The comparison of methylene blue and vitamin E in prevention of abdominal postoperative adhesion formation in rat uterine horn models. Biochemical and histopathologic evaluation[1]

Comparação do azul de metileno e vitamina E na prevenção de aderência abdominal pós-operatória em corno uterino de ratos. Avalição bioquímica e histopatológica

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ABSTRACT
Purpose: To compare the effects of vitamin E and 1% methylen blue solutions on prevention of experimentally induced adhesions in rats. **Methods**: Thirty seven female Spraque Dawley rats were randomized into four groups. First group was kept as sham operated group. An adhesion model was constituted on the left uterine horn of the other groups. The lesion areas of rats from the second, the third and the fourth groups were coated with 2 ml 0.9 % saline solution (C group), 10 mg vitamin E (VE group) and 1% methylen blue solutions (MB group), respectively. **Results**: Histopathologically, adhesion scores, mononuclear cell infiltration, oedema and fibrosis were more prominent in the MB group compared with C and VE groups. There were no significant differences between the groups in tissue glutathione peroxidase (GPx), catalase (CAT) activities and glutation (GSH) level, these parameters were slightly increased in group with VE supplementation though. The administration of VE and MB significantly decreased NO ($P<0.01$) levels when compared to the C group. The level of malondialdehyde (MDA) in the VE group was significantly lower ($P<0.05$) than those of the Sh and C groups. **Conclusion**: Intraperitoneal methylen blue solutions treatments were more effective according to vitamin E in preventing the formation of intra-abdominal adhesion in a rat uterine horn model.
Key words: Vitamin E. Methylene Blue. Tissue Adhesions. Uterus. Rats.

RESUMO
Objetivo: Comparar os efeitos da vitamina E e 1% da solução de azul de metileno na prevenção de aderências induzidas em ratos. **Métodos**: Trinta e sete ratos fêmeas Spraque Dawley foram distribuídos em quatro grupos. O primeiro grupo foi mantido como grupo *sham*. O modelo de aderência foi realizado no corno uterino esquerdo nos outros grupos. As áreas da lesão dos ratos do segundo, terceiro e quarto grupos foram revestidas com 2 ml de solução salina 0,9% (Grupo C), 10 mg de vitamina E (Grupo VE) e solução de azul de metileno 1% (Grupo MB), respectivamente. **Resultados**: Histopatologicamente, o escore das aderências, infiltração celular mononuclear, edema e fibrose foram mais proeminentes no grupo MB em comparação aos grupos C e VE. Não houve diferença significante entre os grupos na peroxidase da glutatione do tecido (GPx), atividade da catalase (CAT) e o nível de glutation (GSH). Estes parâmetros foram ligeiramente aumentados no grupo com suplemento da VE. A administração da VE e do MB diminuiu significantemente os níveis quando quando comparada ao Grupo C. O nível de malondialdeído no grupo VE foi significantemente mais baixo do que nos grupos *sham* e C. **Conclusão**: A administração intraperitoneal da solução de azul de metileno foi mais eficaz de acordo com a vitamina E na prevenção de aderências intra-abdominais no corno uterino de ratos.
Descritores: Vitamina E. Azul de Metileno. Aderências Teciduais. Útero. Ratos.

Introduction

In intrabdominal surgery, development of peritoneal adhesions is a serious postoperative complication. Postsurgical adhesions are a consequence of incision, cauterization, suturing or other means of trauma. They lead to small-bowel obstruction, chronic abdominal and pelvic pain, and female infertility[1]. Several medications, such as non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, progesterone, anticoagulants, fibrinolytics, antibiotics, vitamin E, methylen blue, pentoxifylline, carboxymethylcellulose, dextran 70, promethazine, antihistaminics and prostaglandin synthetase inhibitors are used in order to prevent the development of postoperatif adhesions[2].

Vitamin E presents interesting biological properties and activities for preventing intraperitoneal adhesions. *In vitro* studies have reported that it has antioxidant, anti-inflammatory, anticoagulant and antifibroblastic effects, and playing a role in diminishing the production of collagen[3-5].

In recent studies, it has been suggested that intraperitoneal application of MB can be used as an effective agent in the prevention of postoperative adhesions[6]. MB is known to inhibit the formation of superoxide by competing with oxygen for the transfer of electrons from flavo-enzymes, primarily xanthine oxidase, and thus inhibitis the generation of oxygen radicals. Several mediators of inflamation have been implicated in adhesion formation. These are locally generated free radicals such as superoxides, peroxides and hydroxyl radicals, and therefore could induce adhesions by damaging cellular membranes. MB is used in patients having refractory septic shock or some neurological diseases[7]. In several studies, use of MB and VE have been reported for prevent of formation adhesion[3,4,6]. However, to date, there has been no report on biochemical or histopathological changes in adhesion tissue treated with MB and VE. Therefore, in the present study, we aimed to investigate the comparative effects of MB and VE on healing and adhesion formation models, and discussed the mechanisms underlying the effects of MB and VE on these processes.

Methods

The guidelines for the care and use of the animals approved by the local institution were followed. The local ethics committee aproved this study. A total of 37 Sprague-Dawley (4-5 month-old female and weighing between 200 and 220g) rats were housed in a climate-controlled (relative humidity of 40-60% and temperature of 21 to 24°C) animal care facility, with a 12 hour light/dark cycle. Before and after surgical procedures, the animals were provided with standard rat chow and water, *ad libitum*.

The rats were anaesthetized with IM ketamine (Ketalar; Eczacibasi, Istanbul, Turkey) at 85 mg/kg body weight and xylazine (Rompun Vet; Bayer AG, Istanbul, Turkey) at 6 mg/kg body weight. Following anaesthesia, abdominal skin was shaved and antisepsis was obtained by 10% povidone iodine solution. The laparotomy was performed with a 3 cm midline incision.

The rats were divided into 4 groups and animals each treated as follows: First group was kept as sham group (Sh, n=7), on which only laparotomy was performed and they received 2 ml of 0.9 % saline solution intraperitoneally. In the remaining rats (n=30), the small bowel was retracted and the uterus was exposed. Punctate serosal hemorrhages were generated by scraping with a No. 15 scalpel blade until petechial bleeding emerged at the abdominal sidewall and antimesenteric surface of the left uterine horn to create adhesions. Prior to closure of the abdominal incision, 2 ml saline (0.9 % isotonic solution) to the animals in the control group were given intraperitoneally (n=10). 2 ml of 1 % methylene blue solution was administered to the rats in methylen blue (MB, Sigma, St. Louis, Mo., USA) group (n=10) and 2 ml of Vitamin E (Evigen ampul, Aksu Farma, 300 mg/2ml, al-alpha-tocopheral acetate), which was dissolved in 58 ml of olive oil, and sterilized in an autoclave and cultured before use, was administered to the rats in vitamin E group (VE) group (n=10). The abdominal incision was subsequently closed, by suturing periton and muscular layers with absorbe materials and then skin was closed with 4/0 silk sutures. Abdominal wall integrity and wound healing were checked during the first 2 days after surgery. The animals were allowed to resume their diet until the 14th postoperative day. Rats in all groups were reanaesthetized and relaparatomy was performed.

Microscopic evaluations

The intraabdominal cavity was inspected through a U shaped incision of the anterior abdominal wall, which was retracted caudal, providing maximal exposure. The peritoneal and left uterine horn tissue including the tissue of adhesion were surgically removed and rats were killed. A half of the each adhesion tissue was preserved in 10 % neural buffered formalin for histopathological examination. The other parts were kept for biochemical analysis.

This tissue samples for histopathological examinations were fixed in 10 % neutral buffered formalin, embedded in paraffin wax, and were cut into 5 µm sections and stained with hematoxylin and eosin. The histological sections were examined for the presence of adhesion score, oedema, fibrosis and mononuclear cell infiltration with a light microscope (Nikon optiphot, Kanagawa, Japan) and photographed. The microscopic scoring was graded as follows: on a scale of mild (1), moderate (2) and severe (3).

Homogenate preparation

Tissues were washed twice with cold saline solution, placed in glass bottles, labeled and stored in a deep freeze (-30°C) until processing (maximum 10 hr). After weighing, the tissue (1 gr) were placed on ice, cut into small pieces with scissors, and homogenized (2 min. at 3000 x g) in five volumes (1:5, w/v) of ice-cold Tris-HCl buffer (50 mM, pH 7.4), using a glass-Teflon homogenizer (Caliskan Cam Teknik, Ankara, Turkey). All preparation procedures were performed at 4°C. After addition of butylhydroxytoluol (4µl per ml), the tissue homogenate samples were used for immediate NO, MDA, GPx, CAT and GSH measurement.

Biochemical assays

Lipid peroxidation levels in tissue homogenate were measured with the thiobarbituric-acid reaction by the method of Placer *et al.*[8] The values of MDA were expressed as µmol per g protein. The GSH content of the tissue homogenate was measured at 412 nm using the method of Sedlak and Lindsay[9]. The solution was kept at room temperature for 5 min, and then read at 412 nm on the spectrophotometer. Results were expressed as µmol per gr protein. GSH-Px activities of the tissue were spectrophotometrically measured at 37°C and 412 nm according to the Lawrence and Burk[10]. Results were expressed as µmol per gr protein. The protein content in the tissue was measured by method of Lowry *et al.*[11] with bovine serum albumin as the standard. CAT activity was assayed in tissue homogenate by the Aebi method[12]. The principle of the assay is based on the determination of the rate constant (s−1,k) for H_2O_2 decomposition at 240 nm by the spectrophotometer. Results were expressed as unit per gr tissue. The nitric oxide content of the tissue was determined according to method of Cortas and Wakid[13]. Results were expressed as µmol per gr tissue.

Statistical analysis

Adhesion score, fibrosis, oedema and mononuclear cell infiltration data were analyzed using Kruskal-Wallis test, whereas biochemical data were analyzed using one-way Analysis of Varience (ANOVA). The Duncan test was performed for multiple comparisons using the SPSS 11.0 for Windows. The data were expressed as means±standard errors (SEM). Results were considered statistically significantat $p<0.05$.

Results

All animals recovered from anaesthesia with no evidence of complications. In these rats, adhesions were also found between the depersonalized antimesenteric surface of the left uterine horn and adjacent abdominal walls. The histopathological findings including adhesion scores, oedema, mononuclear cell infiltration and fibrosis scores in all groups are shown in Table 1. There were significant differences beween groups with respect to histopathological scores of adhesions. The histologic findings of adhesions in the C group differed significantly those of MB groups with respect to adhesion scores ($P<0.01$), fibrosis ($P<0.01$), mononuclear cell infiltration ($P<0.01$) and oedema ($P<0.01$). There were no significant difference between the C and VE groups.

In microscopical examination of uterus of the Sh group, no pathological semptoms were found (Figure 1A). Whereas, in microscopical examination of the uterus sections of the C group, the abdominal cavity of uterus was found to be attached to abdominal wall and neighboring organs. When the sections of the adhesions are examined, oedema, thickness and occasionally capillary vascular proliferation were noted in the uterus seroza. There were also microabsce focus, fibrosis and intensive mononuclear cell infiltration in the muscular layers of the uterus (Figure 1B). Similar adhesions to thouse of C group were found in the uterus of rats from VE groups, but the severity of the adhesions were less. The degrees of fibrosis, mononuclear cell infiltration, oedema and capillary vascular proliferation were less in the uterus seroza when compared with those of the C group (Figure 1C). In the rats treated with MB, oedema and mild mononuclear cell infiltration were observed in the uterus seroza, there were no adhesion though (Figure 1D).

The results of tisue GSH, MDA and NO values and CAT and GPx enzyme activities in all groups are shown in Table 2. The highest CAT, GSH and GPx values and the lowest MDA levels were determined in tissue homogenates in VE group, the difference were not significant between the groups though. Tissue MDA values were significantly reduced ($P<0.05$) in group with VE supplementation compared to Sh and C group. Levels of NO in the C group was significantly higher than those in Sh, VE and MB groups ($P<0.01$). The levels of NO in MB treated rats were significantly lower than in Sh and C rats.

Adhesion scores were significantly correlated with oedema, fibrosis and mononuclear cell infiltration grades with p values 0.01, 0.01 and 0.05, respectively. Also, the significance of the correlations between fibrosis and mononuclear cell infiltration, oedema and fibrosis, and mononuclear cell infiltration and oedema grades were determined to be 0.01, 0.01 and 0.01, respectively.

TABLE 1 - The degrees of histopathological lesions of uterus sections in the all groups.

	Groups			
	Sh (n=7)	C (n=10)	VE (n=10)	MB (n=10)
Adhesion score	NI	2.70±0.15	2.00±0.20	0.20±0.13*
Oedema	NI	2.60±0.16	2.10±0.23	1.00±0.21*
Mononuclear cell infiltration	NI	2.80±0.13	2.40±0.22	0.80±0.13*
Fibrosis	NI	2.20±0.13	1.70±0.21	0.40±0.16*

NI = no influence

*Statistically different from control and VE groups ($p<0.01$)

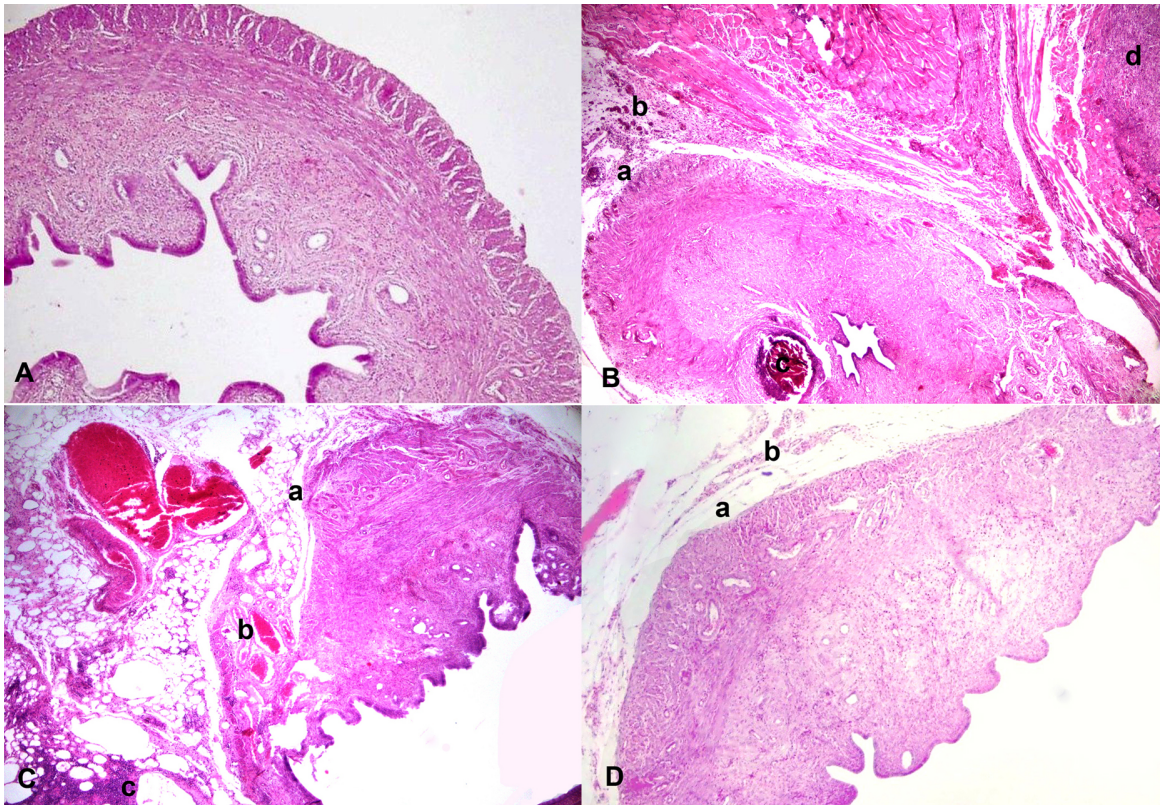


FIGURE 1 – A. Normal histopathological view of uterus sections in Sh group. **B**. Histopathological view of uterus section in C group. a) oedema and thickness in the seroza b) capillary vascular proliferation c) microabsce focus d) mononuclear cell infiltration (H.E. x 100). **C**. Histopathological view of uterus section in VE group. a) oedema and thickness in the seroza b) capillary vascular proliferation c) mononuclear cell infiltration (H.E. x 100). **D**. Histopathological view of uterus section in MB group. a) oedema in the seroza b) mild mononuclear cell infiltration (H.E. x 100).

TABLE 2 – The mean tissue levels of MDA, CAT, GSH, GPx and NO in all groups.

Tissue parameters	Sh (n=7)	C (n=10)	VE (n=10)	MB (n=10)
CAT (k/gr tissue)	18.15±1.71	19.41±1.77	24.59±5.40	18.20±0.67
GSH (µmol/gr protein)	4.59±0.33	5.37±0.48	7.63±1.57	5.24±0.32
GPx (µmol/gr protein)	100.07±3.62	111.63±10.68	128.89±27.11	106.74±5.24
MDA(µmol/gr protein)	2.55±0.10	2.59±0.21	2.06±0.15 *	2.23±0.20
NO (µmol/gr tissue)	33.96±1.25	35.58±0.18**	34.04±0.62	33.17±0.34***

*Statistically different from Sham and control groups ($p<0.05$)
**Statistically different from sham, VE and MB groups ($p<0.01$)
*** Statistically different from sham and VE groups ($p<0.01$)

Discussion

The histologic findings on adhesions showed that the C group had the highest adhesion, fibrosis, oedema and mononuclear cell infiltration scores (Figure 1B). We did not find these results surprising because similar results have been reported previously by authors[14]. As shown in Table 1, histopathological score in MB treated group was markedly diminished in comparison to the C group, whereas no significant difference was found between MB and Sh groups. These findings were in agreement with the results of previous studies, in which similar doses and concentrations of MB were administred for prevention of adhesion formation[6,15]. The results on histopathological changes demonstrate that intraperitoneal treatment of MB decreases the incidence of intra-abdominal adhesion in the experimental model employed fort he current study. Some authors[6,15] reported that MB prevented the formation of intraperitoneal adhesion, possibly by reducing the initial inflammatory response and the subsequent exudation in a rat uterine horn model. Heydrick *et al.*[16] showed that MB inhibits adhesion formation via a mechanism that might involve blocking an oxidative stress-dependent decrease in peritoneal fibrinolytic activity. This effect could be accounted for by the inhibition of inflammatory cells accumulation and in particular scavenging reactive oxygen radicals by MB in the adhesion tissue.

The recent studies[3,4,17] using intraperitonal VE administration showed a significant reduction in postoperative peritoneal adhesions in rats. Our results are in agreement with previous studies reporting on antioxidant agents[17]. Although adhesion, oedema, fibrosis and mononuclear cell infiltration scores have been found to be lower in the VE group than C group, the differences were not statistically significant (Table 1). We think that, morphological changes in uterus were because of adhesion formation, but these changes tended to be considerably mild in intraperitoneal VE treatment. We found that the correlations between fibrosis–adhesion, mononuclear cell infiltration–adhesion and oedema–adhesion were significant, which is also supported by the relationship between adhesion scores and histopathological findings. Similarly, Sanfilippo *et al.*[17] reported that although there was a trend of less fibrosis with VE treatment, there was no statistically significant difference. In addition, Yetkin *et al.*[4] found that neovascularization, inflammation and fibrosis scores regarding histological examination of adhesions in the groups which underwent intraperitoneal VE administration were lower compared to the control groups. These findings obtained in our study suggest that in addition to its properties as an antioxidant, the VE prevents adhesions by inhibition of fibrosis and mononuclear cell infiltration[17].

Vitamin E is a fat-soluble vitamin and one of a number of nutrients called antioxidants. It is particularly important in protecting cells against oxidative damage by induced reactive oxygen species (ROS)[18]. Levels of MDA in the VE treated rats were significantly lower ($P<0.05$) than those in the Sh and C groups. These results shows that MDA may play an important role in the adhesion formation. In the rats treated with VE, tissue GSH content and CAT and GPx activities were slightly higher compared to the other groups. However, there were no significant differences between the any of the groups. In the literature, there is no report on the effects of VE and MB treatments for the preventation of adhesion formation on tisssue MDA, NO, GSH levels and CAT and GPx activities in rats. Masugi and Nakamura[19] concluded that VE decreased the level of lipid peroxidation but did not change activity of the enzymatic antioxidant system such as SOD, GPx and CAT. On the other hand, administration of intraperitoneal of VE in rats with diabetes[20] and nehprotoxicity[21] caused a decrease in levels of lipid peroxidation in the liver and kidney, although SOD, CAT and GPx activities increased. In addition, when the rats were fed with a VE-deficient diet, concentrations of MDA in the liver and lung tissues were increased, but CAT and GPx activity were significantly decreased[22]. Our results are in accordance with previous reports[19-21]. Vitamin E probably reduces the afflicting effects of oxidative stress in living cell, cleaning the free radicals created locally as other antioxidants[3]. The results of this study shows that VE action is due to its antioxidative effects as shown by the decrease in tissue MDA concentration, and the slight increase in CAT and GPx activities and GSH level. However, we observed that VE has stronge antioxidative effects as well as antiinflammatoir and anticoagulant properties for prevention of adhesion formation.

NO is produced from L-arginine by the catalytic action of nitric oxide synthases (NOS). In physiological levels, NO participates in a variety of physiological processes consisting of neurotransmission and regulation of the blood vessel wall. But increased NO, especially associated with oxidative stress, is a harmful condition for tissue[23]. In the present study, the level of tissue NO increased ($p<0.01$) significantly in C group when compared with Sh group. This indicates that adhesion formation may lead to the induction of inducible nitric oxide synthase, resulting in an increased production of nitric oxide[24,25]. Also, NO level decreased significantly in VE groups when compared with C groups in the present study. This result suggests that VE blocks the occurrence of NO and therefore it has a supportive effect on the antioxidant system. It was reported that intraperitoneal administration of melatonin[24] and resveratrol[25] decreases the incidence and extent of peritoneal adhesions and causes a decrease in NO values. Gurel *et al.*[26] reported that administration of VE decreased NO concentrations in both ipsilateral and contralateral renal tissues compared to ischemia-reperfusion group. In addition, VE administration caused a significant decrease in NO metabolite levels after cerebral ischemia-reperfusion injury[27]. Results were in agreement with other reports[26,27]. The lowering effect of VE on NO level presumably resulted from its antioxidative effects.

The effect of MB on oxidative stress controversial. Liver and kidney CAT activity and GSH level did not change after administration of 1 mM MB to rats for 30 days[28]. In addition, MB when administrated either to intact or to diabetic rats did not significantly change oxidative stress parameters (MDA and SOD)[29].

Moreover, it is reported that MB exposure for 5 hours on either ipsilateral or contralateral testes did not ameliorated biochemical or histological parameters in unilateral spermatic cord torsion model[30]. On the contrary, systemic administration of MB has been shown to reduce oxidative stress in kidney of ciclosporine treated rats[31]. Aksu *et al.*[32] reported that administration of MB (2 mg/kg i.p.) to rats with bile-duct ligation for 14 days led to significant increases in SOD activity and decrease in MDA and NO levels, indicating that MB can attenuate hepatic damage by reducing oxidative stres.

In the present study, there were no significant difference in the activities of tissue CAT and GPx, and levels of tissues MDA and GSH in MB treated rats between Sh, C and VE groups. The results obtained in the present study are similar to those reported by some researchers[28-30] but differ from those reported by Rezzani *et al.*[31] and Aksu *et al.*[32]. The differences may be due to the difference in organ systems used, the dose and mode of application of the MB used in these studies. MB had no effect on tissue CAT and GPx activities and GSH and MDA levels. The explanation of this study results is thus rather difficult. It is possible that the effect of MB might be transient and/or that it might have a partial inhibitory influence on the development of adhesion formation. *In vivo* effects of MB in the current study could be influenced by the interaction of other redox systems. Another possibility is that the antioxidant effect of MB is dose-dependent. The dose administrated in our study could be inappropriate for inducing the antioxidant effect. Detailed studies are needed to clarify the effects of MB at different doses, as well as frequency and duration of administration on adhesion formation in rats.

In the present study, the levels of NO in MB treated rats were significantly lower than in Sh and C rats. Dinc *et al.*[15] reported a marked elevation in NO levels in the rats with adhesion formation, but significant reduction after intraperitoneal MB application. On the basis of our results and earlier literature[15,32], it is fair to state that MB inhibitis adhesion formation by inhibiting NOS activity and decreasing NO levels in tissue. Several mechanisms may contribute to reduced levels of NO in MB treated rats. For instance, the main effect of MB is inhibition of soluble guanylate cyclase and inducible NO synthase[33]. It competes with molecular oxygen for the transfer of electrons from flavoenzymes and so inhibits the generation of oxygen radicals[7].

Conclusion

Our results showed that MB had a protective effect on the development of adhesion damage according to the histopathological evaluation, biochemical findings did not support its ameliorating effects though. The interesting finding in the current study is that VE-treatment resulted in significant improvement of biochemical and mild histopathological recovery at the adhesion tissue. Treatment of MB have more advantage according to VE in terms of prevention of postoperative adhesions.

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