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Increasing anastomosis safety and preventing abdominal adhesion formation by the use of polypeptides in the rat

Short title: Anastomosis protection and adhesion prevention by polypeptides

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Abstract

Background and aims: Postoperative adhesions can potentially be reduced using different anti-adhesive agents, though these drugs, however, tend to compromise healing of an intestinal anastomosis. No method that significantly increases anastomosis safety is known at present. The aim of the study was to develop a concept of preventing postoperative adhesions using differently charged bioactive polypeptides, also considering healing and safety of an intestinal anastomosis.

Methods: An ileocolic anastomosis was performed under both "clean" and "septic" conditions in the rat. The treatment group received intraperitoneal poly-L-lysine and poly-L-glutamate while controls received sodium chloride. Abdominal adhesions, anastomosis leakage and burst pressure were analyzed after 1, 3, 5 and 7 days in the "clean" anastomosis model and after 7 days in the "septic" model.

Results: A significant decrease ($p<0.01$) in the amount of adhesions was seen in animals treated with polypeptides after 1, 3, and 5 days, while no difference was seen after 7 days. The anastomosis demonstrated a significantly higher burst pressure as evaluated day 1 and 3 ($p<0.05$ and $p<0.01$, respectively) in the polypeptide treated animals while no difference was seen between the groups at days 5 or 7.

C*onclusion*: The use of differently charged polypeptides administered intraperitoneal after surgery resulted in a significant decrease in the extent of postoperative adhesions. Furthermore, an increase in intestinal anastomosis safety, based on improved burst pressure during the first 3 days, i.e. the critical period during the healing process, was noted. No adverse effects were seen in surgery during "septic" conditions.

Key Words: adhesions, anastomotic healing, polypeptides, burst pressure.

Introduction

Colonic surgery is still associated with a comparably high risk of postoperative complications. Among others, postoperative small intestinal obstruction, septic complications, wound infections, abdominal abscesses and anastomosis leakage are complications that frequently lead to prolonged hospital stay, potential mortality and readmission[1]. Furthermore, ileus and leakage represent main causes for reoperations. Ways to significantly increase intestinal anastomosis safety are not yet known[2]. The incidence of postsurgical adhesions have been reported to be decreased through different ways[3], though an increased risk of intestinal anastomosis leakage has been noted following prevention of abdominal adhesions using hyaluronic acid derivatives[4]. The aim of the present study was to describe a mode of intervention that both increase intestinal anastomosis safety after colonic surgery and effectively decrease the postoperative adhesion rate. An ileocolic anastomosis under normal ("clean") and septic (following cekal ligation and puncture, CLP) conditions was performed in the rat. Prevention included the intraperitoneal application of positively and negatively charged polypeptides. In this pilot study, anastomosis strength, tissue morphology and adhesion formation were chosen as evaluation parameters.

Materials and methods

Animals

One hundred and ten male Sprague-Dawley rats weighing about 250g were used including a separate pool of twelve animals for histological studies. The animals were kept under standardized conditions, had free access to pellets and tap water and were fasted twelve hours prior to the operation. The study was conducted with approval of the local ethical committee.

Chemicals

Osmotic balanced (2,54w% glycerol) aqueous solutions (0.5%) of poly-L-glutamate (pG) and poly-L-lysine (pL) were freshly prepared on the day of the experiment and stored in the refrigerator until used. FITC labelled pL was mixed with pL (1:10 wt %) to track the route of pL by fluorescent microscopy. All chemicals and liquids used for cell culture were purchased from Sigma-Aldrich (St Louis, MO, USA).

Equipment

Regular infusion tubes were used as plastic tubes for the anastomosis burst experiment (Becton Dickinson Infusion Therapy AB, Helsingborg, Sweden). An infusion pump (Syringe Mynder, Critikon Ltd., Basingstoke, UK) served the pressure load of the intestine, which was recorded by an electronic pressure sensor connected to a computer (PowerLab, ADInstruments, Castle Hill, Australia) and processed using specific software (Scope for Windows v.3.6.3, ADInstruments).

Model

The "clean" anastomosis: Anesthesia was induced by ketamine 60 mg/kg (Ketalar, Pfizer, NY, USA) and xylazine 16 mg/kg (Rompun Vet, Bayer AB, Gothenburg, Sweden) by an intramuscular injection. After disinfection, an approximately 5cm long midline laparotomy was performed. The cecum was exposed and removed following dissection of the mesenterium close to the intestinal wall. An end-to-end ileo-colostomy was prepared with eight single interrupted sutures using 7/0 polypropylene (Prolene; Ethicon, Johnson & Johnson, Somerville, New Jersey, USA) thread. The abdomen was closed in two layers using a 4/0 polypropylene suture.

"Septic" conditions: Anesthesia was induced as above. Sepsis was induced by the traditional cecal ligation and puncture (CLP) model[5]. Briefly, the cecum was exposed through a midline laparotomy, and the distal part was ligated about 5mm from the ileocecal junction in order to avoid its obstruction. The ligated part was then punctured with an 18 G needle at both sides and gently squeezed to extrude some stool prior to placing the cecum back to the abdominal cavity and closing the abdomen. Twenty-four hours later, the animals were reanesthetized and the abdominal cavity was washed out with 10 ml isotonic sodium chloride. The cecum was then resected and an ileo-colostomy was performed as described above. The abdomen was closed in two layers using a 4/0 polypropylene suture. Tobramycin was given at a dose of 1.5 mg/kg in 10 ml isotonic saline, administered subcutaneous.

Experimental design

Adhesion study

The animals were randomly divided into 10 groups based on treatment and evaluation time (Table 1). The control groups had an intraperitoneal injection of 6 ml (0.9 %) sodium chloride solution. The five treatment groups received three ml pL and one minute later 3ml pG solution prior to abdominal closure. A treatment group and the respective control were sacrificed on day 1, 3, 5 and 7. The animals from the "septic" experiment were sacrificed on day 7. At the evaluation time, the adhesions were counted and expressed as a cumulative adhesion score[6].

Anastomosis burst

A 6 cm long intestinal segment was resected with the anastomosis area located in the middle[7]. If adhesions were present they were not dissected but harvested together with the anastomos

since dissection could diminish the strength of the anastomos[8]. A silicon tube was inserted into the ileum, while the colonic side was ligated at the end. The intestinal segment was placed immediately under isotonic sodium chloride and during a standard speed of 1.65 ml/min, methylene blue stained saline was infused into the intestinal segment and the intraluminar pressure was monitorized (Fig.1). The maximum pressure prior to anastomotic burst was recorded as the burst pressure. In case of very small leakage, the pressure at the appearance of methylen blue around the anastomosis was considered as the burst pressure despite that the pressure increased further by time in some cases. Evaluation was performed on days 1,3,5 and seven and the anastomotic burst pressure was chosen as parameter since it has been suggested to be a sensitive marker in early anastomotic healing[9].

Histology

Six animals were injected with FITC labelled pL + pG, and two-two animals were sacrificed on postoperative day 1, 3 and five respectively. The anastomosed segment was excised. The specimen was rapidly frozen and embedded, after which the block immediately underwent consecutive cutting to 7µm wide slices. The slides were let to dry in dark for 30 minutes at room temperature after which the cells were stained with 100ng/ml 4´6´-diamino-2-phenylindole hydrochloride (DAPI) [10] solution for 10 minutes. Fluorescent microscopy was performed with both FITC and DAPI filter, and images were digitally merged (OpenLab, Improvosion Inc., Cranberry Hill, Lexington, MA, USA). Ordinary histology was not performed due to problems with normal fixation of the polypeptide complex noted in previous studies (data not shown).

A additional pool of six animals (three treated and three control animals) were operated and kept for 5 months until sacrifice. Hematoxyline and eosin staining were performed to examine the healing of the anastomosis in long term.

Statistical analysis

Kruskal-Wallis test was performed to determine differences between the groups and Mann Whitney U test was used to compare the burst pressures and the adhesion scores between the individual groups. A p-value less than 0.05 were considered statistically significant.

Results

The mortality did not differ between the control and treated groups, though 60% of the CLP animals died prior the 7-day evaluation.

Burst pressure

Significantly higher pressure resistance ($p \leq 0.05$ and $p \leq 0.01$, respectively) was detected in animals receiving pL+pG as evaluated on the first and third days following treatment as compared to controls (Fig.2). There were no statistical differences in burst pressure after five and seven days, neither in "septic" or non-septic animals.

Adhesions

The animals subjected to polypeptide treatment developed less adhesions as compared to controls at all time points studied ($p \leq 0.01$; $p \leq 0.001$ and $p \leq 0.01$ at 1,3 and 5 days, respectively) in both models though the difference was not statistically significant at the seventh day between the non-septic groups (Fig.3). Significantly less adhesions ($p \leq 0.05$) were recorded in polypeptide treated animals that underwent ileocolostomy in peritonitis as seen seven days after surgery.

Histology

Using fluorescent microscopy, the histology (Fig.4) demonstrated that a protecting layer of pL+pG complex was located in the anastomosis from the first day. The deposit formed in the

wound was covered by an increasing number of cells by time and was almost rebuilt till the fifth day. The area in green colour represents the pL+pG complex, while blue stands for all nuclei.

In the animals kept for 5 months histology with Heamatoxyline-eosin staining showed normal healing in both controls and treated animals (Fig.5).

Discussion

Anastomotic insufficiency and postoperative ileus due to abdominal adhesions represent major complications following intestinal surgery, conditions associated with substantial morbidity and mortality[11]. We have previously shown that the combination of poly-L-lysine and poly-L-glutamate significantly reduces adhesions in an experimental model on abdominal adhesions[12]. Results from the former study and other pilot studies (data not published) demonstrates that the use of differently charged polymers decrease the incidence of abdominal adhesions and in addition improved the anastomotic safety as noted in the present study. Controversies exits whether to use commercial antiadhesive products available today clinically when there is need for an intestinal anastomos[13]. Icodextrin 4% as well as hyaluronic-acid-carboxymethylcellulose membrane has, however, in animal studies not been noted to have any adverse effect on the anastomos[14, 15].

We used bursting pressure to test the intestinal anastomosis strength[9, 7]. By applying methylen blue in the irrigation fluid and placing the specimen under isotonic solution, the method proved sensitive during the entire examination period. Considering that this method required removal of the intestinal segment from its circulation, the specimen had to be analyzed immediately. Other authors have reported significant differences in bursting pressure, time dependently, up to day five, explaining why we chose this method of testing

the anastomosis. Breaking strength is after this a more sensitive method to test the anastomos. Breaking strength is noted to increase progressively from day 7 to 28[16], but the clinical relevance of this could be discussed.

The combination of poly-L-lysine and poly-L-glutamate increased intestinal anastomotic strength during the first postoperative days. This represents the most critical period regarding postoperative anastomosis insufficiency, clinically symptomatic after 5-7 days[2]. In animals kept for 5 months histology showed a normal healed anastomos.

Poly-L-lysine has a strong positive charge while poly-L-glutamate has a negative charge. The mixture of these appositively charged polypeptides resulted in a non-or low soluble complex and the application in the peritoneal cavity represent a new concept for prevention and treatment. To some extent, mechanisms can be suggested from simple physical-chemical arguments. A mixed solution of one polycation, like poly-L-lysine, and one polyanion, like poly-L-glutamate, will strongly associate due to electrostatic interactions. This association may have a number of macroscopic consequences, the two most important being increased viscosity, with often a gel-like appearance, and an associated phase separation. The latter can lead to formation of precipitates of the two oppositely charged polyions. The injured peritoneum, exposing among other phospholipids, will be negatively charged or neutral[17]. A polycation is expected to associate to all anionic surfaces. An important general mechanism leading to the beneficial behaviour of the mixed polyanion-polycation systems lies in their association. For example, the formation of association structures and an increased viscosity will reduce the possibility of surrounding surfaces to approach, as well as to adhere to each other. A partial association of the polycation to the surfaces may further assist in obtaining the observed effect. We hypothesized that administration of poly-L-lysine prior to poly-G-glutamate allowed poly-L-lysine to adhere to the injured peritoneum and was then neutralized by poly-G-glutamate. This could explain why the poly-L-lysine/poly-L-glutamate complex is

located inside the wound. Obviously, this explanation is quite oversimplified for these complex systems but will be further tested in future studies. Concerning the migration of the compound to the wound, it has been shown that poly-L-lysine is able to migrate through lipid bilayers[18, 19] which could decrease the poly-L-lysine/poly-G-glutamate ratio locally in the wound. If so, more poly-L-lysine molecules can adhere to the wound, resulting in an accumulation of the poly-L-lysine/poly-L-glutamate exclusively at the wound site. The poly-L-lysine/poly-L-glutamate amount could rapidly be taken up and digested by phagocytes[12], although their carrier function in the process is less probable considering the rapid development of the protecting layer (within 1day). This idea is supported by our findings that no visible residual compound was located anywhere else over the surrounding peritoneum. The question why the anastomosis containing the poly-L-lysine/poly-L-glutamate complex is stronger than the one without still remains to be elucidated.

Poly-L-lysine is used to improve biocompatibility of foreign particles, implants[20]. This is in accordance with our observation, as the free surface of the poly-L-lysine + poly-L-glutamate deposit was rapidly covered and subsequently rebuilt by cells. This process can be the cause of the rapid isolation of the wound from the other peritoneal surfaces and subsequently protects against intraperitoneal adhesions. In a pilot study we have shown that a mesothelial cell layer developed and completely covered the compound within 5-7 days following the injury, while the injury site was completely covered with poly-L-lysine + poly-L-glutamate within one day (data not shown). As soon as the protective layer and the mesothelial cell layer exists, no further adhesions develop[21]. A simple ileocolic anastomosis does not lead to extensive peritoneal damage, but a concomitant peritonitis results in a large inflamed peritoneal surface area that may be responsible for the difference in extent of adhesions noted between “septic” and “non-septic” animals. The peritoneal adhesions decreased significantly at all time points evaluated though not at one week in the “non-septic” group as compared to

controls. This can partly be explained by the fact that relatively limited adhesions were seen also in controls after a normal anastomosis. Neither bursting pressure nor the amount of adhesions were studied at earlier time points than 1 week in the CLP model because of the high mortality during the first days[22]. The operative management of the colon in coexisting peritonitis has recently changed with an increasing percentage of resections performed with primary anastomosis[23]. Doubts has also risen whereas to use antiadhesive agents at all during peritonitis since this could potentially diminish the abdominal host defense capacity of restraining infection and e.g. demarcate abscesses as a normal healing procedure[24]. Due to this reason and considering that septic complications cannot be excluded after colonic surgery, we tested the polypeptides also during peritonitis. The increase in anastomosis burst strength, even though not significant after one week, implies that the treatment could be safely applied also in an inflamed environment. We have no information yet about a potential benefit when applying these polypeptides concerning outcome during the early postoperative days in sepsis. Further studies have to be conducted on this.

This pilot study demonstrating a significant higher tolerance to burst pressure using differently charged polypeptides lead to more questions concerning the healing. The neutrophil recruitment, collagen concentration and tension breaking strength should be investigated in future studies even though the validity of these parameters is debated[9, 25]

No antiadhesive agents today have been proven safe for use in clinical situations with an infected abdomen or when an intestinal anastomosis has been performed. The need is thus large for an antiadhesive agent that safely could be applied in all conditions and circumstances. The polypeptide concept, as demonstrated in the present experimental study, could be of interest. Toxicology studies are being planned in order to further process these possible drugs into potential human studies.

In summary, we have demonstrated that a low soluble polypeptide complex, consisting of the positively charged poly-L-lysine and the negatively charged poly-L-glutamate increased the safety of a colonic anastomosis during the first postoperative days, resulting in a significant pressure tolerance. Furthermore, a decrease in post-surgical adhesions could be shown using this treatment concept.

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	Animals	Treatment	Condition	Time of evaluation
Group 1	Ten	Poly-L-lysine + poly-L-glutamate	Clean	1 day
Group 2	Ten	Sodium Chloride	Clean	1 day
Group 3	Ten	Poly-L-lysine + poly-L-glutamate	Clean	3 days
Group 4	Ten	Sodium Chloride	Clean	3 days
Group 5	Ten	Poly-L-lysine + poly-L-glutamate	Clean	5 days
Group 6	Ten	Sodium Chloride	Clean	5 days
Group 7	Ten	Poly-L-lysine + poly-L-glutamate	Clean	7 days
Group 8	Ten	Sodium Chloride	Clean	7 days
Group 9	Twelve	Poly-L-lysine + poly-L-glutamate	Septic	7 days
Group 10	Twelve	Sodium Chloride	Septic	7 days

Table 1. Experimental design and group division of rats

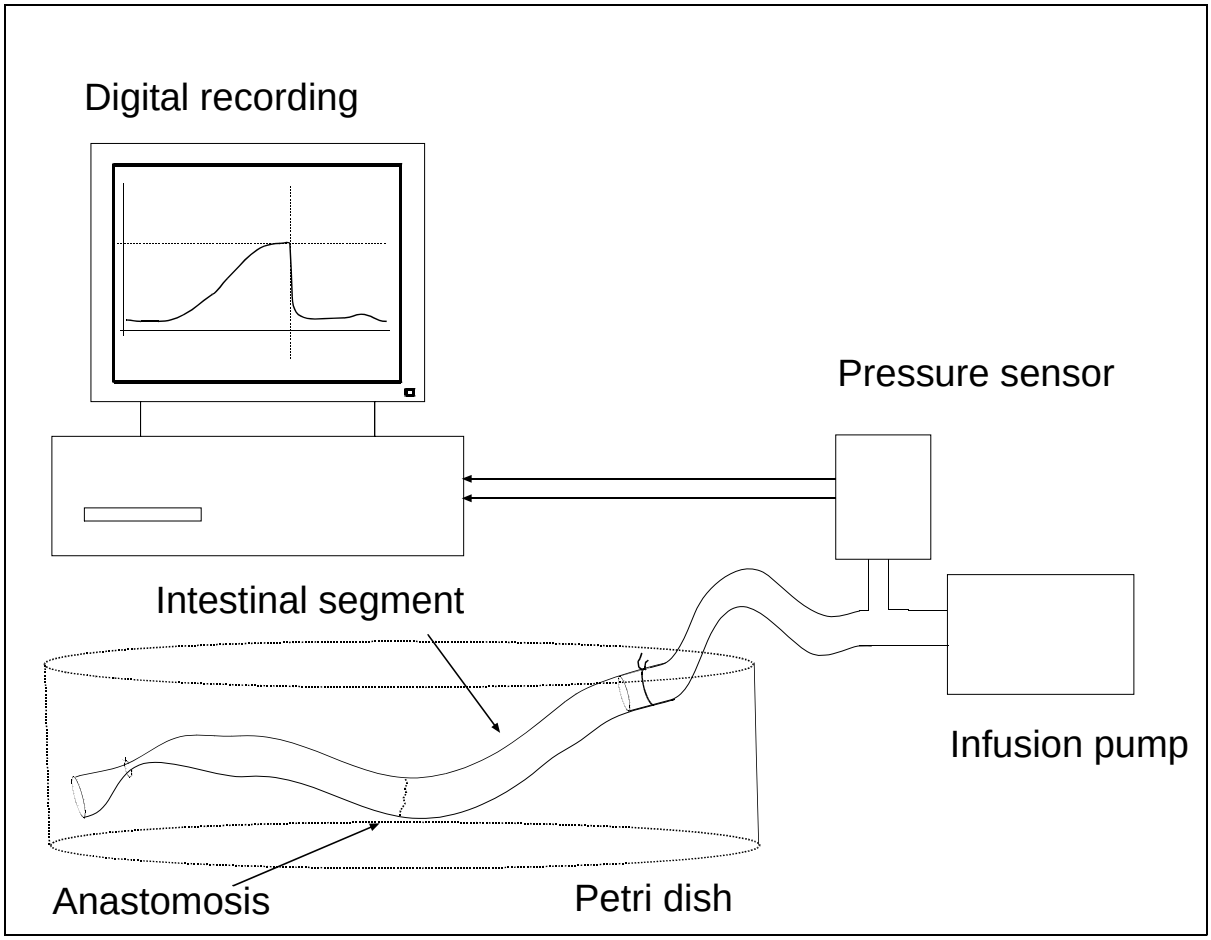

Fig. 1.
Experimental design for checking the bursting pressure

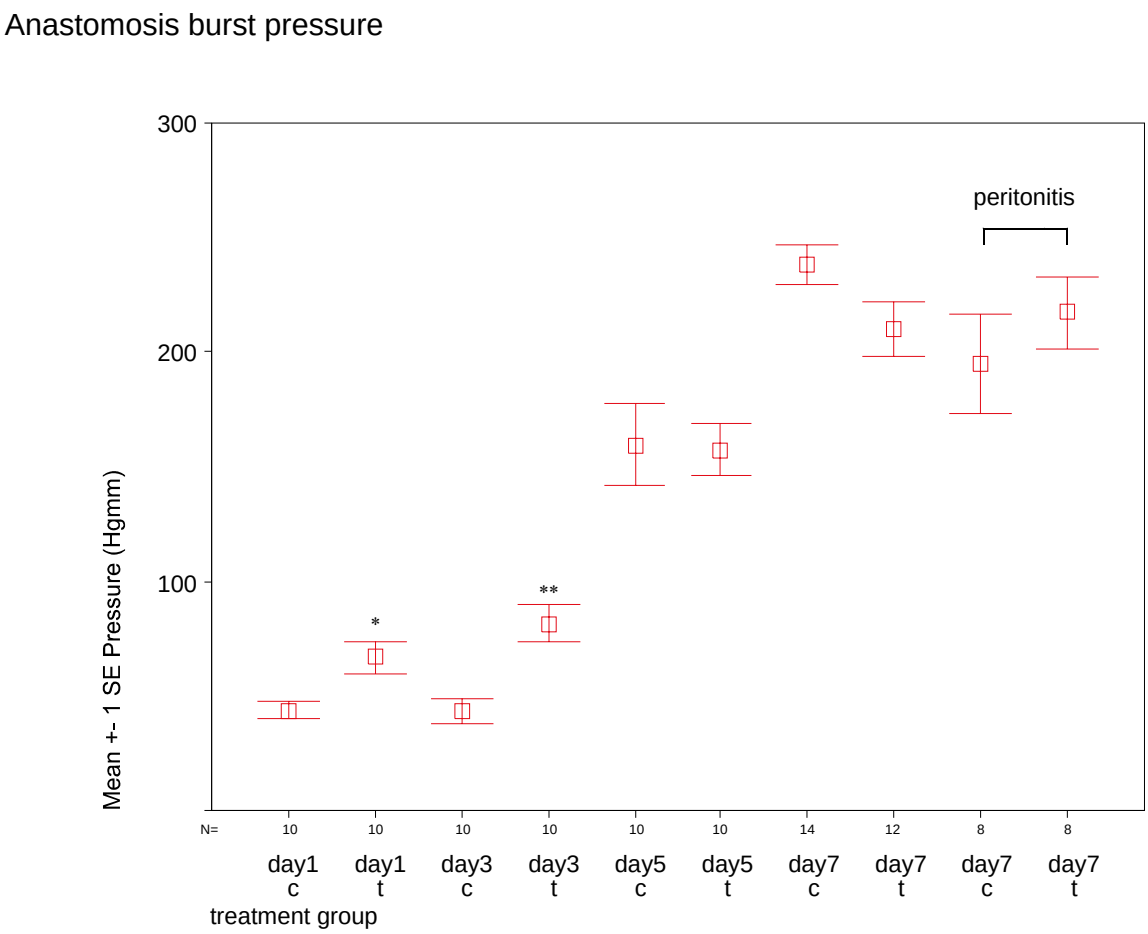

Fig. 2. The anastomosis burst pressure significantly increased 1-3 days after surgery while no significant differences were recorded later in time. *p<0.05; **p<0.01 vs. controls. c=control; t=treatment

Peritoneal adhesions

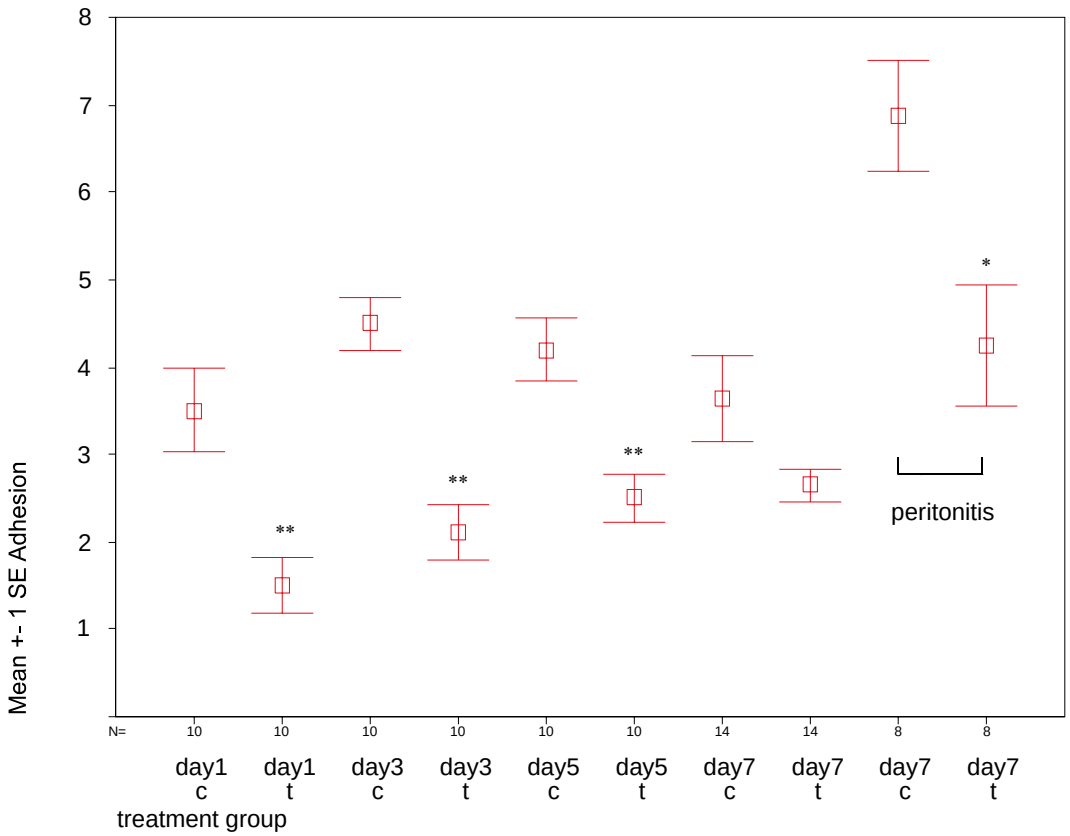

Fig. 3. Peritoneal adhesions. Less adhesions were seen in the treated group in both the "non-septic" and "septic" anastomosis groups. *p<0.05; **p<0.01 vs. controls.
c=control; t=treatment

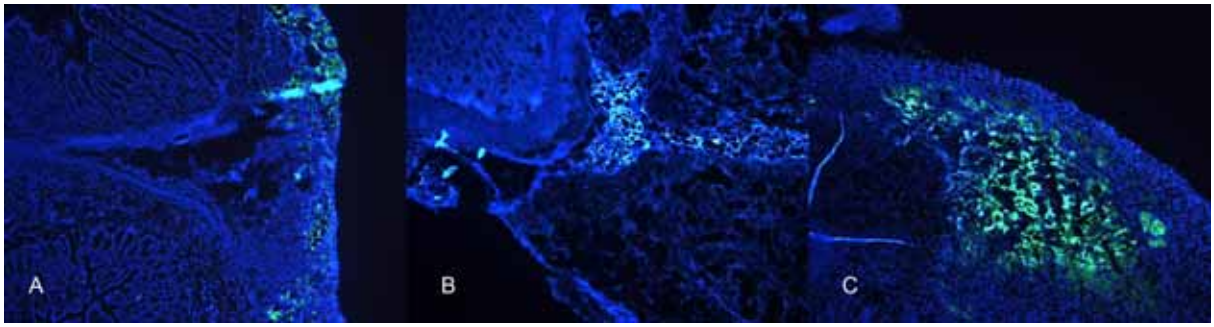

Fig. 4. Histology: Fluorescence activity of the labelled poly-L-lysine + poly-G-glutamate complex is visible 1(A), 3(B) and 5(C) days following intraperitoneal administration.

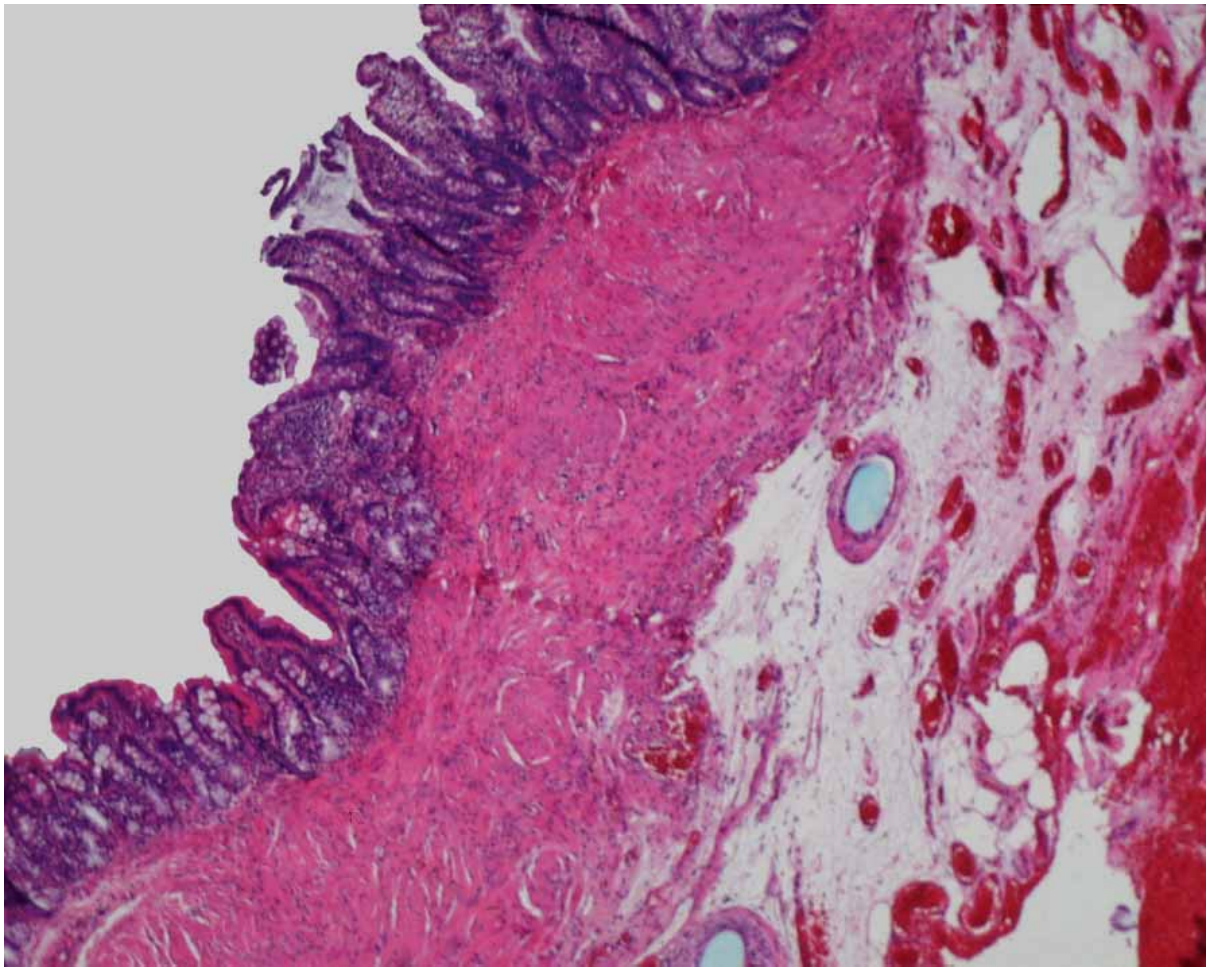

Fig. 5. Histology with healed anastomos in animal treated with poly-L-lysine and poly-L – glutamate after 5 months.