Is There any Effect of lycopene's preventing peritoneal adhesion formation in rats: An Experimental Study

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Abstract

Objective Purpose of the study; to demonstrate the effects of lycopene on the prevention of intraabdominal adhesions in rats with biochemical, histological and macroscopic parameters. Material methods Twenty eight rats were divided into four groups consisting of 7 rats each. Group 1 [only adhesion], Group 2 [adhesion+corn oil], Group 3 [adhesion+ 5 mg/kg lycopene], Group 4 [adhesion+20 mg / kg lycopene]. Macroscopic adhesion score, histopathological examination, Vascular endothelial growth factor (VEGF) H-score, malondialdehyde, total anioxidant capacity and VEGF values were measured in the groups. Results There were significantly higher extend [$P < 0.05$], severity [$P < 0.05$], degree [$P < 0.05$] and total adhesion [$P < 0.05$] scores in the control group and corn-oil group than in the low lycopene group and high lycopene group. VEGF H-scores were significantly lower in lycopene-given groups, regardless of dose. When low lycopene group and high lycopene group were compared in terms of anti VEGF H-score, no significant difference was observed. Malondialdehyde levels were statistically significantly lower in the control group and high lycopene group [$p<0.05$]. Conclusion Biochemical parameters, histopathological examination, and adhesion scoring revealed that lycopene significantly reduced adhesion formation.

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[lycopene and adhesion]

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Twenty eight rats were divided into four groups consisting of 7 rats each. Group 1 [only adhesion], Group 2 [adhesion+corn oil], Group 3 [adhesion+ 5 mg/kg lycopene], Group 4 [adhesion+20 mg / kg lycopene]. Macroscopic adhesion score, histopathological examination, Vascular endothelial growth factor (VEGF) H-score, malondialdehyde, total anioxidant capacity and VEGF values were measured in the groups.

Results

There were significantly higher extend [P < 0.05], severity [P < 0.05], degree [P < 0.05] and total adhesion [P < 0.05] scores in the control group and corn-oil group than in the low lycopene group and high lycopene group. VEGF H-scores were significantly lower in lycopene-given groups, regardless of dose. When low lycopene group and high lycopene group were compared in terms of anti VEGF H-score, no significant difference was observed. Malondialdehyde levels were statistically significantly lower in the control group and high lycopene group [*p<0.05]* .

Conclusion

Biochemical parameters, histopathological examination, and adhesion scoring revealed that lycopene significantly reduced adhesion formation.

Key words: adhesion, lycopene, peritoneum, rat, Vascular endothelial growth factor **(** VEGF)

WHAT'S KNOWN? (what is already known about this subject?):

Lycopene is a pigment belonging to the carotenoids family, naturally found in vegetables and fruits. The antioxidant, anti-inflammatory, antiproliferative and antineoplastic effects of lycopene have been reported in various experimental and epidemiological studies.

WHAT'S NEW? (what does this study contribute to the literature?):

To the best of our knowledge, there are no in vivo studies on the anti-adhesion effect of lycopene. The need for in vivo studies was emphasized as a limitation in the only in vitro study conducted on this subject (1). Therefore, it is thought that this molecule is worth studying in the prevention of adhesions and will contribute to the literature as the first in vivo study. In this observational rat study, we investigated the effectiveness of lycopene use in preventing adhesions after gynecological surgery

Introduction

Although our knowledge about peritoneal cavity physiology and peritoneal healing mechanism is increasing, postoperative adhesions continue to be a problem for surgeons from different disciplines. In different studies, the direct relationship between intra-abdominal surgical interventions and adhesion formation has been revealed, and it has been stated that the most important cause of intestinal obstruction is adhesions due to previous surgeries (2-5). Postoperative adhesions are fibrous connections that can be seen between organs that are not normally combined with each other and are surrounded by serous membranes following injury or surgical operations (2). Intra-abdominal adhesions can cause complications such as abdominal pain, intestinal obstruction and infertility in women. Approximately 30% of intestinal obstructions develop due to intra-abdominal adhesion and postoperative peritoneal adhesion occurs in more than 90% of all laparotomies (2). These adhesions sometimes require major abdominal surgery interventions or cause prolongation of operations performed for another reason. In addition, they lead to postoperative mortality, morbidity and cost increase due to their ability to extend the length of hospital stay (6). Despite advanced surgical techniques and medical treatment options, postoperative intraadominal adhesions are still an unsolved problem. When the literature is examined, it is seen that many experimental, clinical studies and theoretical reports on adhesion prevention have been published since the beginning of the century (7). Various chemical agents using as adhesion inhibitors prevent fibrin organization by inhibiting fibroblastic proliferation. Therefore, many agents such as non-steroidal anti-inflammatory drugs [NSAIDs], corticosteroids, calcium channel blockers, histamine antagonists, antibiotics, fibrinolytic drugs, antioxidants and vitamins have been tried to inhibit this proliferation (8). Lycopene is a pigment belonging to the carotenoids family, naturally found in vegetables and fruits. The antioxidant, anti-inflammatory, antiproliferative and antineoplastic effects of lycopene have been reported in various experimental and epidemiological studies (9, 10). In this observational rat study, we investigated the effectiveness of lycopene use in preventing adhesions after gynecological surgery.

Material methods

This study was approved by Our University Animal Experiments Local Ethics Committee [26.04.2017, Protocol No: 2017/011]. Twenty eight female wistar albino rats weighing 160-250 g were included in the study.

Experimental animals were kept in our University Animal Laboratory at a room temperature of 24 ° C and a 12/12 hour day and night cycle. Fed with standard rat food and water without restrictions. Each rat was anesthetized with ketamine hydrochloride [40 mg/kg iv]. Before surgery, The abdominal area of the rats was shaved and wiped with 1% povidone iodine and prepared for the operation, approximately 4 cm lower midline laparotomy was performed on the umbilical region. Before surgery, the rats were randomly divided into four groups each consisting of 7 rats. The operations performed in groups of rats were as follows:

1. Control group [CG]; a standard adhesion was created, no adjuvant was given
2. Corn oil group [CoG]; After the injury, 1 ml of corn oil was administered intraperitoneally. The same dose was continued intraperitoneally for 14 days.
3. Low-dose lycopene group [LLG]; After the injury, 5 mg/kg lycopene was administered intraperitoneally. The same dose was continued intraperitoneally for 14 days.
4. High-dose lycopene group [HLG]; After injury, only a single dose of 20 mg / kg lycopene was administered intraperitoneally.

The dose of lycopene was chosen based on other studies (11-13)

After a 4 cm abdominal incision, the uterine horns were exposed and a lesion was created on the antimesenteric surface of each uterine horn surface with the help of 10 wolt bipolar cautery. At the same time, extra adhesion was created by scraping operations on the visceral surface until serosal bleeding occurred. After the procedure was completed, the abdomen was closed continuously using 4.0 vicryl. The rats were then allowed to recover for two weeks. Two animals in the LLG and two animals in the HLG died after the first surgical procedure, so these groups were evaluated out of 5 animals. After the recovery period, the animals were sacrificed and evaluated for adhesion formations. The researchers who evaluated the adhesions had no prior knowledge of which group the rat belonged to. Adhesion scoring based on extend, severity, and resistance to applied force is summarized in table 1. The sum of the three parameters was used as the total score for each group.

Tissue samples taken from the peritoneum and adhesion area were sent for histopathological examination. Adhesion examples are shown in figure 1.

Fixation and Tissue Tracking

Tissues were fixed with formaldehyde for 48 hours. The tissues were washed with Phosphate-Buffered Saline (PBS) at pH 7.2 / 7.4 after fixation. Tissues were first passed through a series of ethanol increasing from 80% to 95% and 2 times 96% respectively. It was held for 45 minutes in each ethanol series. In the transparentization step, the tissues were xylolized twice for 1 hour. The tissues whose transparency is completed are embedded in paraffin for 2 times for 1 hour and then blocked (14). The tissues were kept at + 4ºC after the blocking process. 5 µm sections were taken with a rotary microtome.

Hematoxylin-Eosin [H-E] staining method

With this method, it is aimed to determine the morphological properties of the preparations at the light microscope level. After the preparations were deparaffinized, they were rehydrated with decreasing concentrations of ethanol [99.6%, 96%, 90%, 80%, 70%, 50%]. Tissues held for 2 minutes on each alcohol batch. After staining with hematoxylin for 3 minutes, it was soaked in tap water for 5 minutes and removed by immersion in ammonia water. Then the preparations were stained with eosin for 3 minutes and washed with distilled water for 5 minutes. Tissues were kept in pure xylene solution for 1 minute after waiting for 2 minutes in increasing concentrations of ethanol series [50%, 70%, 80%, 90%, 96%, 99.6%]. Sections closed with enthallen were examined with Nikon Eclipse 80i image analysis system (15).

Anti-VEGF Immunohistochemistry [IHC] Staining

After the preparations were deparaffinized, they were rehydrated with decreasing concentrations of ethanol [99.6%, 96%, 90%, 80%, 70%, 50%]. Each alcohol batch was held for 2 minutes. Hydrogen peroxide [3%] [H2O2] was applied at room temperature for 5 minutes, followed by 3 washes with PBS. For permeabilization,

after incubating with 0.1% Triton-X 100 for 10 minutes at room temperature, it was washed again 3 times with PBS. After 20 minutes of application with protein block, it was incubated with anti-VEGF primary antibodies at the dilutions recommended by the company at + 4 ° C for 1 night without washing. After washing with PBS 3 times the next day, secondary antibodies biotin [30 minutes] and then streptavidin [30 minutes] were applied. Washing was done with PBS 3 times between two applications and after the last application. The diaminobenzedine [DAB] chromogen was applied for 3-6 minutes to ensure the visibility of immunoreactivities. Afterwards, the preparations washed with distilled water at least 3 times were counter-stained with Mayer's hematoxylin for 1 minute, then washed again 3 times with distilled water and covered with enthallen. Control staining was done to test whether the immunoreactivities were specific. Experiments were studied in 3 repetitions independently from each other and each group was 3 samples (16).

Evaluation of immunohistochemistry staining

Immuno stained preparations were evaluated under light microscopy [Nicon Eclips 80i, Japan]. The randomly determined areas in the preparations were examined by the researcher blindly [without reporting the groups]. The staining intensity was scored as no [-] [0], light [1] [+], medium [2] [++], strong [3] [+++] and thus the number of reactive cells was determined. After scoring, photos were taken from the sections with the image analysis system. H-Score values were calculated after counting positive stained cells in each 100 cells in 3 randomly selected fields for each group [H-Score: [?]Pi [i + 1] [Pi:% number of positively stained cells; i: staining intensity].

Biochemical analysis

Measurement of lipid peroxide level

Measurement of lipid peroxides in tissue samples was made by Uchiyama and Mihara methods (17). MDA levels in the samples were calculated from the calibration curve prepared from standard solutions. Results are given as nmol / mg protein.

Measurement of VEGF level

Measurement of VEGF levels in tissue samples was carried out with ELISA kit [Sun Red, Shanghai Sunred Biological Technology Co., Ltd.]. First of all, standard samples were prepared. Standard prefixes of 50, 100, 200, 400, 800 and 1600 ng / L were prepared from the stock solution. 0.05 ml standard and 0.05 ml HRP-streptavidin were added to the wells determined in the ELISA plate. Samples were pipetted. The plate was covered and incubated at 37 deg C for 90 minutes. For tissue samples, 0.04 ml sample, 0.01 ml VEGF antibody and 0.05 ml HRP-streptavidin were added. ELISA plate was incubated at 37 C for 60 minutes. At the end of the period, all samples were removed from the ELISA plate with a pipette and washed 3 times with washing buffer. At the end of the washing process, chromogen A [0.05 mL] and B solutions [0.05 ml] were added to the wells and the plate was incubated at 37 deg C for 15-30 minutes. At the end of the period, the reaction was stopped by adding 0.05 ml of stop solution to the wells. The optical density of the resulting yellow color was read in a microplate reader [Thermo Scientific(r) Multiskan Go] at 450 nm wavelength. Results were evaluated according to the standard calibration curve.

Measurement of total antioxidant capacity [TAC]

Measurement of total antioxidant capacity in tissue samples was performed spectrophotometrically with a commercial kit [Rel Assay Diagnostics]. According to the principle of this method, the antioxidant molecules in the studied sample cause the formation of a lighter colored compound by reducing the dark blue-green colored ABTS radical. The absorbance of the resulting color is read in the spectrophotometer at a wavelength of 660 nm (18). The level of antioxidant molecules in the sample is calibrated with the stable antioxidant compound Trolox Equivalent [a vitamin E analog] present in the kit.

Statistical analysis

SPPS 25 [IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.] statistical package program was used to evaluate the data. Histopathological changes between

experimental groups were analyzed comparatively. Experiments were done independently in 6 replicates. Variables are expressed using mean +- standard deviation, percentage and frequency values. Variables were evaluated after checking the preconditions for normality and homogeneity of variances [Shapiro Wilk and Levene Test]. For comparison of three or more groups, the one-way anova test and the Tukey HSD test, one of the multiple comparison tests, were used. Categorical data were analyzed with Fisher's Exact Test and Chi-Square test. In cases where the expected frequencies are lower than 20%, an evaluation was made with the "Monte Carlo Simulation Method" to include these frequencies in the analysis. For the significance level of the tests, p <0.05 and p <0.01 values were accepted.

Results

The results obtained from the present study are shown in Table 2. The total adhesion scores were 5,8571+-1,21499 for the CG, 6,2857+-1,49603 for the CoG, 2,6+-1,14018 for the LLG, 2+-1,41421 for the HLG. There were statistically significant differences between macroscopic adhesion scores between groups. A statistically significant decrease was found in all adhesion scores in rats given lycopene compared to groups not given lycopene regardless of dose. There were significantly higher extend [P < 0.05], severity [P < 0.05], degree [P < 0.05] and total adhesion [P < 0.05] scores in the CG and CoG than in the LLG and HLG. Although some adhesion scores [severity, degree and total adhesion score] were lower in the HLG compared to the LLG, these differences were not statistically significant. p values were 0.73, 0.967 and 0.891 respectively. There was no significant difference between the CG and CoG in terms of all scoring.

When histopathological findings are evaluated, a high level of inflammation was observed between the perimetrium and smooth muscles in the CG and CoG groups. Hyperemia and intense inflammatory cells have been detected in vascular structures. Moderate edema and thickening were also detected in the perimetrium. Similar to CG and CoG groups, high-level inflammation, hyperemia and moderate edema were observed in the perimetrium in the LLG. Inflammation and edema decreased statistically significantly [p <0.05] in the HLG group compared to the other groups, including the LLG group. Symptoms of hyperemia almost disappeared [Table 2, Figure 2]. Anti-VEGF involvement was observed around the vascular structures and perimetrium in the anti-VEGF immunohistochemistry staining of the uterine sections of the experimental groups. It was observed that VEGF H-score values of CG and CoG groups were statistically significantly different from LLG and HLG groups [*p<0.05*]. VEGF H-scores were significantly lower in lycopene-given groups, regardless of dose. When LLG and HLG were compared in terms of anti VEGF H-score, no significant difference was observed [Table 2, Figure 3]. There was no significant difference between the groups in terms of VEGF and TAC in biochemical parameters except for MDA [Table 2]. Compared to the CoG group, MDA levels were statistically significantly lower in the CG and HLG [*p<0.05]* .

Discussion

Postoperative adhesions are a result of the cellular and biochemical response that occurs after peritoneal trauma while attempting to repair the peritoneum. Many methods have been used to reduce adhesion formation, such as reducing the initial inflammatory response, preventing fibrin formation, increasing fibrinolysis, preventing collagen deposition, and using a barrier against adhesion formation, and many studies have been conducted on them (19, 20) but today there is still no drug or method that can be used alone that can prevent adhesion formation satisfactorily. Many studies have shown that inflammatory cells and reactive oxygen species play a role in the formation of adhesion (21, 22). Adhesions are cellular and vascularized structures containing oxidants, angiogenic factors, and inflammatory cells (23). The anti-adhesion effects of antioxidants have been tested in various animal models (24, 25). In this study, we tried to examine the potential effect of lycopene, a potent antioxidant, antiproliferative, anticarcinogenic, and anti-inflammatory, on adhesion formation in rats with traumatized uterine serosa (9). To our knowledge, the present study is the first study that used lycopene as an adhesion inhibitor in an animal model.

MDA is produced by cells involved in the inflammatory response. It is a by-product formed by oxygen radicals breaking down lipid-containing structures such as plasma and cell membranes. It is a parameter used in evaluating both tissue damage and inflammation severity (26). In our study, a significant difference

was observed between the HLG and CG in terms of MDA levels [p-0.007]. Although MDA levels were found to be lower in the HLG than in the LLG, this difference was not statistically significant.

VEGF is an angiogenic cytokine that participates in the process of adhesion formation through the formation of new vessels (27). In this study, although there was no statistical difference between the groups in terms of VEGF release, numerically, more VEGF positive cells were encountered in the treatment groups compared to the CG.

Substances that prevent oxidations caused by free radicals and have the ability to capture and stabilize free radicals are called 'antioxidants'. The total effect of all antioxidants in body fluids is called total antioxidant capacity [TAC] (28). In this study, there was no significant difference between the groups in terms of TAC. Higher TAC levels were detected in the HLG but it was statistically insignificant.

Various methods have been used for grading intraabdominal adhesions. The systems that are made according to the percentage of traumatized adherent area used by Links et al. Or Leach et al.'s systems consisting of three different parameters are the most commonly used methods. (29). We used the system of Leach et al. In this system, adhesions are scored in three separate categories according to type, prevalence, easy or difficult separation. In our study, adhesion scores were significantly lower in the lycopene groups compared to the control group, which indicates the effectiveness of lycopene alone in reducing adhesion formation, in line with other studies (1). In in vitro studies, it was found that vitamin E has antioxidant, anti-inflammatory, anticoagulant and antifibroblastic effects; It has also been shown to reduce collagen production and suppress fibrin production (30). Lycopene has similar properties with vitamin E in terms of its antioxidant, anti-inflammatory and antifibroblastic effects. Corrales et al. applied vitamin E intraperitoneally and showed that its anti-adhesion effects were as much as bioresorbable membranes containing carboxymethyl cellulose. However, the same effect could not be shown after intramuscular injection (31). In another study, Yetkin et al. demonstrated that intraperitoneal vitamin E injection and human amniotic membrane separately reduced postoperative adhesion in rats; however, a synergistic increase in their effects could not be demonstrated with their co-administration (32). Similarly, colchicine, which has antifibrotic, anti-inflammatory, antihistamine, membrane stabilization and lipid peroxidation inhibition effects, is a plant-based drug. In cases where peritoneum is damaged, adhesions can be prevented by changing neutrophil migration to the region and the distribution of adhesion molecules on neutrophil and endothelial cells by using colchicine (33). The results of our study are consistent with these studies.

Our study had some limitations. First; It was the route of application of the anti-adhesion agent. We chose this method in our study because the intraperitoneal route is generally used in the literature. Another limitation is that the dose of intraperitoneal lycopene required to prevent adhesion is unknown. In this study, we used the doses used in animal models of oxidative stress.

Conflict of Interest: The authors declare that they have no conflict of interest.

Conclusion

Histological and mechanical parameters obtained in our study suggest that lycopene, which we use as an anti-adhesion agent, is effective in preventing intra-abdominal adhesions and does not negatively affect wound healing.

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Table-1: Macroscopic adhesion scoring method

Score	Extend	Severity	Degree
0	No	No	No
1	Less than 25%	Filmy avascular	Detached with gentle traction
2	Between 25-50%	Opaque, translucent, avascular	Detached with moderate traction
3	Between 50-75%	Vascular or opaque	Detached with sharp traction
4	More than 75%	Opaque, thick veins available	-

The adhesion score is equal to the sum of the scores from each part of the adhesion. The highest possible score is 11.

Variable	**Mean ± standart deviation**	**Mean ± standart deviation**	**Mean ± standart deviation**	**Mean ± standart deviation**	**P-value***	**p-value****
	CG	**CoG**	**LLG**	**HLG**		
VEGF	283,75±69,88	351,42±96,47	313±45,4	295±86,44	p-0.427	
TAC	0,77±0,32	0,58±0,13	0,68±0,37	0,74±0,32	p-0.636	
MDA	13±3,82[a]	20,48±5,29[a,b]	16,08±4,27	11,47±1,47[b]	P-0.005	p[a]-0.014 p[b]-0.007
Adhesion score	**Adhesion score**	**Adhesion score**	**Adhesion score**	**Adhesion score**	**Adhesion score**	**Adhesion score**
Extend	2,71±0,48[b,c]	2,85±0,37[d,e]	1,4±0,54[c,d]	1,4±0,54[b,e]	p-0.000	P[b]-0.001 p[c]-0.001 P[d]-0.001 P[e]-0.001
Severity	1,71±0,48[b]	2±0,81[d,e]	0,8±0,44[d]	0,4±0,54[b,e]	p-0.001	p[b]-0.007 p[d]-0.015 p[e]-0.001
Degree	1,42±0,78[b]	1,42±0,78[e]	0,4±0,54	0,2±0,44[b,e]	p-0.007	p[b]-0.029 p[e]-0.029
Total	5,85±1,21[b,c]	6,28±1,49[d,e]	2,6±1,14[c,d]	2±1,41[b,e]	p-0.000	p[c]-0.002 p[b]-0.000 p[d]-0.001 p[e]-0.000
Histopathological findings and VEGF H-Score	**Histopathological findings and VEGF H-Score**	**Histopathological findings and VEGF H-Score**	**Histopathological findings and VEGF H-Score**	**Histopathological findings and VEGF H-Score**	**Histopathological findings and VEGF H-Score**	**Histopathological findings and VEGF H-Score**
Inflammation	4,21±1,3	4,01±2,30	3,9±1,36	1,10±2,1*		
Edema	2,42,0±2,2	2,33±0,26	2,212±0,72	0,11±0,64*		
VEGF H-Score	271,30±2,41	265,12±0,96	152,23±1,10#	132,56±1,12#		

Table 2: Macroscopic adhesion score, biochemical, histopathological findings and VEGF H-Score results

CG: control group, CoG: corn oil group, LLG: low lycopene group, HLG: high lycopene group, VEGF: vascular endothelial growth factor, TAC: total antioxidant capacity, MDA: malondialdehyde

P[a]: comparison between CG and CoG, P[b]: comparison between CG and HLG, P[c]: comparison between CG and LLG, P[d]: comparison between CoG and LLG, P[e]: comparison between CoG and HLG

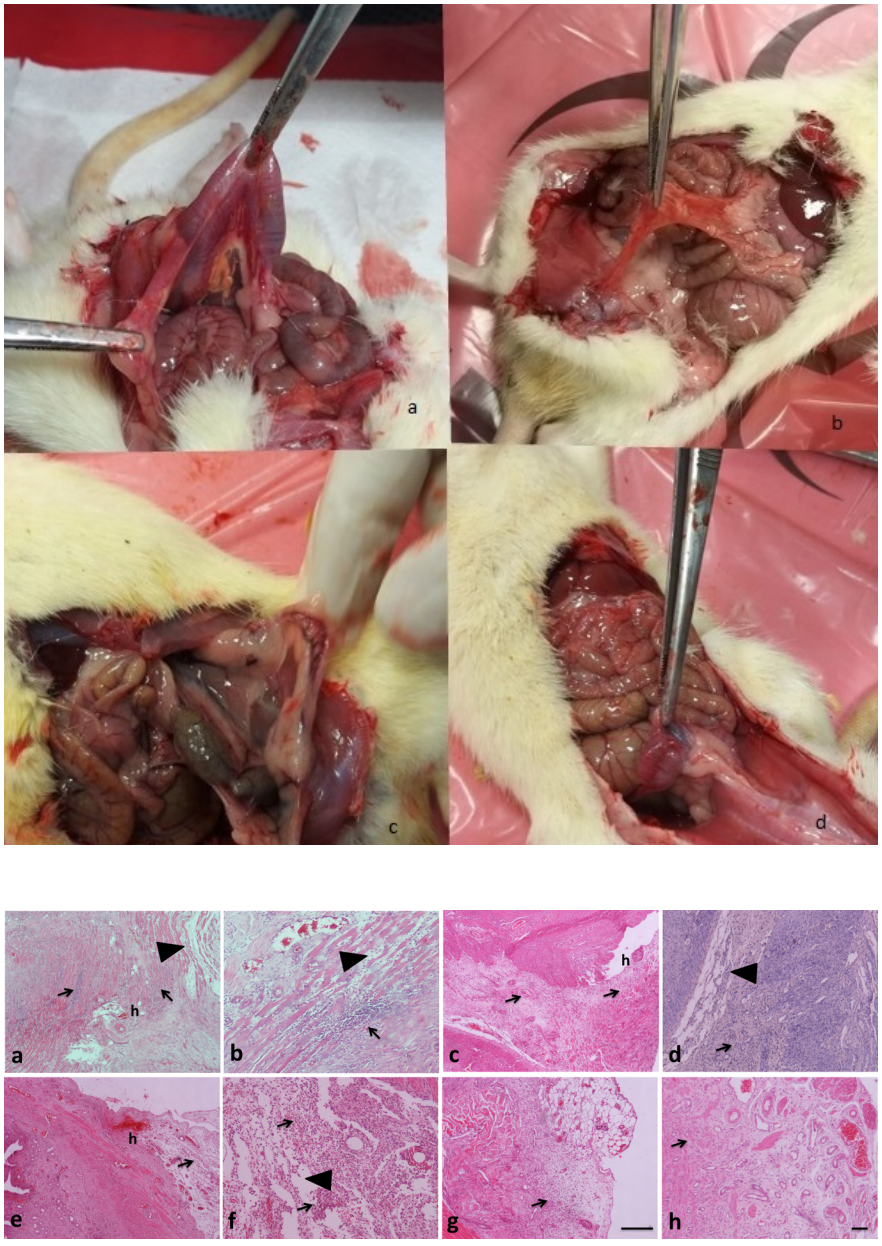
* Statistically significant difference compared to CG, CoG and LLG [p <0.05]. # Statistically significant difference compared to CG and CoG [p <0.05].

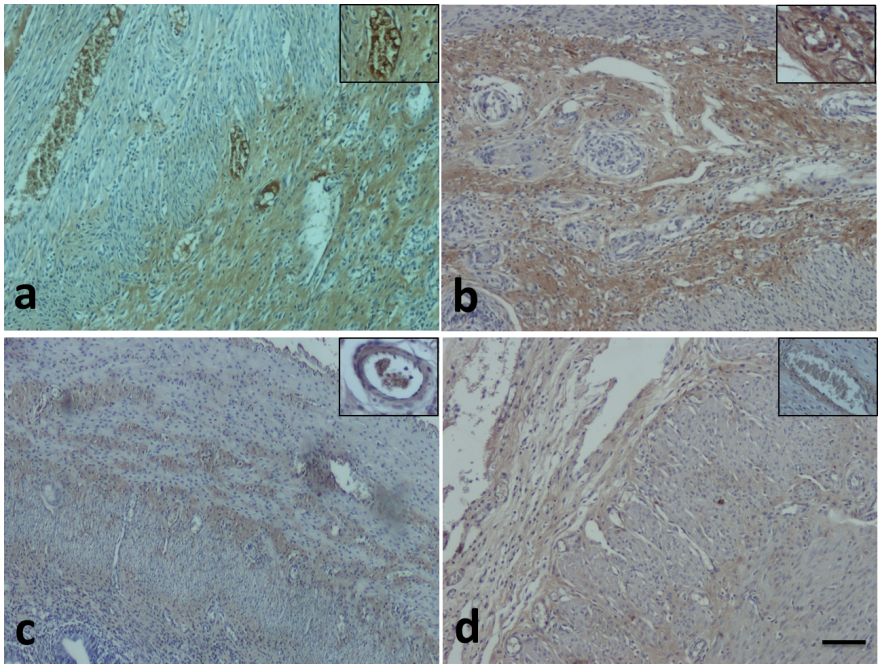
*Data was analysed using oneway anova test,**Data was analysed using Tukey HSD test

Figure 1: Adhesion examples; **a:** adhesions between bowel and uterine horn, **b:** fibrous adhesion between bowel and uterine horn, **c,d:** Complete adhesion to the anterior abdominal wall and bowel

Figure 2: a: CG *black arrow* ; high level of inflammation in the perimetrium and between smooth muscles,*arrowhead;* moderate edema, *h* ; hyperemia magnification 4X, **b:** CG *black arrow* ; high level of inflammation in the perimetrium and between smooth muscles,*arrowhead;* moderate edema, magnification 10X **c:**CoG *black arrow* ; high level of inflammation in the perimetrium and between smooth muscles, *h;* hyperemia magnification 4X, **d:** CoG *arrowhead;* moderate edema, magnification 10X, **e:** LLG *black arrow* ; high level of inflammation in the perimetrium, *h;*hyperemia magnification 4X, **f:** LLG *black arrow* ; high level of inflammation in the perimetrium, *arrowhead;*medium edema magnification 10X, **g:** HLG *black arrow* ; low level inflammation in perimetrium 4X, **h:** HLG*black arrow* ; low level inflammation in perimetrium, magnification 10X, Hematoxylin Eosin Staining.

Figure 3: Anti VEGF immunohistochemical staining. **a** : CG, **b:** CoG, **c:** LLG, **d:** HLG, magnification of large pictures is 10X, thumbnails represent vascular structures belonging to groups.





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