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Research article

MULTIPLICATION OF THE ENTEROCYTE MASS BY SEROSAL PATCH TECHNIQUE

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Damage to the small intestine and impairment of the intestinal epithelium occur in various diseases, resulting in a need for new epithelium. Therefore, bioengineering of the small intestine is becoming an attractive field of research where all contributions are highly appreciated.

The purpose of this study was to determine the possibility of the multiplication of the enterocyte mass using the technique of serosal patch with the application of hematopoietic stem cells, as well as the assessment of the quality of newly formed mucosa.

Sixty *Mill Hill* hooded rats were divided in 4 groups, 15 animals each. In the control group animals, the patch was not created. In the other three groups, the animals were operated on and in each group 8 parietal and 7 visceral patches have been created. One of the groups with operated animals (Group NS) was not postoperatively treated. The second group of operated animals (Group G) was stimulated with granulocyte colony-stimulating factor (G-CSF). The third group of operated animals (Group GM) was stimulated with recombined humane granulocyte-macrophage colony-stimulating factor (rHuGM-CSF).

In the group of animals that were not stimulated, epithelium proliferated slowly. In the group of animals stimulated with G-CSF stimulants, the epithelium initially proliferated rapidly, but appeared atrophic after eight weeks. Stimulation by rHuGM-CSF led to faster epithelization, and epithelium showed signs of advancing proliferation after eight weeks.

We confirmed the possibility of enterocyte mass multiplication by using the serosal patch technique, as well as that stimulation with rHuGM-CSF is more effective than stimulation with G-CSF.

Key words: bioengineering, colony-stimulating factors, enterocytes, small intestine, patch technique.

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INTRODUCTION

The small bowel is possibly the only segment of the digestive tract that may be considered as absolutely essential to life [1]. The need for new intestinal tissues arises from many disorders including intestinal ischemia, tumors, and inflammatory bowel disease [2]. Bioengineering of the small intestine is becoming an attractive field of research where all efforts and investments are undoubtedly justified [1,3-5].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic growth factor which promotes the activity of neutrophils and macrophages, e.g. by upregulation of reactive oxygen species and expression of MHC class I molecules, and also increases phagocytotic activity and the expression of pro-inflammatory cytokines, adhesion molecules and co-stimulatory molecules [6]. The recombined humane granulocyte-macrophage colony-stimulating factor (rHuGM-CSF) is obtained by genetically modifying *Escherichia coli* bacteria and no pharmacological difference could be shown *in vivo* and *in vitro* between these two items. Several studies have demonstrated its positive impact on intestinal regeneration, epithelial cell migration and mucosal proliferation [7]. The granulocyte colony stimulating factor (G-CSF) induces proliferation of neutrophil colonies, differentiation of precursor cells to neutrophils and it stimulates the activity of mature neutrophils [8,9]. In recent years G-CSF and rHuGM-CSF factors are increasingly used in various experimental studies [7,8,10].

Many experiments tried to promote different methods to multiply the enterocyte mass [11-13]. All experiments have been set up so that the short bowel syndrome was induced in experimental animals in order for the multiplication of the enterocyte mass to begin afterwards, which made both the operative procedure and the postoperative care complicated [6]. The purpose of this study was to determine the possibility of enterocyte mass multiplication with the technique of serosal patch by using hematopoietic stem cells stimulators and assessing the quality of the newly formed mucosa.

MATERIALS AND METHODS

Experimental animals: All studies were conducted in compliance with the guidelines established by the Guide for the Care and Use of Laboratory Animals approved by Institute of Child and Youth Health Care of Vojvodina Ethical Committee. This study was preceded by a pilot study with 20 experimental animals based on which the conceptual methodology was established. A sample of sixty *Mill Hill* hooded male rats, age of 2.5 months and mean weight of 259 g was used. The rats were housed in individual cages and were acclimated to laboratory conditions (22°C with 12-hour light/dark cycle). Rats were fasted for 48 hours before the experiment with free access to water.

Surgical procedure: All procedures were done using the aseptic technique. General anesthesia was induced by ketamine chloride (7 mg/kg) administered intraperitoneally. The abdomen was shaved and prepared with Betadine. The abdomen was opened through a midline incision, and the bowel was eviscerated.

In the creation of the parietal patch a longitudinal incision was made in a length of 2 cm in the region of jejuno-ileal transition. The edges of the incision were separated for 1/3 of the circumference of the bowel, and with the extended intestinal suture they were brought together with Prolene 6-0 suture to the parietal peritoneum lateral on the right side from the laparotomy incision (Figure 1A).

When the visceral patch was created, a 2 cm longitudinal incision was made in the region of terminal ileum. The edges of the incision were separated as described for the parietal patch, and sutured in the similar way and with the same suture material to the serosal surface of the caecum (Figure 1B). In both cases the tightness of the patch was examined, and the intestines were returned to the peritoneal cavity. Before the closure of the abdomen, the rats were resuscitated with 2 ml saline and Gentamycin (5 mg/kg) were administered intraperotoneally. In all operations, the abdominal cavity was closed in two layers.

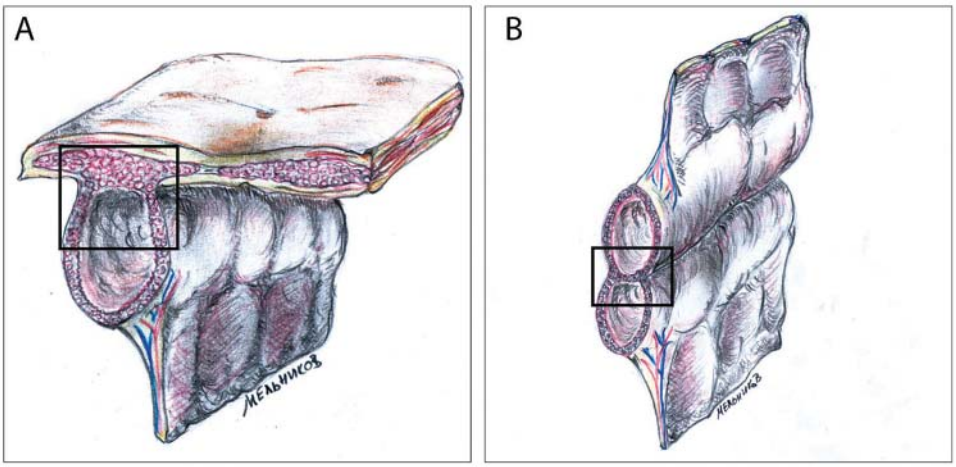

Figure 1. Schematic diagram of patch making (**A** – parietal; **B** – visceral; rectangle is showing about intestinal segment that was used for histology)

Postoperatively, the animals were allowed water ad libitum immediately after the operation and normal chow at the beginning of the first postoperative day. Gentamycin was administered intramuscularly for seven days in the aforementioned dose.

Experimental design: The sample was split into four groups of 15 animals. First three groups of animals were operated on and in each group 8 parietal and 7 visceral patches were created. Group NS: the group of operated animals that were not stimulated postoperatively with any of the stimulators of cell growth. Group G: animals that

were given subcutaneously 30 μg/kg/day of G-CSF (Hoffmann-La Roche, Basel, Switzerland) during 10 days after the surgical creation of the patch. Group GM: animals that were stimulated with recombinant human granulocyte-macrophage colony stimulating factor (rHuGM-CSF, Hoffmann-La Roche, Basel, Switzerland) at a dose of 10 μg/kg/day, subcutaneously for 10 days after the surgical creation of the patch. Group C, control group, contained animals that were not treated surgically for creation of the patch.

The day when the animals in groups G and GM were operated upon is considered to be the beginning of the experiment (1st day of the experiment). After administration of the stimulators, the animals of all four groups were maintained grouped as on the first day, under the same laboratory conditions. Animals in all 4 groups had their weight checked at the beginning of the experiment (on the 1st day of the experiment), as well as after two weeks, four weeks, six weeks and eight weeks from the beginning of the experiment. Half of the animals from each group were sacrificed four weeks after the operation, and the remaining animals were sacrificed after eight weeks. All animals were sacrificed with a ketamine chloride overdose inducing respiration paralysis and the serosal patch material was prepared for histological examination.

Histological processing of the material: From the intestinal segment that corresponded to the place of patch creation (shown as a rectangle on Figure 1) three parallel tissue sections were sampled from all animals for histological preparation. One tissue sample was fixed in 70% alcohol and processed into standard paraffin blocks, then cut into 5 μm sections and stained with hematoxylin-eosin suitable for examination by optical microscope. On the sections examined by optical microscope, features that were followed were existence (or not) of enterocytes and goblet cells in the surface of parietal or visceral peritoneum in the patch segment. In the case that enterocytes and goblet cells were present it was interpreted as a renewal of the mucosa and multiplication of the enterocyte mass. Therefore, it was noted whether there were only enterocytes in one layer, formation and appearance of the intestinal villi, presence and regularity of intestinal glands (crypts) and atrophy of the mucosa. Also, a presence of connective tissue (scar), inflammatory infiltrate or other pathological substrate was noted in the area of the patch. Second tissue sample from the patch segment was fixed in 2% solution of gluteraldehyde in phosphate buffer, and after which it was prepared according to the instructions for processing biological materials for the scanning electron microscope (SEM). Third tissue sample was fixed in 4% solution of gluteraldehyde and prepared for the transmission electron microscopy (TEM). On TEM and SEM slides, the ultrastructural organization of enterocytes and development of microvilli on the apical segment were analyzed.

RESULTS

Out of 45 operated animals in total, two died during the first week after the surgery (4.4%). One of two, from the Group NS (6.7%) with parietal patch created, died

due to its dehiscence and the development of diffuse peritonitis. The other animals, from the Group GM (6.7%) with created visceral patch, died due to the development of ileus. The average weight of all animals on 1st day of the experiment was 256g. Changes in animal weight during the experiment are given in Table 1. All groups of animals showed weight gain during the experiment, and it was statistically significant compared to the control group (Table 1). Although the average weight of animals in Group G was initially higher than in other groups, weight gain in Group G was higher compared to Group NS ($p<0.001$) and Group GM ($p<0.001$) each day when measured. In all groups the weight of the animals after eight weeks was statistically significantly higher than at the beginning of the experiment ($p<0.001$).

Table 1. The weight of animals during the experiment and its statistical significance compared to the control group (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Groups	Weight (g) ($\bar{x}\pm SD$)				
	Beginning	**2 weeks**	**4 weeks**	**6 weeks**	**8 weeks**
Group C	248±12.3	245±18.6	267±21.1	271±15.7	283±13.7
Group NS	212±17.4***	215±15.4***	276±16.8*	288±12.4**	300±17.9*
Group G	313±13.5***	317±22.1***	359±18.9***	379±23.7***	398±19.4***
Group GM	252±20.7*	272±14.6***	305±14.5***	322±19.6***	336±22.3***

Control group (unoperated animals)

Histological analysis of the material sampled from Group C (in the region where the parietal patch has been formed in other groups) showed that mucosa is of jejunal type. A thin submucosa exists, and longitudinal and circular part of the muscle is of the same thickness. Plexus myentericus is present. Epithelium of the villi contains enterocytes and goblet cells (Figure 2A). Histological analysis of the material sampled from Group C (in the region where the visceral patch has been formed in other groups) showed that the mucosa corresponds to the mucosa of the terminal ileum with usual histological appearance (Figure 3A).

The mucosa above the parietal patch

In Group NS (without stimulation) a disruption of all layers of the wall with proliferation of connective tissue was observed even after eight weeks. The connective tissue scar is infiltrated with numerous inflammatory cells, it reaches the surface, and the formation of the mucosa is not restored in these parts. In some areas of the patch mucosa forms short spatular and wide villi. Distribution of the crypts is irregular. Epithelium of crypts is only partially multiplied (Figure 2B). TEM enterocytes in the region of regeneration showed the usual ultrastructural organization of enterocytes and adequately developed apical segment with regular microvilli.

In Group G (stimulated by G-CSF), mucosa has restored its continuity on the entire surface four weeks after the surgery, but it is thin, atrophic, with rare goblet cells. Villi are rare and irregular and crypts are densely arranged. There is a discontinuity in the

muscular layer in the place of the patch, where the connective tissue has proliferated. TEM confirmed good ultrastructural organization of enterocytes and well developed microvilli on the apical segment. After eight weeks, the continuity of the mucosa is restored, and the epithelium of the microvilli and crypts consists of enterocytes and goblet cells. Surrounding focally hyperplastic intestinal villi grew over the smaller area of atrophic mucosa. Mucosa overlaps the proliferated connective tissue. It can be noted that there is no regeneration of muscle layer which is replaced by reparative connective tissue (scar). Blood vessel wall is thickened (Figure 2C).

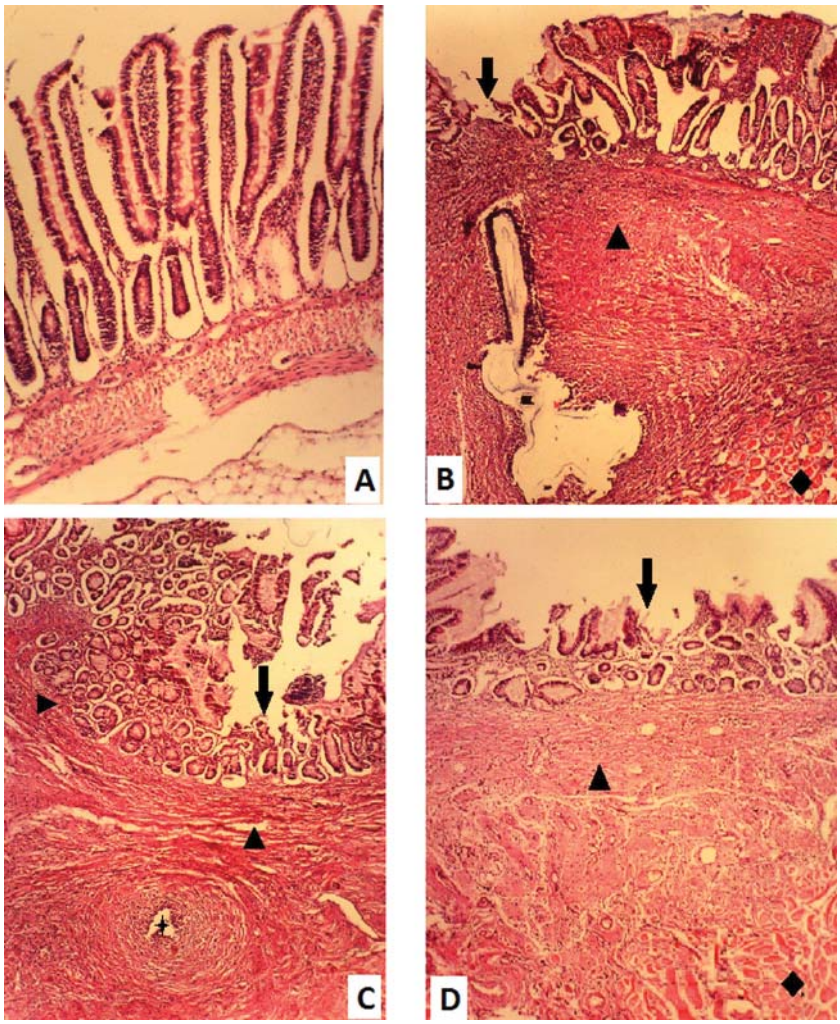

Figure 2. Microphotographs of unresected small intestine wall and areas of parietal patch after eight weeks (**A** – Group C, HE, x100; **B** – group NS, HE, x40; **C** – Group G, HE, x40; **D** – Group GM, HE, x40). Legend: ↓– area of incomplete or complete owergrowth of the mucosa over the parietal patch; ▲– connective tissue with inflammatory infiltrate on the place of interrupted muscular layer of the patch; ► – densly arranged crypts and short villi ♦ – striated muscle of the abdominal wall under the peritoneum of the parietal patch; *– thickened blood vessel

In Group GM (stimulated by rHuGM-CSF) the discontinuity of all layers of intestinal wall, which is not overgrown by mucosa completely, can be observed after four weeks. In the central part of the defect in some sections there are groups of intestinal glands with proliferated epithelium. Inflammatory infiltrate is present in the scar tissue. Eight weeks after the surgery mucosa overgrows the entire length of the defect which is in depth filled with proliferated young connective tissue. Mucosa is thinned, villi and crypts are rare and irregular. The epithelium of the villi and of the crypts is typical intestinal, although the epithelium of the crypts shows regeneration with multiplied cells (Figure 2D).

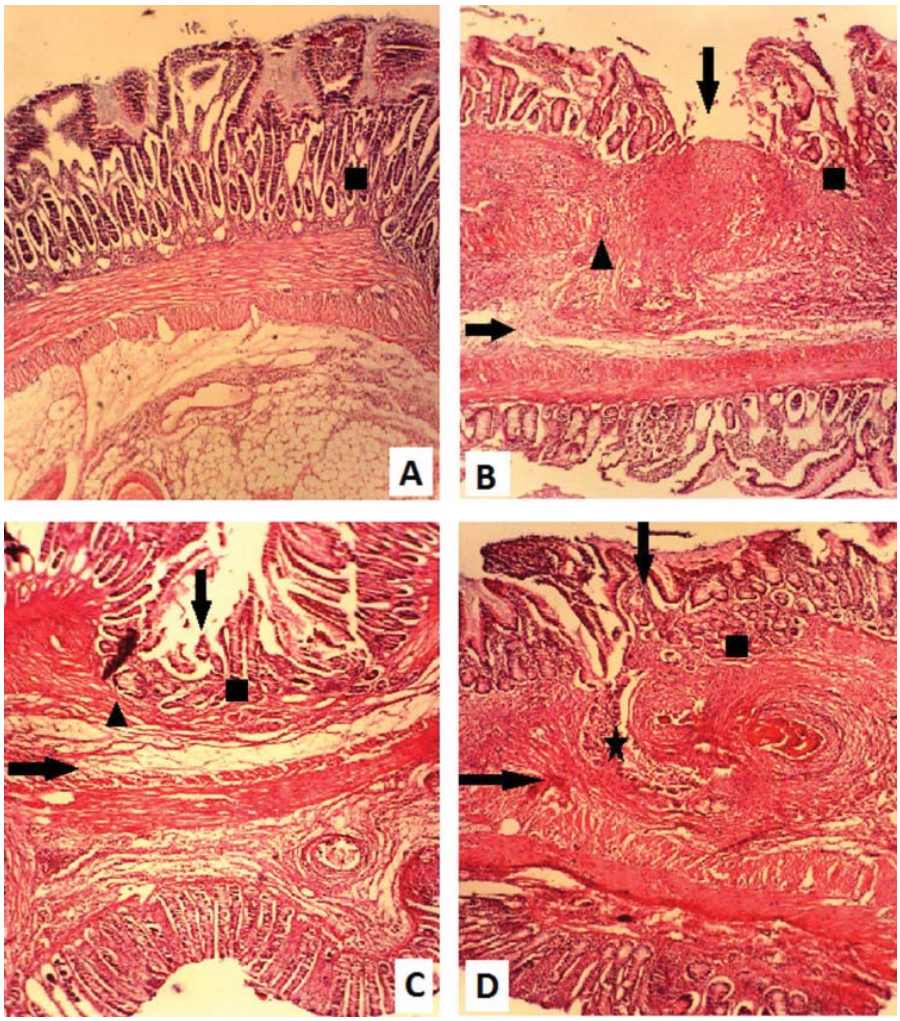

Figure 3. Microphotographs of unresected small intestine wall and areas of visceral patch after eight weeks (**A** – Group C, HE, x40; **B** – Group NS, HE, x40; **C** – Group G, HE, x40; **D** – Group GM, HE, x40). Legend: ↓- area of defect or owergrowth of the mucosa over the visceral patch; ▲– connective tissue (on the right) on the place of interrupted muscular layer (on the left); ■ – crypts; *– diverticula; → – under the horizontal arrow is the intact wall of the caecum on the place of the visceral patch

The mucosa above the visceral patch

In Group NS (without the stimulation), eight weeks after the surgery there is a visible defect of the wall. Atrophic mucosa overgrows the defect incompletely, and the muscle layer is interrupted and replaced by the connective tissue with inflammatory infiltrate. Crypts are rare and irregular (Figure 3B). TEM enterocytes show good ultrastructural organization of the enterocytes, but with less developed and irregular microvilli on the apical segment (Figure 4B).

In Group G (stimulated with G-CSF) the mucosa and epithelium (superficial and glandular) are entirely renewed after four weeks. There is a defect of the muscular layer on the place of incision and suture, and this area is replaced with proliferated connective tissue with inflammatory infiltrate. Eight weeks after surgery the mucosa is also renewed. Intestinal villi are short, and crypts are rare and irregular. Muscle layer is interrupted in the place of incision and suture and replaced with connective tissue (Figure 3C). Sections observed with SEM and TEM showed well developed enterocytes with formed brush border of irregular microvilli and the usual ultrastructure of enterocytes (Figure 4C).

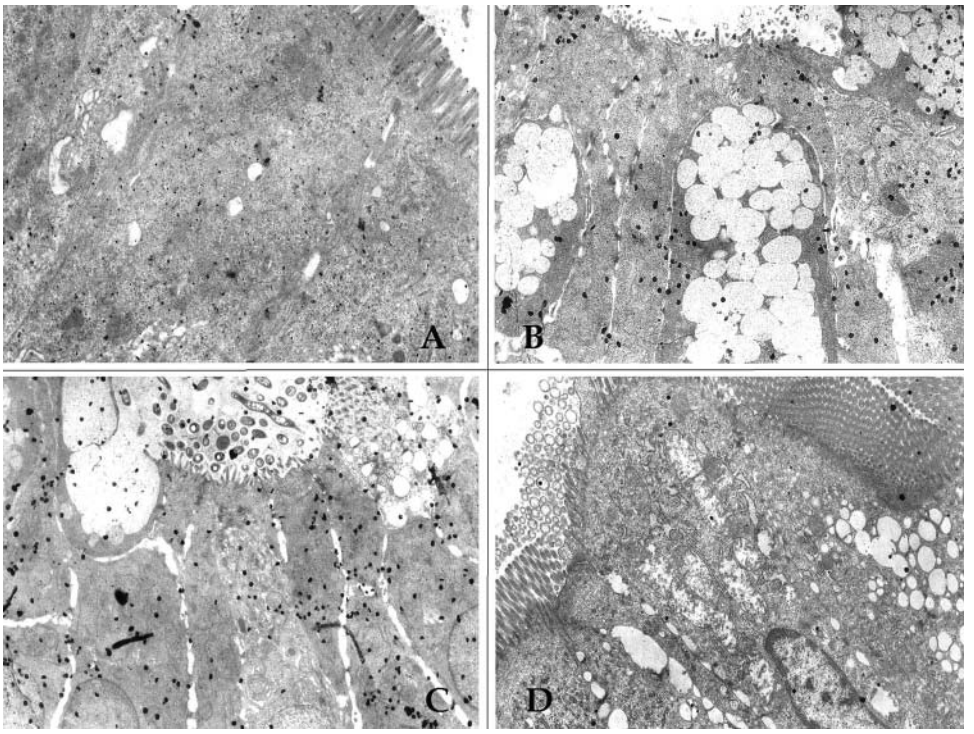

Figure 4. Electron microscope photographs of the enterocytes in the region of visceral patch and control after eight weeks (**A** –TEM, x5000 showing enterocytes in Group C; **B** – TEM, x3300, showing enterocytea in the regenerated region above the visceral patch in Group NS; **C** – TEM, x2600, showing enterocytes in the regenerated region above the visceral patch in Group G; **D** – TEM, x5000, showing enterocytes in the regenerated region above the visceral patch in Group GM)

In Group GM (stimulated with rHuGM-CSF) mucosa overgrows the defect after four weeks. It is mildly atrophic and with deep diverticulum. Villi are short, and the epithelium of the crypt cubical to cylindrical, slightly multiplied. Eight weeks after the creation of the patch, the continuity and appropriate thickness of the mucosa is established in the area of the visceral patch. Villi are pyramidal and spatular in shape. Crypts are of the appropriate characteristics slightly irregular. Muscle layer is interrupted occasionally with diverticula of the newly formed mucosa (Figure 3D). Sections observed with TEM show well developed enterocytes of usual ultrastructure with adequately formed brush border of regular microvilli (Figure 4D).

DISCUSSION

In our experiment, all animals showed weight gain, despite the fact that they were subjected to a relatively serious surgical procedure. This suggests that this procedure is well tolerated and effective, and it could give a fine and fast postoperative recovery. We believe that the results are applicable in larger animals, also.

Tissue engineering is defined as an interdisciplinary field that applies the principles of engineering and life sciences toward the development of functional substitutes that replace damaged or missing tissue. Tissue engineering has several potential advantages over conventional therapies, e.g., avoiding prosthetic materials, the use of autologous cells thereby obviating immunosuppression, the capacity for growth and remodeling, and overcoming donor organ shortage [4]. We strongly believe that the experimental procedure used in our study offers all the above stated advantages. In a relatively short period of time with the appropriate stimulation, it leads to a multiplication of the enterocyte mass and recovery of the mucosal surface. In our study, the mortality of animals during the two months was extremely low (only 2 animals), and considering the body weight gain it could be concluded that general condition of the animals was very good. This newly formed mucosal surface originates from the subject itself, so there is no danger of donor-host reaction and complications.

Various attempts to use synthetic materials for the patch did not give good results [2,11,14]. The first report of creating novel intestinal surface using an animal model came in 1973 when Binnington and colleagues performed an incision on the antimesenteric border of the jejunum and patched this opening using the serosa of the adjacent descending colon. However, some of the animals had persistent bare serosal areas that remained not covered by neomucosa despite waiting up to 36 weeks [15,16]. In our study, we had similar results after 8 weeks in animals with visceral patch that were not stimulated: the areas of atrophic mucosa that incompletely overgrow the area of visceral peritoneum in the patch. But when rHuGM-CSF or G-CSF were used, complete coverage of the area of the patch was accomplished after only four weeks. This implicates significant positive effect of the applied substances. Investigators have also performed studies in rats by using the parietal peritoneum as a base for the regeneration of neomucosa [2]. Erez advocated the use of peritoneum because

fluids and electrolytes can be absorbed through the peritoneum while waiting for the neomucosa to completely cover the patched area [17].

In our study, the serosa of the caecum used for visceral patching appeared to be a better solution. When stimulated with same factor (rHuGM-CSF), the visceral patch showed better neomucosa formation after eight weeks. In the area of the visceral patch, the neomucosa restored its continuity and it had the appropriate thickness, along with other features that resembled histologically normal mucosa. In some sections, after four and after eight weeks, a diverticulum of newly formed mucosa was present. The most probable explanation is that the stimulation with rHuGM-CSF caused proliferation of epithelial cells of the mucosa, but at the same time it caused proliferation of fibroblasts and myocytes which lead to trapping of the newly formed mucosa and the formation of diverticula. If this assumption is correct, visceral patch stimulated with rHuGM-CSF would be a superior method because of this multipotent action on epithelial mucosal cells and fibrocytes and myocytes. However, this could be the topic of a new experiment.

The growth of the neomucosa depends on the location and size of the incision [6,14]. Thompson reported that neomucosal growth was better in the patched ileum as compared to the jejunum, which may be due to differences in the luminal content, local humoral differences and adaptive capacity between the jejunum and ileum [18]. In our study the location of placement of the patch was determined through a pilot study. It showed that medial middle laparotomy is convenient and easy for forming a parietal patch about 2 cm laterally from the peritoneal incision. Creating a visceral patch is even easier because both intestines are exteriorized and the surgical procedure is performed outside the abdomen. The serosa of the caecum was used for a patch in rats because caecum is well developed in these animals. A pilot study showed that the length of incision in rats should not exceed 2 cm and that the edges of the incision can be moved for only one third of the intestinal extent. Conclusions of the pilot study were in accordance with Thompson, who used the colon serosa as a patch in his study, in width that was 1/3 of the ileum [2].

The possibility for stimulation of the intestinal cell proliferation was of special interest in the conception of this study. Through the study of granulocyte growth factors they were found to influence the fibroblasts and to be able to perform simultaneous indirect stimulation of epithelial cell proliferation accelerated by creating an underlying connective tissue and intercellular substance [19-21]. Indirect stimulation was hypothetically expected from G-CSF through the proliferation of fibroblasts, i.e. the quickened process of connective tissue creation. Indirect stimulation was also hypothetically expected from rHuGM-CSF through the proliferation of fibroblast, but a minimal paracrine stimulation of enterocyte proliferation through macrophages was not ruled out.

Our study showed that any of the applied forms of stimulation promote and facilitate the formation of the epithelium of the mucosa at a different degree. In animals

that were not stimulated (Group NS), regardless of the patch type, the epithelium proliferated very slowly. It took eight weeks for it to grow over the defect, and even then it stayed atrophic without the potential for further proliferation. In the group that was stimulated with G-CSF (Group G) there was a rapid tissue proliferation, especially in the group with the parietal patch, which gave a good base to the epithelium so it grew over the defect four weeks after the surgery. However, the epithelium itself stayed discretely atrophic after eight weeks, without any signs of further proliferation. Stimulation with rHuGM-CSF (Group GM) confirmed the expected paracrine stimulation of the epithelium. The defect was covered very quickly, and the epithelium, though slightly atrophic, showed signs of further proliferation even after eight weeks. Ring came to similar histological findings after the stimulation with prostaglandin E2 in rats with the parietal patch [6,14]. Without the stimulation in his study, as well as in ours, there is a dominant reparation (connective tissue scar formation) in relation to the regeneration, and the mucosa is atrophic without the tendency for further proliferation. With stimulated animals the histology results very similar to the result in the group stimulated with rHuGM-CSF, regardless of the patch type.

In the conclusion of our study, we emphasize that both types of patches without any stimulation gave incomplete regeneration of the epithelium, which after eight weeks became atrophic, without potential for further proliferation. We showed that there is a possibility of enterocyte mass multiplication, and that some type of stimulation is necessary, not only to speed up the process but also to make the regeneration of the mucosa complete in the histological sense. Stimulation with rHuGM-CSF gives better effects than the stimulation with G-CSF.

Also, according to our study, serosa represents a quality patch base for the regeneration of the epithelium. The serosa is also suitable for surgical procedures due to its accessibility and a fine surface that can relatively easy, with fine surgery, tighten the defect of the intestinal wall.

Although this study was conducted in rats, it was inspired by the need to find an operating procedure that can increase intestinal absorption surface and allow the survival of patients suffering from short bowel syndrome in human medicine. We believe that the visceral patch technique presented here is an excellent method, and in combination with these or some new stimulators, it could bring a solution for many patients.

Acknowledgments

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UMNOŽAVANJE ENTEROCITNE MASE TEHNIKOM SEROZNOG *PATCH*-A

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Do oštećenja tankog creva i crevnog epitela dolazi u brojnim oboljenjima, i dovodi do potrebe za novim epitelnim tkivom creva. Stoga je bioinženjering primenjen na tanko crevo postalo interesantno područje intraživanja, u kome je svaki doprinos dobrodošao.

Svrha istraživanja bilo je ispitivanje mogućnosti umnožavanja enterocitne mase tehnikom seroznog *patch*-a, uz primenu faktora stimulacije kolonija.

Ukupno šezdeset *Mill Hill Hooded* pacova podeljeno je u četiri grupe od po 15 životinja. Životinje u kontrolnoj grupi nisu operativno tretirane. U preostale tri grupe je operativnom tehnikom u svakoj grupi kreirano po 8 parijetalnih i 7 visceralnih *patch*-eva. Životinje u jednoj grupi nisu postoperativno tretirane (Grupa NS). Životinje u drugoj grupi (Grupa G) stimulisane su faktorom stimulacije kolonija granulocita (*granulocyte colony-stimulating factor, G-CSF*). Treća grupa (Grupa GM) je stimulisana rekombinovanim humanim faktorom stimulacije kolonija granulocita i makrofaga (*recombined humane granulocyte-macrophage colony-stimulating factor, rHuGM-CSF*).

U grupama životinja koje nisu stimulisane faktorima stimulacije kolonija, epitel je sporo proliferisao. U grupama stimulisanim G-CSF, epitel je inicijalno ubrzano proliferisao, ali je nakon osam nedelja postajao atrofičan. Stimulacija sa rHuGM-CSF dovela je do brze epitelizacije, a epitel je i nakon osam nedelja pokazivao znake dalje proliferacije.

Rezultati našeg istraživanja potvrđuju mogućnost umnožavanja enterocitne mase tehnikom seroznog *patch*-a. Takođe, stimulacija sa rHuGM-CSF pokazala se mnogo efikasnijom od stimulacije enterocita sa G-CSF.