ORIGINAL RESEARCH

Prevention of postoperative peritoneal adhesions by O-carboxymethyl chitosan in a rat cecal abrasion model

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Abstract

Purpose: Post-surgical adhesion formation can result in significant morbidity and mortality. N,O-carboxymethyl chitosan (N,O-CMC) has been previously shown to be effective in the prevention of postsurgical adhesion formation. In this study, we evaluated the ability of O-carboxymethyl chitosan (O-CMC), another chitosan derivative generated by carboxymethylation of chitosan's oxygen centers, to reduce postsurgical adhesion development.

Methods: Twenty male Sprague-Dawley rats (250 ± 20 g) were divided into two equal groups: O-CMC group and saline (control) group. All rats underwent a midline laparotomy and the cecum was abraded to cause petechial hemorrhages. Following peritoneal injections of either saline or O-CMC, the incisions were closed. Seven days after surgery, the animals were killed and adhesion formation was scored. Tissue samples from the adhesions were examined histochemically.

Results: Adhesion formation was significantly decreased in the O-CMC group ($P < .001$) in comparison with the control group. Furthermore, significantly less collagen ($P < .001$) and fewer inflammatory cells and fibroblasts were detected in the O-CMC-treated animals. Additionally, a significantly

($P < .05$) lower level of TGF- β 1 expression was found in the O-CMC group.

Conclusion: O-CMC appears to be effective in the prevention of postoperative peritoneal adhesion formation, which may be attributed to decreased accumulation of inflammatory cells and fibroblasts and reduced collagen synthesis.

Adhesion formation is a very common complication of surgery. Several studies have documented that 68-100% of patients undergoing laparotomies have post-surgical adhesions.^{1,2} The formation of adhesions, probably resulting from mechanical damage, ischemia, and infection by microorganisms,¹⁻³ can increase the morbidity and mortality following surgery.⁴⁻⁶ There are two major strategies for adhesion prevention or reduction: improvement of surgical technique and application of adjuvants.⁷ Modifications in technique alone will decrease but not prevent adhesion formation; thus, adjuvant therapy is essential.⁷ Among

the studied adjuvants, N,O-carboxymethyl chitosan (N,O-CMC) has recently attracted much interest. N,O-CMC is generated by carboxymethylation of chitosan's nitrogen and oxygen centers, which results in enhanced solubility. This water-soluble chitosan derivative has been shown to reduce postoperative adhesion development,⁸⁻¹⁰ which may act as a biophysical barrier and block the adherence of inflammatory cells.¹¹ Another chitosan derivative, O-carboxymethyl chitosan (O-CMC), developed by carboxymethylation of chitosan's oxygen centers, also exhibits remarkable water-solubility and bio- compatibility.¹² Little is known, however, of the potential of O-CMC to protect against adhesion formation. In this study, we investigate whether O-CMC protects against adhesion formation in an experimental peritoneal adhesion model, and suggest possible underlying mechanisms involved.

Materials and Methods

Animal model

A total of 20 adult Sprague-Dawley rats, aged 5-7 weeks and weighing 250 ± 20 g, were purchased from Sun Yat-sen University. All rats were maintained under pathogen-free conditions. Animal experiments were approved by the Animal Care Committee of Sun Yat-Sen University.

An animal model of surgical adhesion formation was created as described by Harris *et al.* with minor modifications.¹³ Briefly, rats were anesthetized with intraperitoneal injection of chloral hydrate (300 mg/kg of body weight; Qingdao Yulong Algae Co., Ltd., Qingdao, China). After hair removal, the abdomen was prepared with 2.5% iodine tincture followed by 75% alcohol using aseptic technique, and a 4 cm mid-line incision was made. The cecum was exposed and abraded by scraping with a scalpel blade until petechial hemorrhages over a 1×2 cm area developed. The animals then received a 2 ml intraperitoneal injection of either 2% O-CMC (Yuhuan Ocean Biochemi-

cal Co. Ltd., China; O-CMC group, $n = 10$) or saline (control group, $n = 10$). Finally, the abdominal cavity was closed by a running suture of 1-0 silk.

Assessment of adhesions

In pilot experiments, adhesion formation were examined at three, seven and 14 days after surgery. At three days postsurgery, some mild adhesions had formed, and more severe adhesions were detected at seven days. The degree of adhesion formation at day seven was similar to that seen at day 14. Based on these preliminary results, we chose seven days as the endpoint for this experiment. At the end of the experiment, the animals were euthanized with an overdose of chloral hydrate and adhesion formation was examined by two investigators who were blind to the underlying treatment. Adhesions were evaluated by scoring the extent, type and tenacity according to the method (Table 1), which has been widely used.^{8,14-16} Total adhesion scores were the sum of the extent, type and tenacity scores of lesions.

Quantitation of hydroxyproline levels in peritoneal adhesions

Surgically excised adhesive tissue samples were immediately frozen and stored at -20°C . Hydroxyproline is a component of collagen, and its concentration is directly correlated with collagen content. The hydroxyproline assay was performed by using a method developed by Reddy and Enwemeka.¹⁷ Approximately

TABLE 1. Adhesion scoring system

Score	Extent of site involvement	Type	Tenacity
0	None	None	None
1	<25%	Filmy, transparent, avascular	Fall apart
2	<50%	Opaque, translucent, avascular	Lysed with traction
3	<75%	Opaque, translucent, capillaries	Sharp dissection required
4	>75%	Opaque, large vessels	

(Kennedy R *et al.* 1996; ref. 8)

100 mg of each sample was homogenized in 500:1 of 2 N sodium hydroxide by sonication. Homogenates were hydrolyzed by autoclaving at 120°C for 20 min; 450:1 chloramin-T (Sigma-Aldrich, St Louis, MO, USA) was added to 100:1 hydrolyzed samples, mixed gently, and kept at room temperature for 25 min to oxidize. Erlich's aldehyde reagent (500:1; Sigma-Aldrich) was then added to each sample, and the chromophore was developed by incubating the samples at 65°C for 20 min. Absorbance of each sample was read at 550 nm using a spectrophotometer (Shanghai Precision Instruments Co., Ltd., Shanghai, China).

Histological analysis

Adhesion-carrying tissues were collected and fixed in a 10% buffered formaldehyde solution. Sections of 4 µm thick were stained with hematoxylin and eosin (H&E) solution (Sigma-Aldrich) for histopathological evaluation or with Masson's trichrome stain (Sigma-Aldrich) for collagen assessment.

Immunohistochemical staining

Immunohistochemistry for transforming growth factor-β1 (TGF-β1) was performed on the consecutive formalin-fixed, paraffin-embedded tissue sections. The sections were deparaffinized, and incubated in methanol and 3% H₂O₂ with 1.5% normal goat serum (Zhongshan Goldenbridge Biotechnology Co., Beijing, China). Following antigen retrieval, the slides were incubated with TGF-β1 rabbit polyclonal antibody (SC-146; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution in a humidified chamber at 4°C overnight. The tissue sections were then incubated for 30 min with 3 µg/ml biotinylated antibody (Santa Cruz Biotechnology). Subsequently, avidin-biotin-peroxidase complex solution (Zhongshan Goldenbridge Biotechnology Co.) was added and incubated for 45 minutes at room temperature. The reaction products were visualized using 3,3'-diaminobenzidine-tetrahydrochloride (Sigma-Aldrich). The

cell nuclei were counterstained with hematoxylin. Negative controls were performed with normal immunoglobulin G (Santa Cruz Biotechnology) in place of the primary antibody. TGF-β1 staining was semi-quantitatively evaluated according to a previously published method.¹⁸ Cells that stained positive for TGF-β1 were counted on four representative high-power fields (magnification, ×400) of each slide. Data were expressed as the average number of TGF-β1-positive cells per high-power field for each experimental group.

Statistical analysis

Data was analyzed by using SPSS11.0 software (SPSS Inc., Chicago, IL, USA) and presented as mean ± standard deviation (S.D.). Significant differences (defined as $P < .05$) between two data groups were analyzed using Student's *t*-test.

Results

Evaluation of adhesion formation

No animal died during or after surgery. Seven days after surgery, the animals were sacrificed and adhesion formation was evaluated. The control animals showed consistent and marked adhesions between the cecum and abdominal wall (Fig. 1). These adhesive bonds were dense and difficult to remove from involved organs. In contrast, O-CMC treatment decreased adhesion formation as manifested by a few thin, filmy, membranous adhesions. Peritoneal adhesion scores for both the O-CMC and control groups are summarized in Table 2. The mean adhesion score was significantly less for the O-CMC group in comparison with the control group (4.8 ± 1.6 versus 9.9 ± 1.2 , $P < .001$).

Histological analysis

Since inflammatory cells and their cytokines have been documented to be involved in adhesion formation,^{19,20} the adhesion-reducing effects of O-CMC

FIGURE 1. The effect of O-CMC on postsurgical adhesion formation in a rat cecal abrasion model. Sprague-Dawley rats underwent cecal abrasion surgery followed by peritoneal injections of either saline or O-CMC. The animals were killed and assessed for adhesion formation seven days later. Less adhesion formation is detected in the O-CMC group compared to the saline group.

TABLE 2. Adhesion scores for O-CMC and saline groups

Subject	O-CMC		Saline	
	Observer A	Observer B	Observer A	Observer B
1	3	3	11	11
2	5	4	9	8
3	6	6	11	11
4	4	4	8	8
5	4	4	11	11
6	4	4	11	10
7	7	6	9	8
8	4	3	10	10
9	8	8	11	11
10	4	4	9	9
Mean $\pm$ S.D.	4.8 $\pm$ 1.6*		9.9 $\pm$ 1.2	

* $P < .001$ versus the saline group.

TABLE 3. Quantitation of hydroxyproline levels in peritoneal adhesions

Groups	Hydroxyproline level ($\mu\text{g}/\text{mg}$ tissue)
O-CMC	0.407 $\pm$ 0.089*
Saline	0.712 $\pm$ 0.074

Data are means $\pm$ S.D. * $P < .001$ versus the saline group.

were investigated to determine whether they were associated with decreased infiltration of inflammatory cells. Histological analysis of peritoneal adhesions revealed that, in comparison with the control group, there was a marked reduction in the number of infiltrated cells including macrophages, lymphocytes, and granulocytes in the O-CMC group (Fig. 2). Moreover, the number of fibroblasts in the O-CMC group was considerably less than that in the control group. Additionally, a lower level of collagen was found in the O-CMC group (Fig. 2 C) compared to the saline group (Fig. 2 D), as evidenced by Masson's trichrome stain.

Collagen assessment

Since collagen is known to play a critical role in the pathophysiology of postoperative adhesions,²¹ the extent of collagen deposition in the adhesion-carrying tissues was determined using the hydroxyproline assay, assuming that 12.5% of collagen is hydroxyproline. As shown in Table 3, the mean of hydroxyproline

FIGURE 2. Histological analysis of the adhesion samples from both the O-CMC and saline groups ($n = 10$ for each). Surgically excised adhesive tissue samples were fixed, embedded, sectioned, and stained with hematoxylin and eosin (H&E) solution for histopathological evaluation or with Masson's trichrome stain for collagen assessment. Representative images for each group are shown. Fewer macrophages, lymphocytes and fibroblasts (A) and a lower level of collagen (blue) (C) were found in the O-CMC group in comparison with the saline group. Original magnification, $\times 400$.

content in the O-CMC group was significantly lower than that in the control group (0.407 ± 0.089 versus 0.712 ± 0.074 $\mu\text{g}/\text{mg}$ tissue, $P < .001$).

Analysis of TGF- β 1 expression

Next, the expression of TGF- β 1, which promotes tissue fibrosis and adhesion formation, was assessed.^{22,23} As shown in Fig. 3, the percentage and intensity of TGF- β 1 positive cells were significantly lower in the O-CMC group compared to the control group ($P < .05$).

Discussion

Peritoneal adhesions are serious complications of surgery and can cause pain, infertility and potentially lethal bowel obstruction. Many different types of materials have been used to prevent or minimize postoperative adhesions. Hyaluronic acid (HA), for instance, has been found to prevent postoperative adhesion in mice after laparotomy.²⁴ A chitosan derivative, N,O-CMC, has also been extensively investigated, and has been shown to exhibit adhesion-reducing properties.⁸⁻¹⁰ In this study, another chitosan derivative, O-CMC, was studied to determine its ability to prevent adhesion formation. As expected, O-CMC treatment resulted in significantly less adhesions in a rat cecal abrasion model in comparison with to the control treatment.

To investigate the possible mechanism(s) of action of O-CMC, the adhesion samples were histologically examined. Notably, O-CMC-treated animals showed a marked reduction in the number of infiltrated inflammatory cells, including lymphocytes and macrophages. Furthermore, the number of fibroblasts in the adhesion samples was considerably less in the O-CMC group versus the control group. Most importantly, collagen synthesis was significantly decreased following the O-CMC treatment. Additionally, exposure to O-CMC was found to significantly decrease TGF- β 1 expression. These findings suggest that the adhesion-reducing effects of O-CMC may be associated with decreased fibrotic responses at the surgical

FIGURE 3. Immunohistochemical analysis of TGF- β 1 expression in the adhesion samples from both the O-CMC and saline (control) groups ($n = 10$ for each). (A) Representative immunostaining images for TGF- β 1. Original magnification, $\times 400$. (B) Average number of TGF- β 1-positive cells per $\times 400$ high-power field (HPF) for each group. $P < .05$ versus the saline group.

sites. Postoperative adhesion formation has been characterized by excessive deposition of fibrin, which likely results from an imbalance between fibrin deposition (fibrinogenesis) and fibrin degradation (fibrinolysis).^{25,26} The infiltration of inflammatory cells and the release of cytokines and chemokines are considered to be early events in this pathological process.^{19,20} Based on our limited observations, O-CMC appears to prevent the adhesion of inflammatory cells to injured sites and attenuate inflammatory responses, consequently leading to less fibrinogenesis. This hypothesis concerning the function of O-CMC is supported by several studies. An *in vitro* study demonstrated that few of fibroblasts and macrophages could adhere to N,O-CMC-coated plates,¹¹ implying that the chitosan derivative inhibits the adhesion of inflammatory cells to the ECM of injured tissues. In another study, intranasal application of water-soluble chitosan was shown to attenuate infiltration of inflammatory cells and markedly decrease production of inflammatory cytokines such as tumor necrosis factor- α and interleukin 6.²⁷ An alternative mechanism

for the O-CMC function is direct interference with proliferation and activation of inflammatory cells; however, this seems unlikely. In most cases chitosan and its derivatives display good biocompatibility, although the polysaccharides at higher concentrations may inhibit cell proliferation.²⁸ The exact mechanism(s) underlying the O-CMC function needs further study.

Two questions remain to be addressed in future work. Firstly, does O-CMC have advantages in the reduction of postoperative adhesions in comparison with other chitosan derivatives such as N,O-CMC? Secondly, is O-CMC safe? Although N,O-CMC has not been shown to interfere with postsurgical healing,⁹ the effect of O-CMC on wound healing is still unknown. In our future research, we will clarify this issue using a model of bowel resection. Nevertheless, our data indicate that O-CMC is able to prevent adhesion formation in an experimental peritoneal adhesion model. This biomaterial may have therapeutic implications as adjuvants during peritoneal surgery.

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