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ORIGINAL ARTICLE

Effects of extensive mobilization and tension anastomosis in anorectal reconstruction (experimental study)

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Abstract

Purpose Anorectoplasty and pull-through procedure can be performed with extensive mobilization or tension anastomosis, which can compromise bowel blood perfusion. We aimed to analyze the hypoxia biomarker values and histopathological findings in both conditions to correlate the occurrence of anal stenosis and defecation disorders in experimental models.

Methods We created anorectal reconstruction models with impaired vascularization of the anorectum (group I) and tension anastomosis (group II) in rats. A third group of animals underwent sham operation (group III) and another as controls (group IV). Hypoxia biomarker values were assessed in all groups. The histopathological changes on the postoperative days 3 and 35, anal stenosis and defecation disorders on day 35 were compared.

Results Hypoxia biomarker values confirmed postoperative ischemia in groups I–III compared to control. Group I and II rats had a similarly pronounced ischemia with histopathologic changes in the anorectum on the postoperative day 3 and accompanied by severe fibrosis on day 35. Compared to the sham operation, both groups showed defecation disorders with significant anal stenoses.

Conclusion Extensive rectal mobilization to about the same extent as tension anastomosis has a major impact on postoperative rectal ischemia, resulting in severe fibrotic changes in the anorectum and defecation disorders in the long term.

Keywords Anorectoplasty · Ischemia · Tension anastomosis · Anal stenosis · Constipation · Anorectal malformation · Hirschsprung's disease

Introduction

Despite the development of new operative techniques and improvement in postoperative care of patients with anorectal malformations (ARM) and Hirschsprung's disease (HD), 16–87% of patients still suffer from long-term defecation problems, regardless of the type of defect [1–3]. Postoperative rectal ischemia has been defined as one of the factors affecting the functional outcome of surgery in patients with anorectoplasty and pull-through procedure [4–6]. The lack of rectal tissue required for pull-through presents the surgeon with a choice: extensive mobilization of the rectum with severe circulatory disorders (preservation of the predominantly intramural blood supply to the rectum) or creation of anastomosis under conditions of tissue tension [7]. Although both surgical approaches are associated with some degree of postoperative rectal ischemia, their impact on the long-term effects, such as fibrotic stenosis of the anorectum and defecation disorders, remains uncertain [4, 5, 7].

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Ischemia and, therefore, decreased oxygen supply to the tissues trigger the processes that provide adaptation to hypoxia, including activation of hypoxia-inducible factor 1-alpha (HIF-1α) stimulating the expression of vascular endothelial growth factor (VEGF) [8]. The upregulation and role of these biomarkers of response to hypoxia under ischemic conditions have been demonstrated in various tissues, including the colon [9–11].

In the present study, we created two original experimental models of postoperative ischemia of the anorectum in rats—the model of impaired vascularization of the anorectum and the model of tension anastomosis of the anorectum—to study and compare the effects of these two surgical factors on severity of rectal ischemia, fibrotic changes, and resulting anorectal dysfunction. The biomarkers of tissue ischemia (HIF-1α and VEGF-C) were assessed in the early postoperative period, while histopathological changes were studied in the early and late postoperative periods.

Materials and methods

Animals and experimental models

The study was approved by the Institutional Review Board, and the animals were cared for and handled in accordance with the National Institutes of Health guidelines. The study included 70 12-week-old male outbred rats weighing 370–420 g that were purchased from the vivarium of Sechenov University (Moscow, Russia) and housed separately (one rat per cage). The rats were divided into four groups: group I ($n=20$), the model of impaired vascularization of the anorectum; group II ($n=20$), the model of tension anastomosis of the anorectum; group III ($n=20$), sham operation; group IV ($n=10$), intact control rats.

Group I–III animals were anesthetized by intramuscular injection of 2 mg/kg Zoletil 100 (Virbac, France). A monopolar coagulator at 12 W output power was used for both coagulation and sphincter identification during surgery. Surgical procedure started by putting holding sutures to the anal verge and a circular incision of the skin around the anus at a distance of 1–2 mm, then mobilization of the rectum started at the skin level.

The model of impaired vascularization of the anorectum: The rectum was mobilized to the sphincter very close to the intestinal wall, leaving no mesenteric collaterals. The serosal layer of the mobilized rectum was coagulated circumferentially continuously at the sphincter level, leaving the distal portion with only intramural blood supply. After the resection of 1–2 mm of cutaneous distal part of the mobilized rectum bearing the holding sutures, the anorectal anastomosis was created by eight 6/0 Vicryl interrupted sutures (Fig. 1).

The model of tension anastomosis of the anorectum: The rectum was carefully mobilized to the sphincter without damaging the serosal vasculature, and one half of the mobilized rectum was resected, and the anorectal anastomosis was created under tension by eight 6/0 Vicryl interrupted sutures (Fig. 2).

Sham operation: The rectum was carefully mobilized to the sphincter without damaging the serosal vasculature, and 1–2 mm of cutaneous distal part of the rectum bearing the holding sutures was resected. Then anorectal anastomosis was done in the proper place using eight 6/0 Vicryl interrupted sutures. This procedure is intended to demonstrate the effect of the surgical intervention.

All surgeries were performed by the same surgeon (the first author). The methods of creating the experimental models of postoperative ischemia of the anorectum were patented and registered in the State Register of Inventions of the Russian Federation: No. 2719661 and No. 2719663 dated April 21, 2020.

Following surgery, animals were maintained in individual cages and fed a standard rodent chow diet and water ad libitum. No medication and anus dilatation were used in any of the groups in the postoperative period.

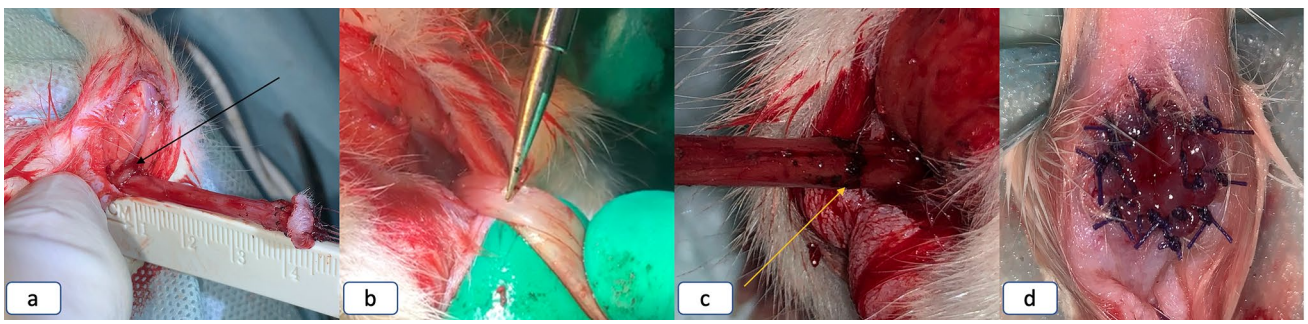

Fig.1 The creation of "Impaired vascularization of the anorectum" model. **a** Mobilizing the rectum to the sphincter muscle (black arrow); **b**, **c** the serous layer of the mobilized rectum was coagulated circumferentially at the sphincter level (yellow arrow); d—formation of the anus by applying interrupted sutures

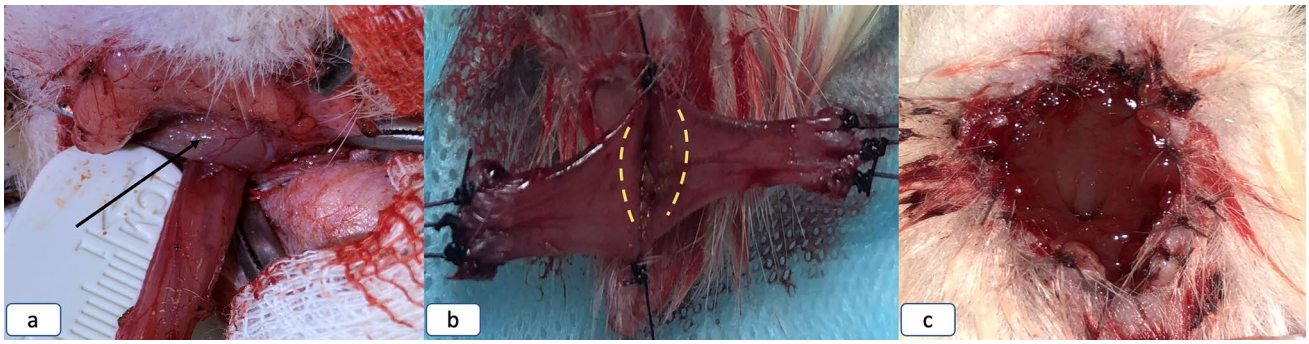

Fig.2 The creation of "Tension anastomosis of the anorectum" model. **a** mobilization of the rectum to the sphincter (black arrow); **b** resection of half of mobilized rectum (resection line—yellow dotted line; the first two anorectal sutures are placed at 12 and 6 o'clock); **c** formation of the anus under tension

In groups I, II and III, the rats were randomly divided into subgroups A and B (10 rats each). In subgroups A, the severity of anorectal ischemia was assessed by measuring the VEGF-C and HIF-1α levels in the rectal tissue homogenates and by histopathological examination on postoperative day 3. In subgroups B, anorectal function was assessed between postoperative days 30–35, anal caliber was measured immediately before killing and obtaining the rectal tissue samples for histopathological examination on postoperative day 35 (Fig. 3). The rats were killed by intramuscular injection of a lethal dose of Zoletil 100 (Virbac, France).

Measurement of ischemia biomarkers

On postoperative day 3, tissue samples from the anorectum were taken from anesthetized rats of the experimental groups (IA, IIA and IIIA) and control group (IV). The samples were homogenized in ice-cold 1.15% KCl buffer (pH 7.4). The homogenate was then centrifuged at 14000 g for 10 min and

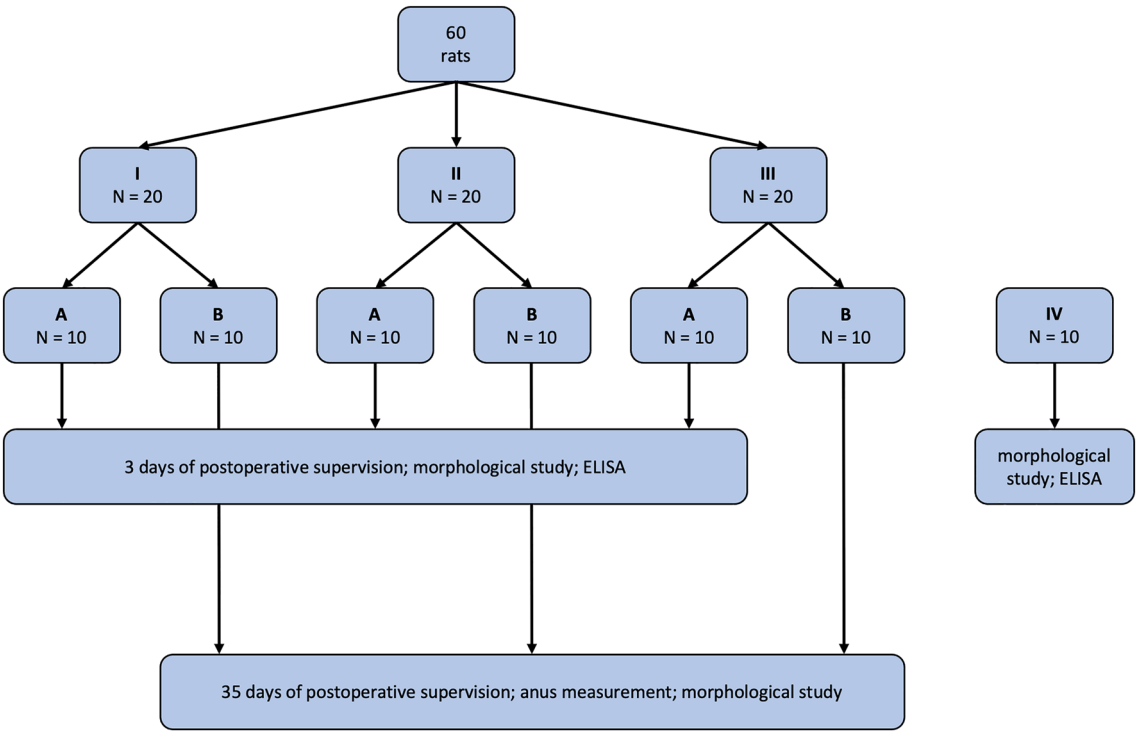

Fig. 3 Study design. I—rats with "impaired vascularization of the anorectum" model; II—rats with "tension anastomosis of the anorectum" model; III—rats with "sham operation" model. **A** subgroup of 3 days of postoperative supervision; **B** subgroup of 35 days of postoperative supervision; ELISA—enzyme-linked immunosorbent assay

the supernatant was collected in separate Eppendorf tubes and stored at − 25 °C for no longer than 1 month prior to testing. The VEGF-C and HIF-1α levels we measured in the supernatant by enzyme-linked immunosorbent assay (ELISA) using Stat Fax 2100 analyzer (Stat Fax, USA) using the Rat VEGF-C (Bender Medsystems, Austria) and HIF-1A SEA798Ra (Cloud-Clone Corp., USA) ELISA kits according to the manufacturer's instructions. All specimens were measured in duplicate wells, and the mean of the two values was taken for analysis.

Histopathological examination

Rectal tissue samples were obtained from each sacrificed rat, fixed in 10% natural buffered formalin, and subjected to routine tissue processing. The tissue sections were stained with hematoxylin and eosin and examined with a light microscope (Leica DM2000, Germany). The observed histological changes were classified as mild, moderate, or severe based on the severity of acute inflammation (density of infiltration of neutrophils and eosinophils), circulatory disorders (blood clots, hemorrhages, congestion of veins, edema), and necrosis on postoperative day 3 (subgroups A) as well as based on the changes in the histotopography of intestinal wall layers, the degree of sclerosis, chronic inflammation, and angiogenesis on postoperative day 35 (subgroups B). All samples were studied by the same pathologist.

Postoperative assessment of anorectal function and anal caliber

Anorectal function was assessed in subgroups B between postoperative days 30 and 35 according to the following criteria: stool consistency, number of fecal grains in a cage, and cleanliness of the perianal region. Characteristics of normal anorectal function were determined in the control group. A five-day observation revealed that healthy control rats excrete 20–30 grains of feces per day, have a clean perianal area, and have no fecal grains retained in the anus. Therefore, the number of fecal grains of less than 15 per day was recorded as constipation. The occurrence of unformed feces and the presence of fecal matter adhered to the perianal area was recorded as fecal incontinence.

Rat anal caliber was measured preoperatively and on postoperative day 35 by insertion of Hegar dilators ranging from 8.5 to 5.5 mm in diameter (in 0.5 mm decrements) into the anus of anesthetized rats, starting from the 8.5 mm dilator. If the anus was not patent, the next smaller dilator was used. The anal caliber matched the maximal diameter of Hegar dilator that could be effortlessly inserted into the anus. Because preoperatively all rats had an anus patent to a size 8.5 mm Hegar dilator, postoperative anal stenosis was defined as failure to insert the 8.0 mm or smaller Hegar dilator.

Statistical analysis

The data were processed using R-statistics software version 3.3.0. Throughout the work, continuous variables were presented as median (Me) and interquartile range [LQ; UQ], categorical variables were presented as counts. The Wilcoxon signed rank test was used to compare rat anal caliber before vs. after the surgery within the same group. The Kruskal–Wallis test followed by Dunn's post hoc test was used to compare biomarkers levels in the rectal tissue homogenate and rat anal caliber between the study groups. Two-tailed p values of less than 0.01 were considered statistically significant.

Results

All rats were in a satisfactory condition after the surgery, there were no immediate postoperative complications or animal deaths. During the 35-day follow-up, the rats showed normal physical activity, urination and defecation, good food intake (all rats had a stable weight, which was measured weekly).

VEGF-C and HIF-1α levels in rectal homogenate

On postoperative day 3, in subgroups IA, IIA, and IIIA, the levels of VEGF-C and HIF-1α in the rectal homogenate were greater than in group IV (control) ($p < 0.01$ for all). There was no significant difference in the levels of these biomarkers between the subgroups (Fig. 4).

Histopathological findings

On postoperative day 3

In subgroup IA, all rats demonstrated signs of severe microcirculatory disorders and inflammation. The lesion of submucosa and serosal layer of the rectum was characterized by dense infiltration of neutrophils and eosinophils, formation of blood clots and small hemorrhages, and congestion of veins and venules. Necrosis of the serosal layer with fibrin deposition was noted in seven rats (Fig. 5a).

In subgroup IIA, there were signs of microcirculatory disorders with less pronounced inflammation. Extensive areas of hemorrhage with deposition of fibrin mass and perifocal inflammatory reaction were noted in the muscle and serosal layers. There were also significant edema and loosening of the submucosa (Fig. 5b). Small areas of necrosis of the serous layer were found in only two rats.

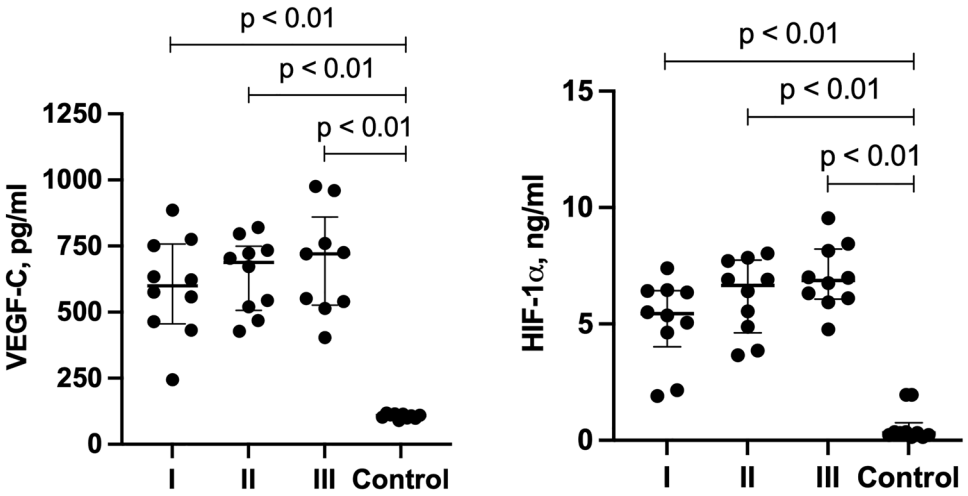

Fig.4 VEGF-C and HIF-1α levels in rectal tissues. I—group "impaired vascularization of the anorectum"; II—group "tension anastomosis of the anorectum"; III—group "sham operation". Data are presented as median (Me) and interquartile range [LQ; UQ] and analyzed using Kruskal–Wallis test followed by a post hoc Dunn test to contrast between pairs of samples

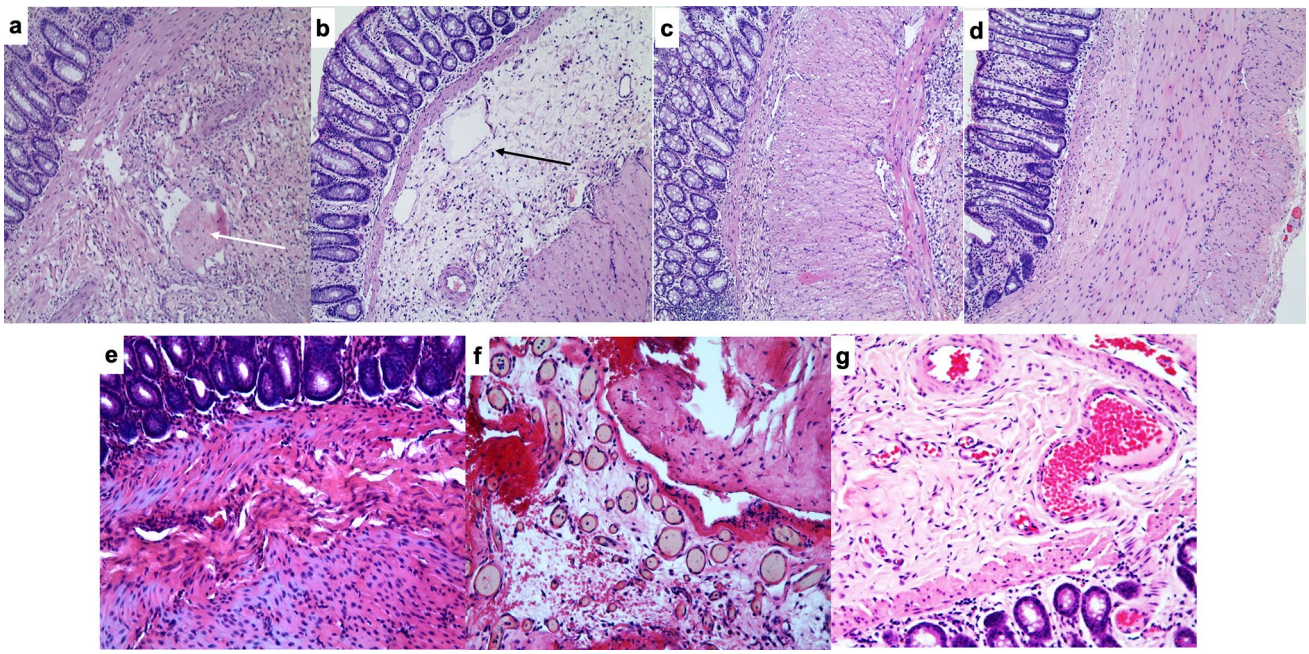

Fig.5 Histopathological changes in the rectal tissue on postoperative day 3 and postoperative day 35. **a** IA group: infiltration of the neorectum submucosa with neutrophils and eosinophils, filling of veins and venules, formation of blood clots (white arrow), small hemorrhages (HE×100); **b** IIA group: extensive areas of hemorrhage with loss of fibrin mass and perifocal inflammatory reaction in the muscular and serous layers, edema and loosening of the submucosa (black arrow) (HE×100); **c** IIIA group: slight inflammatory infiltration near the serous membrane (HE×100); **d** control group: normal histological structure of the rectum (HE×100). **e** IB group: violation of the layered topography of the rectum, severe sclerosis of the submucosal and muscular layers with accumulations of lymphocytes (HE×400); **f** IIB group: signs of sclerosis of the submucosal layer, subserosal zone, perivascular sclerosis, active angiogenesis (HE×400); **g** IIIB group: focal mild fibrosis of the submucosa with neoangiogenesis (HE×400)

In subgroup IIIA, changes in the intestinal wall were minimal. There were areas of mild inflammatory infiltration in the serous layer (Fig. 5c).

In group IV (control), the rectal histology was normal (Fig. 5d).

On postoperative day 35

In subgroup IB, eight rats demonstrated altered histotopography of the rectal wall layers due to obliteration of the submucosal layer. Severe sclerosis of submucosal and muscular

Table 1 Histopathological findings in rectal tissues on day 3

Day 3	Presence of inflammation *n* (%)	Circulation disorders *n* (%)	Areas of necrosis *n* (%)
Group IA	10 (100) ↑↑↑	9 (90) ↑↑↑	7 (70) ↑↑
Group IIA	10 (100) ↑↑	10 (100) ↑↑↑	2 (20) ↑
Group IIIA	6 (60) ↑	0 (0)	0 (0)
Group IV	0 (0)	0 (0)	0 (0)

*↑—mild; ↑↑—moderate; ↑↑↑—severe

Table 2 Histopathological findings in rectal tissues on day 35

Day 35	Disruption of the topographic layers *n* (%)	Signs of sclerosis and chronic inflammation *n* (%)	Poor angiogenesis *n* (%)
Group IB	8 (80) ↑↑↑	10 (100) ↑↑↑	9 (90)
Group IIB	2 (20) ↑	10 (100) ↑↑	3 (30)
Group IIIB	0 (0)	1 (10) ↑	0 (0)

*↑—mild; ↑↑—moderate; ↑↑↑—severe

layers with accumulation of lymphocytes was observed in all rats of this subgroup. There were no signs of angiogenesis in most rats. The repair process in this subgroup could be considered as a prolonged inflammatory reaction with a pronounced giant cell response (Fig. 5e).

In subgroup IIB, all layers of the rectal wall were preserved, and their topography was undisturbed in most rats. There were severe signs of sclerosis in submucosal and subserosal layers in all rats. The chronic inflammation was weak, compared to subgroup IB rats. The repair process in this subgroup could be considered as active angiogenesis near the serous membrane (Fig. 5f).

In subgroup IIIB, the rectal wall demonstrated focal mild fibrosis with active angiogenesis in the submucosal layer and almost complete absence of the inflammatory cell infiltration (Fig. 5g).

The histopathological findings in rectal tissues on postoperative days 3 and 35 are summarized in Table 1 and Table 2.

Anorectal function and anal caliber

On postoperative days 30–35, constipation was noted in four subgroup IB rats and in three subgroup IIB rats. Fecal incontinence was not observed in any of the rats in subgroup IB and was observed in one subgroup IIB rat. Neither constipation nor fecal incontinence was noted in subgroup IIIB or group IV rats.

Preoperatively, the 8.5 mm Hegar dilator could be effortlessly inserted into the anus of all subgroup IB, IIB, and IIIB rats. On postoperative day 35, the anal caliber significantly decreased to 7.0 mm [6.3; 7.3] in subgroup IB and to 6.5 mm [6.3; 7.3] in subgroup IIB, compared to the preoperative size of 8.5 mm [8.5; 8.5] ($p < 0.01$ for both). In contrast, in subgroup IIIB, the anal caliber was 8.0 mm [7.8; 8.5], which did not differ from its preoperative size ($p > 0.05$). The median postoperative anal caliber in subgroups IB and IIB was statistically significantly less, compared to the caliber in subgroup IIIB ($p < 0.01$ for both), while the difference in the anal caliber between subgroups IB and IIB was not significant ($p > 0.05$).

Discussion

Surgical correction of ARM and HD often requires extensive mobilization of the rectum and/or formation of anastomosis with rectal tissue tension [12]. In clinical practice, it is very difficult to reliably assess the role of these factors in the treatment outcome, given the relatively small number of such patients available at a single clinical center and significant variability of other factors also affecting the outcome. Researchers have previously created models similar to ours, but the impairment of rectal vascularization due to rectal mobilization was not standardized and severity of rectal ischemia and rectal tissue tension was not described [13]. In one study, researchers performed anorectal mobilization followed by a 2 or 4 cm resection of the rectum in dogs to evaluate the role of the internal anal sphincter in fecal continence. However, such an extent of resection is unlikely to result in anorectal tissue tension in dogs, and rectal ischemia has also not been evaluated [14]. Therefore, to investigate the impact of impaired vascularization of the rectum and the impact of rectal tissue tension during the formation of anorectal anastomosis on postoperative morphology and function of the rectum, we have created two original rat models separately reproducing these two surgical factors.

To evaluate early response of the rectal tissue to hypoxia after the creation of anorectal anastomoses, we measured the levels of VEGF-C and HIF-1α in the rectal tissue homogenate on postoperative day 3. In all experimental groups, the levels of both biomarkers were increased to a similar extent. On one hand, it suggests similar severity of the rectal tissue hypoxia in these groups; on the other hand, it may be explained by high sensitivity of these biomarkers to tissue hypoxia, so that even mild ischemia could induce their maximum synthesis in the early postoperative period. Increased levels of HIF and VEGF in the intestinal tissue have been also demonstrated on the 3rd day after the induction of experimental high-altitude intestinal ischemia in rats [15] and on the 2nd day after colitis modeling in mice [11], but in none of these studies a surgical intervention was performed. Thus, the detected elevation of the VEGF-C and HIF-1α levels in our models indicated the development of ischemia

in the rectal tissue in the early postoperative period but did not allow us to differentiate its severity. The value of measuring biomarkers of hypoxia to assess postoperative rectal ischemia remains a subject of further investigation.

Histologic examination of the rectal tissue samples obtained on postoperative day 3 revealed severe rectal microcirculatory disorders and signs of inflammation in both group I and group II rats and minimal changes in group III. On postoperative day 35, group I rats demonstrated severe fibrosis, lymphocyte infiltration, and minimal or no angiogenesis; group II rats also showed pronounced fibrosis in the rectal tissue but, in contrast to group I, exhibited milder inflammatory cell infiltration and potent formation of new blood vessels; group III rats had normal healing findings without fibrosis. Thus, despite the differences in the activity of angiogenesis and severity of chronic inflammation, the degree of rectal fibrosis was similar in both experimental models, which is in line with similar levels of hypoxia biomarkers in the rectal tissue homogenate detected in the early postoperative period.

The frequency of postoperative anorectal dysfunction and severity of anal stenosis in the long-term were similar in group I and group II rats. These experimental findings are in accordance with the data that both surgical factors are associated with the risk of defecation disorders and anal strictures in patients who have undergone a repair of ARM [7]. Moreover, it has been shown that boys with ARM, compared to girls, have higher incidence of postoperative complications in the long-term, which can be explained by the fact that the males require a higher degree of rectal mobilization to reach the perineum and, therefore, have a greater risk of severe tissue ischemia [16]. Another study suggested that higher incidence of defecation disorders in patients after multi-stage correction, compared to patients after single-stage correction, may be caused by greater initial severity of ARMs that implied a more extensive mobilization of the rectum and its tension [17]. In patients with HD requiring pull-through procedures, special care must be taken to ensure that the descending colon is not under tension and that after mobilization, which inevitably involves the intersection of a number of vascular collaterals, the blood supply of the intestine remains sufficient [18, 19]. Collectively, these clinical studies suggest that anorectal ischemia associated with surgical repair of congenital colonic defects plays an important role in the development of postoperative complications in the long-term. Our data not only confirm these observations but also indicate that incidence and severity of the complications do not depend on whether repair was associated with extensive mobilization of the rectum or creation of anorectal anastomosis under conditions of tissue tension.

The strength of our study is that, to our best knowledge, this is the first study demonstrating and comparing the contribution of extensive rectal mobilization and of rectal tissue tension to the long-term outcome using two original experimental models of postoperative anorectal ischemia in rats. The models allowed us to evaluate the severity of early postoperative ischemia by measuring the levels of hypoxia biomarkers specifically in the rectal tissue homogenate and to correlate it with the severity of histopathological, anatomical, and functional abnormalities in the anorectum observed in the late postoperative period. Although the models were created using adult rats without pre-existing malformations or functional disorder and, therefore, may not fully reflect the anatomical and functional characteristics typical of children with ARM and HD. In addition, the possible thermal tissues damage to the thin rectal wall could have some effects on histologic pictures in the model of impaired vascularization of the anorectum, which can be viewed as a limitation of our study. Also, there were not tools to quantify tissue tension during the experimental surgery in group II and the absolute length of the resected part could differ in each particular rat due to differences in the size of rats, but the length ratio between the resected and mobilized parts of the rectum was similar in all rats. It allows us to assume that the degree of tension was also similar. However, we believe that they represent a useful tool for further investigation of postoperative ischemia of the anorectum and development of new methods for fibrosis prevention and treatment.

In summary, we have shown experimentally that extensive rectal mobilization to about the same extent as tension anastomosis has a major impact on postoperative rectal ischemia, resulting in severe fibrotic changes in the anorectum and defecation disorders in the long-term. This finding signifies the need for assessment and mitigation of intra- and postoperative rectal tissue ischemia in children with ARM or HD regardless of whether anorectal anastomosis is created in conditions of extensive mobilization of the rectum or rectal tissue tension. We believe that intra- and postoperative assessment of rectal ischemia and control of the degree of rectal tissue tension is a promising topic for future research aimed at improving surgical outcomes in children after anorectoplasty or pull-through procedures.

Author contributions Conceptualization: DM and OM; methodology: DM and OM; formal analysis and investigation: DM, TM, and LS; writing—original draft preparation: DM; writing—review and editing: HÖ and IB; supervision: DM. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Ethics approval The study was approved by the Institutional Review Board and the animals were cared for and handled in accordance with National Institutes of Health guidelines.

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