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In vivo evaluation of *in situ* polysaccharide based hydrogel for prevention of postoperative adhesion

Weiwei Lou [a], Hualin Zhang [b,1], Jianfeng Ma [a], Dafeng Zhang [a], Chuantong Liu [a], Siqian Wang [a], Zhennan Deng [a], Haihong Xu [a], Jinsong Liu [a,*]

[a] *Department of Prosthodontics, School & Hospital of Stomatology, Wenzhou Medical College, Wenzhou 325027, China*
[b] *College of Stomatology, Ningxia Medical University, Yinchuan 750004, China*

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ABSTRACT

In this paper, the carboxymethyl chitosan/oxidized dextran hydrogel was developed and its potency application in the prevention of postoperative adhesion was investigated. The developed hydrogel showed porous and interconnected interior structure with pore size about 250 μm, which was sensitive to lysozymic solution (1.5 μg/ml) with almost complete degradation after 4 weeks of *in vitro* incubation. *In vivo* study suggested that the developed hydrogel showed the great capacity on the prevention of postoperative adhesions in rat model. According to the result of histopathological examination, it clearly showed that the mesothelial cell layer of abdominal wall and cecum were completely recovered after 7 days of surgery in 3% carboxymethyl chitosan/oxidized dextran hydrogel group, while obvious adhesion between abdominal wall and cecum was observed as treatment with saline solution or 3% carboxymethyl chitosan solution after 1 day of surgery. All these results suggested that the developed biodegradable hydrogel might have potential application in the prevention of postoperative adhesion.

1. Introduction

Postoperative abdominal adhesions were very common, that were the main causes of postoperative morbidity and mortality(Alpay, Saed, & Diamond, 2008; Ward & Panitch, 2011; Yaacobi, Israel, & Goldberg, 1993; Zeng, Yu, You, & Zhang, 2007). It occurred at almost in all abdominal surgery. Although the adhesions could be lysed surgically, they typically recur with equal or even greater severity (Duron, 2007). In past three decades, several strategies including pharmacological approaches, barrier-based approaches were developed to prevent the postoperative abdominal adhesions (Bolgen, Vargel, Korkusuz, Menceloglu, & Piskin, 2007; Liu, Shu, & Prestwich, 2007; Meisel et al., 2011; Wei et al., 2009). For the pharmacological approach, several drugs against fibrinous adhesion were tested to prevent the postoperative abdominal adhesions, but few were entered into clinical trials because of its severe side-effects (Imai, Takagi, Matsunami, & Suzuki, 2010; Tarhan, Barut, & Sezik, 2008). Recently, more and more attention has been oriented to the barrier-based approach. Polymer solution, hydrogels, membranes and the solid-sheet were developed as the barrier-based system to prevent the postoperative abdominal adhesions (Bolgen et al., 2007; Wei et al., 2009). For the polymer solution such as hyaluronic acid (HA), carboxymethyl chitosan (CMC) and etc, it was greatly limited by the short residence time at the site of the administration, yet resulting in failure in the effectiveness (Kennedy, Costain, McAlister, & Lee, 1996; Yeo et al., 2006). Alternative approach is to use the films/membranes that can physically separate the wound tissue during the healing process (Wei et al., 2009; Zhang et al., 2011). But it was very difficult to completely cover the peritoneal wound surfaces during the surgery. Additionally, application of these formulations can be complexed by the difficulties in handling and fixation to tissue, which might also compromise their effectiveness as barrier systems (Alpay et al., 2008).

To overcome these shortcomings, smart hydrogels made from natural/synthetic polymers that were sensitive to temperature, light, ionic strength, etc have been developed as the barrier for prevention of post-operative adhesion (Nguyen & West, 2002; West & Hubbell, 1995; Yang et al., 2012; Zhang et al., 2011). These hydrogels could effectively separate the traumatized peritoneal surfaces during the critical period of adhesion development at 3–5days after surgery. Recently, one such system based on photo-crosslinking hydrogel has been developed as barrier for the application (Matsuda, Se, Ikada, & Iwata 2002). Although this photo-crosslinking hydrogel exhibited the excellent effectiveness against the postoperative adhesion, UV illumination could be rather tedious, and the safety of UV usage in the

* Corresponding author at: School & Hospital of Stomatology, Wenzhou Medical College, 113 Xueyuan Road, Wenzhou, China. Tel.: +86 577 88066088; fax: +86 577 88063088.
E-mail address: jinsliuliu@163.com (J. Liu).
[1] Hualin Zhang was co-first author with Weiwei Lou.

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surgical process remains to be documented. A recent study of hydrogel made from the chemically modified hyaluronic acid and polyethylene glycol demonstrated the effectiveness in the application of prevention of postoperative abdominal adhesions (West & Hubbell, 1995). However, the relatively long gelation time might be impracticable. Therefore, it is extremely necessary to develop a kind of anti-adhesion hydrogel, which was not only effectiveness during the whole healing process and easy to applied via laparoscopic and open surgical procedures, but also had relative shorter gelation time and suitable retention time.

In this paper, a covalently cross-linked polysaccharide based hydrogel was successfully developed and potential application in the prevention of postoperative adhesion was evaluated in a rat model. Dextran and carboxymethyl chitosan have been approved by Food and Drug Administration for various biomedical applications (Jayakumar et al., 2010). Herein, we pre-functionalized dextran with the reactive functional groups (—CHO) to cross-link the carboxymethyl chitosan to develop a covalently cross-linked polysaccharide based hydrogels. The detailed characterizations including micro-structure observation, *in vitro* degradation test were carefully performed before its further *in vivo* applications.

2. Materials and methods

2.1. Materials

Dextran (Mw = 70,000) and carboxymethyl chitosan (the degrees of substitution of carboxymethyl groups on chitosan skeleton were approximately 80%) were purchased from Aladdin-Reagent Co. Ltd. (China). Sodium periodate ($NaIO_4$) and *t*-butyl carbazate were kindly provided by Sigma–Aldrich (USA). Ultrapure water from Milli-Q water system was used to prepare the aqueous solutions.

2.2. Synthesis of oxidized dextran

The oxidized dextran was successfully synthesized by the periodate oxidization method as previous report (Weng, Romanov, Rooney, & Chen, 2008). Briefly, 0.35 g of $NaIO_4$ (pre-dissolved into 50 ml distilled water solution) was added into 100 ml of dextran solution (1%, w/v) for reaction at room temperature for 12 h. Twelve hours later, the equimolar amount of diethylene glycol was added to the reaction system to quench the unreactive $NaIO_4$. After that, the mixture was transferred into a dialysis bag (Molecular cut-off, 3500) for dialysis against distilled water 3 days, followed by lyophilized the resulting solution to obtain the oxidized dextran. The oxidization degree of dextran was detected by measuring the number of aldehydes in these polymers using *t*-butyl carbazate as previous report (Boontheekul, Kong, & Mooney, 2005).

2.3. Preparation of in situ cross linking hydrogel

Solution of 30 mg/ml of oxidized dextran and carboxymethyl chitosan were prepared separately in 10 ml PBS solution (pH = 7.4). To prepare the hydrogel, 0.5 ml of each polymer solution with weight ratio of 1:1, were mixed at room temperature for 2 min to obtain the cross-linked hydrogel. The phase state is defined as "sol" when the mixture could flow freely, and the phase state is regarded as "gel" while the mixture could not flow down when the test tube is inversed.

2.4. Scanning electron microscopy (SEM) observation

The morphological characterization of cross-linked hydrogel was performed by a scanning electron microscopy (JSM-5900LV, JEOL, Japan) at 20 kV. The lyophilized of hydrogels were quenched with liquid nitrogen and cut-off, and the cross-section were sputtered with gold before the observation.

2.5. In vitro degradation study

In vitro degradation behavior of hydrogels was measured by the method as the previous report (Yang et al., 2010). Briefly, 1 ml of hydrogel was pre-prepared in 24-well plate followed by weighting and recording, and then transferred into 15 ml BD tube. Subsequently, 5 ml PBS solution or 1.5 μg/ml lysozyme aqueous solutions were added as the degradation medium for the period of 4 weeks study. The lysozyme solution was refreshed daily to ensure continuous enzyme activity. At predetermined time points (1–4 weeks), the samples were carefully withdrawn from the degradation medium, and the water delayed on the surface of hydrogel was cleared by using the filter paper. The mass of hydrogel was weighted and the degree of *in vitro* degradation was expressed by the weight loss as follows formula:

$$\text{weight loss}\,(\%) = \frac{W_o - W_t}{W_o} \times 100$$

where W_o was the original weight of hydrogels, and W_t was the mass of hydrogels as weighted at specific time *t*.

2.6. In vivo application of hydrogels

Adult male SD rats weighting of 120–150 g were selected for the animal experiment. Animals were cared for in compliance with protocols approved by the Institutional Animal Care and Use Committee of Wenzhou Medical College. The abdominal hair of mouse was removed by using chloral hydrate after anaesthetization. After that, about 5 cm long, midline incision was made along the linea alba on the abdominal wall, and the peritoneum was opened. Post surgical peritoneal adhesions model were developed by the previous report with little modification (Yang et al., 2012). Briefly, the cecum was first identified, and then the ventral side of the cecum was abraded with surgical gauze until an area of 3 cm^2 wound was developed. Meanwhile, on the opposing parietal peritoneal, the defect of abdominal wall was developed by using a scalpel with area of 2 cm × 1.5 cm. Finally, the two injured surfaces were approximated with suture line. Twenty-seven mouse were assigned randomly to three groups, nine mouse for each group: (1) the mouse was treated with 1 ml PBS solution (pH = 7.4); (2) the mouse was treated with 1 ml of carboxymethyl chitosan solution (30 mg/ml) and (3) the mouse was treated with 1 ml of carboxymethyl chitosan/oxidized dextran hydrogels (polymer concentration was 30 mg/ml). According to the report of Phillips and Dudley (1984), the grading standard could be divided into five levels as follows: Grade 0: no adhesions; Grade 1: the ratio of adhesive area/the total treated area in the vermiform processes <20%; Grade 2: the ratio of adhesive area/the total treated area is about 40%; Grade3: the ratio of adhesive area/the total treated area is about 60%; Grade 4: the ratio of adhesive area/the total treated area is ≥60%. On 3rd, 5th and 7th of post-operation, three mice from each group were sacrificed, and the peritoneum was opened and observed.

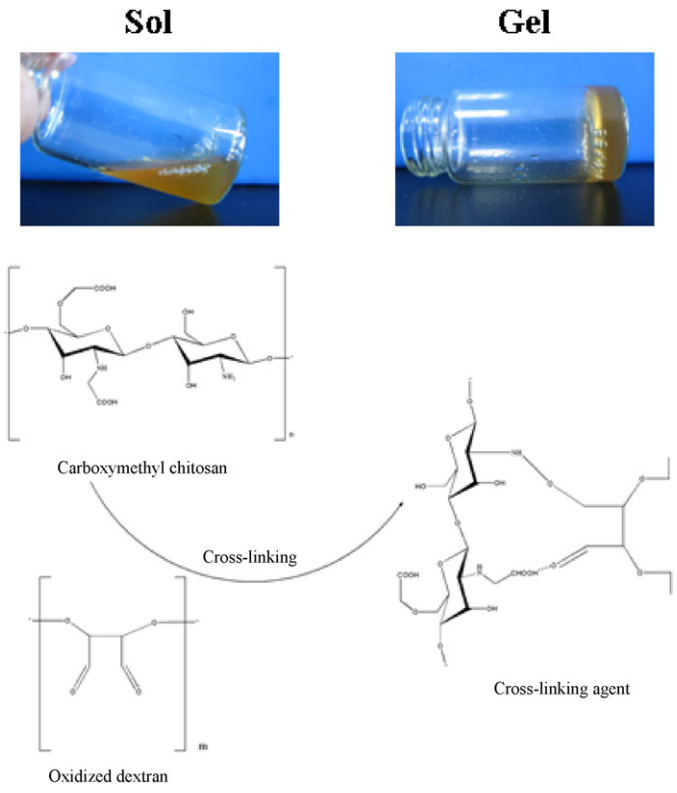

Fig. 1. Sol–gel transition of 3% carboxymethyl chitosan/oxidized dextran solution (weight ratio 1:1) at room temperature.

2.7. Histopathological examination

For the histopathological examination, the tissue from each group at specific time point was collected and fixed immediately in 4% paraformaldehyde in PBS solution (pH = 7.4) for 72 h and embedded into paraffin. Finally, the fixed tissues were sectioned and stained with hematoxylin and eosin (H&E) for microscopic observation.

3. Results

3.1. Synthesis of oxidized dextran

As well known to us, the periodate oxidation could specifically cleave the vicinal glycols in polysaccharide to form their dialdehyde derivatives that could serve as a non-toxic macromolecular cross-linker (Pescosolido et al., 2011). As measured by *t*-butyl carbazate assay, the oxidation of dextran was 20.4%, which could be served as a macromolecular cross-linker suitable for further *in vivo* applications.

3.2. Preparation and characterization of hydrogels

Because of the coexistence of abundant amino groups, hydroxyl groups and carboxylic groups associated with carboxymethyl chitosan and plentiful aldehyde groups as well as hydroxyl groups along the oxidized dextran, the Schiff base and hydrogen bond formation was expected as mixing these two polymer solution. As presented in Fig. 1, it is clearly observed that initially the mixture (polymer concentration 30 mg/ml) was a yellow fluid while turned into a non-flowable yellow hydrogel with the time proceeding.

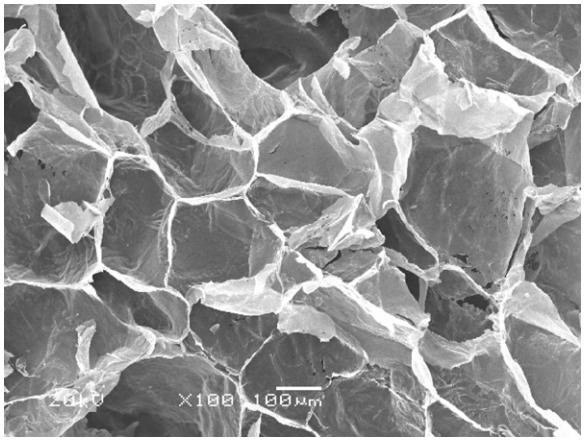

Fig. 2. SEM observation of 3% carboxymethyl chitosan/oxidized dextran hydrogel (weight ratio 1:1).

3.3. SEM observation

Fig. 2 depicts the SEM image of a fractured, lyophilized hydrogel prepared from a 3% carboxymethyl chitosan/oxidized dextran (weight ratio 1:1) solution. As presented in Fig. 2, it clearly observed that the developed hydrogel had a highly porous and interconnected interior structure with an average pore size of approximately 250 μm, which showed no obvious distinction with other chitosan based hydrogel(Weng et al., 2008). Due to its highly porous and interconnected interior structure, we inferred that the encapsulated drugs such as macromolecules could diffuse freely into it.

3.4. In vitro degradation test

In this paper, the comparative *in vitro* degradation test of the effects of PBS solution and 1.5 μg/ml lysozyme aqueous solutions on the degradation behavior of 3% carboxymethyl chitosan/oxidized dextran (weight ratio 1:1) hydrogel was investigated. It is well known that, in human serum, chitosan was mainly depolymerized enzymatically by lysozyme, but not by other enzymes or other depolymerization mechanisms (Muzzarelli, 2010). *In vitro* degradation of chitosan based hydrogel and films

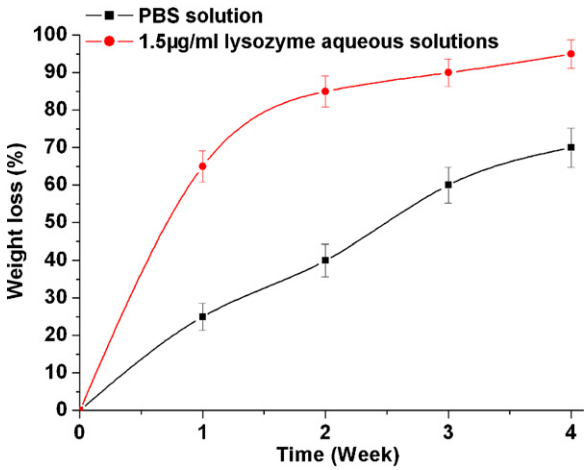

Fig. 3. *In vitro* degradation behavior of 3% carboxymethyl chitosan/oxidized dextran hydrogel (weight ratio 1:1) in PBS solution (pH = 7.4) and 1.5 μg/ml lysozyme aqueous solutions.

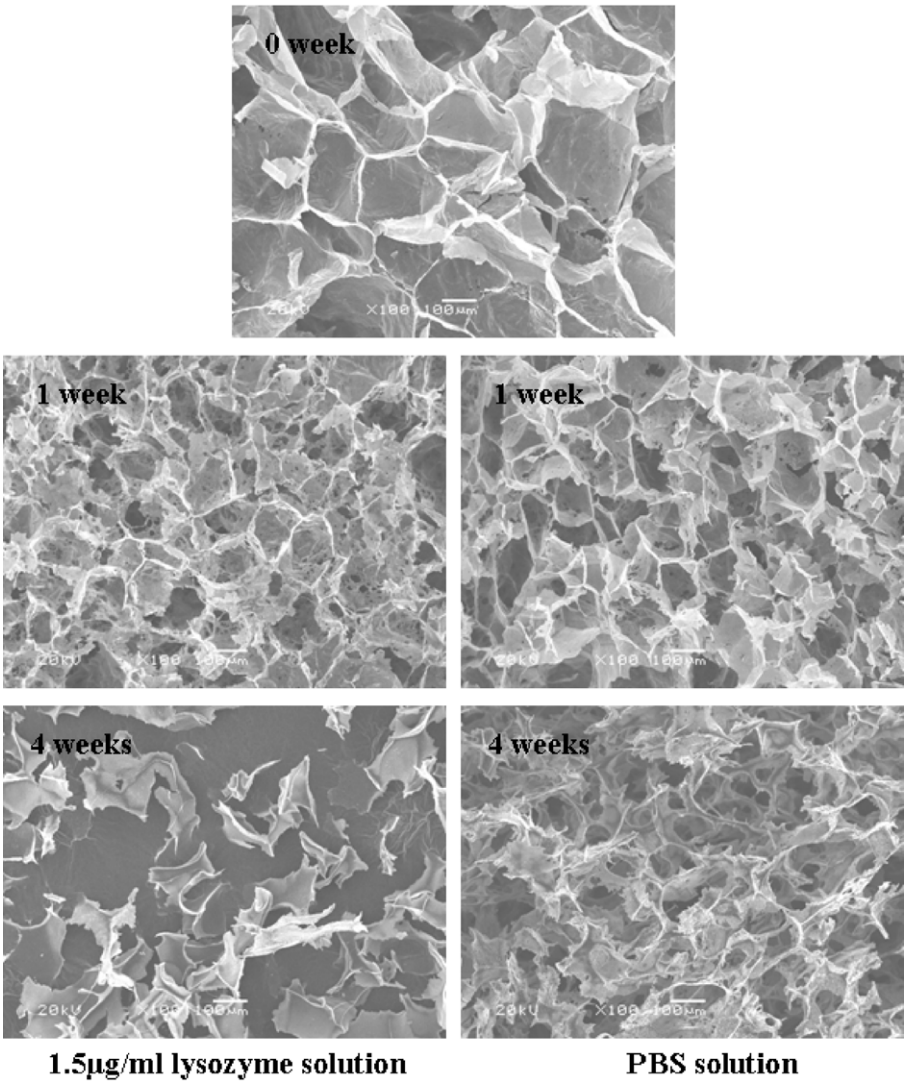

Fig. 4. SEM images of 3% carboxymethyl chitosan/oxidized dextran hydrogel (weight ratio 1:1) exposing in PBS solution (pH = 7.4) and 1.5 μg/ml lysozyme aqueous solutions after 1 week and 4 weeks.

have already been performed by a number of studies. However, most of the studies were performed at accelerate conditions using low pH condition as well as high enzyme concentration solution(Li et al., 2010). As depicted in Fig. 3, it is clearly observed that the hydrogel underwent relatively fast initial degradation in both PBS solution and 1.5 μg/ml lysozyme aqueous solutions at first week. The obvious weight loss of the hydrogel in PBS solution at first week might be induced by the leakage of uncross-linking components (carboxymethyl chitosan and oxidized dextran). However, the weight loss of hydrogel exposing in 1.5 μg/ml lysozyme aqueous solutions (approximately 65% weight loss) was greater than that exposing in PBS solution (only about 25% weight loss), indicating that the developed hydrogel was more susceptible to the lysozyme solution as compared with PBS solution, which was in agreement with the previous study (Weng et al., 2008). Four weeks later, it was clearly observed that the hydrogel exposing in 1.5 μg/ml lysozyme aqueous solutions was almost degraded completely, whereas the hydrogel exposing in PBS solution was still keeping intact with 40% of initial weight. Furthermore, the morphology of hydrogel as a function with time was monitored by a SEM observation. As shown in Fig. 4, we could obviously observe that hydrogel exposing in 1.5 μg/ml lysozyme aqueous solutions showed significant degradation with collapse micro-structure after 1 week of incubation. After 4 weeks of incubation, the micro-structure of hydrogel was complete disaggregation into several fragments. For the morphology of hydrogel exposing in PBS solution, it clearly observed that although the degradation occurred with the time evolution, the intact porous micro-structure of hydrogel still be observed even after 4 weeks of incubation. Therefore, all these results suggested that the developed hydrogel with good biodegradability in enzymic solution might have potential application in biomedical filed.

3.5. *In vivo application of hydrogel*

In this paper, the developed carboxymethyl chitosan/oxidized dextran hydrogel was developed to evaluate the potential application in the prevention of postoperative adhesion. As presented in Table 1, on the 7 days of post-operation, the percentage of non-adhesion from carboxymethyl chitosan/oxidized dextran hydrogel group showed significant differences as compared with that of control group and carboxymethyl chitosan group. ($p < 0.05$). More specifically, all nine animals treated with 1 ml of PBS solution showed a score 4 adhesion. In the case of 3% carboxymethyl chitosan solution group, six animals out of nine animals had

Table 1
Comparison of abdominal adhesion among PBS solution group (control), carboxymethyl chitosan solution group and carboxymethyl chitosan/oxidized dextran hydrogel group.

Group	Control ($n = 9$)	3% Carboxymethyl chitosan solutions ($n = 9$)	3% Carboxymethyl chitosan/oxidized dextran hydrogel ($n = 9$)
Grade 0	0	3	8
Grade 1	0	1	1
Grade 2	0	1	0
Grade 3	0	2	0
Grade 4	9	2	0
Percentage of non-adhesion (%)	0	33.3	88.9

Comparison of peritoneal adhesion between experimental and control groups in groups.

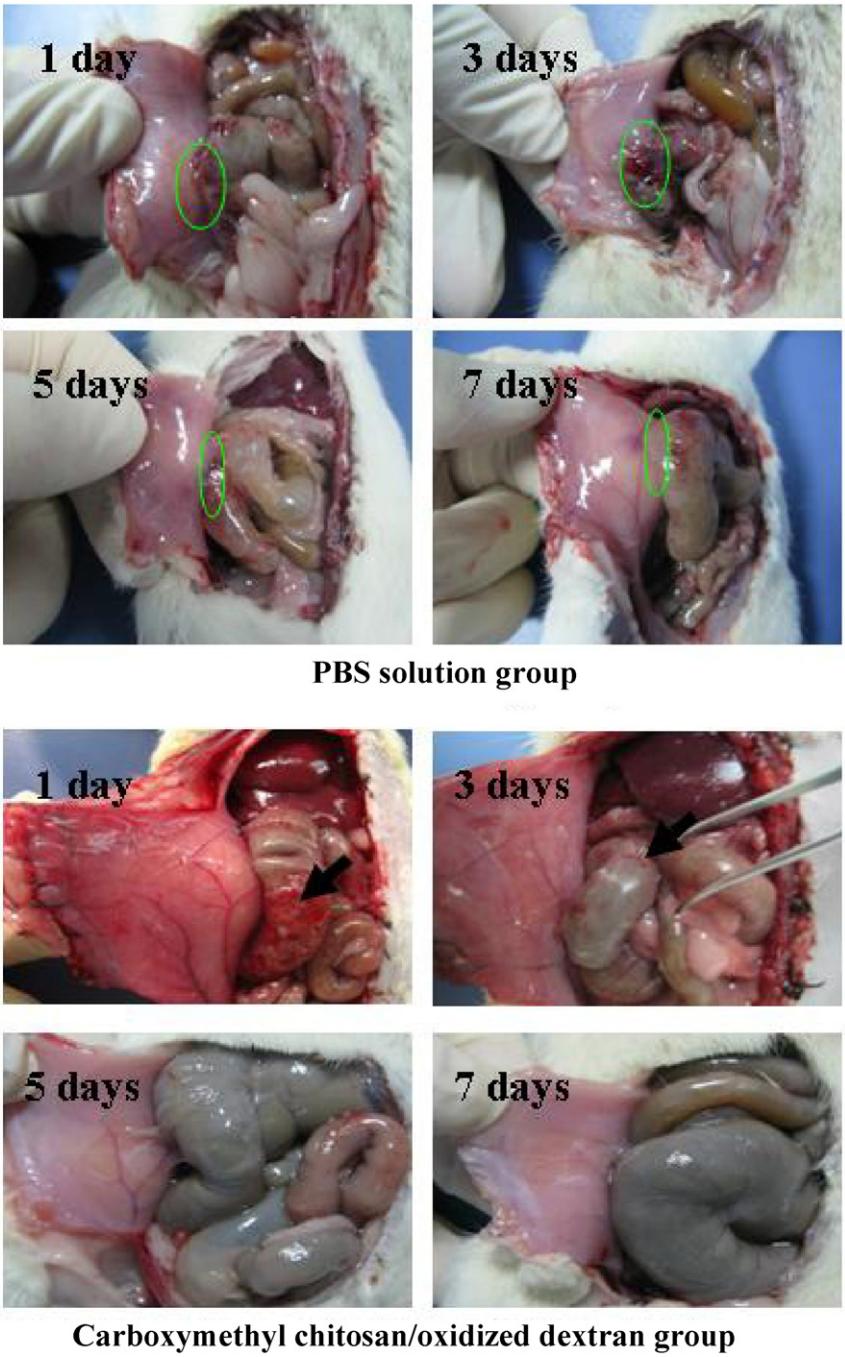

Fig. 5. Photographic observation in PBS solution group and carboxymethyl chitosan/oxidized dextran hydrogel group at different time point (ellipse, adhesion region: arrow, resident hydrogel).

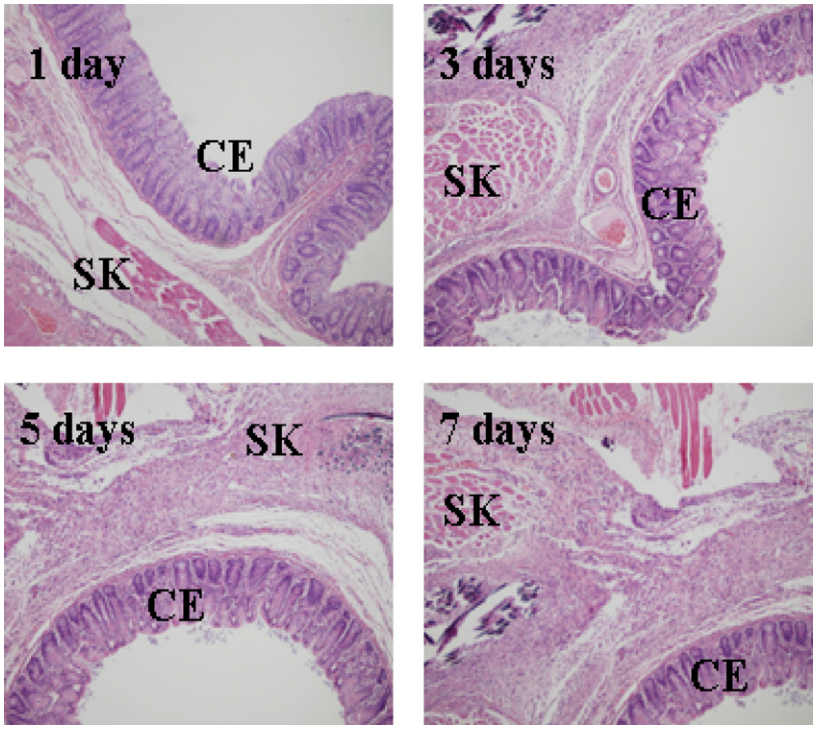

Fig. 6. Histological examination of PBS solution group at different time point (×100). CE: cecum; SK: skeletal muscle.

adhesion (score 2–4), which showed the disappointment effectiveness for prevention of adhesion. However, none of animals except one animal developed into adhesions in 3% carboxymethyl chitosan/oxidized dextran hydrogel, suggesting the effectiveness of developed hydrogel for adhesion prevention. As depicted in Fig. 5, we could observe that the adhesion was occurred in PBS solution group on 1 day after surgery. Meanwhile, a small amount of peritoneal exudate was observed at 1 day after surgery. The adhesion formation was also observed on the 3th day, 5th day and 7th day of post-operation, indicating that the irreversible pathological process of adhesion was occurred. Conversely, in the 3% carboxymethyl chitosan/oxidized dextran hydrogel group, the hydrogel was mixed with punctate hemorrhage, appeared red and adhered to the parietal and visceral wounds as discrete solid-like membranes at 1 day after surgery. Three days later, the hydrogel was gradually degraded/adsorbed from the surface of injured abdominal wall and injured cecum, while the punctuate hemorrhage of injured cecum was disappeared. On 5th day of surgery, it was clearly observed that the injured abdominal wall and cecum was completely recovered with no adhesion formation, suggesting the excellent barrier system for prevention of adhesion formation.

3.6. Histopathological examination

According to the results of light microscopy, tissues taken from the adhesion site in PBS solution group showed the close apposition of the muscular layers of the cecum to the abdominal wall (Fig. 6), with varying thickness of fibroblasts and inflammatory cells at the early stage of wound healing. Conversely, the samples taken from the injury site in 3% carboxymethyl chitosan/oxidized dextran hydrogel groups, it is clearly observed that the mesothelial cell layer of abdominal wall was disappeared at 1 day of surgery. With the time evolution, the mesothelial cell layer of abdominal wall was completely recovered after 7 days of surgery (Fig. 7). The similar results were also observed in the injured cecum tissue (Fig. 7), indicating that application of developed hydrogel could effectively prevent the postoperative adhesion by improving the remesothelialization.

4. Discussion

Peritoneal adhesion is an inevitable result of surgical trauma to the peritoneum. Generally, the formation of peritoneal adhesion needs the following steps: Step1 is formation of fibrinous gelatinous matrix, which happens at about 3 h after the injury. Step 2 occurs between 1 and 5 days after surgery with formation of a large number of fibroblasts and collagen fibers. Step 3 takes place during 5–10 days after injury with the regeneration of mesothelial cells layer (Zhang, Xu, & Zhou, 2006). Therefore, the early intervention of injury wound was very essential for the prevention of progress of peritoneal adhesion. Over past few decades, numerous formulations such as membrane, hydrogel, polymer solution have been developed to prevent the postoperative adhesion. But few were success in the subsequent clinical trials (Kennedy et al., 1996; Matsuda et al., 2002; Ward & Panitch, 2011).

An ideal barrier system for prevention of abdominal adhesions not only be easy to applicable through the laparoscope, but also can provide unrestricted coverage of the affected peritoneum throughout healing. The developed carboxymethyl chitosan/oxidized dextran as a barrier was suitable for the prevention of abdominal adhesions. As we all known that chitosan without any chemical modification can accelerate the healing progress, ant its main mechanism involved in prohibition of fibroblast proliferation (Yang et al., 2010; Yeo et al., 2006). Furthermore, previous study has been demonstrated that carboxymethyl chitosan was an effective anti-adhesion agent than hyaluronic acid at inhibition adhesion formation in rat model (Kennedy et al., 1996). Therefore, the developed hydrogel composed of carboxymethyl chitosan and oxidized dextran might be a desirable formulation for prevention of abdominal adhesion compared with that of native carboxymethyl chitosan

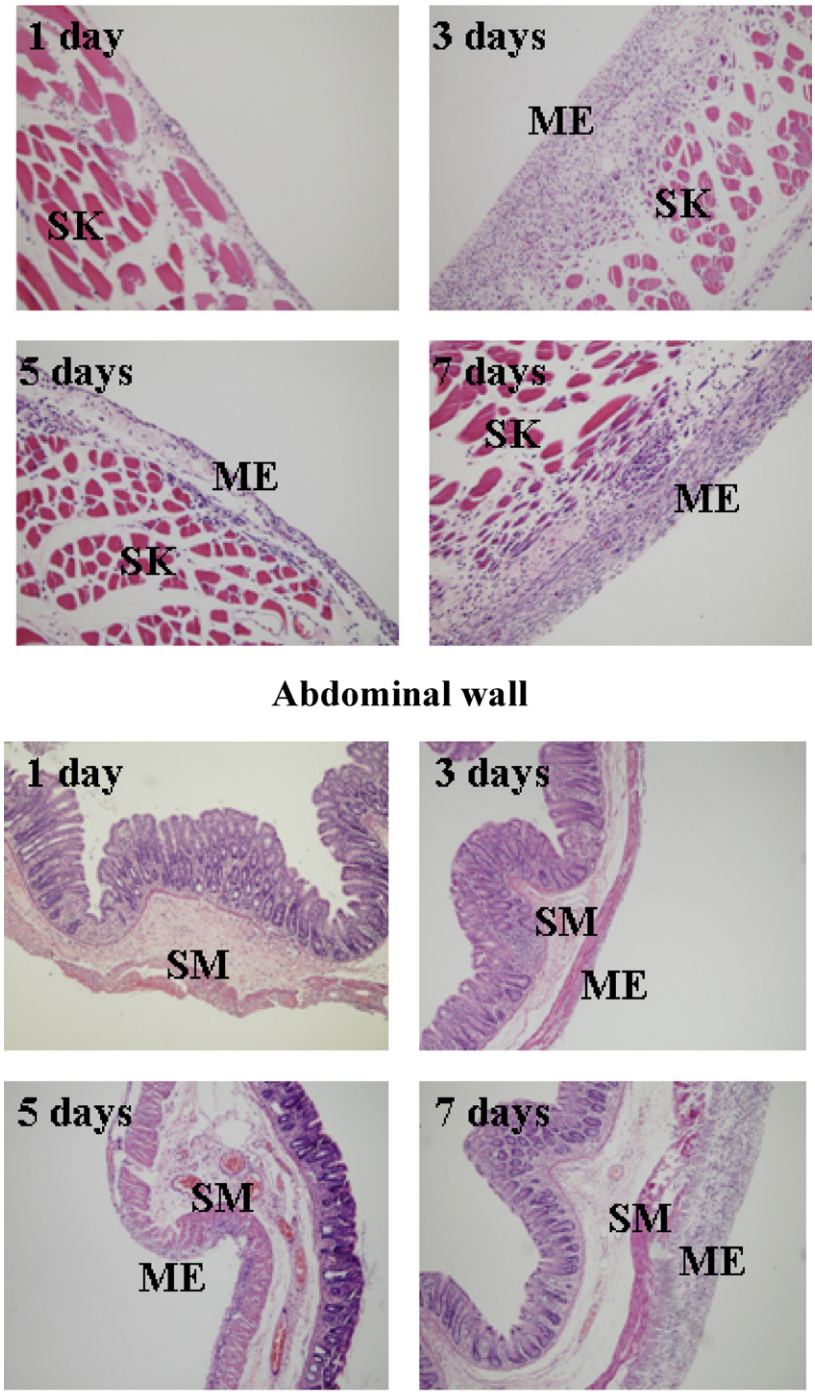

Fig. 7. Histological examination of carboxymethyl chitosan/oxidized dextran hydrogel group at different time point (×100). CE, cecum; SK, skeletal muscle; ME, mesothelial cell layer.

solution. First, the developed hydrogel can be easily applied via a needle and adhere to the injured site without any additional instrument such as UV illumination, which was suitable for laparoscopic applications. Secondly, the hydrogel could be degraded or adsorbed from the abdominal cavity in a short time after the remesothelialization, which would not effect on the healing process. Finally, although the punctuate hemorrhage occurred after the surgery, the capability of the hydrogel to prevent the abdominal adhesion was not hampered.

5. Conclusion

In this paper, a covalently cross-linked polysaccharide based hydrogel (carboxymethyl chitosan/oxidized dextran hydrogel) was successfully developed by a simple mixing carboxymethyl chitosan and oxidized dextran solution without using any other cross-linkers. The developed hydrogel showed porous and interconnected interior structure with pore size about 250 μm was suitable for application in the peritoneal cavity. Meanwhile, the developed hydrogel was sensitive to lysozyme solution with almost

complete degradation after 4 weeks of incubation *in vitro* rending it as a promising anti-adhesion agent. *In vivo* application of hydrogel suggested that the developed hydrogel showed the great capacity on the prevention of postoperative adhesions as compared with that of saline solution or viscous carboxymethyl chitosan solution by promoting the re-mesothelialization of abdominal wall and cecum. Therefore, the biodegradable, *in situ* injectable carboxymethyl chitosan/oxidized dextran hydrogel might have great application in the prevention of postoperative adhesion.

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