Reduction in postoperative adhesion formation and re-formation after an abdominal operation with the use of *N, O* - carboxymethyl chitosan

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Background. *Postoperative adhesions have proven to be intractable complications after abdominal operations. This study assessed the efficacy of* N, O *- carboxymethyl chitosan (NOCC) to limit adhesion formation and re-formation in a rabbit abdominal surgery model.*

Methods. *In study 1 (adhesion formation), injuries to the large bowel, cecum, and abdominal sidewall were generated in rabbits. The rabbits (10/group) were randomly assigned to 1 of 5 treatment groups: Group A received no NOCC treatment; in group B, NOCC gel was applied directly to the injured site and NOCC solution was applied throughout the abdominal cavity; in group C, NOCC gel was applied near the injured site and NOCC solution was applied as above; in group D, NOCC gel was applied distant to the injury and NOCC solution was applied as above; in group E, a mixture of NOCC gel and solution was applied at the injured site. Adhesions were evaluated 14 days later. In study 2 (adhesion re-formation), adhesions were generated as above but were then lysed by careful dissection. After adhesiolysis, the rabbits (9/group) were treated with NOCC gel and solution at the site of adhesiolysis or left untreated. Adhesion re-formation was assessed 14 days later. In study 3 (mechanism of action), sterile tissue culture plates were coated with NOCC and adhesion of cultured, radiolabeled murine fibroblasts to the plates was assessed.*

Results. *In study 1, animals treated with NOCC gel and solution showed reduced adhesion formation (*$P < .01$*). NOCC gel was equally efficacious if applied on the site of injury or near the site of injury but less efficacious if applied at a site distant to the injury. In study 2, animals treated with NOCC gel and solution showed less adhesion re-formation compared with the untreated control animals (*$P < .01$*). In study 3, murine fibroblasts did not adhere to NOCC-coated tissue culture plates.*

Conclusions. *NOCC gel and solution can reduce adhesion formation and re-formation in this rabbit model. The inability of fibroblasts to adhere to NOCC solution—coated surfaces suggests that NOCC may act as a biophysical barrier. (Surgery 2004;135:307-12.)*

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POSTOPERATIVE ADHESIONS OFTEN OCCUR after abdominal operations, and they can be responsible for complications such as chronic pain, bowel obstruction, and infertility.[1] Although adhesions can be lysed surgically, they typically recur with equal or greater severity.[2] A treatment that would reduce or prevent adhesion formation would eliminate the need for repeated invasive and costly operations to resolve adhesions and the complications resulting from the operative intervention.

Various approaches to reduce or prevent adhesion formation have been studied in animal models; some approaches have been the subject of clinical trials.[3,4] A common approach has been to establish a physical barrier between injured surfaces by the application of films or gels. Many of these potential therapeutic products have used hyaluronic acid (HA) as a base. However, there have been problems associated with the use of HA. For example, HA degrades quickly and disappears from the injured site soon after application, which limits its efficacy as an adhesion preventative therapy.[5] Attempts have been made to increase

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the stability of HA by cross-linking it with various agents, which has resulted in enhanced efficacy in animal models.[5] Clinical trials of such HA-based products have yet to provide evidence of substantial efficacy in human beings. Products using carboxymethylcellulose have also been reported to prevent adhesion formation in experimental models.[6,7] However, carboxymethylcellulose is a known inflammatory agent,[8] and its widespread use in the clinical setting is unlikely.

In this study, we use *N, O* - carboxymethyl chitosan (NOCC) to provide a physical barrier between surgically damaged surfaces. NOCC is a nontoxic, absorbable, long-chain polysaccharide. We have previously shown that this product, when used as a combination therapy of gel and solution, can reduce the incidence and severity of postoperative adhesions in preliminary rat models.[9,10] In this study, we assess the effect of NOCC gel and solution on adhesion formation and re-formation in the more widely accepted rabbit intestinal injury model. Our results demonstrate that the application of NOCC gel and solution inhibits the formation of abdominal adhesions and also inhibits the re-formation of adhesions after adhesiolysis.

MATERIAL AND METHODS

Animals. Female New Zealand white rabbits (2.5-3.0 kg) were purchased from Charles River, maintained in the Dalhousie University Faculty of Medicine animal care facility, and given free access to food and water. All animal experimentation was undertaken in compliance with the guidelines of the Canadian Council of Animal Care.

NOCC. A cross-linked 1% NOCC gel formulation and a non−cross-linked 2% NOCC solution were provided by Chitogenics Ltd. (Halifax, Nova Scotia, Canada). Both gel and solution were prepared and used in a sterile manner.

Abdominal injury model. This experimental model was modified from Johns and coworkers' model.[5] Rabbits were anesthetized by means of inhaled halothane. After preparation for aseptic surgery, a midline laparotomy was performed. The right abdominal sidewall was exposed, and a 3 × 5-cm section of peritoneum and transverse abdominal muscle was removed to make a sidewall injury. The large bowel and cecum were exteriorized, and a 10-cm length of large bowel was scraped (approximately 40 times) with a No. 10 scalpel blade until punctate bleeding occurred. A 5-cm length of cecum was abraded by applying digital pressure with gauze (approximately 40 times) until punctate bleeding occurred. After the injury was completed, animals were treated with 14 mL NOCC gel and 12 mL NOCC solution or were left without treatment.

After treatment, the midline incision in the muscle was closed with a 3-0 polyglycolic acid (Dexon, Tyco Healthcare Group, Pointe-Claire, Quebec, Canada) suture using a horizontal mattress stitch. The midline skin incision was closed with skin staples. All the animals received analgesic (buprenorphine, 0.03 mg/kg by subcutaneous injection) and antibiotics (enrofloxacin, 5 mg/kg, by subcutaneous injection) at the time of surgery and a second dose of analgesic 12 hours later. Animals were recovered in heated cages, with frequent observation, and received food and water freely. At 14 days after the operation, animals were killed by means of a lethal injection of sodium pentobarbitol (340 mg/mL), and adhesion formation was assessed on the basis of the percentage of the 3 × 5-cm sidewall injury that was involved in adhesion formation with the cecum and/or colon.

A numeric value for the extent of adhesion formation was obtained by measuring the size of the adhesion to the 3 × 5-cm sidewall site (length and width if roughly square, and diameter if roughly circular), calculating the area of the adhesion on the basis of that measurement, dividing this area by 15 cm^2 (the area of the injured sidewall site), and multiplying this value by 100 to obtain the percentage of the 15-cm^2 sidewall injured site that was involved in adhesions.

In all studies, animals were assigned randomly to treatment groups (or a control group) before the operation. The surgeon performing the operation (T.D.G.L.) was blinded to the assignment. All animals were ear tattooed by animal care personnel immediately before the operation. After the abdominal injury was complete, the NOCC gel and solution were applied by a technician who was aware of the treatment assignment and tattoo number but not otherwise involved in the operation. On the day the animals were to be killed, they were presented randomly for evaluation by the animal care staff. Evaluation of adhesions was performed by the operating surgeon. The surgical assistant (J.Z.) noted the tattoo number and the evaluation. After all the animals were evaluated, adhesion data were provided to the treatment technician who then matched the tattoo numbers recorded for the treatment groups with the adhesion data.

Experimental plan. *Study 1: Evaluation of NOCC on adhesion formation:* Five groups of 10 rabbits were subjected to abdominal injury as above. After the injury, group A received no NOCC treatment;

group B received NOCC gel applied directly on the injured site and NOCC solution applied throughout the abdominal cavity; group C received NOCC gel near the injured site and NOCC solution as above; group D received NOCC gel applied distant to the injury and NOCC solution as above; and group E received a mixture of NOCC gel and solution applied at the injured site. Adhesions were evaluated 14 days later.

Statistical analysis: The Wilcoxon rank sum test was used to assess the data since it did not follow a normal distribution.

Study 2: Evaluation of NOCC on adhesion reformation: Twenty rabbits were subjected to abdominal injury as above. No NOCC gel or solution was applied to any of these animals. Twenty days later, the rabbits were randomly assigned into 2 groups of 10 by their tattoo numbers. All animals were anesthetised and underwent a second operation. During the second operation, any adhesions to the sidewall injury site generated from the first operation were evaluated. The adhesions were then lysed by careful dissection. Any animal with an adhesion score from the first surgery of less than 25% was excluded from the experiment.

Animals that were assigned to the control group received no further treatment before closure and recovery. Animals that were assigned to the experimental group received NOCC gel and solution before closure. NOCC gel was applied at the site of the lysed adhesions, and the solution was applied generally throughout the abdominal cavity. Closure of the muscle and skin was performed as above. Animals were killed 14 days later; the extent and severity of the adhesions were evaluated as above.

Statistical analysis: The Wilcoxon rank sum test was used to assess the data since it did not follow a normal distribution.

Study 3: In vitro fibroblast adherence assay: Mouse 3T3 fibroblasts were purchased from ATCC (Manassas, Va) and maintained in RPMI culture medium (ICN Biomedicals, Aurora, Ohio) supplemented with 10% fetal bovine serum, 20 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and 50 μmol/L 2-mercaptoethanol (all from Life Technologies, Burlington, Ontario). Fibroblasts were prelabeled with chromium (^{51}Cr) in the form of $Na_2{}^{51}CrO_4$ (Amersham, Piscataway, NJ) for 1 hour, washed extensively in RPMI, and added (in triplicate) to wells of 96-well tissue culture plates (NUNC, Life Technologies) at a concentration of 2×10^4 cells/well. The wells in the tissue culture plates were either precoated with 0.1% NOCC solution or not coated. Precoating was achieved by placing 100 μL of 0.1% NOCC (in serum-free RPMI) in wells for 18 hours. NOCC-precoated wells were then washed with saline extensively to remove unbound NOCC.

After the cells were added to the wells, they were allowed to incubate for 90 minutes at 37°C. The plates were then washed extensively with saline to remove nonadherent cells. The remaining (adherent) cells were lysed with 10% sodium dodecyl sulfate (Sigma, St Louis, Mo) and the lysate collected. The percentage of cells adhering to each well was calculated by the following formula: radioactivity in the lysate/radioactivity in lysate of 2×10^4-labeled fibroblasts $\times 100$.

Statistical analysis: The Student *t* test was used to analyze in vitro data.

Table I. Extent of adhesion formation to sidewall injury, expressed as a percentage of the injured area

	% of sidewall injury involved in adhesions in each treatment group				
*Animal No.**	*A (Control)*	*B (Injured site)*	*C (Near)*	*D (Distant)*	*E (Comb)*
1	55	0	0.5	66	55
2	100	0	0	0	0
3	80	0	0	0	0
4	85	0	0	1	0
5	100	0	0	85	0
6	100	0	0	5	0
7	25	0	0	50	2
8	100	0	0	0	0
9	100	0	0	0	0
10	100	0	0	0	0
		$P < .01$	$P < .01$	$P < .01$	$P < .01$

Comb, Combination.
*Animals in the control group (group A) underwent surgery but did not receive NOCC gel or solution. In NOCC–treated groups, NOCC gel was applied on the injured site (group B), near the injured sidewall site (group C), or distant to the injured sidewall site (group D). NOCC gel was followed in these 3 groups by delivery of NOCC solution throughout the abdominal cavity. The animals in group E were treated with a single-step application of a NOCC gel and solution combination applied directly on the injury sites.

RESULTS

Study 1: Effect of NOCC on prevention of adhesion formation. The results shown in Table I demonstrate that if NOCC gel was applied directly on the injured site and NOCC solution was applied throughout the abdomen, there was a decrease in adhesion formation as compared with untreated control animals (group B compared with group A) ($P < .01$). If the NOCC gel was applied near, but not directly on, the injured sidewall site, there was

Table II. Extent of adhesion re-formation to the injured sidewall site after the second operation

	% of sidewall injury involved in adhesion re-formation in each treatment group	
*Animal No.**	*Control*	*NOCC gel/sol*
1	100	0
2	100	0
3	100	0
4	100	100
5	100	1
6	100	0
7	100	0
8	100	30
9	50	0
		$P < .01$

Sol, Solution.
*All animals had adhesions rated as 100% before adhesiolysis. The extent of adhesion reformation was expressed as a percentage of the injured area. NOCC gel was applied after adhesiolysis over the involved area, and NOCC solution was delivered into the abdominal cavity. Animals in the control group did not receive any NOCC treatment after adhesiolysis.

also a reduction in adhesion formation (group C compared with group A) ($P < .01$). There was no reduction in efficacy when the gel was applied near the injured site as opposed to applying the gel directly on the injured site (group B compared with group C) ($P > .05$). If the NOCC gel was applied at a site distant to the injury and the solution was applied throughout the abdomen (group D), the efficacy was reduced, but treatment still showed an effect when compared with the control untreated group (group D compared with group A) ($P < .01$).

In the last group (group E), NOCC gel and solution were mixed together instead of applied separately. This mixture of gel and solution was applied on the injured site and throughout the abdominal cavity. The mixture also showed efficacy at reducing adhesion formation (group E compared with group A) ($P < .01$).

Study 2: Effect of NOCC on prevention of adhesion re-formation. After primary surgery, 18 of the 20 animals developed adhesions that involved the entire injury area of the 15-cm^2 sidewall (ie, all 18 had adhesion extent ratings of 100%) and were subjected to adhesiolysis. Two animals showed adhesion extent ratings of less than 25% after primary surgery and were excluded from the study. All animals in the control group re-formed extensive adhesions (Table II). In the control group, 8 of 9 animals re-formed adhesions that were as extensive as the lysed adhesions (rated at 100%). In contrast, in the group treated with NOCC gel and solution, only 1 of the 9 animals re-formed adhesions as extensive as the lysed lesions. Furthermore, in this group, 6 of the 9 animals showed no re-formation of adhesions at all, and 1 showed limited adhesion re-formation (rated at 1%). NOCC treatment was able to reduce adhesion re-formation ($P < .01$).

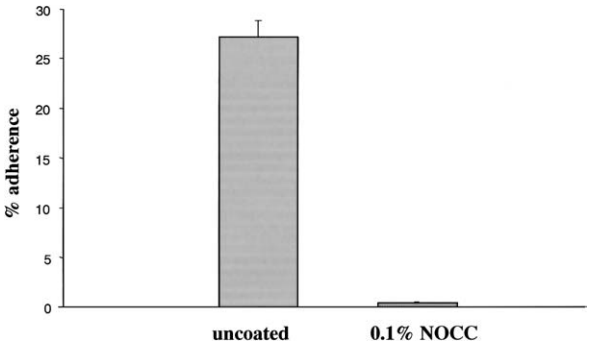

Figure. Adherence of 3T3 fibroblasts to tissue culture plastic, either uncoated or coated with 0.1% NOCC solution. Cells were plated in RPMI media. Results shown are mean ± SD, n = 3.

Study 3: Effect of NOCC on fibroblast adhesion in vitro. The coating of tissue culture plates with a 0.1% NOCC solution blocked the adhesion of mouse 3T3 fibroblasts (Figure). In control cultures, more than 25% of the fibroblasts were found to be strongly adherent to uncoated plates after 90 minutes of incubation. In contrast, less than 1% of the fibroblasts were found to be adherent to the NOCC solution−coated plates.

DISCUSSION

In previous experiments in rats, we found that administration of NOCC gel alone or NOCC solution alone decreased the incidence and intensity of abdominal adhesions. The best results, however, were obtained by application of NOCC gel followed by NOCC solution.[10] In the rabbit experiments described here, we assessed the ability of the NOCC gel and solution to prevent adhesion formation and re-formation in a widely used rabbit abdominal sidewall injury model—a preclinical model with predictive value for clinical use.

The data reported here confirm that NOCC gel and solution significantly reduce adhesion formation after an intra-abdominal operation in rabbits. NOCC gel was equally efficacious whether it was applied on the site of injury or near the site of injury. The data also show that efficacy was retained, but at a reduced level, when the gel was applied distant to the injured site. These findings are important for three reasons. First, it is often

difficult to apply adhesion barrier substances directly on injured sites, especially under conditions of laparoscopic surgery. The ability to apply the gel in the general area of the injured site increases ease of use markedly. Second, it is hard to know the limits of minor abrasive damage during an operation; therefore, a product that has some efficacy outside of the area of application would be of clinical benefit. Third, the reduced efficacy of the NOCC gel when applied far from the site of injury provides insight into the importance of the NOCC gel versus NOCC solution in blocking adhesion formation. Since the positive effects fall off dramatically when the gel is applied far from the injured site, even though the solution coats the entire abdominal area, including the injured site, it would seem that the NOCC gel is the product primarily responsible for the prevention of adhesions in these experiments.

The ease of application of antiadhesion products is an important factor in defining the success of their widespread use. The application of NOCC gel and solution as an efficacious single-step formulation would therefore be of clinical benefit. The data presented here suggest that a single-step application of NOCC gel and solution may retain the efficacy of the original two-step protocol. The one caveat to this conclusion is that 1 animal in this test group did have extensive adhesions. Although that occasionally happens in groups treated with the two-step protocol, further testing would have to be performed to see if the single-step application fails more often than the separate application of the products.

Abdominal adhesiolysis surgery is a common procedure, but it usually provides only temporary relief from symptoms because of adhesion reformation. A product that would prevent adhesion re-formation after adhesiolysis would be of great benefit. Adhesion re-formation models are much more challenging but are of crucial clinical relevance. The data reported here show that the application of NOCC gel and solution markedly reduced adhesion reformation after adhesiolysis. Moreover, during adhesiolysis, significant bleeding occurred because of sharp dissection of the adherent tissue. This bleeding was controlled before closure, but some blood remained in the abdominal cavity during and, for a short time, after application of the gel and solution. The success of NOCC gel and solution under these conditions confirms that they remain effective in the presence of blood in the abdomen.

Although the mechanisms responsible for the observed effect of NOCC gel and solution on adhesion formation are unknown, a likely hypothesis is that the NOCC gel and solution provide a physical barrier between the injured tissue surfaces. There is evidence to suggest that the presence of such a barrier between injured tissue surfaces during the early stages of healing limits tissue apposition, minimizes the deposition of the critically important fibrin matrix, and minimizes adhesion formation.[11] The injured surfaces are most susceptible to adhesion formation between 16 to 36 hours after the operation;[12,13] NOCC gel and solution could function as barriers during this critical period. Our finding that fibroblasts are unable to adhere to NOCC-coated surfaces supports this hypothesis. Fibroblast activation and deposition at the site of tissue injury are known to be pivotal events in the initial stages of adhesion formation.[11] Blocking this important deposition step could be the mechanism by which NOCC gel and solution prevent adhesion formation and re-formation. It is very possible that NOCC functions in vivo to block fibroblast attachment to the injured surfaces, thus interrupting fibrin matrix formation. Of interest is the ability of NOCC solution to prevent the deposition and adherence of fibroblasts onto surfaces even in the presence of extracellular matrix proteins such as vitronectin (in the supplemented media).

The use of physical barriers to prevent postoperative adhesion formation and re-formation has been demonstrated in many experimental models.[3,14-17] An ideal physical barrier should not affect wound healing, should not evoke fibrosis, should be stable during the initial stages of adhesion formation but then degrade, and should be efficacious in the presence of blood and body fluids.[13,18] NOCC appears to possess all of these properties. We have previously demonstrated that NOCC does not limit healing in both a rat skin-healing model and a large bowel anastomosis model.[10] Our in vivo evidence to date strongly suggests that NOCC does not stimulate fibrosis and rapidly degrades.[10] The data from the experimentation presented here confirm that NOCC is efficacious in the presence of blood in the surgical field. Taken together, these data suggest that NOCC gel and solution satisfy the requirements for an ideal physical barrier to prevent postoperative adhesions.

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