# Effects of melatonin and phospholipid on adhesion formation and correlation with vascular endothelial growth factor expression in rats

## Melatonin ve fosfolipidin sıçanlarda deneysel peritoneal adezyon oluşumu üzerine etkisi ve vasküler endotelyal büyüme faktörü ile ilişkisi

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***BACKGROUND***

The aim of this experimental study was to investigate the effects of melatonin and phospholipid on adhesion formation and the correlation with vascular endothelial growth factor (VEGF) expression in rats.

***METHODS***

Sixty Wistar-Albino rats were divided into four groups as sham, control and two study groups, each including 15 rats. In the sham group, laparotomy was the only procedure. Left lower parietal peritoneum was abraded after laparotomy and serosal defects formed on the cecum, ileum and right uterine horn in the study and control groups. Ringer lactate was then applied to the control group, while melatonin and phospholipid suspension were applied separately in the two study groups. Relaparotomy was performed in all groups on the 15th day to score and evaluate the adhesion formation.

***RESULTS***

Adhesion formation was significantly lower in the sham, melatonin and phospholipid groups than in the control group ($p<0.05$). VEGF staining was significantly higher in the control group with adhesion areas compared to the other groups ($p<0.05$). When VEGF staining was compared, there was no significant difference between VEGF-stained and normal areas in the melatonin and phospholipid groups.

***CONCLUSION***

Melatonin and phospholipid decreased the adhesion formation in an experimental adhesion model in rats. There is a correlation between adhesion severity and VEGF expression.

***Key Words:*** Melatonin; peritoneal adhesion; phospholipid; vascular endothelial growth factor.

***AMAÇ***

Deneysel adezyon formasyonu oluşturulan sıçanlarda, melatonin ve fosfolipidin adezyon gelişimine etkilerinin vasküler endotelyal büyüme faktörü (VEGF) ekspresyonu ile ilişkisi araştırıldı.

***GEREÇ VE YÖNTEM***

Çalışmada 60 adet Wistar-Albino cinsi sıçan, rastgele 15'erlik sham, kontrol ve iki adet çalışma gruplarına ayrıldı. Sham grubuna sadece laparotomi yapıldı. Kontrol grubu ve çalışma gruplarında sağ alt kadranda pariyetal peritonda abrazyon yapıldı, çekum, ileum ve uterusun sağ boynuzunda serozal defekt oluşturuldu; kontrol grubunda periton boşluğuna Ringer laktat verildi. Çalışma gruplarından birine melatonin, diğerine fosfolipid süspansiyonu verildi. Bütün gruplarda 15 gün sonra relaparotomi yapılarak, adezyon gelişimi değerlendirilip skorlama yapıldı.

***BULGULAR***

Kontrol grubuna göre sham, melatonin ve fosfolipid gruplarında adezyon gelişimi belirgin olarak düşüktü ($p<0,05$). Kontrol grubunda yapışıklık olan alanlarda VEGF boyaması diğer gruplara göre anlamlı derecede yüksekti ($p<0,05$). Melatonin ve fosfolipid grubunda VEGF boyaması ile normal alanlardaki VEGF boyaması arasında anlamlı fark yoktu ($p>0,05$).

***SONUÇ***

Deneysel adezyon modeli oluşturulan sıçanlarda melatonin ve fosfolipid adezyon gelişimini azaltmaktadır. Gelişen adezyonun şiddeti ile VEGF ekspresyonu arasında paralellik vardı. Şiddetli adezyon gelişen alanlarda VEGF ekspresyonu artmıştı.

***Anahtar Sözcükler:*** Melatonin; peritoneal adezyon; fosfolipid; vasküler endotelyal büyüme faktörü.

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Peritoneal adhesions, which develop after abdominal and pelvic surgery, have been a great problem from the beginning of modern surgery till today. Peritoneal adhesions lead to clinical problems such as intestinal obstruction, chronic abdominal pain, infertility and chronic pelvic pain. When abdominal surgery is needed repeatedly, organ injuries due to peritoneal adhesions may occur and these in turn increase the morbidity and mortality. Many studies have been conducted in order to resolve this problem, to understand the pathophysiology of adhesion formation and to prevent its formation in the light of this data.[1]

Postoperative adhesions occur between the traumatized serosal surfaces during surgery. Inflammation develops after tissue injury and the wound healing process begins. When the fibrin gel structure, which develops during wound healing, is not broken down by the fibrinolytic activity, permanent fibrous tissue forms and adhesion develops.[1]

Melatonin is a neurohormone that is released from the pineal gland. It has been shown in recent studies that melatonin has antioxidant effects by blocking free hydroxyl and peroxyl radicals.[2,3] Melatonin also has an antiadhesive effect and decreases leukocyte-mediated endothelium injury.[4]

Phospholipid is a surfactant-like substance, and is released by mesothelial cells. Phospholipid secretion forms a slippery, oligolamellar layer on the parietal peritoneum and abdominal organs. Tissue trauma during surgery causes lesion on this layer and also decreases phospholipid secretion from mesothelial cells, thus the protective layer on the serosal surfaces dissolves. Regarding this data, it has been thought that application of phospholipids into the peritoneal cavity after surgery may decrease adhesions.[5]

Vascular endothelial growth factor (VEGF) is the most effective angiogenesis factor ever defined as being different from the other angiogenic factors. The most important feature of VEGF is that it only targets endothelial cells. It has been shown that VEGF has effects on early wound healing and fibrosis formation.[6]

The adhesion-preventing effect of melatonin after pelvic surgery was studied only once.[7] The adhesion-inhibiting effect of phospholipid was previously shown in many studies.[5] In this study, its efficacy was compared with melatonin for the first time and melatonin's suitability or not for use in this area was investigated. VEGF expression in experimental adhesion models was evaluated previously.[8] In our study, the VEGF expression in adhesion-positive and normal areas was compared. VEGF expression in adhesion-positive areas in all groups was also investigated and its relation with adhesion severity was evaluated.

## MATERIALS AND METHODS

The study was performed at Erciyes University Hakan Çetinsaya Experimental Research Center. Ethical consent was taken from the medical school's ethical committee.

### Experimental Design

Sixty female Wistar-Albino rats bred from the same research center, weighing between 190 and 230 grams and aged 24 to 32 weeks, were used for this study. Rats were randomly divided into four groups as sham, control and study (n: 2; melatonin or phospholipid) groups. Each group included 15 rats.

### Surgical Procedure

Rats were fasted for 12 hours preoperatively. The animals were anesthetized by intramuscular injection of ketamine hydrochloride (10 mg/kg). The skin was cleansed with povidone-iodine after shaving. Laparotomy was performed with midline incision. Then, 4 cm² of serosa from the cecum and ileum and 2 cm² of serosa from the right uterine horn medial part were removed. Abrasion was also performed in order to create spot hemorrhage on an approximately 6 cm² area of the parietal peritoneum in the lower right and lower median quadrants. Surgical procedure was terminated in the sham group rats after laparotomy and palpation of right lower quadrant organs, periadnexal region and the parietal peritoneum. The abovementioned experimental model was applied to the control, melatonin and phospholipid group rats in order to obtain an adhesive formation.

Melatonin solution and phospholipid emulsion were applied into the peritoneal cavities of each study group. The melatonin powder (Sigma; St. Louis, MO, USA) was thawed with 99% ethanol and diluted with 2 cc physiologic saline (0.9% NaCl) solution in order to obtain 1 mg melatonin in 1 cc serum (10 mg/kg, approximately 2 mg/2 cc melatonin per rat). The ethanol concentration was set to 0.5%.[7] Phospholipid emulsion was given as 75 mg/5 ml/kg (2 ml phospholipid emulsion per rat).[9] After the surgical procedure, the fascia and the peritoneum were closed together with 4/0 catgut, the skin was closed

with 4/0 silk and the incision site was cleaned with povidone iodine. In the early postoperative period, oral intake of water and food was allowed.

One rat each in the control and melatonin groups died during the study, and new rats were taken into the study. Rats were sacrificed with a lethal dose of pentobarbital after 15 days and the adhesion formation was evaluated by a laparotomy with left paramedian incision. In all groups, tissue samples were taken from all serosal surfaces on which adhesion had developed (including the serosal surfaces of the adhesion between the two visceral peritonea and/or the visceral and parietal peritoneum). Additional samples were taken from the serosal surfaces on which there was no adhesion for comparison purposes in the control group. The tissues with adhesion were kept in formalin for pathological evaluation.

**Parameters**

***A. Adhesion score***

The scoring system defined by Linsky et al.[10] was used for adhesion scoring. In this system, the prevalence and severity of adhesion were evaluated separately, then the two values were added and the total adhesion score was calculated. Scoring according to this system was done as follows: prevalence scoring: no adhesion=0, adhesion on 25% of the traumatized area=1, adhesion on 50% of the traumatized area=2, and adhesion on the whole traumatized area=3. Severity scoring: adhesions that can be separated without any resistance=0, adhesions that can be separated using moderate power=0.5, and adhesions that can be separated only with sharp dissection=1. Two scores were summed for each rat and the total score was calculated, which ranged between 0 and 4.

***B. Histopathological investigation***

*VEGF:* For VEGF antibody, staining density and severity were evaluated in the areas where the stained cells were mostly found in both adhesion-positive and normal tissues. Results were evaluated as follows: 0= no staining, 1= suspected, 2= mild, 3= moderate, and 4= strongly positive.[11]

*Immunohistochemical staining procedure:* after paraffin was saturated in the tissues, which were stored in 10% formalin, 5 µm-thick sections were obtained from the specimens and were put on poly-L-lysin-coated slides. Specimens that would be stained by VEGF were kept at 60ºC for an hour, then exposed to xylol and decreasing alcohol series and then washed with water. They were then incubated with EDTA for 20 min in microwave oven and were left to rest for cooling. Specimens were washed with distilled water before and after hydrogen peroxide 3% application for 10 min. Tissues were washed with saline solution with phosphate buffer (PBS) for 10 min. Then, 1/10 diluted VEGF (Neomarkers®, ready to use) antibody was applied to tissues. They were washed with PBS for 10 min. Biotin was dripped to tissues and waited for 10 min, then washing with PBS for 10 min was repeated. Streptavidin was dripped and waited for 10 min, then specimens were washed with PBS for 10 min. Chromogen was dripped and waited for 10 min, and they were washed with distilled water again. Specimens were kept in Meyer-hematoxylin for 3 min, then were washed with distilled water again, passed from increasing alcohol series. After resting in xylol for 15 min, preparations were closed by closing solution.[11]

**Statistical Analysis**

The data are presented as median. Nonparametric Kruskal-Wallis test was used to compare the histopathological scores. The Dunn test was used as the post-hoc test. The data were analyzed with the Statistical Package for the Social Sciences (SPSS) for Windows (version 13.0). Significance was set at $p<0.05$.

## RESULTS

*Adhesion score results:* There was no adhesion in two of the rats in the sham group (13%). In 11 out of 13 rats in this group, in which adhesion developed, adhesion was between the omentum and the incision line. In the control group, there was adhesion in all rats and it was between the omentum or organs and the incision line in 12 out of 15 rats. There was no adhesion in 6 rats in each of the melatonin and phospholipid groups (40%).

The total adhesion score in sham, melatonin and phospholipid groups were significantly low compared to the control group ($p<0.05$), while there were no significant differences between the sham, melatonin and phospholipid groups ($p>0.05$) (Fig. 1).

When the adhesion severity and prevalence scores were compared separately, the sham, melatonin and phospholipid groups had significantly less severity than the control group ($p<0.05$). There were no significant differences between the sham, melatonin and phospholipid groups ($p>0.05$) (Fig. 2, 3).

*VEGF staining results:* No samples were taken for VEGF staining from the rats in the sham, mela-

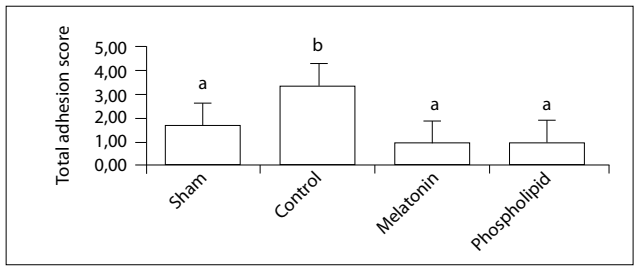


**Fig. 1.** Total adhesion score values. *Values represent median. Values with different letters indicate significant difference.*

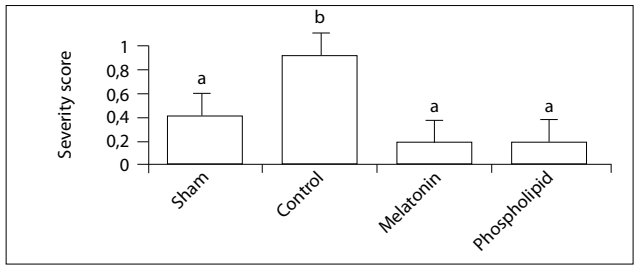


**Fig. 2.** Adhesion severity score values. *Values represent median. Values with different letters indicate significant difference.*

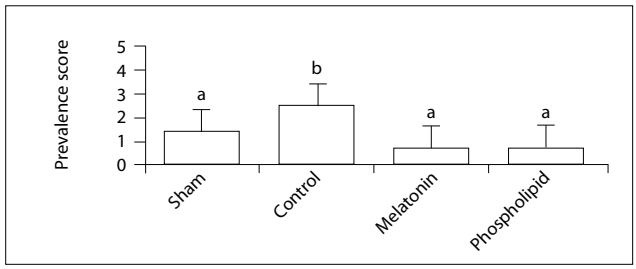


**Fig. 3.** Adhesion prevalence score values. *Values represent median. Values with different letters indicate significant difference.*

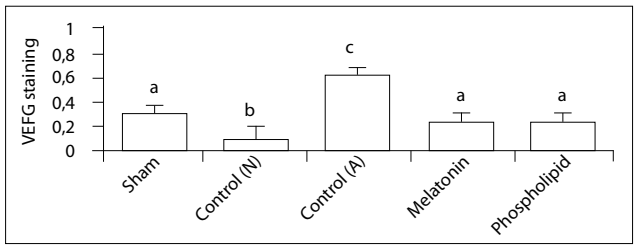


**Fig. 4.** VEGF staining values. *Values with different letters indicate significant difference. VEGF: Vascular endothelial growth factor; N: Normal areas; A: Adhesive areas.*

tonin and phospholipid groups in which there were no adhesions, and these rats were excluded from the statistical comparison. In the control group, VEGF staining in normal areas was significantly low compared with the adhesion- positive areas in the control and sham groups ($p<0.05$). There was no significant difference between the VEGF staining results of the normal areas in the control group and the VEGF staining results of the adhesion areas in the melatonin and phospholipid groups ($p>0.05$). The VEGF staining results of the adhesion areas in the control group were significantly higher than those of the other groups ($p<0.05$). There was no significant difference between the sham, melatonin and phospholipid groups ($p>0.05$) (Fig. 4, 5).

## DISCUSSION

Peritoneal adhesion is actually the result of normal wound healing. However, it is different from classical wound healing due to its localization on serosal surfaces. The key step, which initiates the peritoneal adhesion, is the damage of the mesothelial cells on the serosal surfaces. Serosanguineous exudate occurs after emergence of the subserosal connective tissue, and this in turn forms the soft fibrin gel matrix within 72 hours. This fibrin gel matrix is normally fractionated and removed by the fibrinolytic activity within the mesothelial cells. However, when there is a decrease in the fibrinolytic activity or if the fibrin gel formation is too much for the fibrinolytic activity, dense fibrinous and vascular changes develop within 15 days.[1] Thus, we decided on the relaparotomy time in our study as the postoperative 15th day.

After understanding the pathophysiology of the adhesion formation, in order to break its cascade, various agents that are effective on different steps of this cascade have been used. Fibrinolytic agents,[12] crystalloid solutions,[13] corticosteroids,[14] heparin,[15] hyaluronic acid,[16] non-steroidal antiinflammatory agents,[17] calcium channel blockers,[18] progesterone,[19] nonspecific immunostimulating beta-glu-

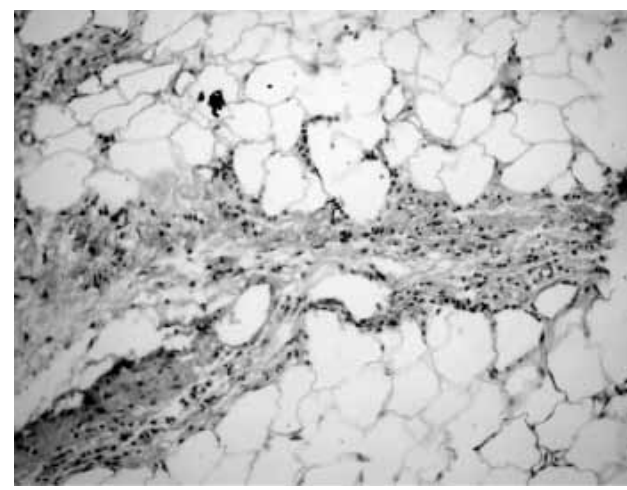

**Fig. 5.** VEGF staining in the control group.

can,[20] and barrier methods[21] were used. Although effective results have been obtained with these agents, several determined side effects that lead to serious clinical problems have restricted their usage.

In our study, although we only performed laparotomy and palpated the right lower quadrant organs and the parietal peritoneum and then closed the abdomen within 3-4 min in the sham group, adhesions developed in 13 out of 15 (86%) rats in this group. The sutures used on the parietal peritoneum might have contributed to the adhesion on the incision line by increasing ischemia and the VEGF expression secondarily. These results support studies that have suggested not closing the parietal peritoneum using suture materials.[22] The parietal peritoneum was closed with catgut in our study; catgut might also have contributed to the adhesions on the incision line due to its tissue reaction. Having no serosal defects or hemorrhage led to low severity adhesions (average severity score 0.43±0.31). These results also show that even just touching the organ and exposing the abdominal cavity to air may lead to adhesions. In the control group, there were adhesions between the omentum and the incision line in 12 out of 15 rats (80%), and there were adhesions between the right lower quadrant organs and the abdominal wall in all of them (100%). These results are consistent with the data that serosal defects and the accompanying clots increase both the prevalence and the severity of the adhesions.

One of the most important effects of melatonin is its antioxidant activity. *In vitro*[23] and *in vivo*[24] studies showed that melatonin has highly toxic hydroxyl radical and other oxygen-based radical-scavenging effects. Melatonin was found to be more effective than other known antioxidants (such as mannitol, glutathione, vitamin C, vitamin E).[25] Melatonin concentration should be more than its midnight peak value in order to observe the antioxidant effects. Thus, the antioxidant effect in humans is only possible at pharmacological doses.[26]

Free oxygen radicals are molecules that are released from neutrophils and macrophages and are efficient in the early period of inflammation. Free radicals target DNA proteins and lipids. They attach to membrane lipids and cause peroxidation and lesion of the membrane, which in turn increase microvascular edema. It has been shown in recent years that oxygen-derived free radicals and their metabolites play a role in leukocyte-related inflammatory reactions and increased cell and tissue damage.[27] Considering that melatonin may lead to decrease in adhesion formation with its antioxidant effect, Ozçelik et al.[7] applied melatonin in various doses, durations and means in an experimental pelvic adhesion model in rats and reported that melatonin significantly decreased adhesion formation in all study groups compared to the control group. The results of this study support that melatonin significantly decreases adhesion formation. There was no significant difference between the melatonin and sham groups with respect to adhesion formation, and severity scores were lower in the melatonin group (0.2±0.25), which suggests that melatonin blocks the inflammatory cascade. Experimental and clinical studies with melatonin showed that the dose range is wide and it has no important side effects. Ozçelik et al.[7] showed that adhesion formation was blocked by either local or systemic application. In light of these advantages, melatonin may be a possible agent for clinical usage as an antiadhesive.

Phospholipids are the components of the peritoneal fluid, which is normally found in the peritoneal cavity. These surfactant-like substances form a slippery layer on the peritoneal surfaces.[28] Following trauma, the phospholipid layer on peritoneal surfaces decreases as does the release of phospholipid due to inflammation. Yet, phospholipid covers the surfaces of serosal defects as a thin film layer and prevents adhesion formation by blocking the damaged opposing surfaces. The adhesive blocking effects of phospholipids have been shown in many studies and no important side effect has been reported. In our study, a significant decrease in adhesion formation was detected in the phospholipid group compared to the control group, while no difference was found between the sham and phospholipid groups. In view of the absence of side effects, their physiologic nature and ease of application, phospholipids are hopeful agents for clinical usage.

No significant difference was detected between the melatonin and phospholipids groups ($p>0.05$); no adhesions necessitating sharp dissection formed in either group. Efficacy of phospholipids has been shown by many studies to date.[29] In this study as well, the efficacy of melatonin was found to be similar to that of phospholipids, which suggests that melatonin may be an efficient agent to prevent adhesion formation.

VEGF is the most powerful angiogenetic factor

ever known. Its most important difference from the other factors that stimulate angiogenesis is its being specific only to the endothelium.[30] VEGF expression increases during wound healing.[31] Angiogenesis is necessary for adhesion formation, which is actually a result of wound healing.[6] Thus, investigators have thought that adhesion formation may be prevented by the inhibition of angiogenesis.[32,33]

In our study, the staining score for VEGF on normal serosal surfaces was found to be quite low while the VEGF staining on adhesion-positive areas in the control group was significantly higher than in those of the sham, melatonin and phospholipid groups ($p<0.05$). This result shows that VEGF expression increases during adhesion formation and has a great contribution to adhesion formation. Interestingly, there was no difference between the normal areas and the VEGF staining of the adhesion-positive areas in the melatonin and phospholipid groups. This result might be due to the blockage of rich-in-vessel, severe adhesion formation or decrease in the accumulation of fibroblasts and macrophages on the damaged area, and to the decrease or blockage of the release of the mediators that are necessary for VEGF expression, by blockage of the inflammatory cascade with melatonin and phospholipid.

In conclusion, melatonin and phospholipid decreased the adhesion formation in an experimentally created adhesion model in rats, and there was a correlation between adhesion severity and VEGF expression. Because of the advantages of melatonin and phospholipid, such as no side effects, ease of application and physiologic nature, they are possible agents for clinical usage as antiadhesives.

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