International Journal of Surgery 10 (2012) 537–541

Contents lists available at SciVerse ScienceDirect

International Journal of Surgery

journal homepage: www.theijs.com

Original research

Macroscopic and pathological assessment of methylene blue and normal saline on postoperative adhesion formation in a rat cecum model

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A R T I C L E I N F O

Article history:
Received 2 February 2012
Received in revised form
15 August 2012
Accepted 18 August 2012
Available online 27 August 2012

Keywords:
Intra-abdominal adhesion
Methylene blue
Cecum
Postoperative adhesions

A B S T R A C T

Background: Adhesion formation after abdominal surgery is a major cause of postoperative bowel obstruction, infertility, and chronic abdominal pain. In this study, we evaluated the effect of normal saline and methylene blue (MB) on postoperative adhesion formation in a rat cecum model.
Methods: A total of 30 Wistar female rats in 2 treatment and 1 control groups underwent midline laparotomy and standardized abrasion of the visceral peritoneum. Normal saline and methylene blue were administrated intraperitoneally at the end of the surgical procedure in 2 treatment groups. Fourteen days after surgery, a re-laparotomy was performed for macroscopic and pathological assessment.
Results: The adhesion grade and extent of the normal saline group was lower than control and MB groups in macroscopic assessment ($P < 0.05$ for both). A comparison of adhesion stages in pathological assessment showed increment in abdominal adhesion by usage methylene blue 1% and demonstrated significant difference between MB and 2 other groups ($P < 0.05$).
Conclusions: Administrated normal saline individually reduce the adhesion grade near cecum. Conversely, usage of methylene blue 1% may unpredictably increase risk of adhesion formation.

1. Introduction

Postoperative abdominal adhesions are bands of tissue that form between structures in the abdomen following surgery, trauma, infection and other harmful events.[1] Postoperative adhesions remain a significant complication of abdominal surgery and can result in pain,[2] infertility[3] and potentially small bowel obstruction.[1] Therefore, preventive measures against adhesion formation are of considerable clinical importance. Physical separation for adhesion prophylaxis can potentially be achieved by using solid or mechanical barriers or by intraperitoneal solutions. When the peritoneum is traumatized, the site of the trauma becomes inflamed and a fibrin matrix that temporarily connects raw injured surfaces is formed. Inflammation is characteristic and is indeed part of the healing process. Fibrin restores tissue integrity and serves as a scaffold into which reparative cells migrate and exert their functions. Fibrinous adhesions are normally reversible, but the ultimate removal and resolution of the deposit is necessary for adhesion-free healing in the peritoneal cavity. This physiological removal of fibrin is accomplished by the fibrinolytic system.[4] Consequently, the fibrinolytic activity in the peritoneum normally degrades fibrin and peritoneal regeneration can take place but prolonged local failure of peritoneal fibrinolysis is a unifying pathogenetic mechanism in permanent adhesion formation caused by several types of peritoneal injury.[4] Intraoperative lavage is strongly established in surgical practice to optimize the cleansing of the abdominal cavity, as it facilitates aspiration of contaminants and reduces the concentration of bacteria and other substances.[5] Normal saline is commonly used for this purpose. Peritoneal administration of normal saline was mostly served as control in previous investigations. Several studies found no influence of normal saline on peritoneal adhesion formation.[6–9] However, an adhesion preventive effect has also been reported [10–14]. On the other hand, methylene blue (MB), a low molecular weight, partially liposoluble vital dye, has been proposed as a new therapeutic option in the reduction of surgery-induced peritoneal adhesions by Galili et al.[15] They found that MB has preventing effect on formation of peritoneal adhesions in contrast to the study of Prien et al.,[16] which documented increased adhesions with MB. Rasa showed low dose of MB caused inhibition of postoperative adhesion

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http://dx.doi.org/10.1016/j.ijsu.2012.08.009

formation, however, with a higher dose, this effect disappeared and even MB actually induced adhesion formation at the highest dose.[17] Heydrick also showed that MB (30 mg/kg) has no effect on adhesion formation at 24 h after surgery, but MB could inhibit adhesion formation by increasing peritoneal fibrinolytic activity after 7 days.[18] On the other hand, some investigations showed positive effect of MB in reduction of intra-abdominal adhesion after surgery,[19,20] however, data on the effectiveness of peritoneal lavage with MB are inconclusive. Hence in this study, we evaluated the effect of normal saline and MB 1% on postoperative adhesion formation in a rat cecum model.

2. Materials and methods

2.1. 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 ethics committee of Baqiyatallah University. Animals were obtained from the animal breeding unit of Baqiyatallah Medical University and the guiding principles in the care and use of laboratory animals were strictly adhered throughout the entire study. Thirty female adult Wistar rats weighting 200–250 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. Animals were randomly divided into three equal groups (10 rats in each group): Control group, methylene blue (MB) group and normal saline group.

2.2. Surgical procedure

Rats were anesthetized with 90 mg/kg ketamine hydrochloride and 8 mg/kg xylazine hydrochloride intramuscularly. The skin of each rat abdomen was shaved and disinfected with 1% of antiseptic povidine–iodine. All surgical procedures were carried out under sterile conditions. Following a 3-cm midline incision, antimesenteric border of cecum was abraded with dry sterile gauze until punctate bleeding occurred. Rubbing 5 times typically provided punctate bleeding. One milliliter of normal saline (0.9% NaCl) and 1 ml of methylene blue (M9140, Sigma–Aldrich) at concentration of 1% were administered intraperitoneally into the abdominal cavity of animals around cecum in the normal saline and MB groups. No medication was administered to animals in control group, only cecums were exposed to air for 5 min. Abdominal wall and skin 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 14th postoperative day, when they were killed by an overdose of ether.

2.3. Macroscopic assessment

The abdominal cavity was inspected through a straight incision and adhesions were identified, counted, and graded using the classification described by Bhatia et al.,[12] with some modifications, by one surgeon, who was unaware of to which group the rats belonged (Table 1).

The extent of adhesion formation was quantified by an innovative method. For this purpose the abdominal area was divided into 8 sections through 4 hypothetical lines, 1 vertical through medial line and 3 horizontal lines. Each section had 12.5% of abdominal area. An adhesion score of 100% could thus be achieved maximally if all the abdominal area had adhesion. The extent of adhesions was documented by comparing them by diagram shown in Fig. 1.

Table 1
Adhesion score.

Definition	Adhesion score
No adhesions	Grade 0
The ratio of adhesive area/total treated area in the vermiform processes is <50%, and the adhesion is easily to be dissected.	Grade 1
The ratio is ≥50% and the adhesion is easily to be dissected.	Grade 2
Area of the adhesion is out of consideration. Although blunt dissection for the adhesion can be carried out, it is difficult and the intestinal wall will be impaired after the blunt dissection.	Grade 3
Area of the adhesion is out of consideration. The adhesion is fast and cannot be bluntly dissected. Also may have adhesion to other organs (liver).	Grade 4

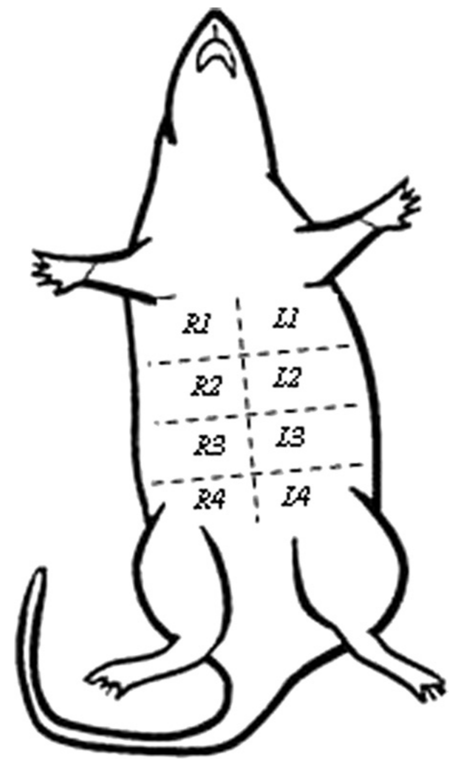

Fig. 1. Hypothetic diagram of abdominal area.

2.4. Pathological assessment

For pathological evaluations samples of adhesive bands were removed, fixed in 10% neutral formalin for 24 h, and were then dehydrated, cleared and embedded in paraffin wax. Paraffin sections were cut at a thickness of 5 mm and stained with hematoxylin and eosin (H&E). Histomorphological findings were assessed with respect to the severity of interstitial fibrosis (IF) and inflammatory cell reaction (ICR) by using the classification described by Mahdy et al.,[20] with some modifications. The extent of ICR was evaluated and 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.

2.5. 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. *P*-value ≤0.05 was considered as significant.

3. Results

3.1. Macroscopic assessment

Most animals survived in the experiment and reached the endpoint of observation in an apparently healthy condition except for four animals in which two rats from control group (died on the 1st and 6th postoperative day) and two rats from MB group (died on the 3th and 8th postoperative day). The induced adhesions were well-formed, filamentous and broad which existed between peritoneum and adjacent intestine. Fig. 2 shows macroscopic scores of adhesion bond around the cecum. In control group, there were five rats with grade 1 of adhesion and another three rats with grade 2 of adhesions. In normal saline group, eight rats has no intra-abdominal adhesion and only one rat with grade 1 and another one with grade 2 of adhesion. In MB group, grades 1, 3 and 4 of adhesion were present in four, one and one animals, respectively and two rats were adhesion-free.

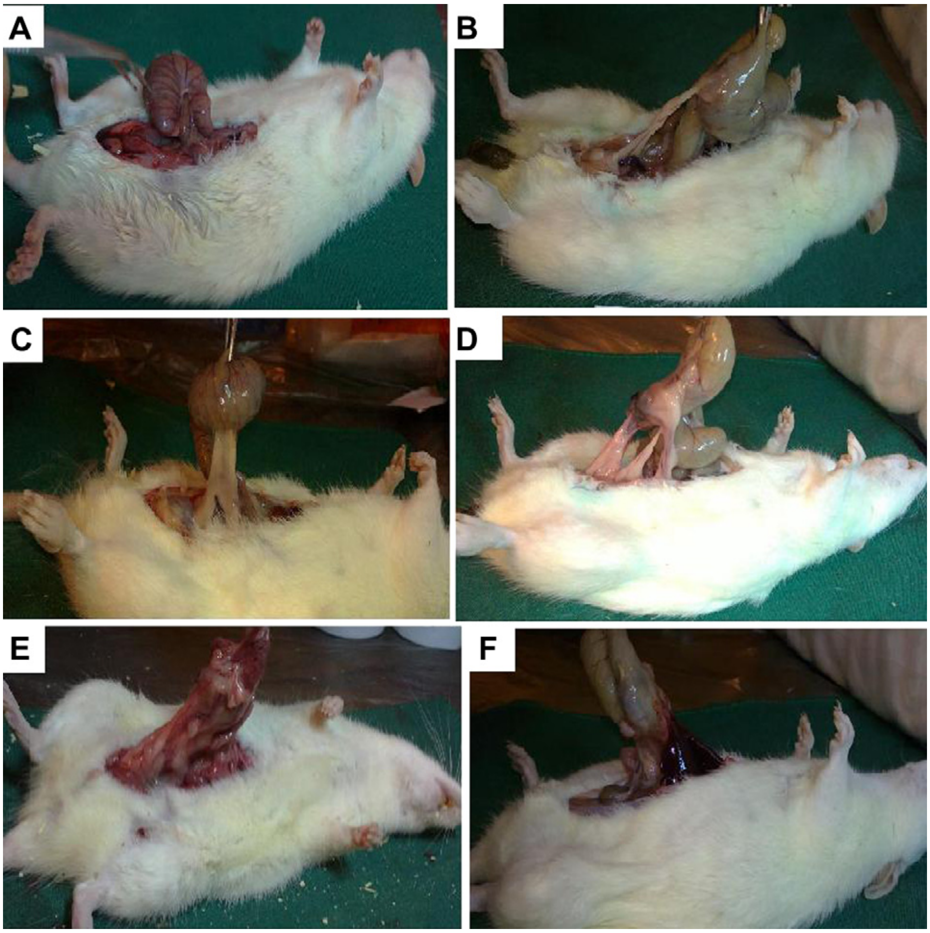

Fig. 2. Macroscopic scores of adhesion bonds around the cecum. (A) No adhesion bond (grade 0). (B) Adhesion bond is appeared but is too short and is easily to be dissected bluntly (grade 1). (C) The ratio of adhesive area/total treated area is more than 50% (grade 2). (D) Area of the adhesion is out of consideration and the intestinal wall will be impaired after the blunt dissection (grade 3). (E) The adhesion is dense and have tightly adherent to intestine and cannot be dissected (grade 4). (F) Adhesion between cecum and other organs (liver).

All macroscopic assessments are shown in Fig. 3. Mean of adhesion scores in normal saline group (0.3 ± 0.21) was significantly lower than control (1.38 ± 0.18) and MB (1.38 ± 0.50) groups ($P < 0.05$). Also mean percentage of adhesion extents in normal saline (3.75 ± 2.67) was significantly lower than control (15.63 ± 2.04) and MB (15.63 ± 5.15) groups ($P < 0.05$). In addition, in all groups, most adhesion area was observed in L4 because cecum is located at the same area and most adhesion bonds created around the cecum.

3.2. Pathologic assessment

Histopathologic findings of adhesion area in all groups are shown in Table 2. MB group showed the highest scores of fibrosis and inflammation. These results differed significantly among the control and MB groups ($P = 0.00005$ in fibrosis score and $P = 0.0002$ in inflammation score) and also between normal saline and MB groups ($P = 0.0001$ in fibrosis score and $P = 0.0002$ in inflammation score). It shows that lavage of MB group had an increasing effect on adhesion formation and caused severe adhesion bonds 14 days after surgery. It seems that normal saline had a slight positive effect on inhibition of postoperative adhesion formation. The results of pathologic finding including fibrosis and inflammation in all groups are shown in Figs. 4 and 5.

4. Discussion

It has been shown that administration of adjuvant promoted to prevent the peritoneal adhesion formation in animal models. The

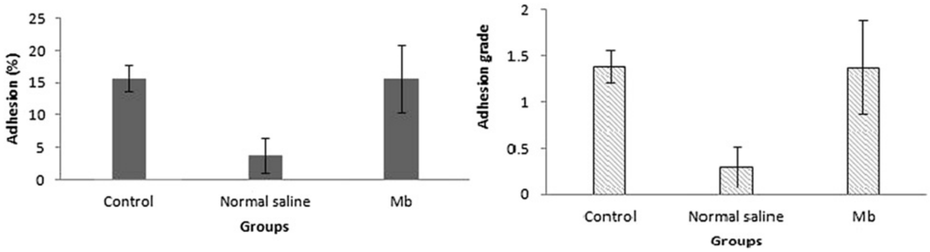

Fig. 3. Adhesion grade and extent (%). Normal saline has lower scores in adhesion grade and adhesion extent ($P < 0.05$) than two other groups. All values are mean ± SEM.

Table 2
Pathologic assessment of adhesion.

Tests	Control	Normal saline (NS)	Methylene blue (MB)
Number of animals	8	10	8
Fibrosis score (mean ± SEM)	0.5 ± 0.19	0.1 ± 0.1	2.25 ± 0.16[a]
Inflammation score (mean ± SEM)	0.38 ± 0.18	0.1 ± 0.1	2.13 ± 0.35[b]

[a] Significantly different from control and normal saline groups in the fibrosis score ($P < 0.0001$).
[b] Significantly different from control and normal saline groups in the inflammation score ($P < 0.0001$).

present study demonstrated that administration of normal saline in abdominal cavity of rats resulted a significant reduction of peritoneal adhesion formation compared to usage of MB 1%. Our results agree with some literatures that reported a beneficial effect of saline in preventing postoperative adhesion formation.[13,14] Larsson et al. showed that intraperitoneal instillation of both 32% dextran 70 and normal saline in women undergoing tubal surgery equally reduced the extent of the intra-abdominal adhesions.[14] Chen et al. also showed that peritoneal infusion of cold saline (4 °C) might decrease postoperative intra-abdominal adhesion formation in mouse model.[21] Otherwise, our findings are in conflict with earlier studies that reported effective usage of MB 1% on abdominal adhesion reduction.

The influence of saline on reduction of abdominal adhesion may be explained by four probable mechanisms: (1) lowering the degree of inflammation, (2) removal of inflammatory mediators that promote fibrin production, (3) separation of small bowel loops, and (4) removal of fibrin from serosal surfaces and subsequently decrease the adhesion formation.[21] It is well known that peritoneum has an inherent fibrinolytic activity similar to that found in vascular endothelium.[22] In adhesion-free peritoneal healing: there is a balance between fibrinogenesis and fibrinolysis. If fibrin exudates overwhelm fibrinolytic activity, organization leading to adhesion rather than resolution of the fibrin–cellular matrix occurs.[23] The use of irrigation with crystalloid solutions either during open or laparoscopic surgery is a popular strategy that is used for adhesion reduction in abdominal surgery. Another widely used method is the instillation of 300–500 mL of crystalloid solution into the abdominal cavity at the end of surgery to allow for “flotation” of the abdominal organs.[4] Otherwise, some researchers showed no influence of normal saline on reduction of abdominal adhesion.[24] They indicated that absorption of water and electrolytes from the peritoneal cavity is rapid and up to 500 mL of isoosmolar sodium chloride absorbed in less than 24 h[24] and because it takes 5–8 days for peritoneal surfaces to form intact mesothelium, a crystalloid solution would be absorbed well before the processes of fibrin deposition and adhesion formation are complete.[25] Accordingly, intraperitoneal crystalloid instillation is not expected to prevent adhesion formation by hydroflotation.

It is interesting to note that as demonstrated previously, administration of normal saline in abdominal cavity may prevent the formation of fibrinous adhesions by modification of fibrinolytic activation. It means by dilution of the PAI-1 in the peritoneal fluid, where fibrinolysis is taking place, the tPA activity is increased. On the other hand, removal of the fibrinous exudates with normal saline instillation may contribute to reduced adhesion formation. Tarhan et al. also reported higher tPA activity after instillation of normal saline in abdominal cavity of rats after 10 days.[4] In fact, although absorption of normal saline in abdominal cavity is fast but it has enough time for dilution PAI-1. MB as another study group had increasing effect on adhesion formation after 14 days. Our results showed that MB 1% not only had no effect in reduction of adhesion formation but also this solution surprisingly increased fibrosis and inflammation near cecum. The inflammatory scores of MB groups showed significantly higher than control and normal saline groups. These results have contrast with published research of Heydrick that injected the same amount of MB (30 mg/kg, in 1 mL of sterile water) into peritoneal cavity of male Wistar rats and sacrificed those after 7 days and reported MB act as antioxidant and may reduce adhesion formation by enhancing peritoneal fibrinolytic activity following surgery.[18] On the other hand, this investigation has confirmation with some other studies. Rasa and his group selected three doses of MB 1 mg/kg, 5 mg/kg and 9 mg/kg for administration in abdominal cavity after cecal serosal abrasions of 75 male Wistar–Albino rats and after 14 days concluded that low dose MB (1 mg/kg) caused inhibition of adhesion formation, but with a higher dose (5 mg/kg), this effect disappeared and MB actually induced adhesion formation at the highest dose (9 mg/kg).[17]

MB, as a nontoxic and safe dye has both antioxidant and prooxidant properties. MB is known to have additional pharmacological actions, including the generation of oxygen radicals, direct inhibition of nitric oxide synthesis (NOS), inhibition of potassium channels, and other nonspecific actions.[19]

Our findings showed that methylene blue not only did not affect the reduction of adhesion formation but also promote the formation and maturation of nascent adhesions. These results in conflict with earlier study by Heydrick who reported methylene blue inhibits adhesion formation via a mechanism that may involve blocking an oxidative stress-dependent decrease in peritoneal fibrinolytic activity.[18] It seems neutrophils and macrophages observed in methylene blue samples of our study secrete inflammatory cytokines and release reactive oxygen species (ROS), which promote adhesion formation. Methylene blue is also absorbed through the peritoneal lining that effects on other organs for inducing adhesion may be expected.

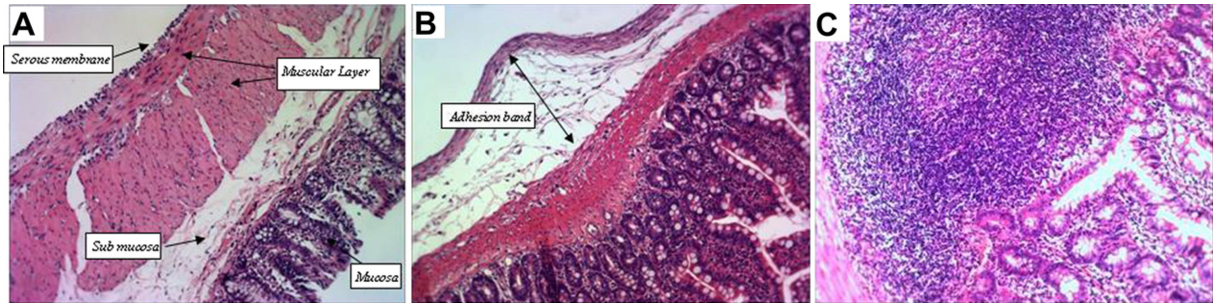

Fig. 4. Photomicrographs of the fibrosis. (A) Normal saline group, normal intestine with no adhesion (grade: Normal). (B) Control group, mild adhesion bonds around intestine (grade: Mild). (C) MB group, fibrous tissue mass with engorged blood vessels (grade: Severe) (H&E ×100).

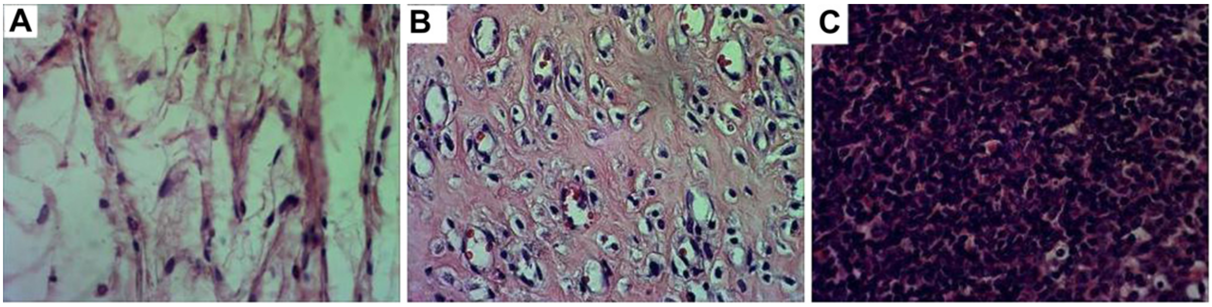

Fig. 5. Photomicrograph of the inflammatory cell infiltration after 2 weeks. (A) Normal saline group, loose collagen fibers and small number of inflammatory cells can be seen (grade: Mild). (B) Control group, red blood cells, neutrophils and fibroblasts with thick collagen fibers sounded them can be seen (grade: Moderate). (C) MB group, activated macrophages and the aggregation of white blood cells and fibroblast can be seen (grade: Severe) (H&E ×400).

One of our limitations in blindly grading adhesion was evidence of blue color found in buttons harvested after 14 days of surgery in MB animals. However, MB is readily transported into the cell and existence of blue coloration in abdominal cavity was insignificant. Also transportation of MB into the cell can be observed by dark mark of microphotographs on MB samples (Fig. 5B).

In conclusion, our results suggest that normal saline administration could reduce postoperative abdominal adhesion, whereas MB 1% increased adhesion formation in abdominal cavity.

ISRCTN

Not applicable.

Ethical approval

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

Sources of funding

None declared.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgment

This study was partly supported by Iran National Science Foundation (INSF).

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