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RESEARCH ARTICLE

Effects of Calcium Dobesilate on Colonic Anastomosis Healing: An Experimental Study

Kalsiyum Dobesilatın Kolon Anastomoz İyileşmesi Üzerine Etkileri: Deneysel Bir Çalışma

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ABSTRACT

Aim: Anastomotic leakage in colon anastomosis (CA) increases morbidity and mortality. Calcium dobesilate (CaD) has angioprotective, antioxidant, lymphatic flow enhancing-and neuroprotective effects. Despite these capillary and cellular effects, there is no data in the literature regarding the effects of CaD on CA healing.

Method: Fifty Wistar-albino rats were randomly divided into five groups. All rats underwent CA after transverse colon transection. CaD was not administered to the control group (Group 1). CaD was administered to the experimental groups (Groups 2, 3, 4 and 5) intraperitoneally or by gavage at doses of 50 or 100 mg/kg/day. CaD was given as a single dose daily during postoperative five days. Bursting pressure values (BPV) and hydroxyproline values (HV) were measured. At the end of histopathological evaluation, polymorphonuclear leukocytes (PNLS), mononuclear leukocytes (MNLS), neovascularization (VS) and collagen fibers (CFS) were scored.

Results: CaD increased BPV and HV in experimental groups. We found a decrease in PNLS, MNLS, VS, and an increase in CFS in experimental groups. These increases seemed to be related to the administration doses of CaD. The decreases in PNLS, MNLS and VS were much more evident in Groups 4 and 5 than the other groups. There was no significant difference in terms of VS between experimental groups.

Conclusion: We found that CaD not only decreased the pathological parameters of inflammation, but also increased the strength of CA mechanically and biochemically. Although VS reduction seemed to have negative outcomes on CA, we know that CaD inhibits over-expression in angiogenesis. As a result, these effects of CaD appear to be dose-dependent rather than the administration methods.

Keywords: Calcium dobesilate, colon anastomosis, bursting pressure, hydroxyproline, antiangiogenesis, collagen fibers

ÖZ

Amaç: Kolon anastomozu (CA) sonrası gelişen anastomoz kaçağı morbidite ve mortaliteyi artırır. Kalsiyum dobesilate (CaD) anjiyoprotektif, antioksidan, lenfatik kan akımını artırıcı ve nöroprotektif etkilere sahiptir. Bu kapiller ve hücresel sahadaki etkilerine rağmen CaD'nin, CA iyileşmesi üzerine etkileri hakkında literatürde veri yoktur.

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Yöntem: Elli adet Wistar-albino sıçan, eşit sayıda ve rastlantısal olarak beş gruba ayrıldı. Tüm sıçanlara transvers kolon transeksiyonu sonrasında CA yapıldı. Kontrol grubuna (Grup 1) CaD uygulanmadı. Deney gruplarına (Grup 2, 3, 4 ve 5), periton içi ya da gavajla ve 50 ya da 100 mg/kg/gün dozlarında CaD uygulandı. CaD, günlük tek doz ve ameliyat sonrası 5 gün verildi. Patlama basınç değerleri (BPV) ve hidroksiprolin değerleri (HV) ölçüldü. Sonunda histopatolojik değerlendirmede (HPE), polimorfonükleer lökositler (PNLS), mononükleer lökositler (MNLS), yeni damar oluşumu (VS) ve kollajen lifler (CFS) skorlandı.

Bulgular: Kalsiyum dobesilat, deney gruplarında BPV ve HV artırdı. Biz deney gruplarında PNLS, MNLS, VS'de azalma, CFS'de ise artış saptadık. Bu artış, ilacın uygulama dozu ile ilişkili gibi görünmektedir. Çalışma gruplarında HPE'de PNLS, MNLS ve VS azalmaktadır ama CFS artmaktadır. Grup 4, 5 PNLS, MNLS ve VS'lerinde saptanan azalma, diğer gruplardan belirgindi. Deney grupları arasında, VS açısından fark yoktu.

Sonuç: Biz, CaD'nin sadece patolojik olarak enflamasyon parametrelerini azaltmakla kalmadığını aynı zamanda mekanik ve biyokimyasal olarak CA'nın gücünü artırdığını saptadık. VS azalması, CA iyileşmesinde olumsuz sonuçlar doğuracak gibi görünmesine rağmen, biz CaD'nin angiogenesisde oversupresyonun inhibe ettiği biliyoruz. Sonuçta, CaD'nin bu etkileri, uygulama şeklinden ziyade doz bağımlı gibi görünmektedir.

Anahtar Kelimeler: Kalsiyum dobesilat, kolon anastomozu, patlama basıncı, hidroksiprolin, antianjiogenezis, kollajen lifler

Introduction

Colon resection (CR) can be performed due to various emergency or elective pathologies. The anatomical integrity of gastrointestinal tract after resection is usually achieved by an anastomosis. Histologically, colonic anastomosis (CA) healing process can be divided into stages, and these stages of the healing in CA are substantially similar to the wound healing stages anywhere in the body.[1,2,3,4,5,6,7,8] The most important factor in anastomotic healing is collagen, which constitutes the stretching force of submucosal connective tissue.[9] The stage with highest risk for anastomotic leakage (AL) is the inflammation stage.[3] AL following a CR is still considered a serious problem for surgical care and has an incidence between 3% and 19%.[2,10] This estimate includes asymptomatic AL with an incidence as high as 50%.[11] In case of AL, the duration of hospitalization is doubled and perioperative mortality is tripled compared to the normal healing process of CA.[2] Many factors affect the healing of CA.[2] Pre-operative colon mechanical cleansing, antibiotic prophylaxis, healthy tissue for anastomosis, surgical technique, indication for surgery (elective or emergency), radiotherapy, hypothermia, advanced age, presence of systemic diseases (obesity, jaundice, anemia, diabetes, chronic renal failure, cirrhosis, malignancies, etc.), nutritional status of the patient (malnutrition, alcoholism and smoking), immune status of the patient, medical prescriptions used by the patient, sepsis and shock are some of these factors.[3,7,10,12,13,14,15] Calcium dobesilate (CaD) (Doxium® 500 mg capsule, Abdi İbrahim İlaç Sanayi ve Ticaret A.Ş, İstanbul, Turkey) is a synthetic agent, which has shown its efficacy at capillary level and which has vasoprotective effects.[16,17,18,19,20] In the experimental studies, it has been shown that CaD has a neuroprotective activity and is an antiangiogenesis in diabetic neuropathy.[21,22] CaD, reduces the over-expression of endothelin-1, intracellular adhesion molecule-1, vascular endothelial growth factor (VEGF) from retinal endothelial cells in diabetic retinopathy and prevents alterations on leukocyte adhesion.[21,23] CaD eliminates detrimental effects of reactive oxygen species (ROS).[24,25,26] CaD increases the nitric oxide synthase activity of capillary endothelial cells and regulates the formation of basal membrane collagen network.[16,19,25] It also regulates the capillary membrane resistance that reduces capillary hyper-permeability and fragility.[18,27] It reduces platelet aggregation and prevents thrombus formation.[25,28,29] Moreover, it also inhibits hyaluronidase, which is responsible for the fragmentation of the matrix mucopolysaccharides in the capillary basal membrane.[16,18,30] CaD reduces transcapillary escape of albumin from peripheral circulation.[18] The antioxidant properties of CaD are attributed to its scavenger activity in lipid peroxidation caused by ROS. It also inhibits the release of inflammatory cytokines, such as platelet activating factor (PAF).[17] Notwithstanding these capillary and cellular effects, there is no data available in the current literature regarding the effects of CaD on healing of CA.

Materials and Methods

Animal Model and Treatment Protocol

The current study was performed using 10-12 week-old male Wistar rats (n=50) weighing 225±25 g. The rats were housed in a temperature-controlled room (20-22 °C) and 55-60% humidity with 12-h light-dark cycles. All rats were fed a standard rodent chow (20% protein, 6% cellulose, 2% fat in 100 g of chow) and given water ad libitum. After an adaptation period of one week, the experimental animals were randomly divided into four experimental groups as Groups 2, 3, 4 and 5, and one control group as Group 1. The treatments were as follows: only CA was performed in Group 1 (n=10); CA was performed and CaD was administered intraperitoneally 50 mg/kg/day in Group 2 (n=10); CA was performed and CaD was administered by gavage at a dose of 50 mg/kg/day in Group 3 (n=10); CA was performed and CaD was administered intraperitoneally 100 mg/kg/day in Group 4 (n=10) and CA was performed and CaD was administered by gavage at a dose of 100 mg/kg/day in Group

5. The design of the experimental groups and control group is shown in Figure 1. An excipient of 0.9% sodium chloride was used for the preparation of various concentrations of CaD for intraperitoneal applications and distillated water was used as the adjuvant for various concentrations of CaD for gavage applications. After 12 hours post-operatively, CaD was administered to the experimental Groups 2, 3, 4 and 5) for five days (Figure 1). All experimental studies were conducted in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised in 1978). The ethical protocol of the current research was approved by Ethics Committee of İstanbul University, İstanbul, Turkey. Institutional Review Board (IRB) (number: 2006/ 30826).

Surgical Procedure

The rats were anesthetized by an intramuscular injection of ketamine hydrochloric acid (HCL) 50 mg/kg (Ketalar®, Eczacıbaşı Pharmaceuticals Marketing, Lüleburgaz, Turkey) and xylazine HCL 10 mg/kg (Rompun® 2%, Bayer, Leverkuesen, Germany). We made a midline incision to expose the transverse colon, which was divided about midway. Integrity was restored with an inverted one-layer end-to-end anastomosis consisting of six or eight interrupted sutures of 6/0 polypropylene (Prolene®; Ethicon, İstanbul, Turkey). The abdomen was closed in two layers with a continuous 3/0 silk suture for the fascia and skin. All rats underwent re-laparotomy at the end of the 5th day and all of them were sacrificed with high-dose ether anesthesia.

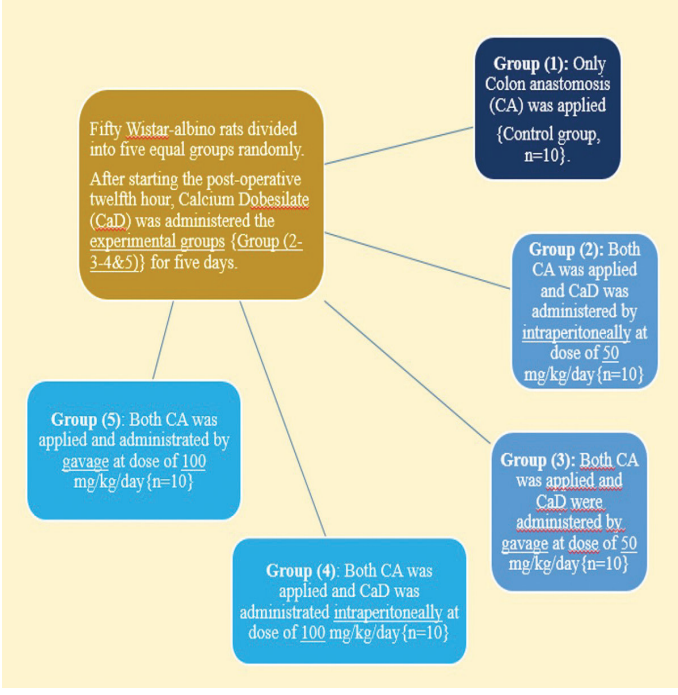

Figure 1. Design of the experimental groups and control group
CA: Colon anastomosis, CaD: Calcium dobesilate

Bursting Pressure Measurements

Bursting pressure value (BPV) was measured *ex vivo*. After re-laparotomy on the fifth postoperative day, anastomotic colonic segments were resected and bursting pressures were measured. The distal end of colon was ligated using a 3/0 silk suture and a catheter was secured into the proximal end and fixed to the bursting pressure apparatus as described elsewhere.[3,31] Through this catheter, the bowel was infused with a continuous flow of air at a rate of 3 mL/min using an infusion pump [Perfuser E (type 871112), B. Braun Meisingen a 6 device]. BPV was defined as the value recorded at the point of an air leakage or gross rupture, and it was noted in mmHg. The site of leakage or rupture during the bursting pressure measurement occurred at the anastomosis area in all rats.

Preparation of Tissue Homogenates

After anastomotic BPV measurement, one centimeter of the colonic segment including the anastomosis site was resected from each subject and half of the specimen was fixated in 10% formaldehyde for histopathological examination. The other half was used in tissue homogenate extraction to determine hydroxyproline levels (HV). The extracted tissues were rinsed in ice-cold PBS (0.02 mol/L, pH 7.0-7.2) to remove excess blood thoroughly and weighed before homogenization. Tissues were minced and homogenized in 6N HCL. The homogenates were then centrifuged at 1.500 x g (or 5.000 rpm) for 15 minutes. Removed supernatant samples were stored at -20 to -80 °C until the assay time for hydroxyproline.

Estimation of Tissue Hydroxyproline Concentrations

The chemicals used for the hydroxyproline assay were of the highest analytical grade available. All of the chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). All reagents were stored at +4 °C and brought to room temperature 20 minutes prior to the usage. Tissue HV were assessed by using the Bergman & Loxley method.[32] The analytic principle of the assay was colorimetric measurement of the colored complex formed with p-dimethylaminobenzaldehyde of pyrrolle after the oxidation of hydroxyl pyroline to pyrrole compound with chloramine T using a spectrophotometer (Shimadzu UV 1601, Tokyo, Japan) at 560 nm. The absorbance of trans-4-hydroxy-L-proline standards was used for the standard curve drawing. Hydroxyproline concentrations were expressed as mg/g of tissue-wet weight.

Histopathological Evaluation

After the samples fixed in 10% formalin solution for 24 hours, they were processed with standard paraffin technique and stained with hematoxylin and eosin. The samples were

then examined under a light microscope. The parameters were evaluated with help of the modified Ehrlich & Hunt scoring scale including polymorphonucleated cells (PNL), mononuclear cells (MNL), neovascularization and collagen fibers (CF). Scores ranged from 0 to 4 as: score 0 (-)=no evidence, score 1 (+)=occasional evidence, score 2 (++)=light scattering, score 3 (+++)=abundant evidence, and score 4 (++++)=confluent fibers or cells.[9,33,34,35]

Statistical Analysis

Categorical variables were presented in percentages and continuous variables were expressed as mean ± standard deviation. Repeated measures ANOVA, Post-hoc tests, Tukey-Kramer test and chi-square test were used to analyze statistical differences between the groups regarding BPV, HV and histopathological evaluation. A p value less than 0.05 was considered significant.

Results

Bursting Pressure Values

Anastomotic BPVs of Group 2 and 3 were significantly higher compared to Group 1 ($p<0.05$). There was no significant difference between Group 2 and 3 in terms of BPV (Figure 2). BPVs of Group 4 and 5 were significantly higher compared to Group 1, 2 and 3 ($p<0.05$). The comparison between Group 4 and Group 5 showed no significant difference (Figure 2). Table 1 shows anastomotic BPVs (mmHg) and HV (mg/g) according to groups.

Hydroxyproline Values

HVs of Group 2 and 3 were significantly higher compared to Group 1 ($p<0.05$). There was no significant difference between Group 2 and 3 in terms of HV (Figure 3). HV of Group 4 and 5 were significantly increased when compared to Group 1, 2 and 3 ($p<0.05$). The comparison between Group 4 and Group 5 showed no significant difference (Figure 3).

Histopathological Evaluation

Polymorphonuclear Leukocyte Infiltration Scores (PNLS)

Group 1 showed a significant increase in scores 2 and 3 ($p<0.05$). There was no statistically significant difference between Group 1, Group 2 and Group 3 ($p>0.05$). Group 4 and 5 showed a significant increase in scores 1 and 2 ($p<0.05$). The increase in score 1 in Group 4 and 5 was statistically significant compared to other groups ($p<0.05$).

Mononuclear Leukocyte Infiltration Scores (MNLS)

Group 1 showed a significant increase in score 2 ($p<0.05$). Group 2 and Group 3 had a significant increase in score 1 when compared to Group 1 ($p<0.05$). There was no statistically significant difference between Group 2 and Group 3 ($p>0.05$). Group 4 and Group 5 showed a significant increase in score 1 compared to Group 1 ($p<0.05$). Groups 1, 2, 3 and 5 showed a significant increase in score 2 compared to Group 4 ($p<0.05$).

Neovascularization Scores (VS)

Groups 1, 2 and 4 did not show any statistically significant difference when compared to each other ($p>0.05$). When

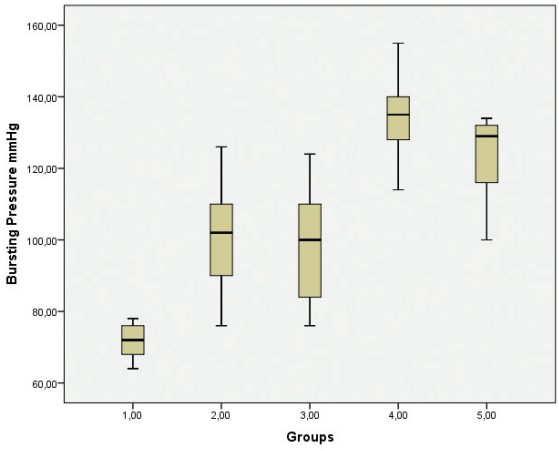

Figure 2. The mean burst pressure values of the groups

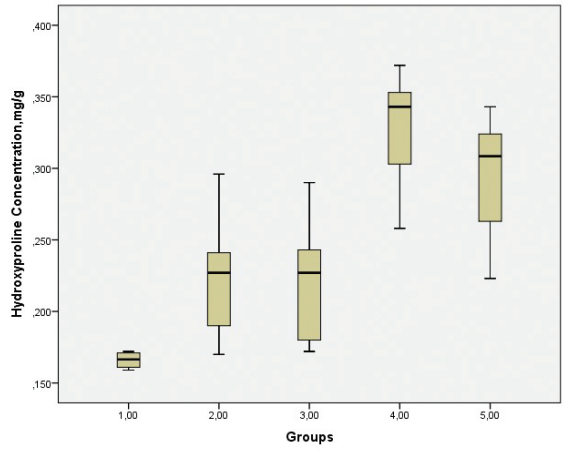

Figure 3. The mean tissue hydroxyproline levels of the groups

Table 1. Burst pressure values and tissue hydroxyproline values of all groups (mean ± standard deviation)

	BPV (mmHg)	HV (mg/g)
Group 1 (n=10)	71.6±4.6	0.166±0.004
Group 2 (n=10)	100.6±15.2	0.222±0.036
Group 3 (n=10)	97.6±15.3	0.218±0.038
Group 4 (n=10)	135.1±11.6	0.329±0.035
Group 5 (n=10)	123.8±10.7	0.295±0.039

BPV: Burst pressure values, HV: Hydroxyproline values

Table 2. Histopathological scores of the groups

	PNLS	MNLS	VS	CFS
Group 1	+++/++	++	++/+++/+	++/+
Group 2	+++/++	++/+	+/++/+++	++
Group 3	++/+++	+/++	++	++
Group 4	+++/++/+	+	++/+++/+	++
Group 5	++/+	+/++	+/++	++

PNLS: Polymorphonuclear leukocyte score, MNLS: Mononuclear leukocyte score, VS: Neovascularization score, CFS: Collagen fiber score

Groups 1, 2 and 4 were compared with Group 3, the increase in scores 1 and 3 were statistically significant ($p<0.05$). When Groups 1, 2 and 4 were compared with Group 5, the increase in score 3 was statistically significant ($p<0.05$). Group 5 showed a significant increase in scores 1 and 2 compared to Group 3 ($p<0.05$).

Collagen Fiber Scores (CFS)

Group 1 showed a statistically significant increase in scores 1 and 2 ($p<0.05$). Groups 2, 3, 4 and 5 had a significant increase in score 2 ($p<0.05$). When Group 1 was compared to Groups 2, 3, 4 and 5, the increase in score 1 was statistically significant ($p<0.05$). Groups 2, 3, 4 and 5 did not show a statistically significant difference when compared to each other ($p>0.05$). Table 2 shows the distribution and comparison of statistically significant scores according to groups.

Discussion

It has been shown that CaD not only has inhibitor activity on VEGF production, but also improves microvascular hemodynamics and shows anti-leakage effects by reducing plasma endothelin-1 levels in experimental diabetic retinopathy.[21,23] Despite its antioxidant and anti-inflammatory effects, studies investigating the effects of CaD on wound healing are limited. Eventually, no studies have investigated the therapeutic efficacy of CaD on colon anastomosis healing. It has been previously reported that CaD inhibits platelet aggregation and prevents thrombus formation.[19,20,24,25,28,29] Both platelet aggregation and thrombus formation are essential for surgical hemostasis during primary hemostasis after the initial injury.[36] In order to avoid any hemostatic problem in our study, we started CaD administration at post-operative 12th hour. BPV has been used as a direct measure of the strength of CA.[33] On the other hand, hydroxyproline is a part of collagen that was demonstrated to be positively correlated with the amount of collagen and healing of CA.[9] Regardless of the administration method, both BPV and HV levels were significantly increased in the experimental groups that were treated with CaD ($p<0.05$). This increase was much more evident when CaD dose was 100/kg per day ($p<0.05$). PNL is known as a potential source of collagenase in the wound healing site and is directly related to collagen catabolism.[4] High collagenase activity plays an important role in anastomotic healing, causing low anastomotic strength early after the formation of an anastomosis because of collagen lysis.[35] In our study, as there was decreased PNLS count and increased CFS accompanied by an increase in both BPV and HV in Groups 4 and 5 compared to Group 1, intraperitoneal administration of 100/mg/kg CaD per day was shown to have positive effect on CA healing. This result might be related to the expected consequences of antioxidant, neuroprotective, anti-inflammatory and capillary effects of CaD. Neovascularization, which promotes collagen synthesis, enhances anastomotic strength.[9] The decrease in VS levels at the given dose seems to be a disadvantage. However, a significant increase in BPV and HV, and their positive effects on CA healing may be associated with suppression of over-expression by CaD on angiogenesis.[22] CaD was reported to exert aforementioned effect through VEGF and endothelin one.[21,23] CaD showed no anti-angiogenic effect when used as gavage at a dose of 100 mg/kg per day; this result may be related to excretion of 50% of the administrated drug via fecal material without any biotransformation. Further studies with higher doses of CaD application are needed to clarify the effects of gavage. In this study, when Group 1 and Group 2 were compared, no significant difference was observed in terms of BPV, HV and CFS ($p>0.05$). We also found the same findings when Group 4 and Group 5 were compared. These findings may be caused by a single daily dose of medication. Previous *in vivo* and *in vitro* studies have shown that repetitive CaD administrations within a day increase both antioxidant effect and lymphatic circulation.[18,27,30] Administration of CaD at a dose of 50 mg/kg/day was found to have no effect in PNLS. However, administration of CaD at a dose of 100 mg/kg/day reduced PNLS ($p<0.05$). This decrease was more prominent in Group 5 than in the other groups ($p<0.05$). The decrease in MNLS was more prominent in the experimental groups than in the control group. The decrease in MNLS was more prominent in Group 4 ($p<0.05$). Finally, with CaD administration at a dose of 100 mg/kg/day, both acute and chronic histopathological parameters of inflammation levels decreased significantly ($p<0.05$). In addition to the above, VS decreased when CaD was administered at a dose of 100 mg/kg/day ($p<0.05$). Moreover, CaD administration increased CFS in experimental groups ($p<0.05$). The aforementioned effects of CaD (reducing

capillary hyperpermeability and fragility by regulating capillary membrane resistance, enhancing plasticity and flexibility of thrombocytes, decreasing blood viscosity and increasing of blood fluidity, augmentation of lymphatic drainage and stimulation of lymph circulation, reducing protein-rich edema by increasing normal proteolysis, etc.) are known to be associated with macrophages and lymphatic transport.[16,18,23,30,36] The decrease in serum protein levels (especially albumin) is critical for the healing of CA. A limitation of our study is the lack of biochemical parameters of blood such as albumin, and further studies are needed in this section.

Conclusion

To summarize, we found that CaD not only reduces pathological inflammation parameters, but also strengthens CA mechanically and biochemically. Although the decrease in neovascularization appears to have negative outcomes on CA, we know that CaD inhibits over-expression in angiogenesis. Finally, these effects of CaD seemed to depend on the administration doses rather than the administration methods. Further researches are needed to clarify this topic.

Ethics

Ethics Committee Approval: Ethical protocol of the current research was approved by Ethics Committee of İstanbul University, İstanbul, Turkey. Institutional Review Board (IRB) (number: 2006/ 30826).

Informed Consent: Not applicable.

Authorship Contributions

Surgical and Medical Practices: S.D., S.D., Ö.S., G.B.D., H.U., Concept: S.D., S.D., Ö.S., G.B.D., H.U., T.İ., Design: S.D., S.D., Ö.S., G.B.D., H.U., T.İ., Data Collection or Processing: S.D., S.D., Ö.S., G.B.D., H.U., T.İ., Analysis or Interpretation: S.D., E.H., A.O., S.D., Ö.S., G.B.D., H.U., E.A., S.Ç., A.T., Z.S., İ.T., T.İ., Literature Search: S.D., E.H., A.O., S.D., E.A., S.Ç., A.T., Z.S., İ.T., T.İ., Writing: S.D., E.H., A.O., S.D., E.A., S.C., A.T., Z.S., İ.T., T.İ.

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