dissolved at 0.5% (w/w) in acetic acid to achieve the stoechiometric protonation of the NH_2 sites. The mixture was filtered successively on 3, 1.2, 0.8 and 0.45 μm Millipore membranes. Then, the polymer was precipitated by means of concentrated ammonia (28% (w/w)). After several washings in deionised and distilled water and centrifugation steps until a neutral pH was achieved, the precipitate was lyophilised. Thus, a chitosan of low DA (2.6%) and very high weight-average molecular weight M_w (close to 540 000g/mol) was obtained, with a polydispersity index (Ip) of 1.6 ± 0.2, and water content of $8 \pm 1\%$ (w/w).

2.2. Characterization of chitosan

DAs of the different samples were deduced from ^1H NMR spectroscopy. Spectra were recorded on a Brucker 250 spectrometer (250 MHz) at 25 °C. About 10 mg of chitosan was dissolved in 1 g of D_2O in presence of HCl. DA was deduced from the method proposed by Hirai et al. [28]. The weight-average molecular weight (M_w) was evaluated by size exclusion chromatography (SEC). SEC was performed by means of an IsoChrom LC pump (Spectra-Physics) connected to a Protein Pack glass 200 SW column and a TSK gel 6000PW. A Waters 410 differential refractometer and a multiangle laser-light scattering detector operating at 632.8 nm (Wyatt Dawn DSP) were connected on line. A 0.15 M ammonium acetate/0.2 M acetic acid buffer (pH 4.5) was used as eluent. Chitosan was dissolved in the buffer (1 mg/mL), filtered on 0.45 μm Millipore membrane then, injected (100 μL). In our case, the refractive index increment dn/dc was evaluated at 632.8 nm and found equal to 0.196 cm^3/g [29]. The water content was determined by means of a thermogravimetric analyser, DuPont Instrument 2950. About 10 mg of chitosan was analysed under a helium flow, operating at a ramp of temperature of 2 °C/min, from 30 to 150 °C.

2.3. Preparation of viscous solutions of chitosan

Before use, chitosan flakes were sterilised. Water steam sterilization is considered as one of the safest and easiest means of sterilising medical devices [30] and was then applied to our raw material. It was operated in