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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 extended mobilization or tension anastomosis, which can compromise the vasculature of the rectum. We aimed to analyze the histopathological findings, hypoxia biomarker values and to correlate the incidence of anal stenosis and defecation disorders of both conditions in experimental models.

Methods

We created anorectal reconstruction models during anorectal mobilization with extended mobilization with impaired vascularization (group I) and tension anastomosis (group II) in rats. Hypoxia biomarker values was assessed in both groups and in sham operated animals (group III). The histopathological changes on the 3rd postoperative day, anal stenosis and defecation disorders on the 35th day are compared with each other and with a control group (group IV).

Results

Hypoxia biomarker levels confirmed postoperative ischemia in I – III groups compared to the control. Groups I, II are accompanied by pronounced histopathological changes in the anorectum on the 3rd postoperative day and accompanied by severe fibrosis on the 35th day. In comparison to group III, both groups showed defecation disorders and anal stenoses.

Conclusion

Extensive mobilization with vascular impairment and tension anastomosis resulted in similar ischemia with histopathologic changes, which in the long term resulted in fibrotic changes associated with defecation disorders.

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 these 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: extended 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 significance in the long-term effects such as fibrotic stenosis and defecation disorders remains uncertain [4, 5, 7].

It is well known that a decrease in oxygen supply to the tissues triggers the processes that provide adaptation to hypoxia, including activation of hypoxia-inducible factor 1-alpha (HIF-1a) stimulating the expression of vascular endothelial growth factor (VEGF) [8]. The expression and role of these markers of hypoxia under ischemia conditions in various tissues, including the colon, have been confirmed in many studies [9–11].

We constructed original experimental models with impaired vascularization by extensive mobilization and with anorectal tension anastomosis to study and compare the effect of these two surgical techniques on the extent of rectal ischemia and on fibrotic changes. Tissue ischemia biomarkers (hypoxia-inducible factor 1-alpha (HIF-1a) and vascular endothelial growth factor C (VEGF-C)) were studied along with histopathological changes in the early and late postoperative period. The frequency of anal stenosis and defecation disorders were evaluated with the clinical findings of anal stenosis associated with constipation and fecal incontinence.

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 National Institutes of Health guidelines. The study included 70 adult (12 weeks old) male outbred rats weighing 370–420 g and all animals were housed separately (one rat per cage). All animals were operated on by the same surgeon (first author) and sample groups were designed based on experience from previous proprietary research and publication data [12, 13].

Anorectal mobilization and the study groups:

All group I–III animals received intramuscular general anesthesia (2 mg/kg of tiletamine hydrochloride and zolazepam hydrochloride, “Zoletil 100”, Vibrac, France). A monopolar coagulator was used at a given power for both coagulation and sphincter electrode identification during surgery. Preoperatively under anesthesia we inserted Hegar dilators in order to check the preoperative caliber of the anus in all rats.

In groups I – III surgical procedure started by putting holding sutures to the anal verge, then a circular incision of the skin around the anus was made at a distance of 1–2 mm. The anorectum was mobilized along its wall to the sphincter. The bowel was then transferred to the perineum after application of either impaired vascularization, tension anastomosis or sham operation, and the bowel was fixed to the anus with 6/0 Vicryl sutures after resection of its end bearing the holding sutures.

Group I-Impaired vascularization of the anorectum (n = 20)

The rectum was mobilized very close to the intestinal wall, leaving no mesenteric collaterals. The serosal layer of the mobilized rectum was coagulated circumferentially at sphincter level, leaving the distal portion with only intramural blood supply (Fig. 1a).

Group II-Tension anastomosis of the anorectum (n = 20)

The rectum was carefully mobilized without damaging the serosal vasculature, and one half of the mobilized rectum was resected and then anastomosed under tension to the anus (Fig. 1b).

Group III-Sham operation (n = 20)

The rectum was replanted after its careful mobilization to demonstrate the effect of the surgery.

Group IV-Control animals (n = 10)

These animals were used as control group with tissue samples and histopathological analyses.

In groups I, II and III, the rats were randomly divided into subgroups A and B (10 rats each). Feeding and watering in the postoperative period began as usual on the day of surgery. No medication and anus dilatation were used in any of the groups in the postoperative period. In subgroups A, the severity of anorectal ischemia was assessed based on VEGF-C and HIF-1α levels in rectal tissue and histopathological examination on day 3 postoperatively.

There were no immediate post-op complications or animal deaths. The animals were sacrificed by lethal doses of tiletamine hydrochloride and zolazepam hydrochloride (“Zoletil 100”, Vibrac, France).

The methods of creating an experimental model of postoperative ischemia of the anorectum are patented and registered in the State Register of Inventions of the Russian Federation: No. 2719661 and No. 2719663 dated April 21, 2020.

Measurement of ischemia biomarkers

Before euthanasia, tissue samples from the anorectum were taken from the experimental groups on the third day (IA, IIA and IIIA) and from animals in the control group (IV). Intestinal tissues were homogenized in 1.15% KCl buffer (pH 7.4) in a pounder on ice. The intestinal homogenate was centrifuged at 14000g for 10 min and the supernatant was collected in separate Eppendorf tubes. Sample aliquots were stored at − 25°C for no longer than 1 month prior to testing. To determine VEGF-C and HIF1-α levels, we used the test systems from BCM Diagnostics and Bender Medsystems (Austria) for the enzyme-linked immunosorbent assay on a Stat Fax 2100 analyzer (Stat Fax, USA). All specimens were measured in duplicate wells and the mean of the two values was taken.

Postoperative assessment of anorectal function and anal caliber

Defecation was assessed between the 30th to 35th postoperative day in group B rats, according to the following criteria: stool consistency, number of fecal grains in a cage and cleanliness of the perianal region. The number of fecal grains of less than 15 per day and visualized retention of feces in the distal

part of the rectum was recorded as constipation. The occurrence of unformed feces and contamination of the perianal area with feces is called fecal incontinence.

Anal stenosis was defined as failure to insert the 8 mm Hegar dilator under anesthesia on postoperative day 35 since all rats had an anus passable preoperatively for the 8.5 mm Hegar dilator.

Histopathological examination

Rectal tissue samples were obtained from each sacrificed rats, fixed in 10% natural buffered formalin, and subjected to routine tissue processing. All samples were studied by a single pathologist (the third author). Samples were subjected to hematoxylin–eosin (HE) staining and examined with a Leica DM2000 light microscope. The blind evaluation of the histological changes was classified as mild, moderate, and severe in subgroups A based on the severity of inflammation, circulatory disorders, and the prevalence of necrosis; and on a degree of disruption of intestinal wall topography, expression of sclerosis and chronic inflammation in subgroups B.

Statistical analysis

Data were processed using R-statistics software version 3.3.0. Throughout the work, the average of the quantitative values is presented as the median (Me) and interquartile range [LQ; UQ]. To assess statistically discernible differences in the indicators of biomarker concentrations between groups, the non-parametric Kruskal–Wallis test was applied, followed by a post hoc Dunn's test to contrast between pairs of samples. Differences were considered statistically significant if $p < 0.01$.

Results

VEGF-C and HIF-1a levels in rectal homogenate in group A animals

In groups IA, IIA, and IIIA, the levels of VEGF-C and HIF-1a in rectal homogenate were greater than in control ($p < 0.01$ for all). There was no significant difference in the levels of biomarkers between groups I, II, and III (Fig. 2).

Histopathological findings

On the 3rd postoperative day:

Signs of severe microcirculatory disorders and inflammation were noted in all Group IA rats. The submucosa and serosal layer of the rectum was observed with dense infiltration of "acute phase" cells (neutrophils and eosinophils). Formation of blood clots and small hemorrhages as well as repletion of veins and venules were observed. Necrosis of the serosal layer with fibrin deposition was noted in 7 rats (Fig. 3A-a).

In Group IIA rats, there were signs of microcirculatory disorders with the less pronounced inflammation with extensive areas of hemorrhage with fibrin mass, and perifocal inflammatory reaction in muscle and serosal layers. There were also significant edema and loosening of the submucosa (Fig. 3A-b). Small areas of necrosis of the serous layer were found in only 2 rats.

In Group IIIA, changes in the intestinal wall were minimal. There were areas of mild inflammatory infiltration in the serous membrane (Fig. 3A-c).

In Group IV, the histopathology of the rectum was normal (Fig. 3A-d).

On the 35th postoperative day:

A comparative analysis of histopathological changes in samples of all group B animals was carried out according to the following features: disruption of the topographic layers, signs of sclerosis and chronic inflammation, angiogenesis.

In Group I, 8 rats suffered damage to the layered topography of the rectal wall due to obliteration of the submucosal layer. Severe sclerosis of submucosal and muscular layers with signs of chronic inflammation and accumulation of lymphocytes was observed in all rats of this group. Angiogenesis in these zones were absent in most rats. A characteristic feature of the reparative process in this group can be considered as a prolonged inflammatory reaction with a pronounced giant cell response (Fig. 3B-a).

In Group II, all layers of the intestinal wall were preserved and their topography was undisturbed in most rats. The chronic inflammation was weak relative to the Group I. We defined signs of sclerosis of the submucosal layer, subserosal zone, in all rats. The characteristic feature of the repair process in this group can be considered as active angiogenesis near the serous membrane (Fig. 3B-b).

In Group III, the intestinal wall consisted of focal mild fibrosis of the submucosa with active angiogenesis and almost complete absence of the inflammatory component **(**Fig. 3B-c**).**

The histopathological findings in rectal tissues on days 3 and 35 are shown in Table 1a&b.

Table 1
a. Histopathological findings in rectal tissues on day 3.

3rd days	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
*↑ - mild; ↑↑ - moderate; ↑↑↑ - severe			

Table 1
b. Histopathological findings in rectal tissues on day 35.

35th day	Disruption of the topographic layers n (%)	Signs of sclerosis and chronic inflammation n (%)	Poor angiogenesis n (%)
I group	8 (80) ↑↑↑	10 (100) ↑↑↑	9 (90)
II group	2 (20) ↑	10 (100) ↑↑	3 (30)
III group	0 (0)	1 (10) ↑	0 (0)
*↑ - mild; ↑↑ - moderate; ↑↑↑ - severe			

Functional and clinical findings and results of anal caliber in group B animals

All experimental animals were in a satisfactory condition after the surgery: they showed physical activity, urination and defecation, good food intake (all rats had a stable weight, which was measured weekly).

Defecation disorders

Constipation was noted in 4 rats in Group IB, and in 3 rats in Group IIB. Fecal incontinence was not observed in any of the rats in group I, and in 1 rat in Group II. Neither constipation nor fecal incontinence was found in either Group IIIB or Group IV animals.

Anal caliber

A Hegar dilator No. 8.5 could be inserted preoperatively in the animals of groups IB, IIB and IIIB. Postoperatively we defined a noticeable decrease in the diameter of the anus in all rats of I and II groups, a No. 8 Hegar dilator could be placed in all Group III animals but not in Groups I and II animals (Table 2).

Table 2
Diameters of anuses on 35th postoperative day.

	I group (No. of Hegar dilator)	II group (No. of Hegar dilator)	III group (No. of Hegar dilator)
1	7,0	6,5	8,0
2	7,0	6,5	8,0
3	7,0	7,0	8,5
4	7,5	6,0	8,0
5	5,5	7,5	8,5
6	5,5	6,0	8,5
7	7,0	6,5	8,5
8	7,5	7,0	8,5
9	7,0	6,5	8,0
10	7,0	7,5	8,0
***** A Hegar dilator No. 8.5 could be inserted preoperatively in all animals			

Discussion

Surgical correction of anorectal malformations and Hirschsprung's disease often requires extensive mobilization of the rectum and/or formation of anastomosis with rectal tissue tension [14]. In clinical practice, it is very difficult to reliably assess the role of these factors in treatment outcome, given the relatively small number of children admitted to a single clinical center and significant variability factors affecting the treatment outcomes. Researchers have previously created models similar to ours, however, the impairment of rectal vascularization due to rectal mobilization was not standardized and assessment of rectal ischemia and rectal tissue tension were not described [15]. There is a study in which researchers performed anorectal mobilization followed by 2 and 4 cm resection of the rectum in dogs to evaluate the role of the IAS in fecal continence. However, such a volume of resection is unlikely to lead to result in tissue tension in dogs, and rectal ischemia has also not been evaluated [16].

The high levels of VEGF-C and HIF-1a in the rectal homogenate in all experimental groups could be explained with their high sensitivity to tissue hypoxia and their maximum synthesis in the early postoperative period. In the literature, we found some studies where the researchers looked at the levels of HIF and VEGF in the gut. In one case, the HIF concentration was assessed on the 3rd day after high-altitude intestinal ischemia modeling, in another study, the concentration of hypoxia markers was measured on the 2nd day after colitis modeling, but in no case a surgical intervention was performed [11, 12]. In this way we confirmed that ELISA results show high levels of VEGF-C and HIF-1a in postoperative

ischemia in rectal tissue, herewith they did not allow us to determine the severity of the ischemia. The value of measuring biomarkers of hypoxia to assess postoperative rectal ischemia remains a subject of further investigation.

In our study, the time-point for the early and late (3rd and 35th postoperative days) were chosen based on published data for determining the intestinal concentration of HIF-1a after the development of ischemia [12]. In addition, we considered the presence of a maximum edema on the 3rd postoperative day as the most pronounced microcirculation disorder in the postoperative area. It has also been reported that the maturation or remodeling phase of wound healing begins at day 21–25, during which time fibroblasts lay down collagen and contribute to scar formation [17, 18]. Therefore, in subgroups B, postoperative surveillance lasted 35 days to assess the extent of defecation disorders and anal stenosis as long-term sequelae.

The early postoperative histopathologic findings suggested that there are differences in tissue repair after rectal ischemia. On the 3rd postoperative day, we found severe rectal microcirculatory disorders and inflammation in groups I and II compared to the sham group. In addition, on the postoperative 35th day, while severe fibrosis was the main finding in these animals, the sham group showed normal healing findings without fibrosis.

In the long-term postoperative period, the histological picture in the intestinal wall after replantation in Group III rats can be interpreted as an adequate reparative process, while the model extensive mobilization with "*impaired vascularization*" is accompanied by severe fibrotic changes in the rectal tissue. It is important that all animals of groups I and II showed pronounced anal stenoses on the 35th postoperative day, which, however, differed morphologically. The fibrotic changes with the disturbance of the layer topography were most severe in rats with extensive mobilization of the rectum. Rats with impaired vascularization showed remarkably poor angiogenesis compared to the morphological picture in intestinal tissues of rats with tension anastomosis.

We also found that in rats after extensive mobilization of the rectum with impaired vascularization and in animals with tension anastomosis of the rectum, in the long-term postoperative period, defecation disorders were found more frequently than in the replantation and the control groups.

The role of operative factors for the long-term outcome are mainly based on the practical experience of surgeons [7]. It was also suggested that the high incidence of anal strictures in a long-term postoperative period in boys with ARM compared to girls may represent the fact that the males required a higher degree of rectal mobilization to reach the perineum, with a greater risk of tissue ischemia [19]. The higher incidence of defecation disorders in patients after multi-stage correction compared to single-stage correction was explained by the initial severity of a type of ARM in these patients that implied a more extensive mobilization of the rectum and its tension [20]. We were also able to find opinions in published articles that with 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 [21]. Furthermore, there are

authors mentioning that the most serious early operative complication as anastomotic leak in patients after pull-through procedures can result from tension at the anastomosis and/or insufficient blood supply up to and including the edge of the anastomosis [6].

Our study aims to create experimental models of postoperative anorectal ischemia in normally developed rats without malformations or functional disorder. We found that extensive mobilization of the rectum as well as tension anastomosis resulted in severe postoperative ischemia on the third postoperative day. The long-term results were marked rectal fibrotic changes, anal strictures, and defecation disorders. To the best of our knowledge, this is the first study evaluating the long-term effect of postoperative rectal ischemia on defecation abnormalities and anal stenosis. The determination of the concentrations of biomarkers of hypoxia in the rectum after surgery is also applied for the first time.

In addition to devascularization, however, the effects of thermal tissue damage are a weak point of our study that can be viewed as a limitation.

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 neorectum and defecation disorders in the long-term. The scientific justification of the importance of these factors in rectal ischemia and long-term outcomes draws the attention of pediatric surgeons to determine the need for intra- and postoperative assessment of rectal tissue ischemia in the surgical treatment of children with ARM or HD. 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 with anorectoplasty and pull-through procedure.

Declarations

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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Conceptualization: Dmitry Morozov, Olga Mokrushina; Methodology: Dmitrii Morozov, Olga Morozova; Formal analysis and investigation: Dmitrii Morozov, Tatyana Marchuk, Lubov Severgina; Writing - original

draft preparation: Dmitrii Morozov; Writing - review and editing: Hüseyin Özbey, Ivan Budnik; Supervision: Dmitry Morozov

All authors read and approved the final manuscript.

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Figures

Figure 1

The creation of models of postoperative rectal ischemia

(a) the impaired vascularization model of the anorectum: 1 – Mobilizing the rectum to the sphincter muscle (black arrow); 2,3 – the serous layer of the mobilized rectum was coagulated circumferentially at

the sphincter level (yellow arrow); 4 - formation of the anus by applying interrupted sutures. (b) - the tension anastomosis model of the anorectum: 1 - mobilization of the rectum to the sphincter (black arrow); 2 - resection of half of mobilized rectum (resection line - yellow dotted line; the first two anorectal sutures are placed at 12 and 6 o'clock); 3 - formation of the anus under tension.

Figure 2

VEGF-C and HIF-1a levels in rectal tissues

I – group “impaired vascularization of the anorectum”; II – group “tension anastomosis of the anorectum”; III – group “sham operation-rectal replantation”; C – control group. 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. *** - $P < 0.01$

Figure 3

A and B

Histopathological changes in the rectal tissue on the 3rd postoperative day.

a (group I) - infiltration of the neorectum submucosa with neutrophils and eosinophils, filling of veins and venules, formation of blood clots (white arrow), small hemorrhages; b (group II) – 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); c (group III) – slight inflammatory infiltration near the serous membrane; d (control group) – normal histological structure of the rectum. (HE×100)

B Histopathological changes of the rectal tissue on the 35th postoperative day

a (group I) - violation of the layered topography of the rectum, severe sclerosis of the submucosal and muscular layers with accumulations of lymphocytes; b (group II) – signs of sclerosis of the submucosal layer, subserosal zone, perivascular sclerosis, active angiogenesis; c (group III) – focal mild fibrosis of the submucosa with neoangiogenesis. (HE×400)