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Use of N,O-carboxymethyl chitosan to prevent postsurgical adhesions in a rabbit double uterine horn model: a randomized controlled design

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In previous studies, N,O-carboxymethyl chitosan has been shown to decrease the incidence and intensity of abdominal adhesions. In the present study, adhesions were induced in 220 rabbits using a double uterine horn model. Rabbits were randomized to receive an operation only or an operation+medical chitosan intraperitoneally. Twenty-two rabbits from each group were euthanized at one of five different times (Day 3, 7, 14, 28, or 42), and adhesion formation was given gross and histopathological scores. Reductions were observed in adhesion extent (P=0.0337) and tenacity (P=0.0271) as well as inflammation (P<0.0001) on Day 3 when medical chitosan was applied. Prior to Day 14, fibrosis was less obvious in the medical chitosan group (P<0.0005). The tenacity scores were significantly lower in the medical chitosan group following Day 14 (P<0.05), while the type scores were lower in the medical chitosan group following Day 28 (P<0.03). Thus, medical chitosan decreased both the gross and the histopathological scores of the induced adhesions.

postsurgical adhesion, adhesion prevention, N,O-carboxymethyl chitosan, rabbit double uterine horn model, randomized controlled trial

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INTRODUCTION

Peritoneal adhesions are primarily induced by surgical procedures in the peritoneal cavity. Despite advances in surgical techniques in recent years, the incidence of postoperative adhesions is still as high as 94% in patients undergoing abdominopelvic operations, even with meticulous operative techniques (Stanciu and Menzies, 2007; Liakakos et al., 2001). Adhesions remain a major source of morbidity and mortality and a substantial financial burden (Wiseman, 2008). Many adhesion-related complications necessitate additional corrective surgeries. Unfortunately, however, additional surgeries can exacerbate adhesions and lead to additional and more severe complications (Tittel et al., 2001).

The pathogenesis of intra-abdominal adhesions has not been entirely delineated. However, evidence suggests that three basic processes are involved: (i) the acute inflammatory response, (ii) the fibrinolytic cascade, and (iii) extracellular matrix remodeling (Cheong et al., 2001). Several therapeutic strategies have been used to prevent adhesion formation, including surgical techniques. However, areview of 27 studies investigating the relationship between various surgical techniques and adhesion-related consequences re-

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vealed no statistical evidence that any specific procedure significantly reduced the incidence of adhesive small bowel obstruction (Ten et al., 2013). Other methods included antioxidant agents, anti-inflammatory drugs, and fibrinolytic agents (Sahbaz et al., 2015; Moraloglu et al., 2011), as well as local intra-abdominal measures such as viscous liquids for hydrofloatation and membrane barriers (Sakai et al., 2015; Hellebrekers et al., 2000). Chitosan has proven beneficial owing to its hemostatic properties; its antimicrobial activity toward a variety of bacteria, fungi, and viruses; and its TGF-β inhibition effect (Ong et al., 2008; Rabea et al., 2003; Krause et al., 1998). N,O-carboxymethyl chitosan (NOCC) is a derivative of chitosan, and the addition of carboxymethyl groups to chitosan's nitrogen and oxygen centers produces a water-soluble, negatively charged, biocompatible polymer that is hydrophilic, lubricious, and vicoelastic.

The objective of this study was to assess the anti-adhesive effect of NOCC in a large-scale randomized controlled study.

RESULTS

The mean ages of rabbits in the NOCC and control groups were (5.30±0.23) and (5.43±0.31) months, respectively, whereas the mean weights of animals in the two groups were (2.43±0.0.31) and (2.53±0.33) kg, respectively. All rabbits were sexually mature and nulliparous. All basic data on the two groups were comparable (P>0.05).

Adhesion assessment

Gross observation

D3. Adhesions at this time were limited and most frequently found between the uterus and bladder. The adhesions were loose, filmy, and avascular (Figure 1A). No NOCC residue was evident around the wounds, and adhesions in the NOCC group were relatively looser.

D7. Adhesions increased in extent and tenacity, but were still easy to separate (Figure 1B). In the NOCC group, adhesions were looser.

D14. The extent and tenacity of adhesions were similar to those observed on D7. Neovascularization was not obvious. Looser adhesions were observed in the NOCC group.

D28. The extent of adhesion increased and obvious neovascularization networks started to form. In the NOCC group, the extent of adhesions was relatively limited, and neovascularization was less prominent (Figure 1C).

D42. The extent and tenacity of adhesions decreased in comparison to D28. In the NOCC group, the extent, type, and tenacity of adhesions all decreased relative to D28. Adhesions in the NOCC group were looser, with fewer and smaller newborn vessels than those of the control group (Figure 1D; Table 1).

Hematoxylin-eosin (HE) staining

D3. Infiltration of inflammation cells was most prominent at this phase. Tissues were edematous with floating fibroblasts or myofibroblasts (Figure 2A). In the NOCC group, there were fewer microabscesses, whereas the inflammatory response was similar to that of the control group.

D7. Chronic inflammation gradually replaced acute inflammatory exudates. In some specimens, newborn microcapillaries comprised of a few or even a single endothelial cell(s) were noted around granulation tissue (Figure 2B). In the NOCC group, proteinoid exudates were more notable than obvious fibrosis.

D14. Infiltration of acute inflammatory cells became sparse and inflammation faded away while fibrous proliferation increased. Fibrosis on the serosa was composed mainly of collagen and a few smooth muscle-like cells. In the NOCC group, the collagen was looser.

D28. Fibrosis gradually became dense, and newborn vessels began to form a network. Many of the vessels had complete structures of arterioles, some of which had diameters greater than 100 μm (Figure 2C). In the NOCC group, diameters of newborn vessels were similar to those of the control group.

D42. The extent of fibrous tissues and thickness of fibrosis proliferation on the serosa decreased, whereas their density increased (Figure 2D). The number and morphology of newborn vessels remained the same as on D28 (Figure 2E; Table 2).

DISCUSSION

Several studies have investigated the anti-adhesive effect and mechanism of NOCC, but few have combined the results of gross and pathological examinations (Ahmad et al., 2014). Therefore, we performed the present study to investigate the effects of NOCC application in both situations.

Its suitable size for operation and observation, along with a fibrinolytic system close to that of humans, made rabbit an appropriate species for exploring the use of NOCC in adhesion prevention after abdominopelvic surgery (Häggroth et al., 1984). The 72 h after the operation are vital for fibrin absorption, which forms the basis for later fibrosis adhesion. It takes seven days for the peritoneal mesothelium to heal completely. Collagen volume reaches its peak on Day 14. After four weeks, all anti-adhesion materials should be completely absorbed; thus, observations on Day 42 should show whether the anti-adhesive effect persisted when no anti-adhesive materials remained.

In previous experiments in rats, N,O-carboxymethyl chitosan (NOCC) has shown significant potential for decreasing the incidence and intensity of adhesions in abdominal surgeries (Costain et al.,1997; Zhou et al., 2004). In the present study, inflammation scores in the NOCC group were significantly lower than those of the control group on D3. Evidence suggests that inflammatory response, includ-

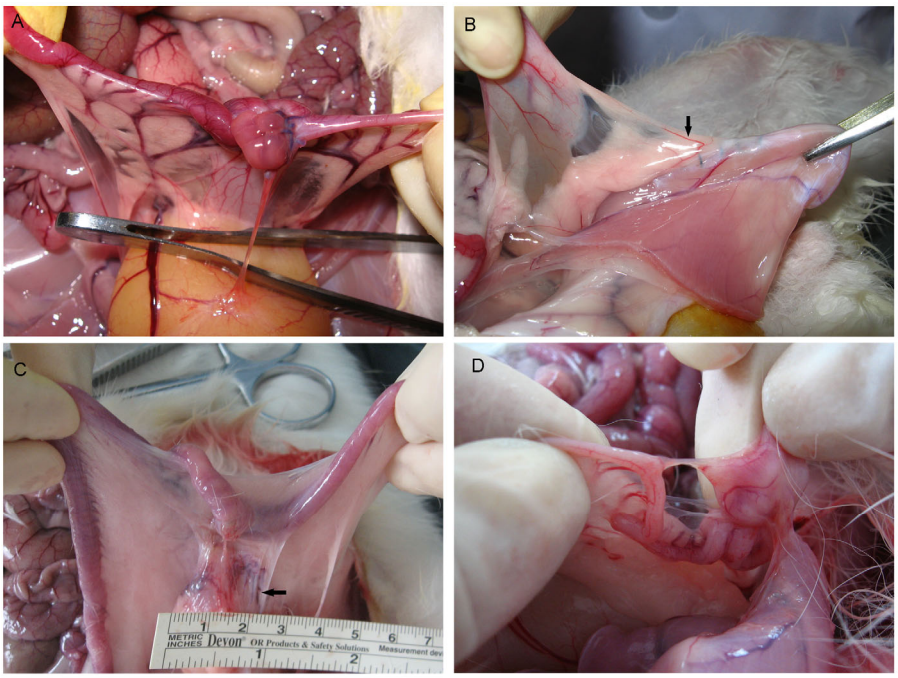

Figure 1 Gross examination of pelvic adhesions. A, Loose, filmy adhesion between the uterus and bladder on D3. B, Neovascularization was observed in one case on D7. C, Neovascularization network formed on D28. D, In the NOCC group, adhesions were looser and newborn vessels were smaller.

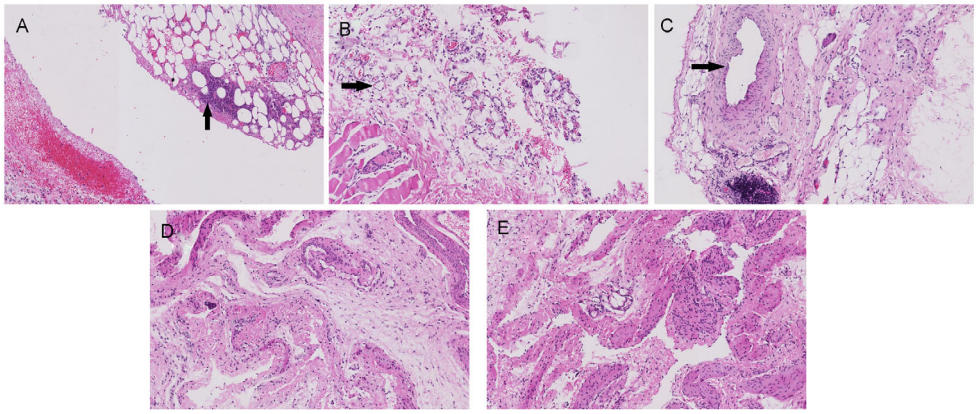

Figure 2 Pathologic examination of pelvic adhesions with HE staining (original magnification 150×). A, D3 in the control group: the arrow indicated the microscopic appearance of microabscess. B, D7 in the control group: the arrow indicated a newborn microcapillary. C, D28 in the control group: the arrow showed a big capillary with complete structures, below which chronic inflammatory cells infiltration could be observed. D, D42 in the control group. E, in the NOCC group.

ing fibroblast activation and deposition at the site of tissue injury, is a pivotal event in the initial stages of adhesion formation (Wang et al., 2010). The injured surfaces are most susceptible to adhesion formation 16 to 36 h after the operation (Ricketts et al., 1999; Harris et al., 1995). NOCC could function as a barrier during this critical period. The physical separation of traumatized serosal areas that localized inflammation, together with the dilution of inflammatory exudates, which reduced the numbers of regional inflammatory cells and cytokines, mitigated the inflammatory response (Yeo et al., 2006) and thus decreased the gross scoring for the extent and tenacity of adhesions.

In the first postoperative week, inflammatory response was more severe in the NOCC group, but the difference was not significant. This suggests that though it was biocompatible, NOCC was still treated as foreign material by the body. Collagenization, which is an indicator of permanent fibrotic formation, occurred during the physiological response to peritoneal damage. Our study found that collagen was most prominent on Day 7, which is consistent with findings of other studies. Collagen synthesis, which reached its highest level between the fifth and seventh postoperative

Table 1 Gross observation scoring for both groups at five different time points after the operation[a)]

Time post-surgery	Characteristics of adhesions	Median (Quartiles)		*P*
		control	Medical chitosan	
D3#	Extent	2 (1~2)	1 (1~2)	0.0337*
	Type	2 (2)	2 (2)	0.7638
	Tenacity	2 (1.25~2)	1 (1~2)	0.0271*
D7#	Extent	2 (1~2)	2 (1~2)	0.4257
	Type	2 (2)	2 (1.25~2)	0.4609
	Tenacity	2 (1.25~2)	1 (1~2)	0.1026
D14#	Extent	2 (1~2)	1.5 (1~2)	0.4693
	Type	2 (2)	2 (1~2)	0.1142
	Tenacity	2 (1~2.75)	1 (1~2)	0.0410*
D28#	Extent	2.5 (1~2)	2 (1.25~2)	0.5006
	Type	2 (2)	2 (1~2)	0.0210*
	Tenacity	2 (2)	1 (1)	<0.0001*
D42#	Extent	2 (1~2)	1 (0~1)	0.1475
	Type	2 (1~2)	1 (0~1)	0.0027*
	Tenacity	2 (1~2)	1 (0~1)	0.0002*

a) #, the number of days after operation; *, scores significantly different between the two groups.

Table 2 HE staining scoring for both groups at five different time points after the operation[a)]

Time post-surgery	Pathology of adhesions	Median (Quartiles)		*P*
		control	Medical chitosan	
D3#	Inflammation	5 (4~6)	3 (1~3.75)	<0.0001*
	Fibrosis	4 (3~4)	3 (2~3)	0.0004*
	Vascular proliferation	0 (0)	0 (0)	1.0000
D7#	Inflammation	2 (1~3)	2 (0~4)	0.9044
	Fibrosis	3 (3~3.75)	1 (1~2)	<0.0001*
	Vascular proliferation	2 (2~4.75)	2 (2~3)	0.2717
D14#	Inflammation	1 (0.25~2)	1 (0~1)	0.8226
	Fibrosis	3 (3~4)	2 (0.25~2.75)	0.0002*
	Vascular proliferation	4 (1.25~5)	2 (1~3.75)	0.1012
D28#	Inflammation	0 (0~1)	1 (0~2)	0.1468
	Fibrosis	3 (3~3.75)	3 (2~3)	0.4036
	Vascular proliferation	2.5 (2~4)	3 (2~3)	0.8155
D42#	Inflammation	0 (0~1)	1 (0~1)	0.4109
	Fibrosis	3 (2.25~4)	2 (2~3)	0.0501
	Vascular proliferation	4 (2~4.75)	3 (2~3)	0.2626

a) #, the number of days after operation; *, scores significantly different between the two groups.

days (Haney and Doty, 1994; van Hinsbergh et al., 1990), allowed for adhesion maturation (Hickey, 2001; Milligan and Raftery, 1974).

After 1 week, the fibrosis, which was vital to adhesion formation at this time, was less obvious in the NOCC group. In a study by Costain et al., *in vivo* evidence also strongly suggested that NOCC did not stimulate fibrosis (Costain et al., 1997). This might explain why the tenacity scores were significantly lower in the NOCC group. In their controlled study, Duran and Cetin found that after 14 d, both extent and severity scores in the NOCC group were significantly lower than those of the control and normal saline groups in a rat uterine horn model (Duran et al., 2003; Cetin et al., 2003). Another controlled study performed by Zhou et al. using a rabbit intra-peritoneal operation model also confirmed that application of NOCC could reduce adhesion formation as well as adhesion reformation on Day 14. Their study found that NOCC remained effective even in the presence of blood in the abdomen (Zhou et al., 2004).

After four weeks, not only the tenacity but also the type of adhesions showed significant reductions in the NOCC group as compared to the control, as found in a clinical study by Diamond (Diamond et al., 2003). This suggests that after complete absorption of NOCC, its anti-adhesive effect remained. Tissue recovery was achieved as successfully as in the control in our study. To further assess the effect of NOCC on the healing of surgical incisions, Costain et al. used three different models: an abdominal anastomosis, a large bowel anastomosis, and an abdominal skin incision; and found that in all three models, NOCC treatment

did not adversely affect the strength of the repaired incision (Costain et al., 1997).

The limitation of this study was the inability to include an illustration of collagen alteration, which is still under observation and will be included in future reports.

From the above illustration, we can conclude that NOCC is easy to use, could provide unrestricted coverage between injured surfaces, remains effective throughout the healing process in the presence of blood and body fluids, and is stable during the initial stages of adhesion formation but then degrades. It could significantly reduce adhesion formation without any adverse effect on surgical wound healing. Thus, it could be an effective choice for abdominopelvic surgery. Further clinical investigation is warranted.

MATERIALS AND METHODS

Animals

Female Japanese white rabbits (5–6 months of age, nulliparous, 2.48±0.32 kg in weight; the Institute of Laboratory Animal Sciences, CAMS and PUMC, Beijing) were acclimatized for a minimum of three days before any procedures were initiated. The rabbits were housed at constant room temperature and humidity under 12 h light/12 h dark cycles in separate cages and allowed free access to standard rabbit diet and water. The Institutional Animal Care and Use Committee at the Peking Medical College Hospital, which is a domestic committee, approved this study on September 1, 2011 (register number 2011100123.)

Study design

Two hundred and twenty female Japanese white rabbits were first randomized to either the operation group or the operation+ medical chitosan (N,O-carboxymethyl chitosan, NOCC) group, then further randomized to one of the five time periods (3, 7, 14, 28, or 42 d), with 22 rabbits in each subgroup. All animals underwent the adhesion operation introduced in the surgical model. The animals were euthanized at the scheduled postoperative time for evaluation.

Surgical model

Each rabbit was anesthetized by IV administration of 3% pentobarbital sodium (0.8 mL kg^{-1}). The hair on the ventral abdominal area was shaved, and the skin was prepared with complex iodine disinfection solution and draped in a sterile fashion.

The abdominal laparotomy was performed using a midline incision of approximately 4 cm in length. Double-horn uterine incisions were created as previously described (Ren et al., 2012). In brief, after the midline laparotomy, a 3-cm incision through the serosa into the superficial muscularis of the uterus (on a random side of the bicornuate uterus) was made using a scalpel, and the incision was closed with interrupted sutures using a 3–0 Vicryl absorbable suture (Ethicon Inc., Somerville, USA). In the NOCC group, 1 mL 2% NOCC (Shanghai Qisheng Biological Preparation Co. Ltd., Shanghai) sterile solution was administered after suturing and spread evenly over the uterine incision area before closure. The midline laparotomy was closed in two layers. The skin was closed with interrupted sutures (1–0 Mersilk non-absorbable suture, Ethicon Inc., Somerville, USA). The entire surgical procedure was performed by the same author.

During the postoperative period, all experimental animals were housed in individual cages and observed frequently. The animals received a standard diet and water as well as 400,000 IU penicillin IM twice a day for three days to prevent wound site infection.

Tissue collection

All tissue samples were fixed with 10% buffered formalin for 14 h and embedded in paraffin blocks. Sections of 4 μm thickness were obtained, stained with hematoxylin-eosin, and examined by light microscopy (BX50, Olympus Corporation, Tokyo, Japan). The pathological examinations were performed according to a previously defined method (Adhesion Scoring Group, 1994).

Adhesion assessment

Gross observation

One author, who was blinded to the treatment groupings, scored the adhesions using an 11-point adhesion scoring system that measured three characteristics of adhesion: extent, type, and tenacity. The scoring was based on a system used in gynecological surgery: the American Fertility Society (AFS) adhesion scoring system (Adhesion Scoring Group, 1994; American Fertility Society, 1988). The highest severity score was used in the calculation.

Pathology observation

Two pathologists, who were blinded to the characteristics of the experimental animals and the study groups, simultaneously evaluated the acute inflammation and chronic inflammation parameters of the peritoneal damage, including leukocyte migration and necrosis as well as fibrosis proliferation and neovascularization.

Observations were scored at each of the three locations (uterus, visceral peritoneum, and pouch of Douglas), and an average was calculated for each animal (Ersoy et al., 2009; Hooker et al., 1999).

Statistical analysis

Most of the data failed normality or homogeneity of variance testing. Thus, all data were described as medians and quartiles, and a Kruskal-Wallis test for nonparametric analysis was used for direct comparison of the scores between two groups. All statistical analyses were conducted using SAS 9.1 (SAS Institute Inc., Cary, USA). Groups were deemed significantly different from each other at $P<0.05$.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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