

# PROTECTIVE EFFECT OF PROBIOTICS ON THE HEALING OF COLON ANASTOMOSIS AFTER ISCHEMIA AND REPERFUSION INJURY IN RATS

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## ABSTRACT

**Introduction:** Intestinal transplantation (IT) is one of the important cause of intestinal ischemia and reperfusion injury. It is well known that anastomotic problems seen after IT are mainly related to reperfusion injury. Probiotics are non-pathogenic live microorganisms that are used for various bowel diseases due to their beneficial effects on bowel functions. Wound healing is an important bowel function and impaired healing is a series trouble after IT and ischemic bowel. In this study, we aimed to investigate the effect of probiotics on the intestinal anastomosis healing process.

**Methods:** Forty male SD rats were divided into four groups: Group I (n:10) colonic anastomosis only; Group II (n:10) colonic anastomosis after IR injury; Group III (n:10) probiotic and colonic anastomosis; Group IV (n:10) probiotic and colonic anastomosis after IR injury. Probiotics are given 250 million/day, po. The left colon was cut and anastomosed with continuous 6/0 nylon suture after 60 minutes of reperfusion. Animals were monitored for 7 days, then sacrificed. Macroscopic findings, anastomotic bursting pressures and histopathologic findings were evaluated.

**Results:** Probiotics were well tolerated in the treatment groups (Groups III and IV). Macroscopically, there were no anastomotic leaks or abscess formation in either of the probiotic groups (Groups III and IV).

## RÉSUMÉ

**Effet protecteur des probiotiques sur la cicatrisation de l'anastomose du côlon suite à l'ischémie et à la lésion de la reperfusion chez les rats**

**Introduction:** La Transplantation Intestinale (TI) est une des causes importantes d'ischémie intestinale et de blessure de reperfusion. On sait bien que les lésions anastomotiques apparaissant suite à une TI sont principalement liés à la blessure de la reperfusion. Les probiotiques sont des micro-organismes vivants non-pathogéniques qui sont utilisés pour différentes maladies intestinales, à cause de leur effets bénéfiques sur les fonctions intestinales. La guérison d'une blessure est une fonction intestinale importante et la guérison diminuée est un trouble en série après une TI et l'ischémie intestinale. Dans cette étude, nous avons pour objectif d'investiguer les effets des probiotiques sur le processus de guérison de l'anastomose intestinale.

**Méthodes:** Quarante rats mâles SD ont été divisés en quatre groupes: Groupe I (n:10) anastomose du côlon seulement ; Groupe II (n:10) anastomose du côlon après une blessure IR; Groupe III (n:10) probiotique et anastomose du côlon ; Groupe IV (n:10) probiotique et anastomose du côlon après une blessure IR. Les probiotiques sont administrés en nombre de 250 millions/

Anastomotic bursting pressures of probiotics groups tended to be highly compared with the ischemic control group (Group II), although the difference was not statistically significant. The histologic scores of probiotic groups were very similar to the control groups.

**Conclusion:** The probiotic groups had less adhesions according to the macroscopic findings; anastomotic bursting pressures tended to be high and histologically, they had equivalent healing scores compared with the control groups. We conclude that probiotics have some beneficial effect to impaired anastomotic healing processes, without any side effects on the colon in the colon. Therefore they might be considered as a supportive treatment in IT patients.

**Key words:** probiotics, ischemia and reperfusion injury, intestinal transplantation.

## INTRODUCTION

Intestinal transplantation (IT) is one of the important cause of intestinal ischemia and reperfusion (IR) injury. Early complications, such as bacterial translocation, infections, development of fistulas and anastomosis dehiscence seen after IT, might be attributed to reperfusion injury<sup>1-3</sup>. It is well known that IR injury causes bacterial overgrowth and alteration of the mucosal barrier function. Microflora in the digestive system plays a critical role in human nutrition and health. They are important in a number of normal gut functions, including food digestion, the metabolism of serum proteins, cholesterol, hormones, and vitamins<sup>4</sup>.

When the intestines are damaged for any reason, the composition of the native microflora alters, pathogenic micro-organisms become overgrown, the balance of the microflora deteriorates and, as a result, absorption and other bowel functions are impaired. From the surgical point of view, one of the important bowel functions is the intestinal anastomotic healing process. Extensive bowel resections causing short

bowel syndrome and ischemia and reperfusion injuries of the gut, can impair the intestinal anastomosis healing<sup>5-6</sup>.

Probiotics are beneficial non-pathogenic micro-organisms in the bowel microflora. A wide variety of probiotics are commercially available in standard solutions. Lactobacillus, Bifidobacterium and Enterococcus strains are the most commonly used probiotics. They have been used in medical practice for some time for various bowel diseases, such as inflammatory bowel disease, as well as acute or chronic diarrhea as a supportive treatment. They have beneficial effects that restore the unbalanced microflora of the injured bowel<sup>7</sup>.

We hypothesize that one of the important factors for deteriorating bowel functions after IR injury is the imbalance of the intestinal microflora. Maintaining the balance of the intestinal microflora could play a role in improving bowel functions. Introducing non-pathogenic live microorganisms into the digestive system could prevent bacterial overgrowth and help to keep intestinal microflora intact after IR injury.

**Mots clefs:** probiotiques, lésion ischémique et de reperfusion, transplantation intestinale.

**THE AIM OF THE STUDY** was to evaluate whether probiotics have a beneficial effect on the intestinal anastomosis healing function. For this purpose, we have designed an IR and colon anastomosis model in the rat, since probiotics are predominantly colonized in the colon.

## METHODS

Forty male Sprague Dawley rats, weighing between 200-250 g, were purchased from Baskent University experimental animals breeding center, in Ankara, Turkey. The animals were housed in the Baskent University Experimental Research Centre, in accordance with guidelines established by the Turkish Government.

The rats were divided into four groups. They were anesthetized with intraperitoneal ketamine (60 mg/kg) and xylazine (7 mg/kg). The abdomen was cleansed with povidone iodine and a laparotomy was performed as a midline incision. Room temperature was 23°C during the procedure and the body temperature of each rat was of approximately 36-37°C throughout the surgical procedure. The rats were heated by light and warm pads. Since the proximal part of the left colon is primarily supplied by the superior mesenteric artery in rats, that part was used for the anastomosis. The four study groups were designed as follows:

Group I (n=10). Laparotomy: the left colon was cut and then anastomotized with continuous 6/0 nylon suture, monitored for 7 days, then the rat was sacrificed.

Group II (n=10). Laparotomy: occlusion of the superior mesenteric artery (SMA) with atraumatic micro clamp for 60 min, followed by 60 min reperfusion. The left colon was cut and anastomotized with continuous 6/0 nylon suture, monitored for 7 days, then the rat was sacrificed.

Group III (n=10). Probiotic 250 million/day, po, for 7 days, laparotomy, the left colon was cut and then anastomotized with continuous 6/0 nylon suture, probiotic 250 million/day, po, monitored for 7 days, then the rat was sacrificed.

Group IV (n=10). Probiotic 250 million/day, po, for 7 days, laparotomy, occlusion of superior mesenteric artery (SMA) with atraumatic micro clamp for 60 min, followed by 60 min reperfusion, the left colon was cut and then anastomotized with continuous 6/0 nylon suture, probiotic 250 million/day, po, for 7 days, then the rat was sacrificed.

## Treatment

The probiotics dose administered was of  $1 \times 10^9$  CFU probiotics/kg, as recommended in the

literature<sup>8</sup>. The rats in groups III and IV received 1 ml probiotic solution (250 million live bacteria), which was administered orally by gavage once per day. The rats in groups I and III received 1 ml saline solution daily by gavage. Since colonization of live microorganisms to the bowel requires time, probiotic treatment was initiated 7 days before the surgical procedure. Treatment with probiotics continued after the surgical procedure for 7 more days. The probiotic solution consisted of *Lactobacillus rhamnus* and *acidophilus*, *Enterococcus faecium*, *Bifidobacterium bifidum* and *longum* (NBL Probiotic Gold, Cell Biotech Co. Ltd, S. Korea). Saline administration was initiated and continued as probiotic treatment.

## Evaluation parameters

### Macroscopic findings

The anastomotic area of the colon was quantitatively evaluated by an experienced surgeon in a blinded manner during necropsy. Anastomotic adhesions, stenosis and/or dehiscence and intestinal obstructions were graded from 0-to-3, as modified from Galili et al<sup>9</sup>; Grade 0: No adhesion, Grade I: A single organ, omentum or bowel, adherent to the anastomosis, Grade II: Two organs adherent to the anastomosis. Grade III: More than two organs adherent or complex adhesion around the anastomosis. The scores from each of the groups were then totalized and averaged to give a mean macroscopic score for each group.

### Anastomotic bursting pressure

The strength of each anastomosis was assessed, measuring its bursting pressure. The anastomotic bursting pressures were measured as we previously described<sup>10</sup>. Briefly, the anastomotic colon segment was resected, the proximal end of the resected bowel was connected to a monitor through a pressure transducer, and the distal end of the resected bowel was connected to a fluid pump, operating at 1 ml/min. The pressures were recorded in millimetres of mercury on a monitor. The pressures of the groups were then totalized and averaged to provide a mean bursting pressure.

### Histopathology

Histopathologic evaluation was performed by an experienced pathologist in a blinded manner. Full thickness sections of the intestinal anastomoses were obtained at necropsy, fixed in 10% buffered formaldehyde solution; after routine procedures, embedded in paraffin, cut into sections 4-to-5 µm wide and stained with hematoxylin-eosin. Epithelization, cellular infiltration, neovascularization, fibroblastic proliferation and collagen deposition were graded from

0-2 (0=absent, 1=mild to moderate, 2=marked) for each parameter, as modified from Greenhalgh et al<sup>11</sup>. Scores were then totalized and averaged to give a mean histological score.

### Statistical analysis

The results are expressed as mean  $\pm$ SD. The differences between the groups were analyzed by the Kruskal-Wallis test, followed by the Dunn test. Probability values  $p < 0.05$  were considered significant. The SPSS 17.0 (SPSS Ver. 17.0, Chicago IL, USA) program was used for analysis.

## RESULTS

### Macroscopic finding at necropsy

Animals in Group I had minimal anastomotic adhesion. There was no presence of anastomotic dehiscence, abscess or stenosis in this group of animals (mean scores was  $0.9 \pm 0.1$ ). The IR injury, however, increased the mean macroscopic scores to  $1.4 \pm 0.2$  (Table 1). There were dense anastomotic adhesions as well as partial anastomotic stenosis and bowel dilatation on the proximal side of the anastomosis in most of the animals in this group. The mean macroscopic scores of the probiotic treatment groups (Groups III and IV) were lower than the non-treatment control groups; there was a significant difference between Groups III and II and between groups IV and II ( $p < 0.05$ ). Regardless of the ischemic injury, all animals in the probiotic treatment groups showed almost normal bowel appearance; there was no anastomotic stenosis or dilatation. Four of ten rats in Group III and three of ten rats in Group IV had minimal anastomotic adhesion only.

### Anastomotic bursting pressure

The mean anastomotic bursting pressures of all study groups are shown in Table 1. The mean bursting pressures of the non-ischemic rats (Group I) was  $168 \pm 11$  mmHg. When the rats were treated with probiotics (Group III), this mean pressure value increased to  $190 \pm 13$  mmHg. However, the differences failed

to reach statistical significance ( $p > 0.05$ ). Intestinal ischemia/reperfusion injury (Group II) decreased the mean anastomotic bursting pressure to  $159 \pm 11$  mmHg. Treatment of these animals with probiotics (Group IV) caused the mean pressure to increase to  $167$  mmHg, although the differences did not reach statistical significance ( $p > 0.05$ )

### Histologic evaluation

Histopathologic examination of the anastomosis showed very similar results in all study groups. Moderately thick granulation tissue, moderate neo-vascularization and epithelial migration, as well as moderate to severe cellular infiltration and collagen sediment, were found in all groups. The mean histologic scores of the study groups are shown in Table I. There were no statistical differences between the groups ( $p > 0.05$ )

## DISCUSSION

The pathophysiology and consequences of intestinal IR injuries are well known<sup>12</sup>. Reactive oxygen metabolites are released, epithelial cells are lost, and villus heights and crypt depths are reduced. As a consequence of these cellular changes, bowel functions are impaired, bacterial overgrowth and bacterial translocation occur, leading to infection, sepsis and even death in some cases. Although various treatment strategies are available to prevent these complications, morbidity and mortality are still high in mesenteric ischemia, particularly for elderly and co-morbid patients. Anastomotic complications are also very common in these patients<sup>13</sup>.

Intestinal anastomosis healing is an important process to prevent local and systemic complications after IT and bowel resections. It is well documented that a reperfusion injury can delay healing of intestinal anastomosis<sup>5</sup>. If the onset of intestinal anastomosis is delayed, local complications such as abscesses or adhesions due to transient leakage of intestinal content may occur. One of the main findings of the present study is that the healing of colon anastomosis,

**Table 1.** Mean macroscopic scores, bursting pressures and histologic scores of the groups.

	Macroscopic scores (0 to 3) <sub>w</sub>	Bursting pressure (mmHg)	Histologic scores (0 to 10)
Group I	$0.9 \pm 0.1$	$168 \pm 11$	$8.2 \pm 0.4$
Group II	$1.4 \pm 0.2$ *	$159 \pm 11$	$8.0 \pm 0.5$
Group III	$0.6 \pm 0.1$	$190 \pm 13$	$7.2 \pm 0.4$
Group IV	$0.5 \pm 0.1$	$167 \pm 14$	$7.8 \pm 0.4$
	* $p < 0.05$	$P = 0.4$	$P = 0.3$

\* Groups III and IV were significantly statistically different when compared with Group II.

as measured in the clinical and mechanical parameters, was slightly impaired in rats who had undergone IR injury. Our data indicated that an IR injury may delay the healing process, which was determined by macroscopic evaluation. Rats with an IR injury had significantly more adhesions around the anastomosis compared with the others. However, our data indicated that this effect is transient and the healing process proceeded and was completed with time; the IR injury had no effect on the strength and histologic appearance of the colon anastomosis at the end of the study. This data might be explained by temporary influence of the colon from the ischemic injury. After IR injury, the healing process is delayed, leakage from partial anastomotic dehiscence may occur and it can cause perianastomotic dense adhesions. The colon recovers later and healing is completed leaving almost the same condition as a normal colon. Effective collateral circulation and splanchnic autoregulation of the colon may play a role in protecting the colon from ischemia.

Probiotics have been utilized in some bowel diseases such as inflammatory bowel disease, acute or chronic diarrhea and constipation as a supportive treatment for many years. Probiotics have the potential to decrease the severity of intestinal mucositis injury through the possible reduction of pro-inflammatory cytokines secretion and gene expression, the release of anti-inflammatory cytokines, the inhibition of inflammatory pathways, the improvement of barrier function maintenance of mucin secretion, the prevention of epithelial cell apoptosis and oxidative damage, and the elimination of pathogenic bacteria<sup>14</sup>.

The other main finding of the present study is that probiotics prevent any delay in the onset of colonic anastomosis healing. The rats who had an IR injury and were treated with probiotics showed significantly better macroscopic appearance at necropsy. Their macroscopic scores were significantly lower than the non-treatment IR group. Anastomotic adhesions were very weak and limited, there was no dehiscence, abscess or stricture in this group of animals. Moreover, this probiotic effect was consistently determined in animals who did not have an IR injury. This data support our hypothesis that maintaining the microflora balance in the colon may help to improve bowel functions. Explanation of how probiotics prevent delay in the anastomosis healing process is beyond the scope of the present study. Further studies are needed to explain the mechanism behind this effect. It is well known that IR injuries cause bacterial overgrowth and break down the microflora balance of the gut. They can consequently cause bacterial translocation, systemic inflammatory response syndrome, irreversible intestinal necrosis and remote organ failure. It is therefore postulated that maintaining the microflora

balance is therefore particularly important to prevent all these complications.

In this study, we demonstrated that probiotic treatment has some beneficial effect to prevent local complications of the colonic anastomosis after IR injury in a rat. There are several mechanisms that could play a role in this effect: One of the common mechanisms identified in probiotics is the inhibition of pathogenic enteric bacteria in the bowel<sup>14</sup>. This helps to prevent bacterial overgrowth and bacterial translocation. In this way, probiotics enable maintenance of intestinal microflora balance in the animal. Protection of the microflora balance could lead to improvements in colon anastomosis healing.

One of the other important mechanism inherent to probiotics is the increase of epithelial barrier functions and the regulation of host immunoregulation<sup>15</sup>. It has been previously shown that *Lactobacillus rhamnosus* species have an impact on the elimination of pathogenic bacteria inhibition of cell apoptosis and maintenance of intestinal permeability in the injured bowel<sup>16-18</sup>. *Bifidobacterium* species also have anti-inflammatory effects by inhibiting IL-8 gene expression<sup>19</sup>. One or more of these beneficial effects of probiotics on the bowel could provide an explanation for our results.

In summary, in this study we showed that probiotics were well tolerated by the rats. Probiotic treatment of animals led to improved anastomosis healing results according to the macroscopic findings. Anastomotic bursting pressures tended to be high and histologically, they had almost equivalent healing scores compared with the control groups. In the light of our findings, we would conclude that probiotics have some beneficial effect to prevent anastomotic local complications after intestinal IR injury in the colon. Therefore they might be considered as a supportive treatment in IT patients..

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#### Disclosure of Potential Conflicts of Interest:

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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