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RESEARCH ARTICLE

The Healing Effects of The Topical Mesenchymal Stem Cells Application on Colonic Anastomosis Subjected to Ischemia Reperfusion Injury

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Abstract

Intestinal ischemia reperfusion injury (IRI) is a challenging problem and it adversely affects the healing of colonic anastomosis. Our experimental study aimed to investigate the role of mesenchymal stem cells (MSC) administration in the healing of colonic anastomosis. A total of 33 rats were grouped as Control, IRI and MSC treatment groups. Three rats were reserved for obtaining MSCs. Colonic resection and anastomosis procedure was performed in all groups. Anastomotic line was wrapped with MSCs impregnated spongostan after colonic anastomosis in the rats of the MSC treatment group. All rats were sacrificed and anastomotic line were sampled for examination on the post operative seventh day. Tissue hydroxyproline (HP) levels and anastomotic bursting pressures were statistically compared. Anastomotic bursting pressures were found to be significantly high in MSC treatment group rats. The lowest anastomotic bursting pressure was detected in IRI group rats. Hydroxyproline content of the anastomotic sites were also found to be significantly higher in the rats of the MSC treatment group when compared with the IRI group rats. Our study showed that the detrimental effects of IRI on the healing process of colonic anastomosis in an experimental model may be alleviated with the treatment of MSCs.

Keywords: *Anastomotic leakage, Colonic anastomosis, Hydroxyproline, İschemia reperfusion injury, Mesenchymal stem cell*

Topikal Mezenkimal Kök Hücre Uygulamasının İskemi Reperfüzyon Yaralanmasına Bağlı Kolonik Anastomoz Üzerine İyileştirici Etkileri

Öz

Bağırsak iskemi reperfüzyon hasarı (İRH) zorlu bir sorundur ve kolon anastomozunun iyileşmesini olumsuz etkiler. Deneysel çalışmamız kolon anastomozunun iyileşmesinde mezenkimal kök hücre (MKH) uygulamasının rolünü araştırmayı amaçlamıştır. Toplam 33 sıçan, Kontrol, İRH ve MKH tedavi grupları olarak gruplandı. MKH'leri elde etmek için üç sıçan ayrıldı. Tüm gruplara kolonik rezeksiyon ve anastomoz işlemi uygulandı. Anastomotik hat, MKH tedavi grubundaki sıçanlarda kolonik anastomozdan sonra MKH emdirilmiş spongostan ile sarıldı. Tüm sıçanlar ameliyat sonrası yedinci günde sakrifiye edildi ve inceleme için anastomoz hat örneklendi. Doku hidroksiprolin (HP) seviyeleri ve anastomoz patlama basınçları istatistiksel olarak karşılaştırıldı. MKH tedavi grubu sıçanlarda anastomoz patlama basınçları önemli ölçüde yüksek bulundu. En düşük anastomoz patlama basıncı İRH grubu sıçanlarda tespit edildi. Anastomotik bölgelerin hidroksiprolin içeriği, İRH grubu sıçanlara kıyasla MKH tedavi grubundaki sıçanlarda önemli ölçüde daha yüksek bulundu. Çalışmamız, deneysel bir modelde İRH'nin kolon anastomozunun iyileşme süreci üzerindeki zararlı etkilerinin MKH'lerin tedavisi ile hafifletilebileceğini göstermiştir.

Anahtar sözcükler: *Anastomoz kaçağı, Hidroksiprolin, İskemi reperfüzyon hasarı, Kolon anastomozu, Mezenkimal kök hücre*

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Introduction

There may be a need for anastomosis in bowel surgeries that are reperformed quite frequently. It is a type of surgery with high mortality and morbidity that is feared in surgery clinics. A leak that may develop from the anatomosis can be very risky for the life of the patient. Therefore, studies in this field are frequently encountered in the literature. To increase the safety of the anastomosis, many agents are tried systemic and topical. The main problem that we frequently encounter in the deterioration of the well-being of the anastomosis in clinical practice is the oxygenation and nutritional status of the anastomotic line. An ischemic bowel carries a high risk for anastomosis. Reperfusion injury after oxygenation of the ischemic tissue causes tissue damage and prevents healing in the anastomosis line [1].

The effect of ischemia/reperfusion (I/R) injury on the healing of colonic anastomosis is one of the most investigated topics of experimental surgery. Intestinal ischemia may result from many clinics senarios such as mesenteric vascular occlusion, mechanical obstruction, strangulated hernia or volvulus. Shock and severe cardiopulmonary diseases are also common clinic problems and they constitute the cause of a more prevalent but underdiagnosed type of intestinal ischemia [1]. Removing the necrotic colonic segment and performing colonic anastomosis may be required in these conditions. A major indicator of the outcome of this procedure is the safety of colonic anastomosis. Factors including the degree of ischemia, the length of ischemic bowel segments and the performance status in which the patient plays an important role in the anastomotic healing process. It has been shown that the presence of I/R injury on intestinal anastomosis delays the anastomotic healing process and this may lead to anastomotic leakage and dehiscence. Although anastomotic leakage and dehiscence seem to be local events of I/R injury, the mediators from the ischemic tissue enter the systemic circulation and affect to their organ systems [2]. Endothelial dysfunction, increased free radical production, nitric oxide depletion and released cytokines are the main characteristics of the mechanism of I/R ınjury. These events trigger a local and systemic inflammatory response according to the severity of ischemic insult. Endothelial dysfunction and cytokine release are the main unfavorable factors responsible for tissue damage [3].

Ischemia reperfusion injury is generally an unavoidable challenging problem. Investigations in this field have focused on early detection and have examined the effects of therapeutic agents on tissue damage [4].

Mesenchymal stem cells have beneficial effects on anastomotic safety in the digestive tract in the presence of ischemia. MSCs from adipose tissue have immunomodulatory, anti-inflammatory and anti-apoptotic properties [5,6]. We aimed to investigate the healing effects of MSCs on colonic anastomosis subjected to I/R injury in our study.

Material and Methods

Ethical Approval

This experimental study was approved by Kırıkkale University Animal Experiments Local Ethics Committee on 09.01.2014 with the number 14/14.

Animals

A total of 33 rats were grouped as Control, IRI and MSC treatment groups. Three rats were reserved for obtaining MCSs and the others were grouped as control (n=10), I/R injury (n=10) and MCS treatment group (n=10).

Preparation of MSCs Impregnated Spongostan Layers

Mesenchymal stem cells were obtained from subcutaneous adipose tissue in the abdomen of rats. Stem cells were isolated by using the primary culture method. Fat tissue was collected from three appropriately anesthetized rats. An average of 0.59 g fat tissue was collected per rat ($n = 3$; $n^1 = 0.64$ g, $n^2 = 0.54$ g, $n^3 = 0.59$ g). The fat tissue was transported in an appropriate transport medium (transport medium containing 10% FBS and 0.4% penicillin-streptomycin) and incubated in standard culture medium by splitting into small pieces. The culture medium was changed daily to prevent the possible different effects of various cytokines induced by MSCs. Cells were passaged 4 times using standard trypsinization method and the number of cells was counted using trypan blue staining method when they were passaged. They were then frozen for use. Characterization of the cells was performed using flow cytometry. It was analyzed for CD29, CD90, CD54, MHC class 1, CD45, CD109 and MHC class 2 receptor. $9x10^6/mm^3$ MSCs prepared separately for each transport container were impregnated with layers of spongostan [7].

Anesthesia and Surgery

Rats were anesthetized using intraperitoneal ketamine HCl 90 mg/kg (Ketalar, 500 mg/10 mL Pfizer; USA) and 10 mg/kg xylazine HCl (Rompun, 23.32 mg/mL Bayer, Leverkusen, Germany). Operation sites of the rats were cleaned with povidone-iodine before incision. About 3 cm midline incision was performed in all rats. In control group rats, 0.5 cm colonic segment was resected in distance 5 cm from the ileocecal valve and later anastomosis added. As described by Fink et al.[8] superior mesenteric artery was clamped for about 15 min for ischemia and intestinal tissue was evaluated for pallor and edema, and released for 5 min to ensure reperfusion before colonic resection and anastomosis procedure in I/R injury group rats. The presence of ischemia was confirmed by the color changes. MSCs impregnated spongostan layers ($9x10^6/mm^3$) were prepared as mentioned below for the rats of the MSC treatment group. After subjection to I/R injury, resection and anastomosis procedure was performed and colonic anastomotic line was wrapped with MSCs

impregnated spongostan layers in MSC treatment group rats. All rats were allowed standard rat chow and water as before surgery. On the seventh postoperative day, all rats underwent relaparotomy and 5 cm of anastomotic colon segments were removed for the examination of tissue hydroxyproline levels and for measuring anastomotic bursting pressure.

Measurement of Anastomotic Bursting Pressure

The anastomotic bursting pressure was measured in all rats as described in the literatüre [9]. A 5 cm colonic segment (including the anastomosis in the middle) carefully resected and fecal content was cleaned with saline solution. The proximal end of this segment was ligated by using 2/0 polyglactin suture and the other end was fixed to the infusion pump using a 16G catheter and then infused with isotonic saline solution at 2 mL/min. The intraluminal pressure of the colonic segment was monitored and measured from the anastomotic site until a leak occurred and the pressure was recorded as anastomosis burst pressure (BP) *(Fig. 1* and *Fig. 2)*.

Evaluation of Hydroxyproline Level in Perianastomotic Tissue

After the measurement of anastomotic bursting pressure, wet perianastomotic tissue samples were weighed, then dried for 3 days at 60°C. Dry tissue samples were also weighed. The tissues were hydrolyzed in 7 N hydrochloric acid (HCl) at 110°C for 8 h and centrifuged at 5000 rpm for 20 min to obtain the study material. The absorbance of the formed material was evaluated colorimetric (photometric) at 121°C at 562 nm and the tissue hydroxyproline (HP) level was calculated.

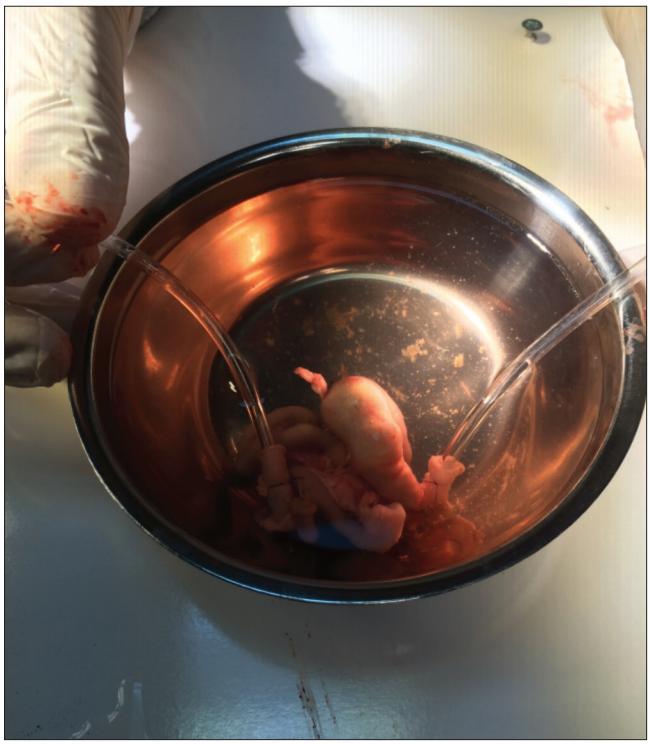

Fig 1. Anastomotic bursting pressure, anastomosis line

Fig 2. Anastomotic bursting pressure, infusion pump

Statistical Analyses

All results are reported as mean ± standard error of the mean. The statistical analyses were performed by using the SPSS® statistical package, version 16.0 for Windows. Due to limited number of rats in each group, non-parametric methods were used for statistical analysis. Kruskal-Wallis variance analysis, which is used to compare the means of three or more groups, was used to determine whether there was a statistical difference between the groups. The Mann-Whitney U test, which is used to compare the means of two groups, was used to determine the origin of the significant difference in terms of groups. P value of less than 0.05 was considered significant.

Results

The experimental protocol was composed of three groups as control, I/R and MSC groups. Ten rats were randomly selected for each group and a total of 30 rats underwent surgery. One rat from the control group, one from the MSC group, and two rats from the I/R group died within the first day after the first surgical procedure of the experiment. Relaparotomy was performed for deceased rats. There was no intra-abdominal adhesion, anastomotic leakage or any

Table 1. *Anastomotic bursting pressure levels of the groups*

Groups	Bursting Pressure		
	Min	Max	Median
Control group	170	230	217.78[a]
I/R group	160	210	199.09[a,c]
MSC group	180	260	236[c]

*Values are presented as median (intrequartile range) *Kruskal-Wallis test,* ***a)*** *The difference between control group and I/R group was statistically significant (P 0.05),* ***b)*** *The difference between control group and MSC group was statistically significant (P<0.05),* ***c)*** *The difference between I/R group and MSC group was statistically significant (P<0.05)*

Table 2. *Hydroxyproline levels of the groups*

Groups	Hydroxyproline Levels		
	Min	Max	Median
Control group	211.39	1113.75	633.38[b]
I/R group	89.87	795.85	476.31[c]
MSC group	671.55	1453.17	1172.97[b,c]

*Values are presented as median (intrequartile range) *Kruskal-Wallis test,* ***a)*** *The difference between Control group and I/R group was statistically significant (P<0.05),* ***b)****The difference between Control group and MSC group was statistically significant (P<0.05),* ***c)*** *The difference between I/R group and MSC group was statistically significant (P<0.05)*

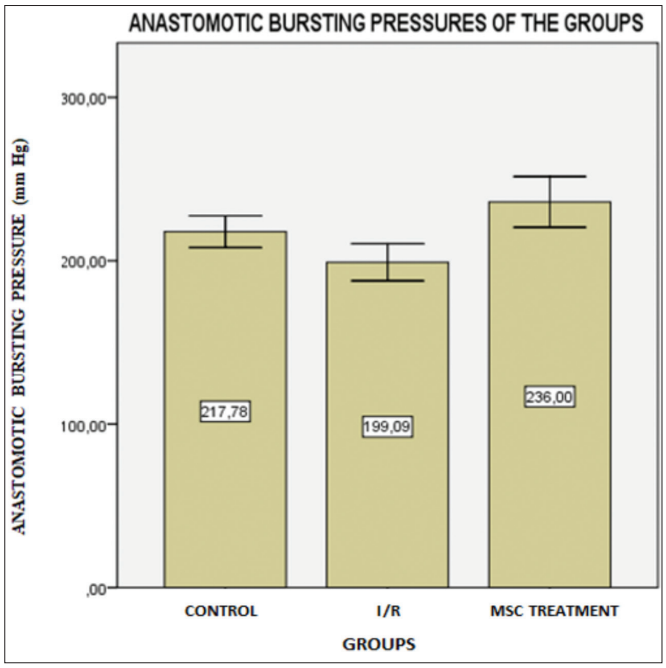

Fig 3. Anastomotic bursting pressure levels of the groups

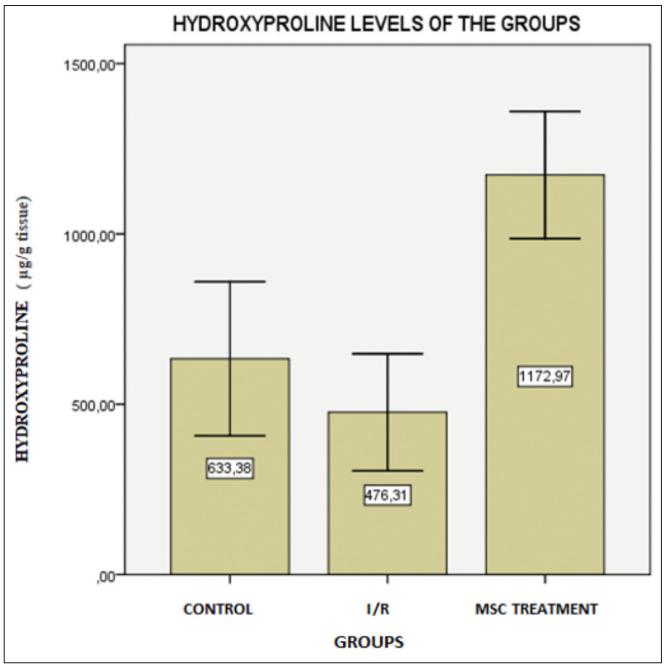

Fig 4. Hydroxyproline levels of the groups

additional surgical pathology. It was excluded from the experiment as it was thought to be caused by anesthesia. No pathology developed in the other rats. The experiment was completed successfully. A 5 cm intestinal segment, including the anastomosis area obtained with the last surgical procedure, was subjected to burst pressure measurements. Then the anastomosis site in the remaining tissue was resected and subjected to hydroxyproline measurements. The results were evaluated statistically. The results are detailed in *Table 1* and *Table 2*.

Mean anastomotic bursting pressure levels were measured as 217.78 mmHg in control group, 199.09 mmHg in I/R Injury group and 236 mmHg in MSC treatment group animals. Compared with control and MSC group animals, anastomotic bursting pressure levels of I/R injury group animals were found to be significantly low (P=0.041 and P<0.001 respectively). There was no significant difference between the control and MSC treatment groups in terms of anastomotic bursting pressure levels *(Fig. 3)*.

Mean hydroxyproline levels were measured as 633.38 in control group animals, 476.31 in I/R group animals and 1172.92 in MSC treatment group animals. There was no significant difference between the control and I/R injury group rats in terms of HP levels. The highest HP levels were noted in MSC group animals. Compared with control and I/R injury group rats, HP levels were found to be significantly high (P=0.002 and P<0.001 respectively) *(Fig. 4)*.

Discussion

Intestinal anastomoses are operations that are frequently performed in surgical clinics. Intestinal resection and anastomosis may be required for many reasons such as ileus, mesenteric ischemia, tumor, bleeding, diverticulum perforations, and stab wounds. In the clinical sense, intestinal structure, blood supply level, intra-abdominal contamination, surgical technique and age of the patient have an effect on anastomosis safety. Anastomotic leakage is

a pathology with high morbidity and mortality. Treatment procedures to maximize anastomosis safety have been tried over time. Academic studies for this purpose are frequently encountered in the literature. The most emphasized parameter in anastomosis safety is the vitality of the intestinal structure, that is, whether it is ischemic or not. It is a known fact that disruption of intestinal oxygenation will adversely affect healing [10]. However, the more effective damage is the destruction caused by the oxygen radicals of the reperfusion that develops after ischemia [1,11,12].

The release of many vasoactive mediators, cytokines and free oxygen radicals and leukocyte activation from reperfused intestines lead to endothelial dysfunction and edema. Reperfusion may lead to more severe injury than the injury results from ischemia itself. A variety of therapeutic agents have been studied in experimental studies to alleviate the adverse effects of I/R injury on the colonic anastomotic healing process. Commonly anti-inflammatory and/or antioxidant agents have been used to reduce ischemia or prevent ischemia-reperfusion injury [1,4,13].

When the experimental studies are examined, it is seen that mostly mechanical and biochemical parameters are used to evaluate the strength of the colonic anastomosis. Mechanically, the measurement of anastomotic bursting pressure is a commonly used method to examine the safety of colonic anastomosis in experimental studies. Christensen et al.[14] showed that bursting pressure is a meaningful parameter, since anastomotic disruption occurs at the maximum bursting pressure point. At such, the bursting pressure is a more accurate parameter to evaluate the bursting strength than the bursting wall tension [14,15]. In our study, we used the bursting pressure measurement method to evaluate the intestinal anastomoses between groups. We examined the significance levels between the data obtained in this way.

On the molecular level, one of the most meaningful parameters to examine anastomotic strength is tissue collagen content. Collagen fibers are the most important component of the wound healing process and primary responsible for the development of strength. Hydroxyproline is found only in collagen and elastin in animals. Therefore, the HP level in animals is a valuable measure in wound healing. On the fifth and seventh days after surgery, collagen synthesis reaches the peak and the wound strength is mainly due to these newly formed, organized collagen fibers [16,17]. In our experimental colon anastomosis model, we measured the HP level in tissue samples taken from the anastomosis line on the 7th day, when collagen synthesis is at its maximum. We compared the level of anastomosis robustness by looking at the statistical significance level of the results we obtained.

Mesenchymal stem cells (MSCs) are multipotent cells and easily differentiate into mesenchymal lineages. Currently MSCs are commonly preferred in the clinical treatment of various diseases due to biologic characteristic. Easy isolation and *in vitro* cultivation of these cells urge investigators to use them commonly. Particularly due to their high immunoregulatory capacity, MSCs are commonly used in diseases associated with immune system alterations. Adas et al.[18] showed that MSCs significantly accelerated the healing parameters for ischemic colonic anastomosis and increased the level of hydroxyproline on the seventh postoperative day. They also stressed that the histological parameters, necrosis and collagen deposition were also found to be important for the healing of ischemic colonic anastomosis. However, they also reported that MSCs did not accelerated angiogenesis in their study. Caziucet et al.[5] found that stem cells increase bursting pressure by elevating the rate of angiogenesis. Stem cells can be obtained from bone marrow or adipose tissue [19,20]. In our study, we used adipose tissue-derived stem cells which have the capability for direct differentiation to endothelial cells as well as indirectly angiogenic growth factor secretion [21,22].

When the literature is examined, we can see that many studies seek an answer to the question of what we can do for anastomosis safety. Similar to the experimental study we used, it was done by trying different substances. Sayin et al.[1] used montelukast and achieved significant results. Akarsu et al.[2] used simvastatin. Pehlivanlı et al.[4] used dexpanthenol or coenzyme Q10. It is seen that the substances used in the studies generally have anti-inflammatory and/or antioxidant properties. We think that the general structure of MSC will provide an effective improvement in the anastomosis line, since it has anti-inflammatory, antioxidant and angiogenic properties, as well as being multipotent and differentiable.

In our study, when the burst pressure measurements were examined, we found that the highest value was in the MSC group. Burst pressure values of the MSC group were significantly higher than the I/R group. When HP values, which are our other parameters, were examined, we found that the results obtained in the MSC group were significantly higher than both the control group and the I/R group. When the data obtained were examined, it was seen that MSC had positive effects on the healing of colon anastomosis.

In conclusion, our results showed that local application of MSCs improve the healing process of colonic anastomosis subjected to ischemia reperfusion injury. Both anastomotic bursting pressure and hydroxyproline levels considerably supported this finding. We think healing effects of MSCs on the wound healing of colonic anastomosis may be due to its anti-inflammatory, antioxidant and angiogenic effects. Of course, further investigations are needed for clinical topical usage of MSCs on colonic anastomosis subjected to an ischemic impact.

Statement of Author Contributions

H.Ö.: work management, article writing, experimental procedure follow-up; G.K.: design, article writing, literature review, statistics; H.B.: biochemical analysis; M.N.: stem cell production, experimental procedure follow-up; M.G.: design, article writing, literature review; Ç.E.D.: background assessment, review of results and final decision

Conflict of Interest

We declare that there is no conflict of interest.

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