

**L-ARGINİN'İN NÖTROFİLLER ÜZERİNDEN POSTOPERATİF
ADEZYON OLUŞUMUNA ETKİLERİ****THE EFFECTS OF L-ARGININE ON POSTOPERATIVE PERITONEAL
ADHESION FORMATION BY ITS EFFECTS ON NEUTROPHILS**

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ÖZET: Cerrahi sonrası oluşan intraperitoneal adezyonlar, infertilite, ağrı ve barsak obstrüksiyonuna neden olan önemli komplikasyonlardır. Bu çalışma L-arginin ve nötrofillerin postoperatif intraperitoneal adezyon oluşumundaki etkilerini araştırmak amacıyla yapılmıştır. 16 sıçan iki eşit gruba ayrıldı; grup I (n=8) 5gün süreyle günlük tek doz % 0.9'luk izotonik sodyum klorür (salin), grup II (n=8) 5 gün süreyle günde tek doz L-arginin aldı ve uterin boynuz ve çekuma cerrahi hasar yapıldı. Adezyon indüklenmesinden sonraki 2 haftanın sonunda adezyon skoru belirlendi. Adezyon dokusu histolojik ve biyokimyasal olarak incelendi. Adezyon oluşumunun ilk günü nötrofil sayısı ve nötrofil adezivite indeksi tesbit edildi. Kontrol grubundaki adezyon gelişimi, L-arginin grubuna göre belirgin olarak daha az idi ($p < 0.01$). Kontrol grubu ile kıyaslandığında, L-arginin grubunda adezivite dokusu hidroksiprolin ve kollajen seviyeleri belirgin olarak artmıştı ($p < 0.05$ ve $p < 0.001$). Sonuç olarak, bu hayvan modelinde, L-arginin postoperatif adezyonları azaltmada yararlı bulunmamıştır.

Anahtar kelimeler: L-arginin, nitrik oksit, nötrofil, peritoneal adezyon.

SUMMARY: Postoperative intraperitoneal adhesions are major postsurgical complications that cause infertility, pain, and intestinal obstruction. The aim of this study was to investigate the effects of L-arginine and neutrophils on the formation of postoperative intraperitoneal adhesion. Sixteen rats, equally divided into two groups; group I (n=8) received daily single dose saline for five days, group II (n=8) received daily single dose L-arginine for five days, and underwent surgical injury to the uterine horn and the cecum. The adhesion scores were determined at the end of the second week after adhesion induction. Adhesive tissue also histologically and biochemically evaluated. Neutrophil counts and neutrophil adhesivity index were determined in the first day of adhesion induction. Adhesion formation in the control group was significantly less than that of L-arginine group ($p < 0.01$). Hydroxyproline and collagen levels of adhesive tissue were significantly increased in the L-arginine group when compared with control group ($p < 0.05$ and $p < 0.001$). We concluded that L-arginine was not beneficial in reducing postoperative adhesions in this animal model.

Key Words: L-arginine, nitric oxide, neutrophils, peritoneal adhesion

Postoperative adhesions after abdominal and gynecologic surgery remain a major clinical concern because of causing intestinal obstruction and infertility. A number of risk factors for peritoneal adhesions have been identified, and a variety of drugs have been used with varying degrees of

success to prevent adhesion formation. However, the pathogenesis of adhesion formation is still not well understood. Studies suggest that 18-20% of the adhesions are caused by inflammation (1). We previously demonstrated that neutrophils, one of the important inflammatory cells, have a role in modulating postoperative adhesion formation (2). Nitric Oxide (NO), synthesized from the amino acid L-arginine, is likely to have a multifaceted role in inflammatory reactions, ranging from the enhancement of vasodilatation and the formation of edema through modulation of sensory nerve endings and leukocyte activity, to tissue cytotoxicity (3). NO is also generated by an inducible nitric oxide synthases in human neutrophils and it plays an important role in neutrophil functions at the inflammatory site (4). But, the

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importance of the interaction between L-arginine - NO pathway and neutrophils in peritoneal healing mechanism is poorly understood.

Although there are limited studies on the effects of L-arginine on postoperative intraabdominal adhesion formation (5,6), the pathogenesis of these effects is not clear. As known, nitric oxide has vasodilatory effect and inhibits neutrophil infiltration (7,8). In this study, we aimed to investigate the role of L-arginine on formation of postoperative peritoneal adhesion by its effects on neutrophils.

MATERIAL-METHOD

Wistar albino rats weighing 190-220 gr were purchased from DETAM (Experimental and Medical Research Center) of Istanbul University and kept on a standard laboratory diet and tap water. The animals were fasted for twelve hours prior to surgery. This study was approved by the ethical committee of Kocaeli University and performed following standard guidelines for the care and use of laboratory animals. The animals were randomly divided into two groups, group I (n=8) receiving saline, group II (n=8) receiving L-arginine (Sigma Chem. Co, St. Louis, USA). Midline laparotomy was performed under ketamine-HCl (60 mg/kg) and xylasin (4 mg/kg) anesthesia using a clean surgical technique. The serosa was stripped from the uterine horn at midportion over 1.5 cm segment and the cecum was grasped and denuded of serosa over a 2 cm length until punctuate hemorrhage occurred. The abdomen was closed at the end of the surgery in two layers with a continuous 2/0 silk (Ethicon) suture (9,10). One ml saline solution for group I and 300 mg/kg of L-arginine in 1 ml saline solution for group II were instilled into the peritoneal cavity after induction of adhesion formation for following five days.

Adhesion Assessment:

The grading system (11) was used to determine the adhesion score at the end of the second week after adhesion induction, which is practical, easy and detailed, by a pathologist who did not know anything about groups.

0, no adhesion;

1, adhesion on 25% of the traumatized area

2, adhesion on 50% of the traumatized area and

3, total area involved. The severity of the adhesions was evaluated according to the scale.:

0.0 no resistance to separation;

0.5 moderate force required for separation;

1.0 sharp dissection needed for separation.

A total score was obtained by adding the two grading scales with a range from 0.0 to 4.0 assigned for each region evaluated. The total score represented both extent and severity.

Histological Assessment

Preparation of tissues for light microscopy

The tissue from adhesions of each animal was fixed with formaldehyde for 24 hours, dehydrated and embedded in paraffin wax. The thickness of the sections was 5 μ m and they were stained with hematoxyline and eosin.

Preparation of tissues for electron microscopy

The abdominal cavity was opened and five tissue samples from adhesions of each group were flooded with 2.5 percent glutaraldehyde in 0.1 M cacodylate buffer at 4°C. Fixation was continued for 24 hours. Primary fixation was carried out using 1 per cent osmium tetroxide in 0.1 M phosphate buffer. Tissue fixed in glutaraldehyde was washed in several changes of buffered sucrose and then post fixed for 2 hours in 1 percent osmium tetroxide in 0.1 M phosphate buffer. The specimens were subsequently dehydrated in graded solutions of ethanol, passed through epoxypropane and embedded in Epon 812. Sections 1 μ m thick were cut on a Reichert microtome. Such sections were stained with uranyl acetate and lead citrate prior to examination through a Jeol 100-c electron microscope.

Biochemical Analysis

Adhesive tissue samples were removed surgically and immediately frozen. These tissue samples were homogenized. Purified acid-soluble collagen (dissolved in 50mM acetate buffer, pH3.5) was included as a test sample for the estimation of hydroxyproline (Hyp). Aliquots of standard Hyp prepared from stock solution and test samples were mixed gently with sodium hydroxide in a total volume of 50 μ l. Samples were hydrolyzed. 450 μ l of chloramine-T was added to the hydrolyzate, mixed gently, and oxidation was allowed to proceed for 25 min at room temperature. 500 μ l of Erlich's aldehyde reagent was added to each sample, mixed gently, and the chromophore was developed by incubating the samples at 65°C for 20 min. Absorbency of each sample was read at 550 nm by a spectrophotometer. The collagen content of adhesive tissue was calculated (12).

Studies on Neutrophil Count and Neutrophil Function

White Cell Count, Neutrophil Count:

Peripheral blood for blood smears and quantitation of the absolute numbers of circulating leukocytes was obtained by tail bleeding. The total circulating white blood cell count/cu mm was determined with a Coulter counter. The percentage and absolute number of circulating white blood cell subsets was determined after performing a differential count of at least 100 cells/smear. Neutrophil Adhesivity: Siliconized glass beads 0.1 mm in diameter were packed to a height of 3 cm over a small piece of glass wool in a siliconized glass syringe 1 cm in diameter, the whole being enclosed in a water jacket at 37°C. Two ml of heparinized whole blood were added to the column, and, after 10 minutes, allowed to flow out from the column. The flow rate was adjusted by controlling the pressure on the plunger of the syringe to 0.1 ml/minute. An absolute neutrophil count (total white-cell count/ mm³ x percent neutrophil) was performed in both influent and

effluent blood and the ratio of the former to the latter termed as the adhesivity index (13).

Statistic

Data in this paper were expressed mean SE. The statistical analysis of adhesion scores and parameters of both groups were carried out using unpaired student t-test. A p value of <0.05 was considered to be statistically significant.

RESULTS

There were no intraoperative and postoperative complications in any of the rats in the experiment.

Adhesion Scores

Adhesion formation was observed in 8/10 and 10/10 rats of saline and L-arginine groups, respectively. The mean combined score of involvement and the character of adhesions were 1.750 ± 0.25 and 3.120 ± 0.23 in saline (group I) and L-arginine (group II) groups, respectively. Adhesion formation in the control group was significantly less than that of L-arginine group ($p < 0.01$) (Table 1).

Hydroxyproline and Collagen Levels

Hydroxyproline and collagen levels in surgically removed adhesive tissue were significantly increased in the L-arginine group ($p < 0.05$ and $p < 0.001$ for Hydroxyproline and Collagen levels, respectively) (Table 1).

Table 1. Adhesion score and tissue hydroxyproline and collagen levels for both groups.

	Adhesion Score	Hydroxyproline Level (G/100g tissue)	Collagen Level (g/100g tissue)
Group I	$1.75 \pm 0.25^{**}$	$2.8 \pm 0.13^*$	$0.17 \pm 0.03^{***}$
Group II	3.12 ± 0.23	3.89 ± 0.21	0.38 ± 0.01

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Histological Results

Light Microscopical Findings

Group I: Abundant fibroblasts and macrophages with several neutrophils and collagen fibers with other inflammatory cells were determined (Figure 1a).

Group II: Inflammatory granulation tissue with several fibroblasts, more neutrophils and macrophages among collagen fibers were observed (Figure 1b).

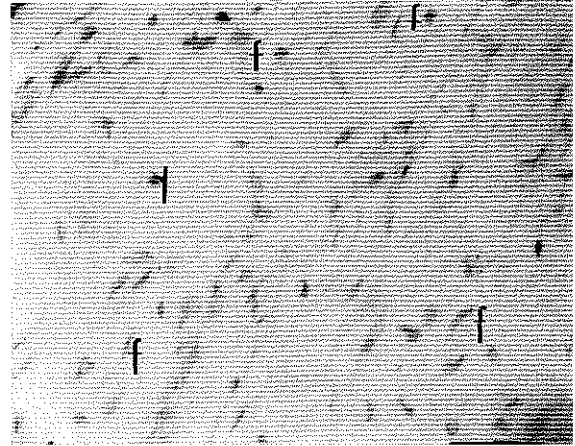
Electron Microscopical Findings

Group I: Several fibroblasts were seen in adhesive tissue. New capillaries are also seen among sparse collagen fibers (Figure 2a).

Group II: Collagen formation was well advanced. Fibroblasts were observed amongst the collagen bundles. Macrophages and other inflammatory cells could be seen in adhesive tissue (Figure 2b).

Figure 1 a) Group I: PMN and mononuclear cells and fibroblastic activity (f: fibroblast) (H&E)

b) Group II: Granulation tissue with abundant PMN, mononuclear cells, fibroblast and vascularization (H&E). (f: fibroblast, b: blood vessels, l: lymphocyte, p: neutrophil, m: macrophage, h: histiocyte, po: plasma cell) (1 bar = 100 μ m)



Neutrophil Counts and Neutrophil Adhesivity Index

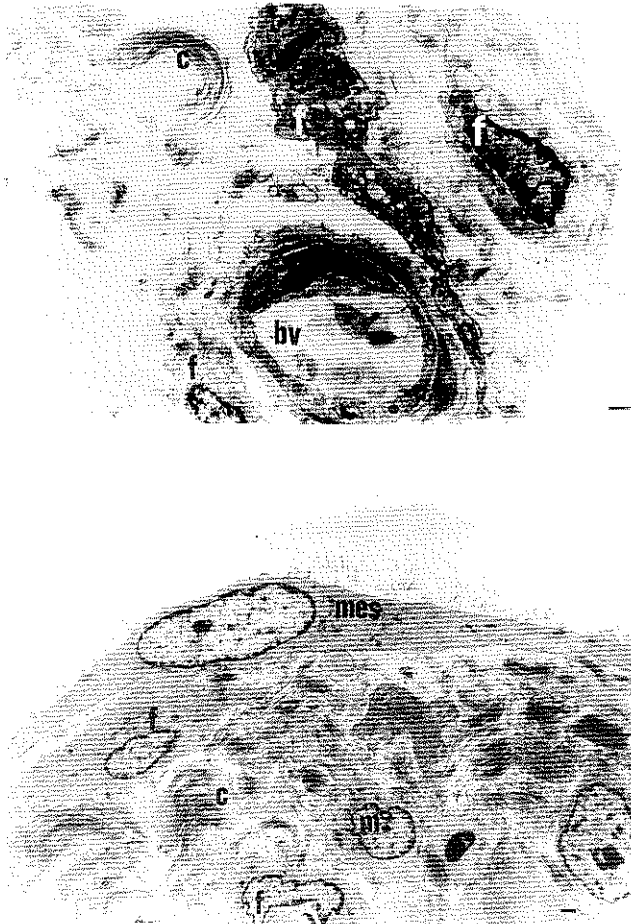
The neutrophil counts were recorded for each group and were found to have increased in L-arginine administrated group II ($p < 0.05$). The neutrophil adhesivity index was found to have increased significantly in group II when compared group I ($p < 0.05$) (Table 2).

Table 2. Blood neutrophil counts and neutrophil adhesivity index for each group

	Neutrophil Counts (/mm ³)	Neutrophil adhesivity index
Group I	3581.6 ± 366.1	26.08 ± 3.13
Group II	$4137.8 \pm 435.2^*$	$45.30 \pm 6.99^*$

* $p < 0.05$

Figure 2 a) Group I: Fibroblasts were abundant in adhesive tissue: A capillary vessel is seen amongst sparse collagen fibers. (f: fibroblast, bv: capillary and c: collagen)
 B) Group II Intact mesothelial cell and two fibroblasts amongst collagen bundles. Macrophages could be seen in tissue. (mes: mesothelial cell f: fibroblast m: macrophages) (1 bar=1m)



DISCUSSION

Postoperative abdominal adhesions represent a major surgical problem. The etiology of pelvic and abdominal adhesions includes previous surgical trauma, infections, hemorrhage, and other types of peritoneal irritation such as endometriosis and foreign body reactions, of these factors operative procedures to be the most important contributing factor (14,15). The postoperative complications due to surgical adhesions has attracted increasing attention by many investigators seeking agents and methods aimed at preventing adhesion formation. In addition to microsurgical techniques to minimize trauma, many substances have been used to prevent adhesion formation, mostly studied substances such as; antibiotics, progestins, dextran-70, corticosteroids, antihistaminic, anticoagulants, anti-inflammatory drugs, mast cell stabilizing agents, sodium carboxy-methyl cellulose, fibrinolytics, and some barrier methods (16,17). In rat uterine horn model, we aimed to compare the adhesion

formation of control group and L-arginine group.

The discovery that mammalian cells generate nitric oxide, was provided important information about many biologic processes. Nitric oxide which is an important mediator of homeostatic processes and host defense mechanisms, is synthesized from the amino acid L-arginine. Changes in its generation or actions contribute to pathological states (3). Since NO exerts powerful biological effects in different tissues and is generated by vascular tissue and by inflammatory cells, it is likely that the formation of NO from L-arginine is involved in the genesis and development of signs and symptoms of inflammation (18,19). Also, neutrophils and macrophages express oxidative L-arginine deiminase (OAD) activity in vitro, metabolizing L-arginine to L-citrulline and reactive nitrogen intermediates including nitric oxide (20). As seen in our previous study (2), the process of inflammation and repair that follows tissue injury is characterized by temporarily ordered cellular response that includes, sequentially, neutrophils, macrophages and lymphocytes and fibroblasts. In present study, we histologically observed abundant neutrophils, lymphocyte, macrophage and fibroblast infiltration in adhesive tissue in group II when compared with group I.

In this study, the adhesion score of untreated control group was significantly lower than that of L-arginine group. There is no report on the usage of L-arginine for the prevention of adhesion formation in the literature except one study. Contrary to our results, Kaleli et al. (5) found that intraperitoneal administration of L-arginine was effective in reducing adhesion formation. This contradiction may be explained as follows. Our histological evaluation demonstrated increased inflammatory cells such as neutrophils, macrophages and fibroblasts in adhesive tissue of both groups especially L-arginine group. The release of an NO like material has also been detected from peritoneal neutrophils, which are themselves inhibited from aggregating by NO. The formation of NO from L-arginine is involved in the genesis and development of the signs symptoms of inflammation (18). In our study; in vitro neutrophil adhesivity index in L-arginine group increased when compared with control group. This results was similar to previous study done by Fukatsu et al. (21) They found that the administration of N-omeganitro-L-arginine methyl ester (L-NAME) which is an antagonist of NO, decreased neutrophil adhesion in the peritoneum. This findings suggest that increase of tissue NO levels causes inflammation. Neutrophil-Endothelial Cells adherence and consequently neutrophil activation and migration into the peritoneum may reduce neutrophil-dependent collagen degradation, which mediated by the generation of oxygen free radicals and release of neutral protease, and results in an increase in adhesion formation (22).

We determined increased hydroxyproline and collagen levels in adhesion tissue in L-arginine treated group when compared control group. In addition the effects of L-

arginineNO pathway on neutrophils and fibroblasts, this increased contents of collagen may be explained via increase the local cellular demand for proline for the synthesis of collagen during repair. Indeed, it has been demonstrated that the size of the available proline pool determines the rate of collagen synthesis under conditions of rapid collagen production, and that the local synthesis of proline from its metabolic precursors, namely ornithine, arginine, glutamate, and glutamine, is enhanced in these circumstances, apparently to compensate for the relative deficiency of proline (23).

Finally, intraperitoneal administration of L-arginine increased the intraperitoneal adhesion formation in an experimental rat model. This demonstrates that external application of L-arginine may be not beneficial for prevention of adhesion formation. This result must make scientists to investigate the role of NO on formation of postoperative intraperitoneal adhesion formation. To explain contradictory results in literature, new and detailed studies must be planned.

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