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

Farzad Panahi, Seyed Homayoon Sadraie, Hadi Khoshmohabat, Elias Shahram, Gholamreza Kaka, Mohammad Hosseinalipour

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: Administered normal saline individually reduce the adhesion grade near cecum. Conversely, usage of methylene blue 1% may unpredictably increase risk of adhesion formation.

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1. Introduction

Postoperative abdominal adhesions are bands of tissue that form between structures in the abdomen following surgery, trauma, infection and other harmful events. Postoperative adhesions remain a significant complication of abdominal surgery and can result in pain, infertility and potentially small bowel obstruction. 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. 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. 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. 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. However, an adhesion preventive effect has also been reported. 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. They found that MB has preventing effect on formation of peritoneal adhesions in contrast to the study of Prien et al., which documented increased adhesions with MB. Rasa showed low dose of MB caused inhibition of postoperative adhesion formation.
formation, however, with a higher dose, this effect disappeared and even MB actually induced adhesion formation at the highest dose.\textsuperscript{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.\textsuperscript{19,20} On the other hand, some investigations showed positive effect of MB in reduction of intra-abdominal adhesion after surgery,\textsuperscript{9,20} 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 povidone–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. The cavity was 5 min, so that the duration of exposure of intestines to air was the same for 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.\textsuperscript{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>
<thead>
<tr>
<th>Grade</th>
<th>Adhesion score</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>No adhesions</td>
</tr>
<tr>
<td>1</td>
<td>The ratio of adhesive area/total treated area in the</td>
</tr>
<tr>
<td></td>
<td>vermiform processes is &lt;50%, and the adhesion is</td>
</tr>
<tr>
<td></td>
<td>easily to be dissected.</td>
</tr>
<tr>
<td>2</td>
<td>The ratio is ≥50% and the adhesion is easily to be</td>
</tr>
<tr>
<td></td>
<td>dissected.</td>
</tr>
<tr>
<td>3</td>
<td>Area of the adhesion is out of consideration.</td>
</tr>
<tr>
<td>4</td>
<td>The adhesion is fast and cannot be bluntly dissected.</td>
</tr>
<tr>
<td></td>
<td>Also may have adhesion to other organs (liver).</td>
</tr>
</tbody>
</table>

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.\textsuperscript{21} 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 3rd 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 had 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.
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
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.\textsuperscript{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.\textsuperscript{14} Chen et al. also showed that peritoneal infusion of cold saline (4°C) might decrease postoperative intra-abdominal adhesion formation in mouse model.\textsuperscript{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.\textsuperscript{21} It is well known that peritoneum has an inherent fibrinolytic activity similar to that found in vascular endothelium.\textsuperscript{22} In adhesion-free peritoneal healing: there is a balance between fibrinogenesis and fibrinolysis. If fibrin exudates overwhelm fibrinolytic activity, organization leading to adhesion formation. Methylene blue is also absorbed through the peritoneal lining that effects on other organs for promoting adhesion formation. MB, as a nontoxic and safe dye has both antioxidant and pro-oxidant properties. MB is known to have additional pharmacological actions.\textsuperscript{19} According to 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.\textsuperscript{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).\textsuperscript{17}

MB, as a nontoxic and safe dye has both antioxidant and pro-oxidant 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.\textsuperscript{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.\textsuperscript{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.

### Table 2

<table>
<thead>
<tr>
<th>Tests</th>
<th>Normal saline (NS)</th>
<th>Methylene blue (MB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Fibrosis score</td>
<td>0.5 ± 0.19</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>(mean ± SEM)</td>
<td>2.25 ± 0.16\textsuperscript{a}</td>
<td>2.13 ± 0.35\textsuperscript{b}</td>
</tr>
<tr>
<td>Inflammation score</td>
<td>0.38 ± 0.18</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>(mean ± SEM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Significantly different from control and normal saline groups in the fibrosis score ($P < 0.0001$).

\textsuperscript{b}Significantly different from control and normal saline groups in the inflammation score ($P < 0.0001$).
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**

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**References**