EVALUATION OF EFFECTIVENESS OF CHITOSAN HYDROGEL AS HAEMOSTATIC FROM DORSAL NASAL VEINS IN RABBITS

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(Received 11 March 2008, Accepted24 April 2008)
key words: hemostasis, , adhesion, biocompatibility.

ABSTRACT

The haemostatic capability, adhesion ability and biocompatibility of chitosan sponges was compared with conventional method as control group. The chitosan sponges were briefly immersed in an aqueous 20% ammonia solution before being applied to a rabbit dorsal nasal vein wound. The total amount of bleeding from the injured veins until hemostasis was similar for both chitosan and control group. The complete hemostasis success rates for both the chitosan and control group were also similar. Under hemostasis, the chitosan sponges strongly adhered to the surface of the rabbit muscles, whereas the control group. Under wet conditions, however, there was no significant difference in the adhesive ability between the two groups.

During implantation, the chitosan sponges were much more flexible and resistant to breakage that good. The biocompatibilities in addition, biodegradation rates of the Chitosan sponges were very different after subcutaneous implantation in rabbit.

INTRODUCTION

Uncontrolled hemorrhaging **is** the leading preventable cause of death both due to accidents and due to surgical procedures [1]. If an injury occurs deep into the tissue accompanied by massive bleeding, suturing the relevant large blood vessels is the first choice to stop the bleeding,

However, in practice further bleeding is often observed, consequently, suturing is not always completely effective in inducing hemostasis, in addition, suturing is not applicable to certain injuries such as those that continuously ooze blood. In such cases, a hemostatic agent or sponge is required, not only to assist the rapid and effective control of bleeding during and after surgery, but also to stop oozing to reduce postoperative complications. Various types of hemostatic agents or sponges have been explored, including light-cured gelatin hydrogel glues [2], cyanoacrylate adhesives [3], microcrystalline collagen and collagen sponges [4,5], gelatin sponge [6,7], fibrin glue [8] and gelatin-poly (L-glutamic acid)-carbodiimide glue [9]. However, each of this procedure has its own shortcomings.

For example, the sources of collagen, fibrinogen or thrombin are from animals or human blood, both of which are expensive and include the risk of viral infection. The adhesive properties of collagen and gelatin sponges to tissues are poor (10).

Therefore, it is of prime importance to develop new and safe biological hemostatic agents applicable for clinical use with improved adhesion and hemostatic properties, as well as reduced tissue response. Chitosan, a (1→4)-linked 2-amino-2-deoxy-s-D-glucan, is prepared by N-deacetylation of chitin, which is the second most abundant polysaccharide found in nature [11–13].

It has been demonstrated that chitosan is an invaluable material in the field of biomedical engineering and biotechnology with a wide variety of applications that range from skin and vascular grafts to substrates for mammalian cell culture [14–16]. Chitosan also has a hemostatic function [17,18]. Hoekstra et al. have used microcrystalline chitosan as a sealant for arterial

puncture sites and found that microcrystalline chitosan is a safe and effective biopolymer for achieving hemostasis [19].

In the present study, we evaluated the feasibility of ammonia solution treated chitosan sponge as a hemostatic agent. Sponges were applied to a bleeding site in rabbits injured with a cutting and punch procedure. Subsequently, the haemostatic effectiveness of the sponge was evaluated by determining the amount of blood released from the injured site after sponge application.

MATERIALS AND METHODS

There are many extraction methods of the chitin from the crustacean shells, the principles of chitin extraction are relatively simple.

The proteins are removed by a treatment in a dilute solution of sodium hydroxide (1-10%) at high temperature (85-100°C). Shells are then demineralized to remove calcium carbonate.

This is done by treating in a dilute solution of hydrochloric acid (1-10%) at room temperature. Depending on the severity of these treatments such as temperature, duration, concentration of the chemicals, concentration and size of the crushed shells, the physico-chemical characteristics of the extracted chitin will vary. For instance, the three most important characteristics of the chitin i.e., degree of polymerization, acetylation and purity, will be affected. Shell also contains lipids and pigments.

Therefore, a decolorizing step is sometimes needed to obtain a white chitin. This is done by soaking in organic solvents or in a very dilute solution of sodium hypochlorite. Again, these treatments will influence the characteristics of the chitin molecule.

The polymer either and made chitosan. The term chitosan is used when chitin could be dissolved in weak acid. When chitin is heated in a strong solution of sodium hydroxide (>40%) at high temperature (90-120°C), chitosan is formed. This harsh treatment removes acetylic grouping on the amine radicals to a product (chitosan) that could be dissolved. It is said that at least 65% of the acetylic groups should be removed on each monomeric chitin to obtain the ability of being put in

Preparation of the Chitosan Sponge

A 1% chitosan solution was obtained by dissolving chitosan in a 2% acetic acid solution. The solution was then poured into Petri dishes.

The dishes were placed within a refrigerator at _20°C for 6h.

The hydrogel-like substance in the dishes was then lyophilized. The porous matrices obtained were immersed in an aqueous 20% ammonia solution for 10 min, washed with distilled water, and then freeze-dried again.

Evaluation Procedure of Hemostasis

After general anesthesia with a mixture of 15% ketamine hydrochloride and xylazine hydrochloride (1mL/kg), twenty adult local rabbits(2.5 kg) were shaved and disinfected in an area over the dorsal nasal veins, short segments of the right and left dorsal nasal veins were exposed and isolated. A small vertical incision wound was made in each of the veins and the wound was enlarged, through the resultant jet spray of blood, the right wound was immediately pressure with finger and the left wound was covered with a 3_3 cm2 chitosan sponge.

RESULTS

Haemostatic Evaluation

To compare the haemostatic capability of the test samples, factors that may influence the results such as differences between individual rabbits and the sizes of wounds were minimized where possible.

***In Vivo* Bioadhesion Evaluation**

It was noticed that the ammonia treated chitosan sponges were much softer and more flexible than the conventional methods and able to adapt to the contours of the wounds. on the muscle directly without bleeding, the chitosan sponges adhered too strongly to the muscle surfaces to be peeled off. However, when up the finger is re-oozing.

When use the conventional method the bleeder off an about 2 minutes and six seconds (fig-2,4).but in chitosan sponge about 30 seconds time required to stopped the bleeding (Fig-1, 3).

The heterophagic vacuoles or small matrix remnants Slight inflammation with some lymphocytes, myofibrils and fibroblastswere also observed. The appearance of myofibrils and fibroblastsindicated that scar tissue was developing (Fig-5).

Two weeks after operation, the encapsulated purulent layer wasenlarged at the periphery of chitosan sponges. More acute inflammatorycells had infiltrated the chitosan sponges and there was no sign ofbiodegradation of the chitosan sponges Newly formed blood vessels appeared in this time (Fig-6).

Four weeks after implantation, the chitosan implants still maintained their porous structure. A much thicker purulent layer and more acute inflammatory cells were found around or in the chitosan sponges (Fig-7).

Six weeks after implantation, most of the chitosan still maintainedtheir scaffold integrity with numerous interspersed purulent cells.

Some purulent cells even formed large channels throughout the chitosansponges It was obvious that chitosan sponges were separated by the channels, (Fig-8)

Eight weeks after the operation, purulent cell infiltrations had further increased in the Chitosan sponge collapsed matrix structures were detected at the outer margins of the implants and more channel structures were found between remnants of chitosan lamellae. (Fig-9).

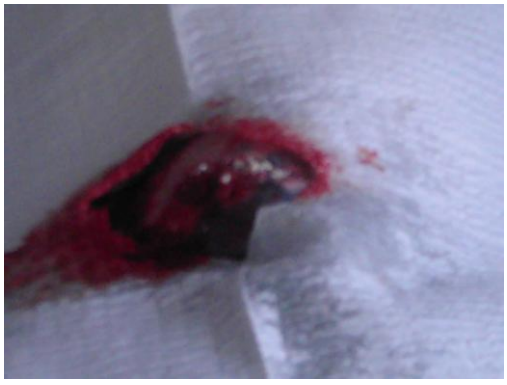


Fig.1: chitosan group after incision

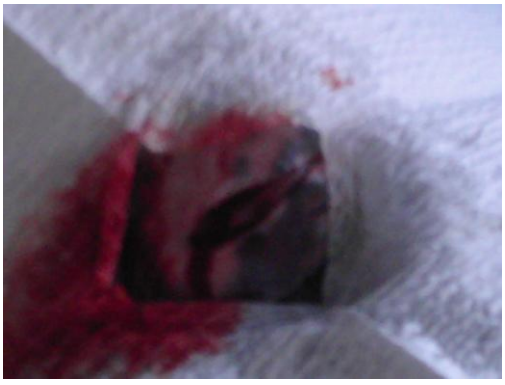


Fig.2: conventional group after incision

Fig.3 chitosan group after application

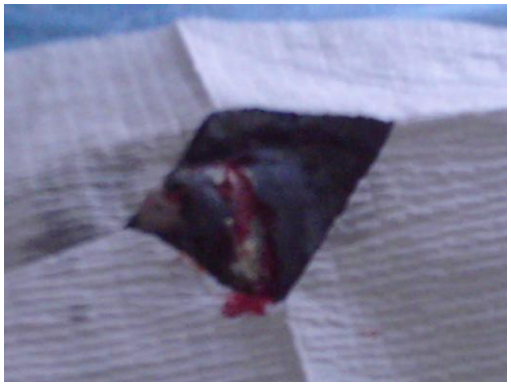


Fig.4: conventional group after application

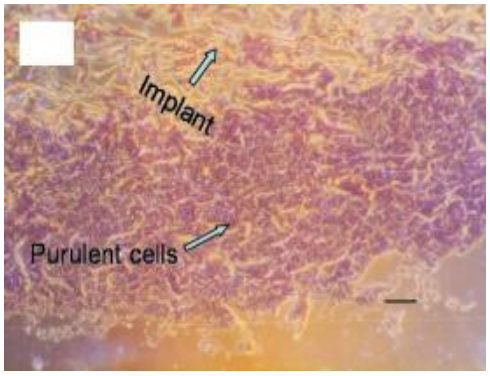


Fig-5: Light microscope evaluation of tissue response to chitosan sponge, 1 week after implantation (X 100) H&E stain.)

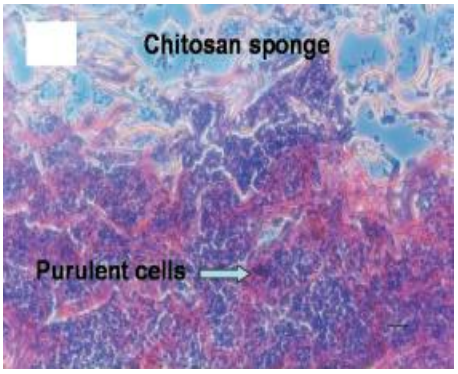


Fig-6: Light microscope evaluation of tissue response To sponge, 2weeks after implantation (X100 H&E stain

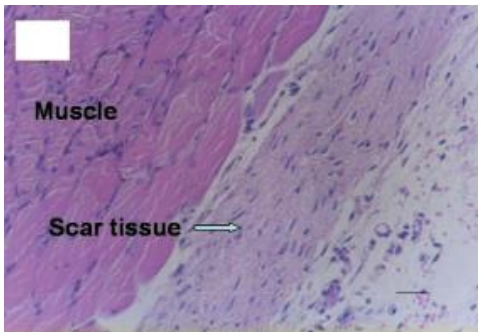


Fig-7: Light microscope evaluation of tissue response to chitosan sponge, 4weeks after implantation (X 100) H&E stain

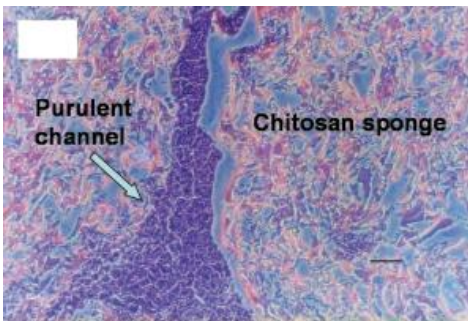


Fig-8: Light microscope evaluation of tissue response to chitosan sponge, 6weeks after implantation (X 100) H&E stain

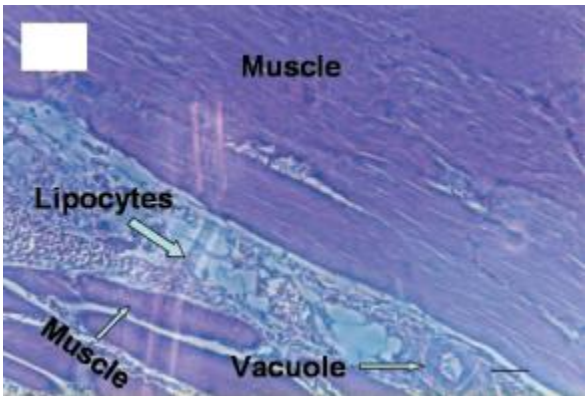


Fig-9: Light microscope evaluation of tissue response to chitosan sponge, 8 weeks after implantation (X 100) H&E stain

DISCUSSION

Hemostasis depends on the successful balance between coagulation,complementary and fibrinolytic pathways, with complex interactionsbetween plasma proteins, blood cells, blood vessel endothelium as wellas blood flow and viscosity [22].

It has been proven that the chitosan films can induce platelet adhesion and aggregation as well as activation of intrinsic blood coagulation The mechanism of hemostasis may, however, is different in each case [23, 24].

platelet induction and soluble coagulant factors, chitosan can induce hemostasis by coalescing erythrocyte cells to one another to form a blood clot. In comparison with animal collagen, natural high molecular weight polysaccharide chitosan has superior merits (such as low cost and non-immunogenicity) as a haemostatic dressing.

Films suitable for *in vivo* wound dressing should preferably be strong however, flexible. In our experiment, the ammonia pretreated chitosan sponges were much softer and more flexible than conventional methods and adapted to the contours of the wounds better. When the sponges were applied directly on the muscle without bleeding, the chitosan sponges adhered to the muscle surfaces much more strongly than the conventional methods (4).

The coagulation procedure using ammonia had little effect on the chitosan structure and bioproperties [18] while it seemed to increase the flexibility of the chitosan sponge, which improved the contactbetween the sponge and the tissue, hence promoting penetration of the polymeric chains into the tissue to form a strong bond leading to an increase in the adhesion strength [19].

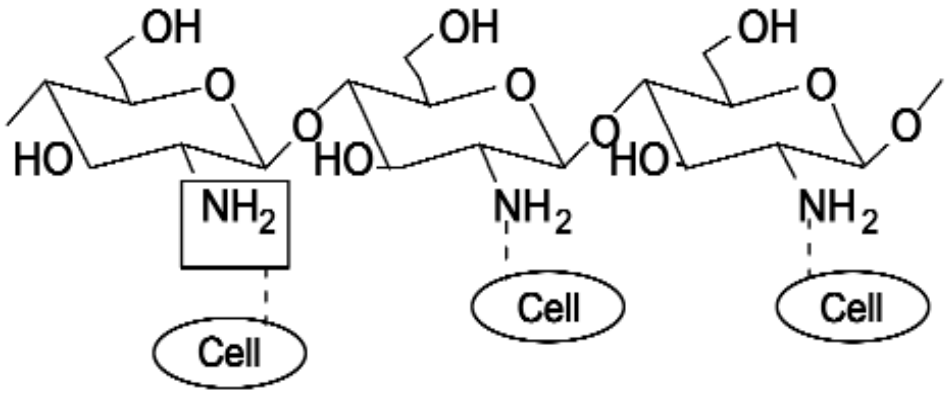
Theoretically, chitosan sponges should biodegrade much faster *in vivo* than sponge since there are more collagenases than lysozymes in animals. This theory does not agree with our *in vitro* biodegradation results where the ammonia treated chitosan was biodegraded very slowly even in lysosomal solutions at 37°C [8].

The chitosan sponges exhibited similar haemostatic capability and complete hemostasis success rate as the conventional methods. Under hemostasis, the chitosan sponges adhere strongly to the surface of

rabbit muscle, The chitosan sponges were much more flexible and resistant to breakage. The biocompatibilities and biodegradation rates of the sponges were different after subcutaneous (1).

When Chitosan was added, an improvement in bioadhesion has been reached. In fact, a polyelectrolyte complex was obtained [19, 20]. Without doubt, increasing of active sites to interact with tissue (negative sites) due to introducing of amine groups of Chitosan important effects strengthening of the bioadhesion forces. Furthermore, effective sealing of dorsal nasal veins puncture at high air pressures could be related to attractive between carboxylic functionality and amine groups of chitosan Forces(24).

The same authors discuss mechanism for coagulation effect of chitosan elsewhere In brief, blood also carries negative charge and in vicinity of any positive charged material will form clot Consequently, with applying chitosan based hydrogel on the bleeding dorsal nasal veins surface or blood would sense oppositely charged substrates as in following diagram.



Electrostatic interaction between erythrocyte and Chitosan

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