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Macroscopic and Microscopic Study of *Allium sativum* (Garlic) Oil Effects on Post-Operative Intra-Abdominal Adhesion in Diabetic and Non-diabetic Male Rats

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Abstract

Backgrounds: Development of peritoneal adhesions has been studied extensively, but to date, no definitive strategy has been implemented to prevent their formation. In the present study, garlic oil has been investigated as a possible option.

Aim: To prevent post-operative intra-abdominal adhesion in both diabetic and non-diabetic rats by using garlic oil.

Methodology: An experimental study including sixty adult male albino rats weighing 200-250 gm. were divided to six equal groups. Diabetes was induced in rats of three groups using a single dose of 120 mg alloxan/kg/BW. All rats underwent laparotomy at which cecal wall abrasion and abdominal wall injuries were induced. Group A (negative control) underwent no procedures; Groups B (positive control) underwent diabetic induction. Group C underwent cecal abrasion procedure and received saline 5 ml/kg/BW intraperitoneally. Group D underwent abrasion and received garlic oil 5 ml/kg/BW intraperitoneally. Group E underwent abrasion, diabetes induction and received saline 5 ml/kg/BW. Group F underwent abrasion, diabetes induction and received garlic oil 5 ml/kg/BW. All rats were sacrificed on day 14 postoperative, and the severity of adhesions was evaluated using histo-pathological fibrosis parameters and immune-histochemical staining to identify the fibro vascular tissue nature.

Results: Significant differences were found between the groups regarding macroscopic adhesion scores, inflammation, fibrosis and neo-vascularization ($p<0.001$, $p<0.001$, $p<0.001$, $p<0.005$, respectively). Macroscopic and histo-pathologic adhesion scores were the lowest in the garlic oil-treated diabetic and non-diabetic groups.

Conclusion: The results of our study revealed the effectiveness of garlic oil in reducing postoperative adhesions in both non-diabetic and diabetic rats.

Keywords: Peritoneum; Diabetic rat; Adhesion formation; Alloxan; Vimentin; Garlic oil

Introduction

Intraperitoneal adhesion remains a significant postoperative complication of abdominal surgery, causing intestinal obstruction, chronic pelvic pain, infertility and may complicate subsequent operations [1].

Peritoneal adhesions prevalence after major abdominal procedures has been evaluated at 63%-97% [2]. Postsurgical adhesions have four major negative impacts on health care outcomes. First, adhesions cause significant morbidity, including intestinal obstruction, infertility and pelvic pain. Second, adhesions are associated with multiple surgical complications. Third, these complications lead to greater surgical workload and utilization of hospital and other health care resources. Fourth, all these negative impacts result in significant economic burden to society [3,4]. Diabetes mellitus (DM) is one of the most common metabolic disorders in the world. The diabetes-associated healing impairment is characterized by a decreased inflammatory response, amount of fibrin, collagen synthesis, tensile strength, angiogenesis, and altered production of cytokines [5-7]. Many studies showed that high glucose concentration inhibits wound healing process associated with prolonged inflammatory phase, defected angiogenesis and diminished fibroblast proliferation [8-10]. *Allium sativum* (Garlic), of the Liliaceae, has been used as both a nutrient and in folk medicine for centuries. The antibacterial, anti-inflammatory, antithrombotic, fibrin lytic, antioxidant, and wound-healing properties of garlic have been well-documented in previous studies [11,12]. The bioactive components of garlic are mainly responsible for the healing properties [13]. The claimed health benefits of chemical constituents present in garlic that treat various disorders have been investigated in both in animals and humans [12]. It has been reported that the major action of garlic is attributed to its sulfur-containing compounds [14].

Materials and Methods

The Human and Animal Research Ethics Committees of the Faculty of Medicine, Suez Canal University, Ismailia, Egypt approved all experimental procedures.

Animals

Sixty adult male albino rats (Sprague- Dawley) with average weight of 200-250 grams per rat were included in our study. They were divided equally into 6 groups. Group (A): A negative control group in which animals were not subjected to any interference. Group (B): A positive control group in which animals were subjected to induction of diabetes. Group (C): Normal saline treated non-diabetic group in which animals were subjected to adhesion formation induction. Saline solution (5 ml/kg/BW) was applied intraperitoneally prior to closing the incision.

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Group (D): Garlic oil treated non-diabetic group in which animals were subjected to adhesion formation induction. Garlic oil (5 ml/kg/BW) was applied intraperitoneally prior to closing the incision. Group (E): Normal saline treated diabetic group in which animals were diabetic, subjected to adhesion formation induction, and saline solution (5 ml/kg/BW) was applied intraperitoneally prior to closing the incision. Group (F): Garlic oil-treated diabetic group in which animals were diabetic, after adhesion formation induction, 5 ml/kg/BW of garlic oil was applied intraperitoneally prior to closing the incision.

Induction of diabetes

Diabetes was induced in rats of three study groups using alloxan (Sigma). Rats were left fasting for 18 hours with free access to water. A single dose of 120 mg alloxan/kg/BW was dissolved in normal saline and administered intraperitoneally to lightly anesthetized rats (with ether). The extent of diabetic induction was monitored after 24 h, based on blood sugar levels using a glucometer and strips (Roche Diagnostics GmbH, Mannheim, Germany). The animals were considered to have diabetes if blood glucose was >280 mg/dl for 2 consecutive days. At regular time intervals, blood samples were withdrawn from the tail vein. The blood glucose was measured and animals were considered diabetics if blood glucose was >280 mg/dl for 2 consecutive days [15].

Induction of adhesion formation

Surgical operations were performed for all forty rats of groups C, D, E and F under maximal aseptic conditions and under the effect of light anesthesia [15]. Animals were secured to the operative table with average room temperature (23-25°C) in a supine position and the animal's abdomen was scrubbed with polyvinyl iodine.

A 3.5 cm surgical incision was made and the cecum was exteriorized and the serosa of the cecum was rubbed with a gauze sponge until punctuate hemorrhage occurred. Thereafter, the cecum was placed in its abdominal location and a 1 cm^2 right-side parietal peritoneum incision was made on the anterior abdominal wall and was sutured by four stitches using vicryl 4-0 [14]. At the end of the procedure the abdomen was closed in double layer in a continuous method (muscle and peritoneum with 3/0 catgut sutures, and skin with 3/0 silk sutures [15]. All operative groups received post-operative wound care, housing in separate cages, feeding, cleaning, and antibiotics in their drinking water (0.1% amoxicillin and 0.015% enrofloxacine) for five days.

Statistical analysis

Data of all groups were studied using the statistical program of social science version 12 (SPSS Inc., Chicago, IL, USA). Differences between experimental groups were tested using ANOVA and chi square tests. The statistical significance of the data was determined by P value ($P<0.05$ was considered significant).

Adhesion scoring

Fourteen days after surgery, a laparotomy was done for all rats after general anesthesia. Tables 1 and 2 showed the severity scoring method and scales for macroscopically evaluations based on Canbaz's severity scoring method [16].

Preparation of tissue specimens and histopathological evaluation

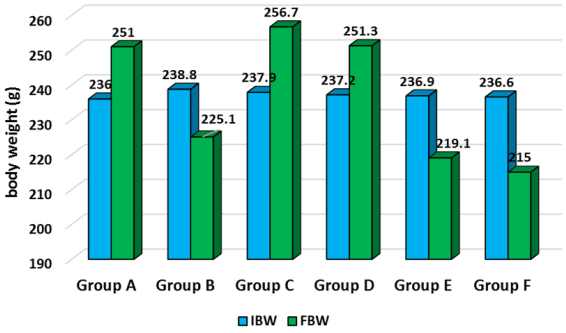
The injured terminal ileum and cecal walls with fibrous adhesions were excised to confirm adhesion formation. The specimens were fixed in 70% alcohol, dehydrated and embedded in paraffin wax. Sections were cut at a thickness of 4-5 µm and stained with haematoxylin and eosin (H&E), or Masson trichrome (MT). Immunohistochemical processing was performed using monoclonal mouse anti-vimentin and anti-podoplanin (D2-40) antibodies. The expression of immunohistochemical parameters ware classified by two independent blinded observers, using a semi-quantitative immunoreactivity score (IRS). The extent of staining was scored as 0 (0-5%), 1 (5-30%), 2 (30-80%) and 3 (80-100%), indicating the percentage of positively stained cells of the area in the section.

Grade	Description
0	No adhesion
1	One adhesion band, no vessel, easily separated
2	Two thin adhesion bands, no vessel, easily separated
3	Three thin adhesion bands, no vessel, easily separated
4	More than three thin adhesion bands, easily separated with no vessel or defused adhesion bands with vessels

Table 1: Canbaz's adhesion severity scoring method.

Score	Extent	Tenacity
0	No adhesions.	No adhesions.
1	Single band of adhesion between viscera or from one viscous to the abdominal wall.	Filmy adhesion, easy to separate by blunt dissection.
2	Two bands, either between viscera or from viscera to the abdominal wall.	Strong adhesion, blunt dissection possible, partly sharp dissection.
3	More than two bands between viscera or from viscera to the abdominal wall.	Stronger adhesion; sharp dissection necessary.
4	Multiple dense adhesions or viscera directly adherent to the abdominal wall, irrespective of number and extent of adhesive bands.	Very strong adhesion between organs; its division by sharp dissection damages organ serosa.

Table 2: Scales for macroscopically evaluations of adhesion formation based on Canbaz's scoring method. Adhesions were macroscopically according to their extent and tenacity.



Graph 1: Comparison between all groups according to the mean of initial (IBW) and final body weight (FBW).

Results

Changes in body weight

As shown in Graph 1, the mean of initial body weight (IBW) in all groups ranged from 236 ± 12.5 gm to 238.8 ± 8.6 gm. The baseline difference between all groups regarding body weight was insignificant. The difference in the final body weight (FBW) between the diabetic and non-diabetic groups was statistically significant ($p<0.005$) (Graph 1).

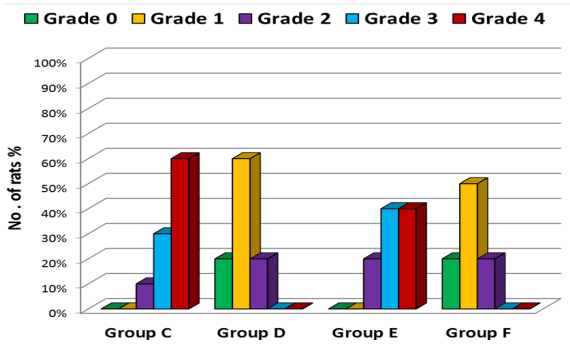
Analysis of gross evaluation of postsurgical adhesions

Results are given in Graph 2 and Figure 1.

In control groups (A and B), 100% of the rats had no adhesions. Group C showed the most severe adhesions in all variables, followed by group E without significant statistical difference. Gross appearance of adhesions in groups D and F indicates formation of different degrees of filmy, transparent, avascular fibrous adhesions which extend to adjacent organs.

In animals of group C the most frequent grade of adhesion was grade four with a mean ± SD of (3.5 ± 0.7) when compared to that of group (D) in which the most frequent grade of adhesion was grade one with a mean ± SD of (1 ± 0.6). The difference between the two groups was statistically highly significant in the severity of adhesion ($p<0.001$). The adhesion score of group (F) was significantly lower than group (E) ($p<0.004$).

Macroscopic adhesion scores were lowest in rats of diabetic groups compared with non-diabetic groups; the adhesion scores were also lower in the garlic oil-treated animals than normal saline treated ones. The difference was statistically significant (Graph 2 and Figure 1).



Graph 2: Grades of Adhesion in the different groups. The effect of allium sativum (garlic) oil on post-operative intra-abdominal adhesion in diabetic and non-diabetic male rats.

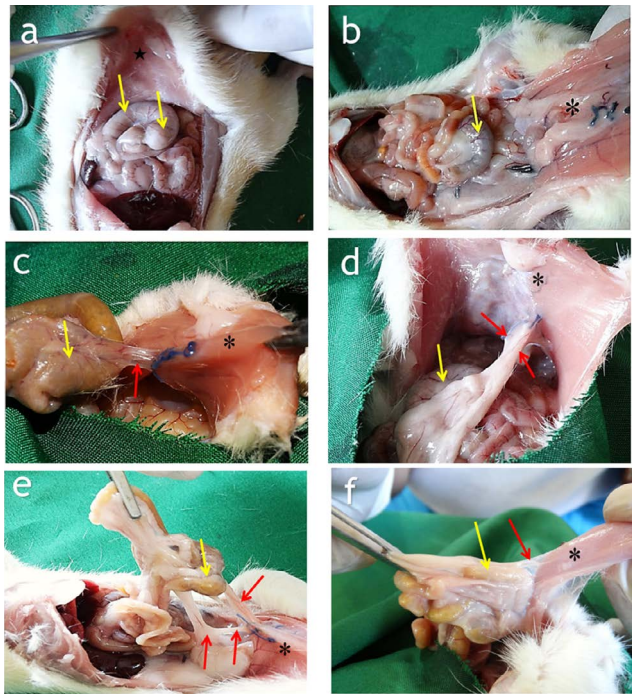


Figure 1: Representative macroscopically photos and adhesion scores. (A-F, Yellow arrows: cecum. Red arrows: adhesion bands. Asterix: abdominal wall).

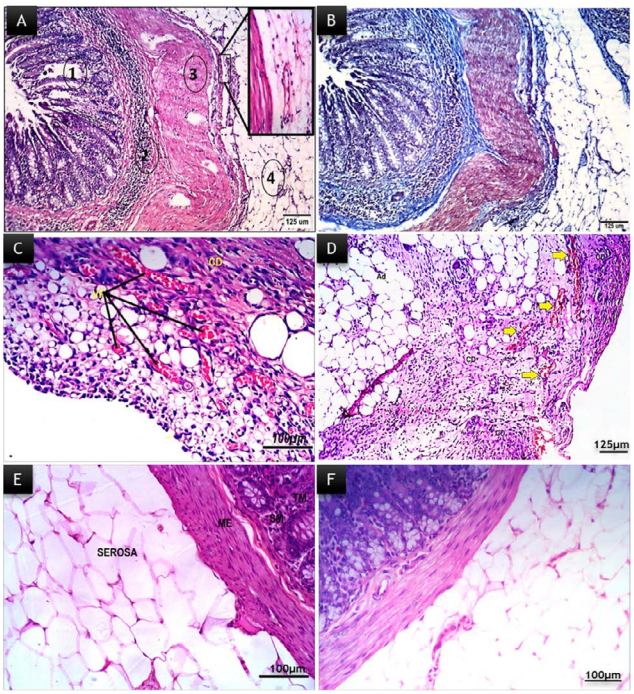


Figure 2: Grades of fibrosis. (A) No collagenous fibrosis in the cecal wall of a rat from negative control group A (H&E X100 bar = 125 µm). (B) No collagenous fibrosis in the cecal wall of a rat from positive control group B (MT X100 bar = 125 µm). (C) Cecal wall of a rat from saline treated non-diabetic group showed massive fibrin strands deposition extends outward with scattered spindle shaped fibroblasts, invasion of different inflammatory cells and newly formed capillary with erythrocytes (arrows) (H&E X200 bar = 100 µm). (D) Cecal wall of a rat from saline treated diabetic group showing moderate degrees of fibrosis, mononuclear cell infiltration, edema and capillary vascular proliferation compared with those of group C, (E) Cecal wall of a rat from garlic oil treated non-diabetic group and (F) of a rat from garlic oil treated diabetic group showing normal serosa with absence of collagenous fibrosis (H&E X200 bar = 100 µm).

Histopathological results

Moderate infiltration of inflammatory cells, Figure 2 accompanied by vascular proliferation (VP) in the fibrous structure without formation of foreign body giant cells were observed in biopsies of group E compared with those of Group C (Figure 3).

Immunohistochemical examination

The garlic treated groups D and F exhibited positive immunoreactions (Figure 4) to D2-40 antibodies within the cells of the mesothelium. No significant increase in D2-40 positive cells was seen in the saline treated groups C and E (Figure 5d) compared to control groups A and B (Figure 5b). On the other hand, garlic treated diabetic rats showed significant increase in D2-40 expression compared to diabetic rats treated with normal saline (Figure 5f).

Cells stained with vimentin as a marker of its mesenchyme origin were statistically significant with higher expression in adhesion areas of the saline-treated groups C and E (Figure 5c) compared with the garlic-treated groups D and F ($P<0.001$) (Figure 5e).

The detection of mesothelium cells as an indicator for reperitonealisation showed no difference between the D2-40 staining values of group C and group E, but the difference between group D and F was statistically significant ($p<0.001$).

The ratio of mesothelium infiltrate (vimentin) was significantly elevated in the garlic oil treated groups in comparison to the normal saline

treated groups in (2.1 ± 0.4 vs. 0. ± 0.4; p = 0.001, 1.9 ± 0.7 vs. 0.3 ± 0.5; p = 0.005, respectively) as shown in Graph 3 (Graph 3).

Discussion

The present study examined the effect of intraperitoneal administration of 5 ml/kg/BW garlic oil in preventing postsurgical adhesion in a rat model. After intervention we noted significant reduction of body weight in diabetes induced groups. This finding was expected as weight loss is one of the cardinal manifestations of diabetes, especially if it is untreated. Intraperitoneal route was more effective in reducing postoperative adhesions compared with oral routes in most of previous studies [17-20]. Possible advantages of intraperitoneal route include formation of a barrier between peritoneal surfaces and a direct effect on the local inflammatory process [20,21]. The adhesion induction rat model used in this study was proved to be very successful in terms of inducing a maximum inflammatory response and dense adhesion induction [22].

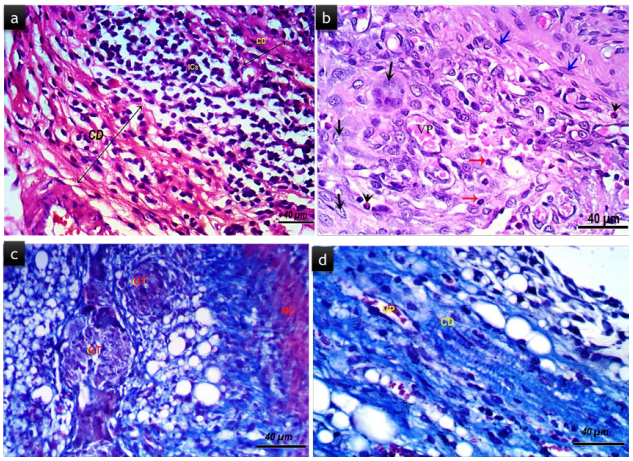


Figure 3: Histological sections (stained with H&E X400 bar = 40 µm). (a) showing severe inflammatory cell infiltrate from group C. (b) invasion of different inflammatory cells primarily polymorph nuclear cells, macrophages(black arrows), plasma cells (red arrows), monocytes (head arrows) and red blood cells filled capillaries (VP) from group E. (c) increase in the number of the foreign body granulation tissue composed of spindle-shaped fibroblasts, new capillaries, dense inflammatory infiltrate and multinuclear giant cells (GT) in group C. (d) foreign body granulation tissue is absent in section of and dilated (VP) micro vessels group E.

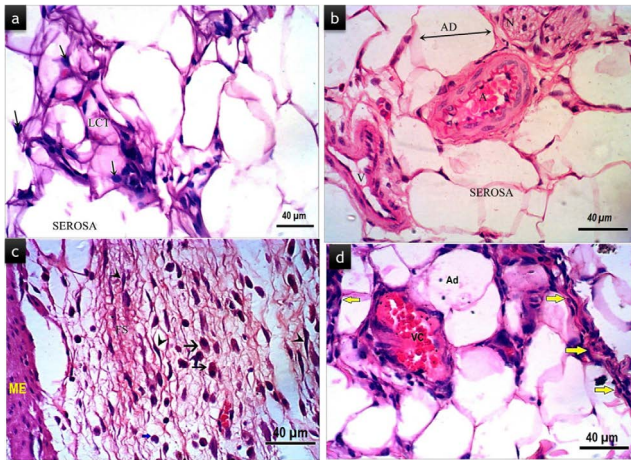


Figure 4: Histological sections (stained with H&E X400 bar = 40 µm). (a) and (b) Showed absence of fibro proliferative tissue and inflammatory cell infiltrate after application of garlic oil in group D. The arrows refer to normal cells of loose connective tissue (LCT). (c) and (d) Mild fibrous bands with mild inflammatory cells infiltrates in group F (yellow arrows), muscularis externa (ME), adipocyte (AD), artery (A), vein (V) and Capillary (VC).

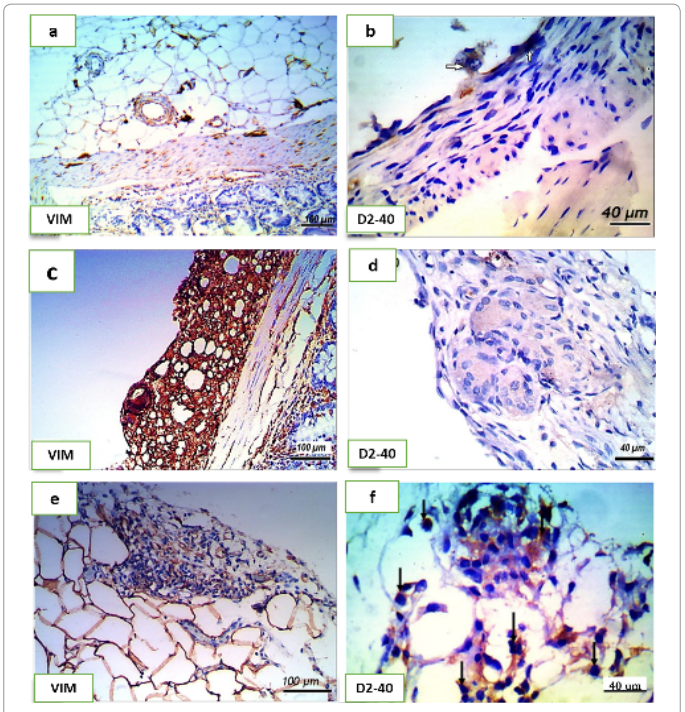
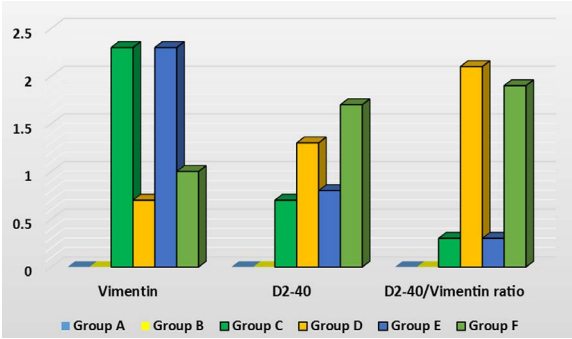


Figure 5: Shows comparison between control, saline treated and garlic oil treated groups according to vimentin and D2-40 immunostaining reactions. (a) Mild brown staining in all layers (positive control) no signs of fibroblastic proliferation in group A. (b) Normal staining of the mesothelial layer, (white arrows), D2-40 immunostained section of group A, (c) Strong brown staining of the fibroblastic proliferative tissue in group C, (d) Negative cytoplasmic staining reaction in adhesive tissue, in group C. (e) Mild immunostaining expression of vimentin in mild fibro vascular tissue from group D. (f) Strong cytoplasmic immunostaining detecting mesothelial cells proliferation (arrows).



Graph 3: Expression of vimentin and D2-40 antibodies and D2-40 / vimentin ratio in diabetic and non-diabetic rat model of postoperative abdominal adhesion.

Saline solution has been evaluated for its ability to prevent peritoneal adhesion in several studies, but the results were controversial. Some studies have reported that saline solution prevents peritoneal adhesion by lowering the inflammatory response and removing fibrin, while, other studies did not show any reduction of adhesion severity with saline solution [17,18,23,24]. The present study revealed a statistically significant higher degree of adhesion in saline-treated animals than in garlic oil-treated groups.

Maximum adhesion formation in the form of multiple multinucle-

ated giant cells and foreign body granulation tissue reaction occurred at the site of surgery and mainly around the vicryl suture knots. This is in line with Elkins et al., who found that the densest adhesion and most severe inflammatory reaction occurred around the surgical knots [25].

Our results on the effect of garlic oil on postoperative adhesion formation are in agree with those of Sahbaz et al. who reported significant inhibition of adhesion formation in garlic oil administered intra-abdominally [26].

Garlic oil applied to surgical regions caused least fibrosis, lesser amount of inflammatory cell infiltration and no vascular proliferation in compared to the normal saline-treated non-diabetic groups. The decrease in inflammatory cell infiltrate can be taken as an index of garlic anti-inflammatory effect. The decreased fibroblast migration is assumed to lead to better wound closure, less fibrosis, and a lesser amount of scar tissue [27].

The anti-inflammatory properties of garlic and its components have been reported in many other studies [12,28]. Garlic exerts anti-inflammatory effects by inhibiting oxidative stress-induced activation of nuclear factor kappa-B (NF-kB) which is the major factor involved in the expression of pro-inflammatory enzymes such as (nitric oxide species) NOS and cyclooxygenase-II [12]. Garlic oil also inhibits production of T-helper cells and inflammatory cytokines [29]. Diallyl thiosulfate (a component of garlic oil) increases corticosteroid levels and thereby acts as an anti-inflammatory and immunomodulation agent [12,29,30].

The present study also revealed that the reduction in adhesion scores (inflammatory cell infiltrate, neovascularization and fibrosis) was significantly greater in non-diabetic groups compared to the diabetic groups.

Oviedo-Socarrás et al. reported similar results, and concluded that the diabetic environment greatly altered the healing response in most of the analyzed parameters [31].

Abbasian et al. reported that, postoperative adhesion formation was more pronounced in diabetic rats [15]. The expression of vimentin by immunohistochemical technique was absent in the control groups. However, normal saline treated groups and garlic oil-treated groups showed intense and mild vimentin expression, respectively. Similar findings were also reported by Klink et al. [32]. Gross finding of the garlic oil treated non-diabetic group showed that garlic oil an adjuvant to adhesion prevention is effective significantly reducing macroscopic and microscopic fibrosis in peritoneal surfaces. Those adhesions appear thin filmy pulled apart easily with minimal digital pressure. Holmdahl et al. stated that adhesion results from normal peritoneal wound healing response and develops as a filmy, transparent, avascular adhesion in the first 5-7 days after injury. On the other hand, most rats of this group showed normal healing of peritoneal injury with no gross signs of adhesive bands [33].

The current study indicated that garlic oil might alter the local repair process. Enhanced accumulation of inflammatory and mesenchyme cells in the saline-treated groups almost disappeared in garlic oil treated groups. This resulted in improving ratio of mesothelium cells to vimentin expression and indicate enhancement of reperitonealisation with a reduced mesenchyme infiltrate.

The preventive effect of garlic oil may be due to the anti-inflammatory effects of its component or to its barrier effect. The garlic oil-treated non-diabetic group showed lower, but insignificant effects compared to those of garlic oil-treated diabetic group which were improved regarding histological parameters. The net effect may not be linked only to the anti-inflammatory effects of garlic oil but, it is likely to be related to many other mechanisms. However, the following three mechanisms could be involved in exhibiting this effect:

(1) An increased healing process due to its antimicrobial, anti-inflammatory and immunomodulation effects.

(2) The inhibition of mechanical contact between surfaces.

(3) The inhibition of the inflammatory process resulting from the reduction in leukocyte adhesion and proinflammatory cytokines.

Conclusions

The current study revealed that garlic oil is effective in reducing postoperative adhesions in both non-diabetic and diabetic rats, but there was a limited effect on the non-diabetic group regarding histological parameters compared to the diabetic group. Further experimental researches are needed particularly to test the best anti-adhesive dose of garlic oil and the possible mechanism in diabetics.

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