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Preparation of Chitosan Derived from Shrimp's Shell of Persian Gulf as a Blood Hemostasis Agent

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ABSTRACT

In this study chitin was extracted from shrimp's shell of Persian Gulf, using different methods and chitosan is derived from chitin as a microcrystalline powder, which is being named as Persian Gulf chitosan (PGC) with deacetylation degree of 86 % and the viscosity-average molecular weight ($\bar{M}_v$) of 3.57×10^5. The purpose of this experiment was to evaluate the effectiveness and safety of PGC as a hemostasis agent with surgical application. Results using PGC in sheep carotid punctured hole model showed significant reduction in manual compression time ($p<0.009$) of the carotid artery. Comparative results were found in sheep, wherein a created wound in the carotid artery could be sealed relatively quickly and easily. There were no significant differences in time length to hemostasis between heparinized and non-heparinized sheep. This work suggests that PGC is a safe and effective biopolymer to achieve hemostasis.

Key Words: chitosan, hemostasis, in vivo, carotid artery, chitin

INTRODUCTION

New technical developments, including arterial characterization, angioplasty and stent implantation often require either arterial sheaths, or intense anticoagulation, or both. Complication at the arterial access site may often occur by catheterization from inadequate hemostasis. Chitin and chitosan have been examined for the uses in a wide variety of biomedical applications such as wound dressing, drug delivery systems and hemostasis.

Chitosan is a polysaccharide derived from chitin, which exhibits numerous interesting physicochemical and biological properties. Due to its biocompatibility, biodegradability and bioactivity, it is more and more considered as a very interesting substance for diverse applications as a biomaterial such as drug delivery systems, surgical thread, bone healing materials and wound dressing [1–2]

In the case of normal conditions of use, clinical

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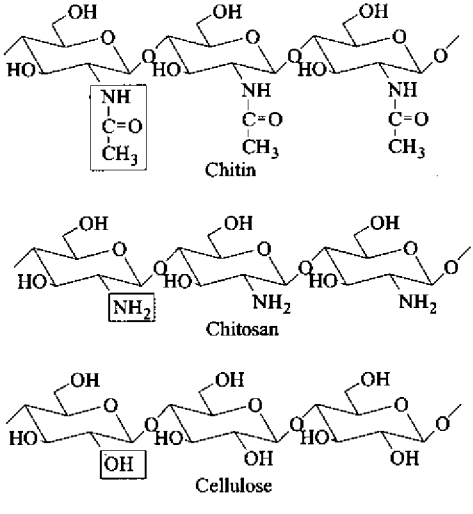
tests, carried out in order to promote chitosan-based biomaterials, do not report any inflammatory or allergic reaction following implantation, injection, topical application or ingestion in the human body [1]. Biodegradation of chitosan leads to the release of aminosugars, which can be incorporated into glycosaminoglycans and glycoproteins metabolic pathways, or they are excreted [1]. In addition, chitosan is a bioactive polymer. It is a hemostatic agent, which presents antithrombogenic properties [3]. It also stimulates the immune system of the host against viral and bacterial infections.

Chitosan is obtained from *N*-deacetylation of chitin (Scheme I). Both these polysaccharides are copolymers of β, (1→4) linked *N*-acetyl-D-glucosamine and *N*-glucosamine units [4]. Deacetylation degree (DD) represents the proportion of *N*-acetyl-D-glucosamine units with respect to the total number of units. It allows us to define the two terms chitin and chitosan. Thus, in the case of chitosan, DD is considered to be above 50%. This value also determines the solubility limit of the polymer in dilute acidic solutions ($2 < pH < 6$).



Structure of chitin, chitosan and cellulose

Scheme I

DD is a structural parameter, which influences physicochemical properties such as the molecular weight, the elongation-at-break and the tensile strength. Results show that the percentage of cell adhesion is strongly dependent on the DD value and increases when this value increases [5].

Chitosan is shown to enhance lingual hemostasis in rabbits treated with a known antagonist of platelet function, such as epoprostenol [6]. Chitosan could also achieve hemostasis and allows the promotion of normal tissue regeneration [7–10].

Materials and Methods

Chitin used in these experiments was extracted from Persian Gulf shrimp's shell according to Kifune et al. Method [11]. The extraction parameters were optimized to mild acidic and alkaline concentrations. At first, Shrimps' shell were washed with water, dried and cut into small pieces. Initial step of chitin extraction was carried out by acetic acid at room temperature for 2 h. This step was followed by filtration, neutralization and washing. Deproteination was performed using alkaline treatment with 2N sodium hydroxide solution at 60–65 °C and then neutralization was carried out by process of washing.

Demineralization was followed by 10% hydrochloric acid at room temperature for 3–5 h.

In order to remove natural pigments in the chitin, acetone was added to the solid under reflux conditions for 2 h. Chitin extraction yield from shrimp's shell was 28–30 %.

Deacetylation was carried out by the process suggested by Kurita et al. [12] and the reaction parameters (reaction duration, temperature and concentration of alkaline reagent) was optimized as follows: A suspension of 1 g chitin in 50 mL of aqueous sodium hydroxide, as deacetylation reagent, (50 % by weight) was mixed at desired temperature (90–95 °C) under nitrogen purging. After 3–5 h, the solid was filtered off, washed with water to neutral pH and methanol. It was then dried at 85 °C. Deacetylation reaction yield was 75–86%.

Determination of Deacetylation Degree

Determination of chitin deacetylation degree (DD) is

essential to study structure-property relationships and possible industrial uses. DD was determined by Takanori et al [13] method using FTIR spectroscopy. FTIR spectrum was obtained with a Brucker IFS-48 spectrometer.

Viscosity and Molecular Weight Measurement

The viscosity and molecular weight measurement were performed using an Ubbelohde viscometer at room temperature. The solvent used for chitin was dimethylacetamide/LiCl and for chitosan, 0.2 M acetic acid/ 0.3 M sodium acetate. The viscosity-average molecular weight $\bar{M}_v$ was obtained from viscosity equation using Mark-Houwink parameters according to the equation:

$$[\eta] = k\ \bar{M}_v^{\alpha}$$

Where $[\eta]$ is intrinsic viscosity and k and α are constants. These parameters have been determined for chitin ($k=2.4\times10^{-1}$ cm^3g^{-1} and $\alpha=0.69$) [14] and chitosan ($k=0.078$ cm^3g^{-1} and $\alpha=0.76$) [15].

Elemental and Crystalline Analysis

The elemental analysis (CNHO) was carried out by Elementar-Analysensysteme Gmbh-Germany analyzer.

Crystalline property of chitin and chitosan were determined by X-Ray diffraction using a Ziemens D 5000 X-RD. Ash content of chitin and chitosan were determined by weight loss of 1 g chitin heating at 900 °C for 2.5 h.

Phosphate Buffered Saline Solution and Water Adsorption

The water sorption capacities of chitosan membranes or powder were determined by swelling the chitosan membranes in pH 7.4 of phosphate buffered saline (PBS) and de-ionized water at room temperature, respectively [2]. A known weight (200 mg) of chitosan membrane or powder was placed in the media for the required period of time. The wet weight of the chitosan membrane was determined by first blotting the membrane with filter paper to remove adsorbed water on the surface, then it was weighed immediately on an electronic balance. The percentage water adsorption of chitosan membranes in the media was then calculated from the formula:

$$E_{sw} = [(W_e - W_0)/W_0]\times100$$

where E_{sw} is the percentage water adsorption of chitosan membranes at equilibrium. W_e denotes the weight of the chitosan membranes at equilibrium water adsorption and W_0 is the initial weight of the chitosan membranes. Each swelling experiment was repeated 3 times and the average value was taken as the percentage water adsorption.

Statistical Analysis

Statistical analysis and student t-test were performed using Macrocal Origin 3.5.

Animals

Eighteen adult female mixed breed sheep (weight range: 40–45 kg) were used in this study. The female sheep were chosen because they are calmer, less aggressive and easier for long-term test. All animals received human care under standard laboratory conditions with free access to food and water.

RESULTS AND DISCUSSION

Characteristics of Chitin and Chitosan

Table 1 shows the specification and Figure 1 indicates

Table 1. Characteristics of chitin and chitosan.

Specification	Chitin	Chitosan
Viscosity-average molecular weight ($\bar{M}_v$)	3.22×10^6	3.57×10^5
Deacetylation degree (DD)	20%	86%
X-ray diffraction	10°	10°
Peaks	19°4'	19°4'
Ash at 900 °C	0.34	
Ca content (wt%)	0.09	
PBS solution adsorption (%)	–	20 (film)
PBS solution adsorption (%)	–	210 (powder)
Water adsorption (%)	–	82 (powder)

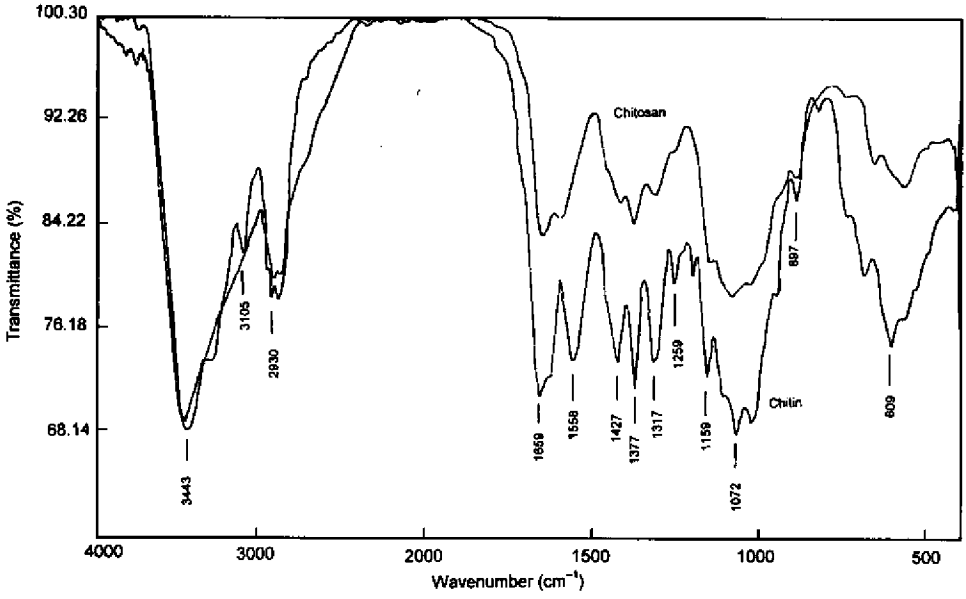


Figure 1. FTIR Spectra of chitin and chitosan (PGC).

FTIR spectra of chitin and chitosan, which have been used in this study.

Surgical Exposure of the Carotid Artery (In Vivo Assessment)

The sheep were sedated with Xylazine hydrochloride (Bayer-Germany, 0.1 mg/kg intramuscularly). The left lateral saphenous vein was cannulated for intravenous access and then sheep were anesthesized with sodium thiopental (Biochemic GMBH, Vienna, 5–6 mg/kg, intravenously). A tracheostomy was performed through a midline cervical incision and ventilation was begun with a volume-cycled ventilator. General anesthesia was maintained with Halothane (Rhodio Ltd. UK, 1.5–3%). Heparine was administered at an initial dose of 65 International urine (IU)/kg in group 2 (Table 2).

After shaving and disinfecting of the neck with 10% povidine-iodine, an oblique incision was made at the anterior border of sternocleidomastoid muscle. The incision was carried directly down to the carotid sheath. A segment of 8–10 cm of the common carotid artery was carefully exposed. Three holes were punctured by a 14-gauge needle (ϕ1.1 mm) as shown in Figure 2 in each animal. This procedure uniformly led to jet bleeding (Figure3). The first hole was used, as a control site before PGC application and two test holes were punctured on appropriate locations. With proximal and distal controls, a small amount (# 1 cm^3) of the PGC on 4×4 sterile gauze was brought upon the puncture hole. Pressure was applied gently to the puncture site (Figure 4). The distal control was lifted very shortly to allow back-flow of blood into the common carotid and PGC. This was immediately followed by a proximal control. The arterial pressure was recorded continuously, and in period of 1 to 3 min after application of the PGC, the bleeding control was assessed (Figure 5). Manual compression on the control holes were continued for 15–17 min to seal the arterial puncture site. In the event of continuing the bleeding after 17 min, suturing of the puncture site was performed using 6–0 proline suture material.

Table 2. Time to hemostasis with PGC.

Type study	Puncture site	Bleeding control time[a]	Number
Time to hemostasis with PGC (group I)	Carotid artery	2.2±1.4 min	24
Time to hemostasis with PGC in heparinized animals (group II)	Carotid artery	3.2±1.2 min	12
Time to hemostasis without PGC (control)	Carotid artery	More than 17 min	18

(a) p value <0.009 compared to the control.

As shown in Table 2 results using PGC in sheep registered significant reduction in manual compression time (p<0.009) of the carotid artery in comparison with the control.

Carotid Patency

The carotid patency was then determined by direct inspecttion, palpation and the wounds were closed. At 1–6 months follow-up no infectious complications at the healed skin wound side and puncture site were detected.

Hemostatic mechanism

PGC induced sealant installation via an arterial sheath

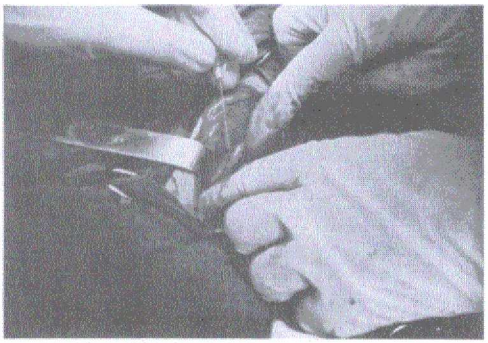


Figure 2. Puncturing of artery with needle G 14.

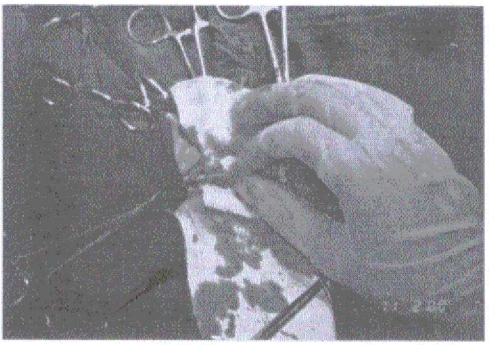


Figure 4. Manual compression of needle hole with PGC.

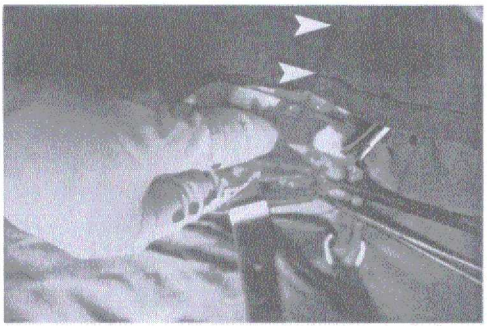


Figure 3. Jet bleeding from needle hole.

Figure 5. Sealing the needle hole with PGC to stop bleeding.

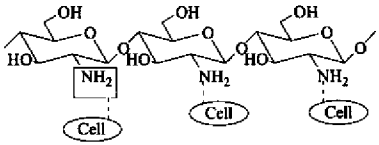


Figure 6. Electrostatic interaction between erythrocytes and chitosan.

at the completion of catheterization.

Coagulation test showed that the hemostatic mechanism of PGC seems to be independent of the classical coagulation cascade and appears to be an electrostatic interaction between the cell membrane of erythrocytes (negative charge) and chitosan (positive charge) [10] as shown in Figure 6.

CONCLUSION

Results using PGC in sheep documented significant reduction in manual compression time ($p<0.009$) of the carotid artery in comparison with the control.

Comparative results were found in sheep, wherein a created wound in the carotid artery could be sealed relatively quickly and easily.

There were no significant differences in time to hemostasis between heparinized and non-heparinized sheep. This work suggests that PGC is a safe and effective biopolymer to achieve hemostasis on the puncture site of the sheep carotid artery.

The results showed that the hemostatic mechanism of PGC seems to be independent of the classical coagulation cascade and appears to be an electrostatic interaction between the cell membrane of erythrocytes (negative charge) and chitosan (positive charge).

The optimization and a better formulation of PGC for hemostatic purpose require further studies.

REFERENCES

1. Chatelet C., Damour O. and Domard A., "Influence of the degree of acetylation on some biological properties of chitosan films", *Biomaterials*, **22**, 261–268 (2001).
2. Mi F.L., Shyu S.S., Wu Y.B., Lee S.T., Shyong J.Y. and Huang R.N., "Fabrication and characterization of sponge-like asymmetric chitosan membrane as a wound dressing", *Biomaterials*, **22**, 165–173 (2001).
3. Hoekstra A., Struszczyk H. and Kivekas O., "Percutaneous microcrystalline chitosan application for sealing arterial puncture sites", *Biomaterials*, **19**, 14671471 (1998).
4. Ravi Kumar M.N.V., "A review of chitin and chitosan applications", *Reactive & Func. Polym.*, **46**, 1–27 (2000).
5. Blair H.S., Guthrie J., Law T.K. and Turkington, "Chitosan and modified chitosan membranes. I. Preparation and characterization", *J. Appl. Polym. Sci.*, **33**, 641–656 (1987).
6. Klokkevold P.R., Subar P.P., Fukayama H. and Bertolami C.N., "Effect of chitosan on lingual hemostasis in rabbits with platelet dysfunction induced by epoprostenol", *J. Oral. Maxil. Surg.*, **50**, 41–45 (1992).
7. William G.M. and Herbert J.Q., "Method of achieving hemostasis inhibiting fibroplasias, and promoting tissue regeneration in a tissue wound", *US Patent* No. 4532134 (1985).
8. Malette W.G., Quigley H.J., Gaines R.D., Johnson N.D. and Rainer W.G., "Chitosan: a new hemostatic", *Ann Thorac Surg*, **36**, 55–58 (1983).
9. Brandenberg G., Leibrock L.G., Shuman R., Malette W.G. and Quigley H., "Chitosan: a new topical hemostatic agent for diffuse capillary bleeding in brain tissue", *Neurosurgery*, **15**, 9–13 (1984).
10. Rao S.B. and Sharma C.P., "Use of chitosan as a biomaterial: studies on its safety and hemostatic potential", *J. Biomed. Mater. Res.*, **34**, 21–28 (1997).
11. Koji K., Inoue N., Katsuhiro and Shigeru M., "Process for the production of chitin fibers", Us Patent 4431601 (1984).
12. Kurita K., Tomita K., Tada T., Ishii S., Nishimura S.I. and Shimoda K., "Squid chitin as a potential alternative chitin source: Deacetylation behaviour and characteristic properties", *J. Polym. Sci. (A) Polym. Chem.*, **31**, 485–491 (1993).
13. Sannan T., Kurita K., Ogura K. and Iwakura Y., "Studies on chitin: 7. IR Spectroscopic determination of degree of deacetylation", *Polymer*, **19**, 458–459 (1978).
14. Roberts G.A.F., ed., *Chitin Chemistry*, Macmillan, London, Ch 3, pp 85–110 (1992).
15. Rhazi M., Desbrieres J., Tolaimate A., Alagui A. and Vottero P., "Investigation of different natural sources of chitin: Influence of the source and deacetylation process on the physicochemical characteristics of chitosan", *Polym. Int.*, **49**, 337–344 (2000).