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Original research

Evaluation of chitosan–gelatin films for use as postoperative adhesion barrier in rat cecum model

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ABSTRACT

Background: Postoperative adhesions remain a significant complication of abdominal surgery and can result in pain, infertility and potentially lethal bowel obstruction. Pharmacotherapy and barrier devices have reduced adhesion formation to varying degrees in preclinical studies or clinical trials.
Materials and methods: In this study, we produced blends between chitosan (Ch) and gelatin (G) with various compositions (Ch/G 100/0, 75/25, 50/50, 25/75 w/w) as candidate materials for prevention of postoperative abdominal adhesion. For in vivo analysis, 30 female rats weighing 200–250 g were divided into 5 groups (One control and 4 treatment groups). Under general anesthesia, the anterior surface of serous membrane in rat was scraped slightly with sterile gauze until obvious congestion and small bleeding drops appeared, then sample films set on the cecum in treatment groups and the intestine was put back into the abdominal cavity, which were then closed. After 4 weeks, the abdominal cavity was reopened and the grades of peritoneal adhesion were studied by macroscopic and pathologic assessments.
Results: Our results showed Ch1/G3 films had an insignificant reduction effect on postoperative adhesion, but surprisingly, the sample with more than 25% by weight of chitosan did not have any effect on reducing adhesion formation but also increased inflammation near the cecum.
Conclusion: Administration of chitosan–gelatin films with higher than 25% weight of chitosan had no effect on reduction of adhesion formation in the rat cecum model.

1. Introduction

Adhesions are described as abnormal fibrous connections that develop between tissues and organs as a result of inflammatory processes, such as infections and inflammation, endometriosis, but most frequently as a sequel to surgical trauma following incision, cauterization, suturing, or other tissue trauma. Adhesions develop after nearly all open abdominal surgical procedures.[1] The relevance of adhesions to gynecology not only relates to infertility and abdominal pain, but also to the occurrence of bowel obstruction.[2] Adhesions complicate future surgery with important associated morbidity, expense and a considerable risk of mortality. Despite advances in surgical techniques in recent years, the burden of adhesion-related complications has not changed.[3]

Pharmacotherapy and barrier devices have reduced adhesion formation to varying degrees in preclinical studies or clinical trials; however, complete prevention of adhesions remains to be accomplished. The barrier systems include polymer solutions pre-formed or in situ cross-linkable hydrogels and preformed solid sheets designed to cover affected organs and reduce contact between adjacent organs.[4] The physical separation of traumatized serosa areas using barriers represents the most important clinical strategy for adhesion prevention. However, the optimal material has not yet been found.[5]

An ideal barrier system should be easy to use via both laparoscopy and open procedures providing unrestricted coverage of the affected peritoneum, and remain effective throughout the healing.[4] Some preformed solid sheet such carboxymethyl cellulose (CMC),[6] oxidized regenerated cellulose (ORC), Expanded polytetrafluoroethylene (ePTFE), and polyethyleneglycol have been reported as antiadhesive agents in experimental models.[7] In addition,

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recently some in situ cross-linkable hydrogel such as hyaluronic acid,[4] dextran-based injectable hydrogel,[8] PEG–PCL–PEG[9] and PCLA–PEG–PCLA hydrogels[10] have been studied.

As another solution, controlled release technology could provide sustained drug levels, and if desirable, could also provide a barrier function.[11] In our past publication, some pharmaceutics were investigated,[12] in current research; some polymeric films are evaluated as postoperative adhesion barriers.

Chitin is a co-polymer of N-acetyl-glucosamine and N-glucosamine units randomly or block distributed throughout the biopolymer chains[13] and Chitosan is produced industrially by alkaline hydrolysis of chitin.[14] Chitosan is currently receiving a great deal of interest for medical and pharmaceutical applications. The main reason of this increasing attention is certainly its interesting intrinsic properties.[15] In addition, chitosan is known as a biocompatible material allowing its use in various medical applications such as implantation.[13] Moreover, chitosan is metabolized by certain human enzymes, especially lysozyme and is considered as biodegradable.[16] Biodegradability and biocompatibility, together with specific interactions with components of the extracellular matrix and growth factors, have led to growing use of chitosan in tissue engineering, such as in the repair of skin, bone, and cartilage.[17,18] Besides, in medical and pharmaceutical applications, chitosan is participated as a component in hydrogels. Recently a new derivative of chitosan – hydroxybutyl chitosan (HBC) introduced as a thermosensitive chitosan-based hydrogel barrier for postoperative adhesion prevention.[19]

On the other hand, gelatin is obtained by thermal denaturation or physical and chemical degradation of collagen. As a biomaterial, gelatin displays several advantages: it is a natural polymer, which has not shown antigenity, it is completely resorbable in vivo and its physicochemical properties can be suitably modulated.[20]

Some studies reported excellent ability of chitosan–gelatin network to be used in human skin fibroblast, keratinocyte transplantation and skin regeneration.[21] Also many studies are developed about using chitosan–gelatin scaffolds in tissue engineering.[22,23] When gelatin and chitosan are blended together, the structure formed can affect the spatial distribution of integrin ligands and polycationic chitosan interaction with the anionic cell surface. These effects influence cell adhesion, cellular bioactivity, tissue remodeling process and ultimately the quality of the regenerated tissue. It seems, this combination can have effect in adhesion prevention with the same reason, something that other researchers reported before.[24]

In this study, as part of our ongoing effort to develop a biodegradable film as anti adhesion barrier, chitosan–gelatin film has been chosen as a novel candidate material. For this, we created a series of chitosan–gelatin composite films by varying the ratio of components. The purpose of this work was to prepare soft and elastic biomaterial that can be used as a barrier. Because of the desirable biological activity of chitosan and gelatin, a combination of these two biopolymers may also have beneficial effects on the biological characteristics of composite films. The major advantages of this combination are its simplicity, low cost and the potentially improved mechanical properties.

2. Material and methods

2.1. Materials

Chitosan, low molecular weight (448,869, DDA = 75% – 85%) and type A porcine skin gelatin (G8150) were obtained from Sigma Aldrich Chemical Co. All other reagents were local products of analytical grade.

2.2. Film preparation

Films were prepared according to the methods of Zhang with minor modifications.[25] For this goal, 0.5 g of chitosan was dissolved in 50 ml of 1% acetic acid solution. Then a weight amount of gelatin was poured into chitosan solution and the mixture was stirred for 2 h at 50 °C. The resultant solution was centrifugally degassed for 10 min to prevent air bubbles from forming. The mixture was cast into plastic Petri dishes with 75 mm diameter, dried at 25 °C for 24 h and washed with 100% ethanol until the films became neutral (pH = 7). The films were then dried and cut to patches of 5 cm × 5 cm in size and 60 μm in thickness. The contents of components are presented in Table 1.

2.3. Animals

After obtaining the approval of the Institutional Review Board of our medical school, all experiments were carried out in accordance with the Guidelines of the Animal Care and use ethics committee of Baqiyatallah University of medical sciences.

Thirty female adult Wistar rats weighting 230 ± 20 g were maintained under standard laboratory conditions. Animals were housed in an environment of 21 ± 0.5 °C with a relative humidity of 50 ± 10% and a 12-h light–dark cycle. Food and water were always available. Rats were randomly divided into five groups ($n = 6$) include: one control and four treatment groups (Chitosan, Ch3/G1, Ch1/G1, Ch1/G3).

2.4. Surgical procedure

Surgical procedure was done according to our previous study.[12] Briefly, rats were anesthetized with 90 mg/kg ketamine hydrochloride and 8 mg/kg xylazine hydrochloride intramuscularly. Following a 3 cm midline incision, antimesenteric border of cecum was abraded with dry sterile gauze until punctuate bleeding occurred. In treatment groups after rubbing 5 times (typically provided punctuate bleeding), films set on cecum surface without using any adhesive material or suture. In control group, no medication was administered, only cecum was exposed to air for 5 min. After administration part, abdominal wall and skin of animals were closed, using 4-0 polypropylene (PROLENE, Ethicon, Edinburgh, UK) continuous sutures, respectively. The duration from opening to closing the abdominal cavity was 5 min, so that the duration of exposure of intestines to air was the same for each rat. The rats resumed their preoperative routine until the 28th postoperative day, when they were killed by an overdose of ether.

2.5. Macroscopic assessments

On reoperation day, the abdominal cavity was inspected through a straight incision and adhesions were identified, counted, and graded using the macroscopic and pathological assessment that was described in our previous study.[12] Therefore, macroscopic assessment carried out by following grading method; Grade 0: No adhesion, grade 1: The ratio of adhesive area/total treated area is

Table 1
Composition of films.

Films	Chitosan (g)	Gelatin (g)	wt_{Ch}/wt_{G}
Ch	0.5	0	100/0
Ch3/G1	0.5	0.165	75/25
Ch1/G1	0.5	0.5	50/50
Ch1/G3	0.5	1.5	25/75

<50% and the adhesion is easily to be dissected, grade 2:The ratio is ≥ 50% and the adhesion is easily to be dissected, grade 3: Area of the adhesion is out of consideration and it is difficult and the intestinal wall will be impaired after the blunt dissection, grade 4: The adhesion is fast and cannot be bluntly dissected, also may have adhesion to other organs (liver).

2.6. Pathologic and quantitative assessments

For morphologic and pathologic assessment, tissues recovered from the necropsy were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin with using standard techniques. Quantitative analysis includes area of fibrous tissue around cecum and numbers of fibroblast cells were done. In addition, histomorphological findings were assessed with respect to the severity of interstitial fibrosis (IF) and inflammatory cell reaction (ICR). The extent of ICR was graded on a scale as follows: (0) for normal; (1) for mild; (2) for moderate and (3) for severe. The intensity of fibrosis was examined in 10 randomly selected high power fields (HPF) .The amount of fibrosis was also scored as follows: (0) no fibrosis; (1) minimal, loose fibrosis; (2) moderate fibrosis and (3) florid dense fibrosis. For evaluating fibroblast cells in adhesion area, 10 randomly selected high power fields (HPF) pictures with 400× zoom of adhesion bond in every samples were taken and average of fibroblast cell numbers were then calculated.

2.7. Statistical analyses

A comparison of the groups was carried out using the non-parametric Kruskal–Wallis test followed by Mann–Whitney U statistics, to detect the statistically significant differences among the groups. Data were presented as mean ± SEM. Analysis was performed using SPSS version 13. A *P*-value ≤0.05 was considered significant.

3. Result

3.1. Macroscopic assessment

Most animals survived in the experiment and reached the endpoint of observation in an apparently healthy condition except for one animal from control group and another one from Ch1/G1group (died on the 3rd and 8th postoperative day). On macroscopic observation, adhesion formation was not observed around cecum in some animals after 4 weeks, however adhesion bonds with pieces of residual films with fibrous tissue are observed around the cecum in some rats of treatment groups (Fig. 1).

Table 2
Macroscopic adhesion grade in groups. Ch1/G3 has lower scores in adhesion grade, however differences between all groups is insignificant ($P > 0.05$).

Adhesion score	Control	Ch	Ch3/G1	Ch1/G1	Ch1/G3
Grade 0	–	–	1	1	2
Grade 1	3	3	–	1	1
Grade 2	1	–	3	1	2
Grade 3	1	2	2	2	1
Grade 4	–	1	–	–	–
Adhesion grade (Mean ± SEM)	1.6 ± 0.4	2.17 ± 0.54	2 ± 0.45	1.8 ± 0.59	1.33 ± 0.5

Macroscopic adhesion assessments of all groups are shown in Table 2. However, mean of adhesion scores in Ch1/G3 group (1.33 ± 0.5) was lower than other groups, but differences were not significant ($P > 0.05$).

3.2. Quantitative analysis

In this study, we created new methods for reporting some quantitative elements related to adhesion bond around cecum. Results of these calculations can be seen in Fig. 2A. Samples in Ch1/G3 group have lowest fibroblast cells number compare to other treatment groups ($P < 0.05$) but compare to control group difference was insignificant ($P = 0.17$). As another quantitative analysis, after preparing scanning picture from laboratory slides of samples, fibrous area around one cm length of cecum was evaluated with using "Motic-Images 2000, release 1.2" software. Experiments were run in triplicate per sample. All data were expressed as means ± SEM for $n = 3$. Results of this assessment are shown in Fig. 2B. Samples with more contents of Chitosan have higher fibrous area around cecum and Ch1/G3 group has lowest adhesion area compare to other groups but compare to control group difference was insignificant ($P > 0.05$).

3.3. Pathological assessment

Histopathologic findings of adhesion area in all groups are shown in Table 3. Ch1/G3 group had lowest fibrosis and inflammation score between all groups but difference was insignificant ($P > 0.05$). It seems, increasing chitosan not only had no effect on reduction of inflammation and fibrosis near cecum but also induce more inflammatory reactions. In addition, histopathological photos of samples showed presence of residual pieces of films in all treatment groups except Ch1/G3 (Fig. 3). Intestinal epithelium is shown in Fig. 3A. Mild adhesion bond around the cecum in control group can be seen in Fig. 3B. Ch1/G3 also induce light adhesion

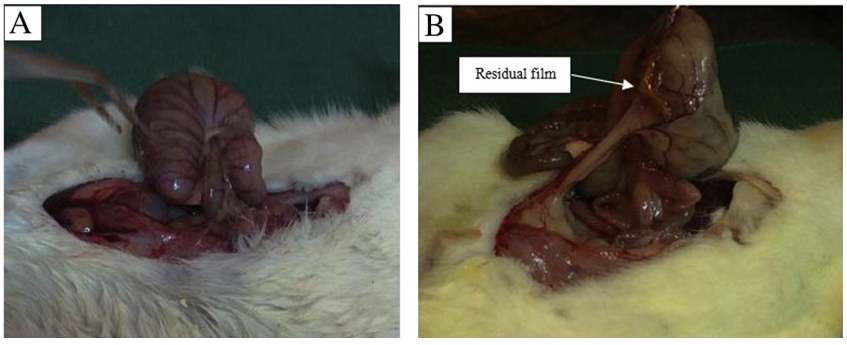

Fig. 1. Macroscopic view of cecum. A) Animal in group Ch1/G3, cecum with no adhesion bond. B) Animal in group Ch3/G1, cecum with adhesion bond and residual pieces of film can be seen.

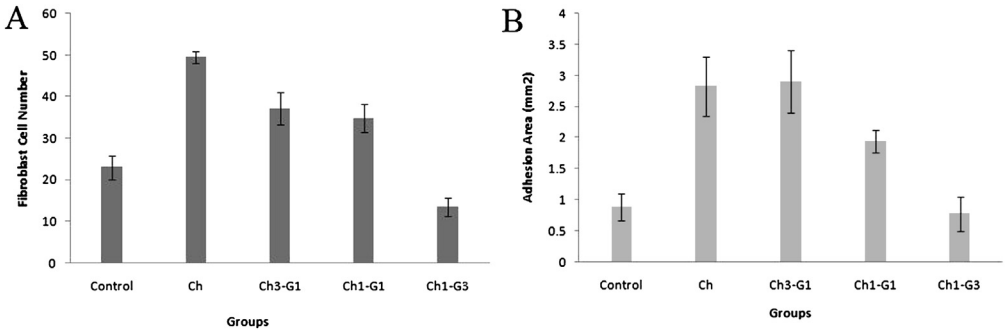

Fig. 2. Quantitative analysis of adhesion. A) Number of fibroblast cells around cecum can be seen in all groups. Samples in Ch1/G3 group have lowest fibroblast cells number compare to other treatment groups ($P < 0.05$) but compare to control group difference was insignificant ($P = 0.17$). B) Adhesion area around one cm of cecum can be seen in all groups. Samples with more content of chitosan have higher fibrous area. All values are mean ± SEM.

bond that can be seen Fig. 3C. Also in Fig. 3D, thick and dense fibrous layers observed around a residual piece of Ch3/G1film. In addition, a compact of connective tissues, numerous leukocytes, blood vessels and anchoring fibroblast cells are observed in this Figure.

4. Discussion

Chitosan–gelatin film had a number of physicochemical properties that are desirable for preventing post-operative adhesions. It was also confirmed by good handling properties during surgery but biological assessment was surprisingly quite different. None of the chitosan–gelatin films prevented adhesions formation and three of them actually seemed to promote adhesion formation. This result obscures the effects of these combinations on prevent of post-operative adhesions. These findings are in conflict with earlier studies that reported effective usage of chitosan on abdominal adhesion reduction.

Zhou et al.[24] prepared gelatinized chitosan film without reporting preparation method and showed the preventive effect of film on peritoneal adhesions induced by wound, ischemia and infection. They reported chitosan prevents peritoneal adhesion by the mechanisms of inhibiting growth of fibroblasts, facilitating reparation of the epithelium, and disinfection. These results contradict our findings that reported inflammatory response and tough fibrous layer surrounded residual films that induce more adhesion bonds in samples with more than 25% weight of chitosan after 4 weeks.

Chitosan and gelatin are derived from natural polymers and have many properties that make them attractive for a wide variety of biomedical applications. When they are mixed together, they form polyelectrolytic complexes in different gelated states depending on their concentrations. These complexes are biodegradable. This degradation involves chitosan degradation and gelatin dissolution. However, the films with more content of chitosan have slower degradation rates because of decrease in reagent groups but it is possible to control the degradation rate by varying the gel formulation.

Table 3
Pathologic assessment of adhesion.

Tests	Control	Ch	Ch3/G1	Ch1/G1	Ch1/G3
Number of animals	5	6	6	5	6
Fibrosis Score (Mean ± SEM)	0.6 ± 0.24	1.83 ± 0.3	2 ± 0.45	1.6 ± 0.25	0.5 ± 0.34[a]
Inflammation Score (Mean ± SEM)	0.4 ± 0.25	1.67 ± 0.33	1.5 ± 0.43	1.4 ± 0.25	0.33 ± 0.21[b]

[a] Significantly different from Ch1/G3 and Ch3/G1 groups in the fibrosis score ($P = 0.027$).
[b] Significantly different from Ch1/G3 and Ch groups in the inflammation score ($P = 0.035$).

The abdominal adhesion develops only several hours after the abdominal surgical operations. At first, the serous fluid exudes from the injured sides of intestinal wall, and then fibrinogen in the serous fluid transforms to fibrin and coagulates; thereby membranous peritoneal adhesion in the injured intestinal wall is formed. Fibrinolytic system is activated and the fibrin is absorbed, thereby the membranous peritoneal adhesion is gradually eliminated. If the fibrin cannot be totally absorbed the left fibrin will be organized and develop fibrinous adhesion. Critical period for formation of postoperative adhesion is 3–5 days after surgery. During this post-surgical period, the fibrin layer is reduced through fibrinolysis and the peritoneal membrane either becomes fully re-epithelialised or not. If fibrinolysis does not occur, an irreversible tissue bridge (adhesion) develops and blood vessels and nerve fibers may be form within the following weeks and months. Therefore, candidate films expected not to be degraded completely in this period. Zhou et al. also mentioned that chitosan could only prevent adhesion during pre-fibrinous stage.[24] As a result, the optimal duration of the films to stay in the abdominal cavity is within 2 weeks and samples that remained in abdominal cavity after 2 weeks (Chitosan, Ch3/G1, Ch1/G1) not only are useless in prevention of adhesion but also induce more inflammatory response because of inducing foreign body reactions. In fact, if the postsurgical initial membranous adhesions cannot be degraded in time, it will form irreversible fibrinous adhesions which cannot be inhibited by chitosan. On the contrary, the intra-abdominal residual of undegraded chitosan film can produce a foreign body reaction and result in the fibrous capsule formation, which facilitates the formation of abdominal adhesion.

These were proved in our study: the adhesion grade in groups with more concentration of chitosan (Ch, Ch3/G1, Ch1/G1) was higher than samples in control group, and histopathologic examination indicated obvious foreign body giant cell reaction at 28 days after surgery. These results agree with reported research of Zhang et al.[26] that investigated preventive effects of chitosan on peritoneal adhesion in rats and concluded exacerbating effect of using chitosan in peritoneal adhesion. Kohane and Yeo also reported an unexpected result of using UV-cross-linked chitosan formulation. In their study, rabbits treated with the UV-cross-linked formulation developed exuberant adhesions, even in the absence of prior peritoneal injury. They found that the modified chitosan and the cross-linked gel increased the expression of proinflammatory cytokines or chemokines such as TNF-a and MIP-2 (a murine IL-8

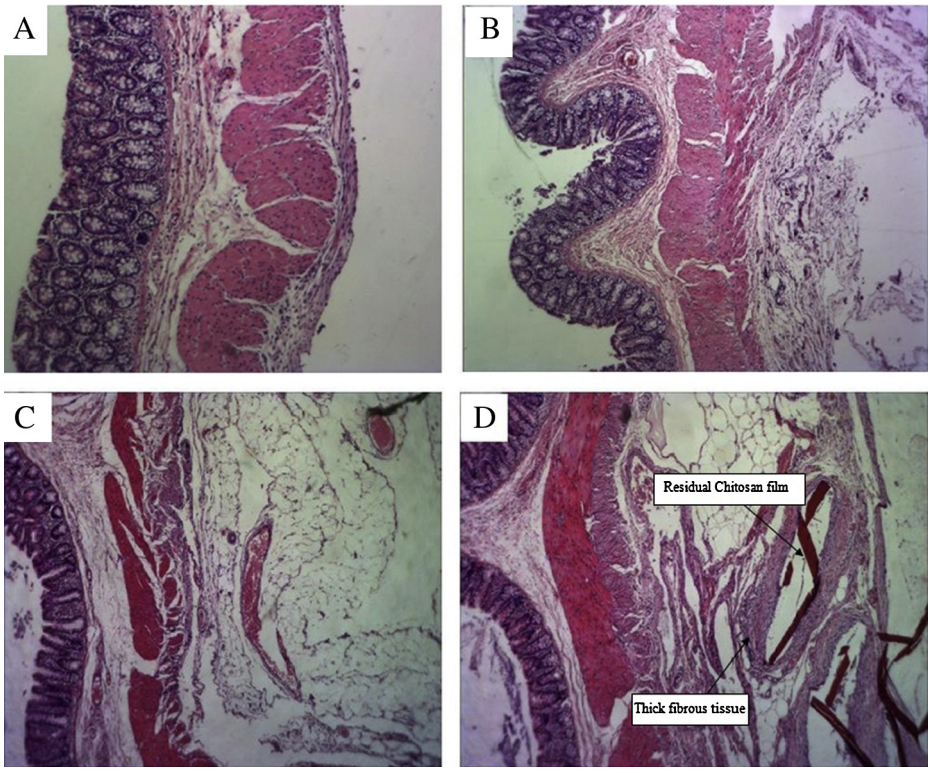

Fig. 3. Microscopic view of cecum. A) Normal cecum. B) Control group, medium adhesion bond can be seen around cecum. C) Ch1/G3 group, light adhesion bond can be seen. D) Ch3/G1 group, severe adhesion bond, residual pieces of chitosan and thick fibrous tissue surround films can be seen (H&E × 40).

analogue) as a cause of adhesion formation.[11] As another potential reason, gelatin is a polypeptide mixture, which is probably antigenic and can cause immunological rejection. This in turn promotes and facilitates the formation of local peritoneal adhesions in Ch1/G3 group and the result was not ideal for using as adhesion barrier. One of our work limitations in blindly grading adhesion was evidence of residual films in tissue on reoperation day.

In conclusion, our results suggest that administration of chitosan–gelatin films with high concentration of gelatin (Ch1/G3) had no significant abdominal adhesion preventive effect compared to control and also films with more than 25% weight of chitosan not only had no effect on reduction of adhesion formation in rat cecum model but also increased inflammatory response and induced more adhesions.

Ethical approval

The article is in accordance with the Guidelines of the Animal Care and use ethics committee of Baqiyatallah university of medical sciences.

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Author contribution

1. Elias Shahram: animal care and film preparation.
2. Seyed Hoomayoon Sadraei: histologic preparation and writing.
3. Gholamreza Kaka: data analysis.
4. Hadi Khoshmohabat: surgery of the animals and study design.
5. Mohammad Hosseinalipour: histopathologic study.
6. Farzad Panahi: surgery of the animals and study design.
7. Mohammad Reza Naimi-Jamal: animal care and film preparation.

Conflict of interest

The authors have no conflicts of interest.

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