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Postoperative anti-adhesion ability of a novel carboxymethyl chitosan from silkworm pupa in a rat cecal abrasion model

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ABSTRACT

N,O-Carboxymethyl chitosan (NOCC) can prevent postsurgical adhesion formation. Here, we described the preparation of a novel silkworm pupa NOCC and its effects on the prevention of postoperative adhesion in a rat cecal abrasion model. The degree of deacetylation (DDA) of silkworm pupa chitosan was only 49.87 ± 0.86%; regardless, it was used as the raw material to construct the novel silkworm pupa NOCC, which had a weaker crystallinity than the NOCC standard. Sixty male Sprague–Dawley rats were divided into three groups and treated as follows: 0.9% normal saline solution as a negative control, medical anti-adhesion gel as a positive control and the silkworm pupa NOCC anti-adhesion solution. Two and three weeks after surgery, the animals were killed and the adhesion formation was scored. The silkworm pupa NOCC solution significantly decreased the levels of WBC, TNF-α, IL-1β, IL-2, IL-6 and IL-8 but had no effect on IL-4. Additionally, a lower level of TGF-β_1 expression was found in the silkworm pupa NOCC group, and significantly less collagen ($P < 0.01$) and fewer inflammatory cells and fibroblasts were detected in the animals of this group. These results suggested that the novel NOCC from silkworm pupa using the method described here have potential applications in the prevention of postoperative intestinal adhesion.

1. Introduction

Adhesion formation after abdominal operations represents an important clinical challenge for surgery. Many procedures can induce adhesion, including cholecystectomy, appendectomy, hysterectomy, colectomy, and abdominal vascular operations [1,2]. Many postoperative complications occur due to adhesion, including chronic abdominal pain [3–5], intestinal obstructions [6,7] and infertility [8,9]. At present, the methods used to prevent postoperative adhesion mainly include the use of a physical barrier and prevention and control *via* drugs. Although some progress has been made, better methods of preventing adhesion are urgently needed.

Chitosan (*poly*-D-glucosamine) is a natural polymer derived from chitin, the second most abundant polysaccharide after cellulose [10]. It has received considerable attention as a functional, renewable, nontoxic and biodegradable biopolymer in the pharmaceutical [11], food [12] and cosmetics [13] industries. Additionally, chitin and chitosan can be easily processed into biomaterials for various biomedical applications, including hydrogels [14,15], nanofibers [16,17], micro/nanoparticles [18–20], membranes [21–24], beads [25,26], scaffolds [27–29] and sponges [30,31]. Chitosan represents a promising material for the prevention of postoperative adhesion because of its exceptional features to be easily processed into different forms.

Carboxymethylation is the most common and effective method for producing the water soluble derivatives of chitosan. Carboxymethyl chitosan has emerged as a promising candidate for various biomedical applications due to its superior biological and physicochemical properties compared to chitosan [32]. It can be divided into O-carboxymethyl chitosan, N-carboxymethyl chitosan and N,O-carboxymethyl chitosan. Among these, N,O-carboxymethyl chitosan (NOCC) is the most widely studied. Early in 1991, Higham et al. found that the carboxymethyl chitosan prevented adhesion and did not require reoperation for their removal because they were degraded and absorbed after completing their function *in vivo* [33]. Kennedy et al. reported that NOCC inhibited experimental peritoneal adhesion in the rat uterine horn and small bowel laceration models and was a more effective and cheaper antiadhesion agent than hyaluronic acid [34]. In the 21st century, Krause et al. revealed that NOCC could markedly decrease adhesion formation after a cardiac surgery without cardiac side effects. Thus, this material may have great therapeutic utility for reducing the morbidity and mortality associated with reoperative cardiac surgery [35]. NOCC has been shown to reduce postoperative adhesion development, possibly by blocking the adherence of inflammatory cells and acting as a biophysical barrier [36]. According to a recent study, NOCC/A-HA-treated

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group had a significant reduction in peritoneal adhesion formation compared with the HA hydrogel group and the normal saline group [37].

China is the largest silk producer in the world, with a yield of over 1×10^5 tonnes of the dry silkworm *Bombyx mori* pupa every year. The solubility of silkworm pupa chitosan in dilute acid is better than shrimp and crab chitosan [38]. Thus, silkworm pupas are more suitable for the preparation of pharmaceutical grade chitosan. Furthermore, most of the commercially available anti-adhesion drugs are expensive, so the development of cheaper and more effective products is critical. Therefore, chitosan from silkworm pupas could be used to develop a series of anti-adhesion products with a wide array of applications in the future.

In our study, we prepared novel silkworm pupa carboxymethyl chitosan and examined its effects in the reduction of postoperative adhesion in a rat cecal abrasion model.

2. Materials and methods

2.1. Materials and animals

Silkworm pupa was heated in an oven at 80 °C for 48 h and then pulverized in a grinder. Medical anti-adhesion gel was obtained from Shijiazhuang Yishengtang Medical Supplies Ltd. Chloroacetic acid was obtained from the Aladdin Industrial Corporation. The Elisa kits and hydroxyproline analysis kit were purchased from the Nanjing Jiancheng Bioengineering Institute.

A total of 60 male Sprague–Dawley rats (250 ± 20 g) were purchased from JOINN Laboratories (Suzhou). All rats were maintained under pathogen-free conditions and were provided *ad libitum* access to food and water. All animals were treated humanely throughout the experimental period.

2.2. Preparation of silkworm pupa carboxymethyl chitosan

The dry silkworm pupa powder (1.0 kg) was added to an extraction vessel, followed by the addition of hexane (4 L) for extraction. The organic solvent was removed, resulting in the production of defatted pupa powder. Then, silkworm pupa chitin was isolated from the defatted pupa powder by treatment with 7% NaOH at 95 °C for 1 h to completely remove proteins, 5% HCl at 70 °C for 1.5 h to remove inorganic salts, and finally with 30% H_2O_2 at 100 °C for 5 min for decolouration. The residue was washed with distilled water and dried in an oven to yield silkworm pupa chitin.

The chitin powder was soaked in 40% NaOH at room temperature for 12 h, resulting in a chitin powder to NaOH solution ratio of 1:4 (W/V). The mixture was sealed in containers and frozen at −20 °C for approximately 24 h, and then heated at 40 °C for 2 h. The temperature cycling treatment was repeated three times to obtain a homogeneous chitin/NaOH solution. Then the final concentration of chitin was diluted to 4% by distilled water. The final solution was centrifuged at 4 °C, 10,000 r/min for 20 min. The supernatant solution was carefully adjusted to pH 7.0 with 1.0 mol/L HCl in an ice-water bath and then dispersed in 70% (V/V) aqueous ethanol to yield a precipitate, which was thoroughly washed with 70% aqueous ethanol. Finally, the samples were dried at 60 °C to yield silkworm pupa chitosan [39].

Table 1
Intestinal adhesion scoring system.

Grade	Adhesion area	Type
0	None	None
I	0–25%	Thin, avascular, transparent
II	25–50%	Thickness, avascular, opaque
III	50–75%	Thickness, capillaries, opaque, sharp dissection required
IV	75–100%	Thickness, opaque, large vessels, sharp dissection required

The chitosan powder was soaked in ethanol at room temperature for 12 h, and then 50% NaOH was added to alkalize the chitosan. Finally, N,O-carboxymethyl chitosan was generated by reacting chitosan and chloroacetic acid under alkaline conditions for 4 h at 70 °C. The samples were dispersed in 70% (V/V) aqueous ethanol and neutralized using diluted HAc. The precipitated samples were filtered and successively washed with 70% and 100% ethanol. Finally, the silkworm pupa carboxymethyl chitosan was dried at 80 °C [40,41].

2.3. Determination of degree of deacetylation (DDA)

The deacetylation degree of chitosan was determined by UV-spectrophotometry [42]. An N-acetyl glucosamine standard solution at five concentrations (0.01 mg/mL, 0.02 mg/mL, 0.03 mg/mL, 0.04 mg/mL, and 0.05 mg/mL) was prepared using 1.0 mmol/L HCl. The light absorption value of the solution series was determined at 202 nm with 1.0 mmol/L HCl as the reference solution. Each concentration was repeated three times to obtain the standard curve.

All samples (10 mg) were dissolved in 100 mL of 0.001 mg/mL HCl. The absorption value at 202 nm was determined. The concentration of acetyl in the sample (C_x) was obtained based on the standard curve. The degree of deacetylation was calculated using the following equation: $DDA\% = (1\text{-}C_x/C_s) \times 100\%$. C_s is the concentration of samples.

2.4. X-ray diffraction (XRD)

XRD patterns were measured with Cu-Kαl radiation using the X'Pert-Pro MPD (PANalytical B.V.) at a voltage and current of 40 KV and 20 mA, respectively. The relative intensities were recorded within the range of 5° to 50° (2θ) at a scanning rate of 5 °/min.

2.5. Surgical procedures

A rat cecal abrasion model was created as described by Zheng et al. with minor modifications [43]. Sixty male rats were randomly divided into three groups as follows: 0.9% normal saline solution (Group A), medical anti-adhesion gel (Group B) and silkworm pupa NOCC anti-adhesion solution (Group C). The concentration of silkworm pupa NOCC anti-adhesion solution was 25 mg/mL made from the 0.9% saline. The solution was disinfected by ultraviolet ray. Rats from each group were killed at 2 and 3 weeks post-operation. All rats were fasted preoperatively for 12 h. The rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg of body weight). After

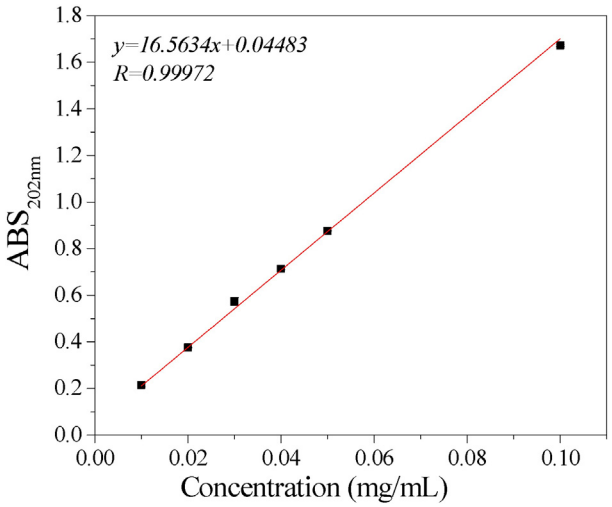

Fig. 1. Acetyl standard curves.