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 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-β1 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.

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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
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 $1 \times 10^5$ 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$_2$O$_2$ 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].

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 ($C_a$) was obtained based on the standard curve. The degree of deacetylation was calculated using the following equation: $\text{DDA} = (1-C_a/C) \times 100\%$. $C$ is the concentration of samples.

2.4. X-ray diffraction (XRD)

XRD patterns were measured with Cu-Kα 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.](image-url)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Adhesion area</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>I</td>
<td>0–25%</td>
<td>Thin, avascular, transparent</td>
</tr>
<tr>
<td>II</td>
<td>25–50%</td>
<td>Thickness, avascular, opaque</td>
</tr>
<tr>
<td>III</td>
<td>50–75%</td>
<td>Thickness, capillaries, opaque, sharp dissection required</td>
</tr>
<tr>
<td>IV</td>
<td>75–100%</td>
<td>Thickness, opaque, large vessels, sharp dissection required</td>
</tr>
</tbody>
</table>
fixation in the supine position and hair removal, the ventral in the lower abdomen was disinfected using 75% alcohol. Then, a 4-cm midline incision was made through the abdominal wall and peritoneum. The cecum was located and elevated into the incision, then abraded until petechial haemorrhage developed over a 1 × 1 cm area. The rats received 1 mL of 0.9% normal saline solution, medical anti-adhesion gel or silkworm pupa NOCC anti-adhesion solution. Finally, the abdominal cavity was closed by a surgical silk suture of 1–0 silk. The postoperative rats were transferred into divided cages for rearing, and the general signs of condition such as activity, faeces, and behaviour pattern were observed.

Fig. 2. X-ray diffraction patterns of the samples a: chitins and chitosans; b: carboxymethyl chitosans.

Fig. 3. Rat intestinal adhesion models with different adhesion grades at two different postoperative time points a/c/e: Group A/Group B/Group C at 2 weeks; b/d/f: Group A/Group B/Group C at 3 weeks.
2.6. Assessment of adhesions

In the pilot experiments, adhesion formation was examined 2 and 3 weeks after surgery. The animals were killed with an overdose of pentobarbital sodium, and adhesion formation was examined by two investigators who were blind to the underlying treatment. Adhesions were evaluated by type and tenacity according to the widely used standard adhesion scoring system proposed by Kennedy (Table 1) [44].

2.7. Analytical method of haematology parameters and inflammatory factors

After 2 and 3 weeks, blood samples were obtained by removing the eyeballs. The white blood cell (WBC), haemoglobin (HGB), red blood cell (RBC) and platelet (PLT) counts were determined using a Full Automatic Biochemical Analyser. The serum levels of TNF-α, IL-1β, IL-2, IL-4, IL-6, and IL-8 were determined using the corresponding Elisa kits.

2.8. Quantitation of hydroxyproline levels

Surgically excursion adhesive tissue samples were immediately frozen and stored at −20 °C. The hydroxyproline levels in adhesion tissues (weighing in the range of 30–100 mg) were determined according to the instructions provided in the manual of the Hydroxyproline Analysis Kit.

2.9. Histopathological analytical method

For the histopathological examination, the adhesion-carrying tissues from each group were collected and fixed in 10% buffered formaldehyde solution for 48 h and embedded into paraffin. Then, the fixed tissues were sectioned and stained with haematoxylin and eosin (H&E) for histopathological evaluation or with Masson’s trichrome stain for collagen assessment. Additionally, immunohistochemistry for TGF-β1 was performed on the paraffin-embedded tissue sections.

2.10. Statistical analysis

Data were presented as the mean ± SD and analysed using the Origin 7.5 software. *p < 0.05 were considered statistically significant.

3. Results

3.1. The DDA of silkworm pupa chitosan

The degree of deacetylation of chitosan was an important indicator for determining the amine (NH₂) content in the chitosan chain. The process of N-deacetylation was investigated using the UV-spectrophotometry method for the determination of DDA. The linear regression equation for acetyl was shown in Fig. 1. The DDA of the chitosan standards and silkworm pupa chitosan were measured using the method. The results showed that the DDA of the chitosan standards was 89.14 ± 0.25%, while the DDA of the silkworm pupa chitosan was only 49.87 ± 0.86%.

3.2. XRD analysis

We chose XRD as the structural analysis method to evaluate the space distribution of the internal atoms in the materials. This method is fast, does not produce pollution or damage the sample and has high measurement precision. Moreover, the use of XRD provides a lot of information about the integrity of the sample crystals.

Fig. 2 showed the X-ray diffraction patterns of different chitin, chitosan and carboxymethyl chitosan samples. The main specific peaks for chitin and chitosan were at 2θ = 9–11° and 2θ = 19–21°, respectively (Fig. 2a). This showed that their crystal structure was an α-helix structure composed of two antiparallel carbohydrate chains. As shown in Fig. 2a, silkworm pupa chitin had obvious diffraction peaks that were the same as the chitin standard. The peaks of two types of chitosan were weakened compared with chitin, suggesting that chitin had a more compact crystal structure. The chitosan standard had only one specific peak at 2θ = 20.2°. In contrast, the silkworm pupa chitosan had specific peaks at 2θ = 9.14°, 19.2°, 21.2°, and 26.1° in a pattern similar to chitin.

Fig. 2b shows the X-ray diffraction patterns of different NOCC. Compared with Fig. 2a, there was only one slight peak in the NOCC, especially in the silkworm pupa NOCC. This result indicated that their crystal structures had been largely destroyed.

3.3. Evaluation of adhesion formation

Two and three weeks after surgery, the animals were sacrificed and adhesion formation was evaluated. Group A rats had different degrees of intestinal adhesion 2 and 3 weeks after the operation. No animal died during or after surgery. These results demonstrated that the rat cecal abrasion model was created successfully.

The results of the abdominal adhesion grade at the different time points were provided in Fig. 3 and Table 2. At 2 weeks, the Group A animals showed consistent and marked adhesion between the cecum and abdominal wall. The formed adhesion strips surrounding the intestines and film were much thicker and required sharp separation. All ten animals treated with 0.9% normal saline solution showed different grades of adhesion. The mean adhesion score was 2, indicating that the adhesion was serious. In contrast, the adhesions of animals in Groups B and C were thinner and only required blunt separation; moreover, the areas of the sticky points were smaller. The mean adhesion score of groups B and C were 0.3 and 0.4, respectively, which indicated a significant reduction in the incidence and degree of postoperative peritoneal adhesion. At 3 weeks, 2 animals in Group A showed strong adhesions (grade II) and 8 animals showed slight adhesions (grade I). The mean adhesion score of Group A was 1.2, while there were no evidence of peritoneal adhesion in Groups B and C.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Scores of abdominal adhesion grades of three groups at two different postoperative time points (2 and 3 weeks).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weeks</td>
<td>Group n</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Haematology parameters in intestinal adhesion rats at different time after drug interventions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weeks</td>
<td>Types</td>
</tr>
<tr>
<td>2</td>
<td>WBC(×10⁹/L) 16.58 ± 2.00</td>
</tr>
<tr>
<td></td>
<td>HGB(g/L) 153.00 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>RBC(×10¹²/L) 7.01 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>PLT(×10⁹/L) 764.00 ± 139.92</td>
</tr>
<tr>
<td>3</td>
<td>WBC(×10⁹/L) 12.95 ± 1.35</td>
</tr>
<tr>
<td></td>
<td>HGB(g/L) 156.67 ± 13.66</td>
</tr>
<tr>
<td></td>
<td>RBC(×10¹²/L) 7.24 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>PLT(×10⁹/L) 953.22 ± 92.54</td>
</tr>
</tbody>
</table>

Note: The data in the table represent average values (±SD) of three-repeated measurements (n = 10).
* P < 0.05 versus Group A.
** P < 0.01 versus the Group A.
3.4. Haematology parameters

The WBC, HGB, RBC and PLT counts in serum were examined 2 and 3 weeks after surgery; the results were shown in Table 3. At 2 weeks, the WBC counts were lower in Groups B and C compared to Group A ($P < 0.05$ and $P < 0.01$). There were no significant differences in the HGB, RBC and PLT counts between the three groups. The results at 3 weeks were consistent with the results at 2 weeks. Only the WBC counts in Groups B and C showed a significant decrease compared to Group A ($P < 0.05$ and $P < 0.01$); there was no difference in the other types of blood cells between the three groups.

3.5. Inflammatory factors

Changes in TNF-α, IL-1β, IL-2, IL-4, IL-6 and IL-8 levels in serum were determined 2 and 3 weeks after the operation. The serum cytokine levels in adhesion rats were shown in Table 4.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Groups</th>
<th>TNF-α(nL/L)</th>
<th>IL-1(β)(nL/L)</th>
<th>IL-2(nL/L)</th>
<th>IL-4(nL/L)</th>
<th>IL-6(nL/L)</th>
<th>IL-8(nL/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>A</td>
<td>43.39 ± 0.07</td>
<td>7.56 ± 0.07</td>
<td>288.22 ± 3.89</td>
<td>94.34 ± 15.16</td>
<td>142.54 ± 0.18</td>
<td>172.74 ± 9.83</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>36.90 ± 1.17</td>
<td>5.07 ± 1.53</td>
<td>274.32 ± 5.432</td>
<td>91.73 ± 2.89</td>
<td>131.82 ± 0.25 **</td>
<td>186.17 ± 3.52</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>35.14 ± 0.47 **</td>
<td>5.17 ± 0.81 **</td>
<td>278.232 ± 1.99</td>
<td>90.97 ± 3.42</td>
<td>112.68 ± 1.67 **</td>
<td>181.18 ± 7.34</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>38.32 ± 0.40</td>
<td>6.34 ± 0.75</td>
<td>295.11 ± 13.60</td>
<td>86.12 ± 3.42</td>
<td>120.42 ± 1.17</td>
<td>178.55 ± 2.48</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>35.83 ± 0.27 **</td>
<td>5.05 ± 0.88</td>
<td>244.14 ± 16.69</td>
<td>86.03 ± 10.24</td>
<td>95.94 ± 2.39 **</td>
<td>166.99 ± 3.81 **</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>34.44 ± 0.45 **</td>
<td>2.71 ± 0.43 **</td>
<td>248.38 ± 11.70</td>
<td>63.24 ± 4.23</td>
<td>112.17 ± 2.12 **</td>
<td>167.20 ± 2.80 **</td>
</tr>
</tbody>
</table>

Note: The data in the table represent average values ($±$SD) of three repeated measurements (n = 10).
* $P < 0.05$ versus Group A.
** $P < 0.01$ versus Group A.

3.6. Hydroxyproline determination

The hydroxyproline levels were shown in Fig. 4. At 2 weeks, the mean hydroxyproline content in Groups B and C were 2.18 ± 0.12 and 2.06 ± 0.05 μg/mg, respectively, which were lower than Group A (2.42 ± 0.18 μg/mg). Group C was significantly decreased compared with Group A ($P < 0.05$). At 3 weeks, the mean hydroxyproline content in Groups B (1.81 ± 0.06 μg/mg) and C (2.04 ± 0.01 μg/mg) was significantly decreased compared to Group A (2.40 ± 0.12 μg/mg) ($P < 0.01$). Thus, the silkworm pupa NOCC anti-adhesion solution significantly decreased collagen synthesis, thereby preventing postoperative adhesion.

3.7. Histological observation

The H&E staining results were shown in Fig. 5. The results for Group A at 2 and 3 weeks are shown in Fig. 5a & b. Desmosplasia and lamainin were clearly visible, while the serosa was seriously damaged and did not possess an obvious structure. There appeared to be an increased inflammatory cell infiltration, even to the level of hyperaemia. The results from Group B were shown in Fig. 5c & d, and Group C was shown in Fig. 5e & f. In comparison with Group A, there was a marked reduction in the number of infiltrating cells in Groups B and C. Moreover, the structures of the rat cecum wall in Groups B and C were clearly visible in contrast to Group A. The mucosa, submucosa, muscular layer, and serous could be distinguished from the innermost to the outermost layers. Additionally, a lower level of fibrous tissue hyperplasia was found in Groups B and C.

The results of Mason’s staining were shown in Fig. 6. The level of collagen deposition and fibroblast activity in the adhesion tissues could be clearly observed following Mason’s staining. The collagen fibres were dyed a different colour, enabling an intuitive observation of the collagen fibre hyperplasia in the postoperative adhesion tissues. The collagen fibres observed under the microscope were blue, while the muscle fibres and cytoplasm were red. Fig. 6 reveals that a higher amount of collagen fibres were found in Group A compared to Groups B and C at 2 and 3 weeks.

The results of the histological analysis indicated that the silkworm pupa NOCC anti-adhesion solution significantly decreased collagen synthesis, thereby preventing postoperative adhesion.

3.8. Analysis of TGF-β1 expression

The results of the expression of TGF-β1 were shown in Fig. 7. Positive immunohistochemical staining for TGF-β1 was represented by brown granules in the cytoplasm and was mainly located in the fibroblasts of the adhesion tissue. At both 2 and 3 weeks the TGF-β1-positive cells were significantly lower in Groups B and C compared to Group A. Based on the results, we concluded that the silkworm pupa NOCC anti-adhesion solution reduced the expression of TGF-β1, thereby reducing adhesion.
4. Discussion

Billions of tonnes of chitin/chitosan are biosynthesized annually, ranking only second in production to the natural polymer cellulose. There is an inexhaustible source of biological resources for chitin.

In our research, silkworm pupa chitosan had good solubility in dilute acetic acid, although its DDA was only approximately 50%. This may be due to the preparation method of the silkworm pupa chitosan described in Section 2.2. Studies have shown that freezing can obviously reduce the crystallinity of chitin and destroy the original order of the hydrogen bonding interactions that are conducive to further chemical modifications of chitin. After the freezing-thawing cycle process, NaOH was able to penetrate the particles inside chitin to moderate deacetylation [45].

The crystal forms of chitin and chitosan have been divided into three classes (α, β, and γ) that are characterized by different physical and chemical properties. Although the crystallinity of silkworm pupa chitin, chitosan and carboxymethyl chitosan are different, the crystal forms were all of the α-type. This result demonstrated that deacetylation and carboxymethylation did not change the natural configuration of the samples. Moreover, the diffraction peak position of the silkworm pupa chitosan was closer to that of chitin. This may be because the DDA of the silkworm pupa chitosan was approximately 50%, allowing it to maintain the bulk of the crystal structure of chitin. The silkworm pupa carboxymethyl chitosan possessed almost no obvious crystal structure, suggesting that carboxymethylation damaged the secondary structure of chitosan and reduced its crystallinity, thereby increasing its solubility in water.

Intestinal adhesions are serious complications of surgery and can cause pain and potentially lethal bowel obstructions. Many different types of materials have been used to prevent or minimize postoperative adhesions. Therefore, the viscous solution is an important research direction for the prevention of peritoneal adhesion [46,47]. NOCC has been extensively shown to exhibit intestinal adhesion-reducing properties. In our study, a novel silkworm pupa NOCC was prepared and studied to determine its ability to prevent intestinal adhesion formation after surgery.

As expected, the silkworm pupa NOCC treatment resulted in significantly reduced adhesion compared with the control treatment. The intestinal adhesion model method we used was stable and easy to generate. Additionally, animal mortality was low and reflected a real surgical situation both directly and objectively. To date, the specific mechanism of the occurrence of postoperative peritoneal adhesion has not been entirely elucidated. Peritoneal trauma can cause a series of inflammatory reactions, resulting in the production of inflammatory cytokines that are involved in the different stages of abdominal adhesion formation, such as TGF-β1, TNF-α, IL-1, and IL-6. Based on the haematology analysis, we concluded that the silkworm pupa NOCC exhibited anti-inflammatory effects in addition to its role as a medical anti-adhesion material. Moreover, the analysis showed that the silkworm pupa NOCC had no effect on the other blood routine indices of rats and had good histocompatibility. TNF-α not only mediates the
inflammatory response but also plays an important role in activating the cytokine cascade response [48]. It also can promote the formation of PAI, and therefore its synthesis and degradation can directly influence the formation of adhesion [49]. In our study, the silkworm pupa NOCC solution significantly decreased the levels of TNF-α, IL-1β, IL-2, IL-6 and IL-8, but had no effect on IL-4. After injury, the secretion of TGF-β1 promotes the expression of fibrous proteins, the accumulation of collagen in the cell matrix, inhibits its degradation, and destroys the balance of fibrous protein synthesis and degradation, eventually leading to adhesion formation [50]. TGF-β1 played a key role in numerous cytokine pathways and has been reported to contribute to the initiation and inhibition of the tissue repair process [51,52]. Researchers studying the application of TGF-β1 antibodies demonstrated significant reductions in the incidence and degree of adhesion [53]. The results in our study suggested that silkworm pupa NOCC solution could decrease the expression of TGF-β1, thereby reducing the formation of peritoneal adhesion.

Tissue adhesion depends mainly on the synthesis of collagen. Hydroxyproline is a precursor of the formation of collagen. Thus, the hydroxyproline content can clearly reflect the degree of adhesion formation. Experimental studies have shown that hydroxyproline levels and adhesion degree exhibit a linear positive correlation [54]. Therefore, the level of hydroxyproline can be used as a strong indicator for the degree of adhesion and wound healing. In our study, silkworm pupa NOCC solution significantly decreased the synthesis of hydroxyproline and inhibited the synthesis of collagen to an extent comparable to the medical anti-adhesion gel. Moreover, the results of H&E staining showed that silkworm pupa NOCC solution also reduced fibrous tissue hyperplasia and inflammatory cell infiltration to a comparable extent as the medical anti-adhesion gel. Masson’s staining can clearly show the presence of collagen deposition and fibroblast activity in the adhesion tissues, including the injured sidewall and cecum. The results of Masson’s staining in our study were in agreement with the H&E staining.

In conclusion, we prepared a novel silkworm pupa NOCC and evaluated its ability to prevent intestinal adhesion in a rat model. The DDA of silkworm pupa chitosan was approximately 50% and was used as a raw material to generate the novel silkworm pupa NOCC, which exhibited weaker crystallinity. The excellent efficacy of silkworm pupa NOCC solution was shown to inhibit inflammatory cell invasion, resulting in reduced formation of fibrous adhesion and TGF-β1 expression.

5. Conclusion

The novel silkworm pupa NOCC studied in this work was the first time to study for the prevention of postoperative peritoneal adhesions in rat model. No in vivo inflammatory response was found which led to less formation of the fibrous adhesion and reducing the expression of TGF-β1. It indicated that the novel silkworm pupa NOCC solution is a kind of safe material with biocompatibility. Therefore, the novel silkworm pupa NOCC is effective in reducing the formation of postoperative intestinal adhesion and considered to be a promising candidate in preventing postsurgical adhesion formation.
Conflict of interests

The authors declare that they have no financial or personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in paper.

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References


Fig. 7. TGF-β1 immunohistochemistry micrograph of adhesion tissues from the three groups (×200). a/c/e: Group A/Group B/Group C at 2 weeks; b/d/f: Group A/Group B/Group C at 3 weeks.


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