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The influence on abdominal adhesions and inflammation in rabbits after exposure to differently charged polypeptides

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ABSTRACT

Background: Abdominal adhesions develop on damaged peritoneal surfaces and constitute a significant health related problem. Previous animal studies have shown promising anti-adhesive effects when administering the polycation α -poly-L-lysine (α PL) and the polyanion poly-L-glutamate (PG) together. The objective of the study was to examine the effect of these differently charged polypeptides when administered by spraying and to evaluate any possible effect on fibrinolysis, fibrosis and inflammation. **Methods:** Rabbits were treated with PLPG after cecal abrasive surgery and analysis from peritoneal biopsies of active tPa/PAI-1 complex and from peritoneal fluid of IL-6 and active TGF β 1 at day 0, 1, 4 and 10 were measured after surgery. Histological specimens were analyzed on day 10 regarding inflammation and fibrosis. Peritoneal adhesions were evaluated by adhesion score. All values were compared to the control group (NaCl). **Results:** PLPG-treated rabbits had a significant diminished adhesion score on day 10 as compared to the control group ($p < 0.005$). Significantly reduced collagen depositions on the peritoneum were seen in the PLPG group when evaluating the histological specimens ($p < 0.05$). No significant differences between the experimental and control groups were seen in peritoneal fluid when analyzing for active protein levels. **Conclusion:** This is the first study to investigate the effect on key parameters in adhesion formation as well as the preventive effect of the PLPG complex on abdominal adhesions in rabbits and also the first study where administration by spraying the polypeptides was used. PLPG was non-toxic in this setting and without significant differences in adhesion formation parameters and a significant reduction in adhesions was observed. This was verified both macroscopically and histologically.

Keywords: Polycations; Abdominal Adhesions; Coagulation; Fibrosis; Inflammation

1. INTRODUCTION

Abdominal adhesions constitute a major clinical challenge causing small bowel obstruction [SBO], abdominal pain, female infertility and a high annual cost for health-care [1,2]. Abdominal adhesions form due to surgery, abdominal infections, foreign bodies and other similar events that may harm the peritoneum [3,4]. The exact mechanism how adhesions form is not elucidated, but the overall picture is quite clear.

Remnants of fibrin due to impaired local fibrinolysis are considered to be the major initiator of abdominal adhesion development [5]. The fibrin residues may result in stable fibrin strands that become invaded by different inflammatory cells and collagen fibers, thus eventually forming stable adhesions [6-8]. Decreased tissue plasminogen activator (tPA) and elevated levels of plasminogen activator inhibitor-1 (PAI-1) resulting in diminished fibrinolysis along with extended fibrosis associated with increased transforming growth factor beta (TGF- β) and higher amounts of inflammatory cytokines such as IL-6 are all part of the local peritoneal adhesion formation [9-11].

Previous *in vivo* studies (mouse and rat) have shown promising results when differently charged polypeptides, *i.e.*, the polycation α -poly-L-lysine and the polyanion α -poly-L-glutamate, were combined together. They form a neutrally charged complex on the injured peritoneal site, thereby reducing adhesion formation [12].

This study aimed to investigate the anti-adhesive effect of the α -poly-L-lysine and α -poly-L-glutamate complex in a rabbit model and its possible effect on local peritoneal adhesion formation by measuring the active tPa/PAI-1 complex, TGF β 1 and IL-6. To minimize the used dose, we aimed to examine whether administration by spraying the differently charged polypeptides decreased adhesions. Furthermore, we aimed to examine if

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the histological picture (inflammation and fibrosis) was influenced when administering the polymer complex on the peritoneal wound site.

2. METHODS AND ANIMALS

2.1. Animals

A total of 28 female Rabbits (Swedish Lop) weighing approximately 2.6 - 3.3 kg were used for induction of peritoneal adhesions. The animals were kept under standardized conditions and had free access to water and pellets. The local ethical committee at Lund University approved the adhesion study (Lund, Sweden M39-10) and the animals received the best animal care in compliance with the guidelines of the Swedish Government and Lund University, Sweden.

2.2. Chemicals

The chemicals α -poly-L-lysine MW > 30,000 kDa (PL) and α -poly-L-glutamate MW 15 - 50 kD (PG) (Sigma Aldrich™, St. Louis, Missouri, USA) were freshly mixed on the day of the experiment with 2.54% glycerol and water in an osmotic balanced solution to a final concentration of 0.5% (5 mg/ml). They were put in separate bottle atomizers that administrated 0.5 ml volume with one dose.

2.3. Model

The animals were anesthetized with 50 mg/kg Ketalar (Parker Davis™ Detroit, Michigan, USA) and Xylazine 6 mg/kg (Rompun: Bayer Sverige AB Sweden) by an intramuscular injection.

A standardized animal adhesion model, cecal abrasion, was performed under sterile conditions. In brief, after a 10 cm midline incision, the cecal area (approx. 2 cm²) was abraded to petechial bleeding using a dry sponge. On the right side of the abdominal wall a square section of 1 cm × 1 cm peritoneum was excised down to the right transverse abdominal muscle and the wound surfaces were gently dabbed with sterile gauze to remove excess blood. The abraded cecum was put back into the abdomen adjacent to the denuded lateral wall.

Treatment was then applied by first spraying the PL and thereafter PG followed by abdominal closure using a running suture (Prolene™ 4-0, Ethicon, Somerville, NJ, USA) in two layers. The animals received subcutaneous saline (0.9%, 10 ml) for resuscitation and buprenorphine for postoperative pain control.

All animals were weighed in conjunction with every operative procedure.

2.4. Experimental Design

The rabbits were randomly and blinded divided at the

beginning of the experiment into different groups according to **Table 1**. Group A (n = 6) received a dose of PL and PG (0.5%, 1 ml of each corresponding to a dose of 2 mg/kg) administered by spray; first the PL was sprayed and after 10 - 15 seconds the PG was given. The control group B (n = 6) received saline (0.9%) 2 ml administered locally by spraying after the surgical procedure but before closure of the abdomen.

In groups A and B peritoneal lavage and biopsies of tissue (size 1 - 2 mm²) in the operation area of the abdominal wall were taken on day 0 (prior to surgery) and days 1, 4 and 10 postoperatively on repeated laparotomies. Biopsies were taken from new areas of the operated area on each occasion. Lavage containing 37°C 20 ml Phosphate Buffered Saline 3 mM EDTA and 25 U/ml Heparin (LEO Pharma AB™ Malmö, Sweden) was collected in tubes by (Becton Dickinson™, San Jose, California, USA) and immediately centrifuged at 1000 G for 4 min at 4°C. The supernatant was allocated and snap frozen up to -80°C. Peritoneal biopsies were homogenized in NaH₂PO₄ buffer containing 0.1% Triton X 100 (Sigma Aldrich™ St. Louis, Missouri, USA) and thereafter centrifuged at 1000 G for 10 minutes at 4°C. Additional peritoneal biopsies from the same area were taken on day 10 in order to stain for inflammatory cells (Hematoxylin and Eosin) and collagen (Massons-Trichrome). Prior to staining, the biopsies were treated with acetaldehyde, fixated in alcohol, paraffin embedded and cut with a microtome.

Group C (n = 8) received PL and PG in the same dose and mode as group A (0.5%, 1 ml of each corresponding to a dose of 2 mg/kg) and group D (n = 8) received saline (0.9%, 2 ml) after the abrasion procedure (described above).

Ten days later the macroscopic peritoneal adhesions in group C and D were noted and carefully dissected. The

Table 1. Experimental design.

Group	Animals	Day 0	Day 1	Day 4	Day 10
A	6	Peritoneal lavage, Peritoneal biopsy	Peritoneal lavage, Peritoneal biopsy	Peritoneal lavage, Peritoneal biopsy	Peritoneal lavage, Peritoneal biopsy, Histology
B	6	Peritoneal lavage, Peritoneal biopsy	Peritoneal lavage, Peritoneal biopsy	Peritoneal lavage, Peritoneal biopsy	Peritoneal lavage, Peritoneal biopsy, Histology
C	8				Adhesion evaluation, Digital photography
D	8				Adhesion evaluation, Digital photography

scar on the lateral abdominal wall area and surrounding tissue was excised. The area covered by adhesions was stained using tissue color and the whole excised operative area was defined and marked. Thereafter, the area was digitally photographed and transferred to a computer.

Straight after that an evaluation for euthanasia was made, all in accordance to AVMA Guidelines on Euthanasia 2007 [13] where, still during anesthesia, the animals received an intravenous over dose of potassium chloride.

2.5. Evaluations

The quality of the peritoneal adhesion was scored according to Lang *et al.* [14]. After that the digital photographs of the defined operated and colour marked adhesive areas were calculated using a CAD program (AutoCad 2011 Autodesk AB, Göteborg, Sweden). Ratios (percent) were calculated between the adhesion area and the total incised abdominal area and analyzed for differences. Histology slides were evaluated. Fibrosis was scored based on collagen deposition on peritoneum and inflammation was scored based on lymphocytes and macrophages [15] (see **Table 2**). Histology evaluation was performed separately by two examiners and in a blinded fashion.

2.6. Biochemical Assays

Determination of active proteins in peritoneal fluid was carried out using commercially IL-6 (R & D Systems™, Abingdon, UK), TGF- β 1 (Promega Biotech AB™ Nacka, Sweden). The active proteins were normalized to total protein (Bio-Rad Laboratories AB Malmö, Sweden).

The tPa/PAI-1 complex was analyzed in supernatant obtained from homogenized peritoneal biopsies using ELISA (t-Pa/PAI-1 Complex ELISA, Haemochrom Diagnostica AB, Mölndal, Sweden,) according to the manufacturer's instructions. Results were also normalized to total protein concentration.

2.7. Statistics

In order to see the distribution of the values in the figures, all results were given in median.

The Mann Whitney U Test was used to analyze dif-

Table 2. Fibrosis and inflammation score.

Score	Fibrosis/Inflammation
0	None
1	Mild
2	Moderate
3	Severe

Evaluation score of histology fibrosis (peritoneal collagen deposition) and inflammation (amount of macrophages and lymphocytes according to Hooker *et al.* [13]).

ferences in adhesion score from digital photos and histology slides (inflammation and fibrosis).

Kruskal Wallis test was used to determine statistic differences in active protein levels of peritoneal fluid and peritoneal biopsy between the control and experiment group. Statistic differences were set to $p < 0.05$ (SPSS v17.0 SPSS Inc., Chicago, Illinois, USA).

3. RESULTS

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3.1. Adhesions

A significant difference in abdominal adhesions could be seen between the experimental and control (group C and D) groups, $p < 0.005$ (**Figure 1**). In the control group, 100% (8/8) of the rabbits had severe adhesions vs. only 9% (1/8) in the PLPG group. In the PLPG group 63% (5/8) had no adhesions and 25% (2/8) had flimsy adhesions.

The digital photo (**Figure 2**) showed that the peritoneal surface was smooth and similar to the rest of the non-traumatized peritoneal surface.

3.2. Inflammation and Fibrosis

Ten days after surgery, hematoxylin/eosin staining for inflammatory cells on submesothelial area did not show any difference after 10 days comparing control and experimental groups as measured according to **Table 2**

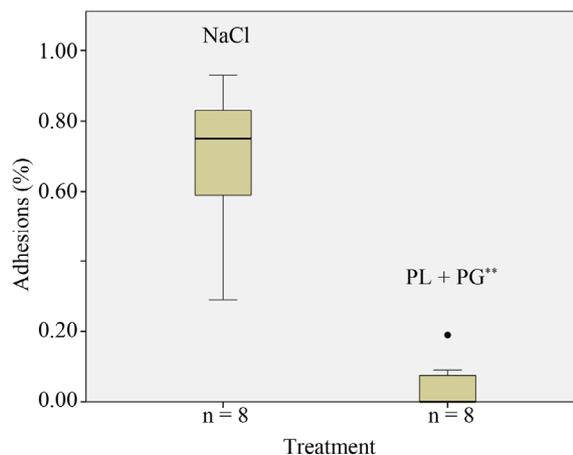


Figure 1. Results of adhesion reduction in animals receiving PLPG. Adhesions in percent of traumatized area in animals treated with saline and PLPG; **Significant reduction; $p < 0.005$.

(**Figure 3**). An increase in collagen deposition on peritoneum was seen on histology (Massons-Trichrome) in the control group compared to the experimental group $p < 0.05$ (**Figure 4**).

3.3. Peritoneal Fluid Measurement

There was no difference between treated animals and controls regarding tPa/PAI-1 complex in peritoneal biopsies.

IL-6 in peritoneal fluid was slightly, although not significant, decreased in experimental animals compared to the control group on day 4 postoperatively ($p = 0.20$).

The amount of active TGF β 1 was slightly decreased on day 4 and 10 in the peritoneal fluid of the experimental group; however, the difference was not statistically significant ($p = 0.20$ and 0.13 , respectively). All animals fared well without signs of any toxic symptoms during the experiments and there was no weight loss recorded.

4. DISCUSSION

The present study is the first one, to our knowledge, that has been done on rabbits with α -poly-L-lysine and poly-L-glutamate (PLPG) as an abdominal adhesion preven-

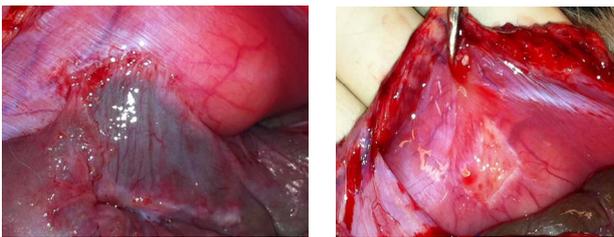


Figure 2. Photographies of treatment effect after 10 days.

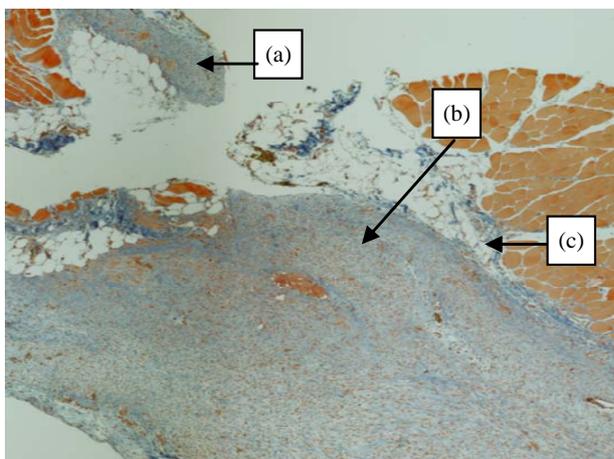


Figure 3. Histology (hematoxylin/eosine) specimen after 10 days. Differences in the fibrosis reaction between (a) (peritoneum treated with PLPG) and (b) (adhesions formation not treated with PLPG) after surgery. (c) is the submesothelial area.

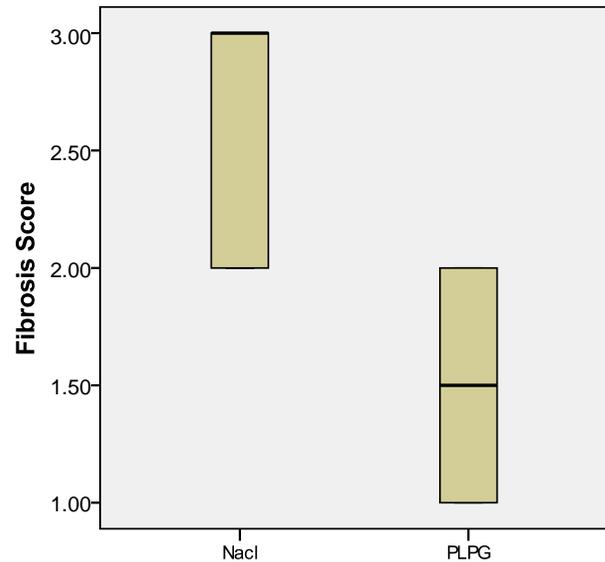


Figure 4. Differences in collagen deposition on the peritoneum in the saline group as compared to the PLPG group; $p < 0.05$.

tive agent, as well as the first study to show effective adhesion control using administration by spraying polypeptides instead of intra-abdominal installation. We have in previous experimental studies shown a strong postsurgical intra-abdominal, anti-adhesive effect of differently charged polypeptides [16]. The best anti-adhesive effect was achieved by combining the two oppositely charged polypeptides α -poly-L-lysine and poly-L-glutamate creating a matrix which serves as a mechanical barrier to prevent adhesion formation [12]. Other combinations of polypeptides have also shown significant reduction in abdominal adhesions though not to the same degree [17]. The PLPG complex has proven to be a biodegradable and non-toxic anti-adhesive agent that accumulates on injured peritoneum and in conjunction with its anti-adhesive effect also decreases parenchymal bleeding. Furthermore, the PLPG complex has no negative effect on bowel anastomoses but rather seem to assist in anastomotic healing [18-20]. The anti adhesive effect of the PLPG complex has been evaluated up to 5 months in rats with the same promising results [18].

The result from the present study shows a significant reduction in peritoneal adhesions, both on histological examination (collagen staining) and the amount of peritoneal adhesions. Adhesions in the control group appeared as rough pale bands on the peritoneum whereas the scarce adhesions in the PLPG group only showed a slightly pale discoloration beneath, an otherwise smooth peritoneum (**Figure 2**). By this finding, in addition to the smooth cecal serosal surface in treated animals, we concluded that the pale color emanated from small amounts (much thinner collagen surface than the control group) of collagen seen on histology (**Figure 3(a)**) indicating that

the PLPG matrix seals off the damaged peritoneal surface. This sealing could contribute to a smaller amount of inflammatory exudate than normally seen in the damaged peritoneum [21]. An indirect sign of this could be the decreased amount of collagen deposition.

These anti-adhesion results are consistent and correspond with previous studies, although not tested in rabbits. In the prior studies PLPG was used as an abdominal instillation in the dose of 40 mg/kg (mice) and the anti-adhesive effect remained at 1.6 mg/kg [22]. In this study, due to the size of the animals, we were able to treat them locally using spray administration by spraying in the dose of 2.5 mg/kg PLPG. Still, a very good anti-adhesive effect was reproduced indicating that local administration of a much smaller dose is possible. For forthcoming human experiments this is crucial. No toxic effects were noted during this experimental study in rabbits.

The normal peritoneal process of healing after trauma and the process of abdominal adhesion formation both involve the mesothelial and submesothelial areas [23-26]. The process of healing, as well as formation of adhesions, is among others mediated by inflammatory cells, mesenchymal cells [27], progenitor cells, tPa, PAI-1, active TGFb1 and inflammatory cytokines (IL1-6). Adhesion formation is a dynamic process where stable adhesions are formed within two weeks. It has previously been shown in rats that active tPa in peritoneal fluid significantly increases 24 h after peritoneal trauma and thereafter returns to normal levels. On the other hand, tPA activity in peritoneal biopsies has been shown to increase up to 72 h after surgical trauma to the peritoneum. PAI-1 in peritoneal fluid has been shown to increase from 4 h up to 72 h after peritoneal surgical trauma [28]. Active TGFb1 in peritoneal fluid was previously shown to gradually increase in peritoneal fluid over a period from 1 to 4 days and thereafter declined, but still remained significantly elevated compared to controls after 7 days [29]. IL-6 has shown to be involved in the process of abdominal adhesion formation. Previous studies have shown IL-6 to be elevated in peritoneal fluid during surgical trauma and antibodies against IL-6 have been reported to decrease abdominal adhesions [30]. The active proteins (mentioned above) are secreted by various cells (mesothelial cells, macrophages, neutrophils, endothelial cells from capillaries in the submesothelial space and fibroblasts) that all plays an important role in the peritoneal adhesion formation and degradation [31-35].

In peritoneal fluid there was no difference in tPa/PAI-1 complex between the control and experiment group before wound inflicting surgery. Individual measured times did not differ statistically measured between the groups. Active TGFb1 and IL-6 showed no differences between the groups.

These results for active proteins in peritoneal fluid could be due to many factors. One of them being that

PLPG has an effect on peritoneal injury that decreases the inflammatory exudate from the injured site (sealing) thus decreasing topical collagen deposition, though without interfering with the submesothelial space. A previous paper showed that the FITC labeled PLPG complex was incorporated in the intestinal wall without interfering with its function [16]. We speculated that this also might be the case for the submesothelial space.

The amounts of inflammatory cells were equal in both groups based on evaluating the histology slides. The inflammatory cells were concentrated in the submesothelial space, which further strengthens our hypothesis that the submesothelial process is not affected by the PLPG complex. We have previously shown that the function of peritoneal macrophages is not impaired when using the PLPG-concept, which is one of the evidences that the PLPG bio absorbable complex does not conflict the crucial steps in the normal peritoneal healing process [12].

5. CONCLUSION

This pilot study of local administration and mechanisms behind the anti-adhesive PLPG effect shows that PLPG significantly diminishes the amount of abdominal adhesions in rabbits using administration by spraying in a low dose. The effect seems to be topical on the peritoneum and without influencing the submesothelial space. No effects were noted in the peritoneal fluid on crucial and central parameters involved in adhesion formation.

6. ACKNOWLEDGEMENTS

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