THE EFFECT OF SURGICAL MUCOSECTOMY ON THE INTESTINE AND ITS POSSIBLE CLINICAL CONSEQUENCES

Ph. D. Thesis

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Full papers related to the subject of the thesis

1. **Urbán D,** Varga G, Érces D, Marei M, Cervellione RM, Keene D, Goyal A, Cserni T. Prolonged ischemia of the ileum and colon after surgical mucosectomy explains contraction and failure of "Mucus free" bladder augmentation. J Pediatr Urol (in press article, 10.1016/j.jpurol.2022.04.015) **IF 1.83**

2. **Urbán D**, Cserni T, Boros M, Juhász Á, Érces D, Varga G. Bladder augmentation from an insider's perspective: a review of the literature on microcirculatory studies. Int Urol Nephrol. 2021;53:2221-2230. **IF 2.37**

3. **Urbán D**, Marei MM, Hajnal D, Varga G, Érces D, Poles M, Imre D, Szabó A, Cervellione RM, Cserni T. Mucosectomy disrupting the enteric nervous system causes contraction and shrinkage of gastrointestinal flaps: potential implications for augmentation cystoplasty. J Pediatr Urol. 2020;16:20-26. **IF 1.622**

4. **Urbán D**, Kőnig R, Cserni T. A rövidbél-szindróma korszerű sebészi kezelése: autológ rekonstrukció és intestinalis rehabilitáció [Autologous reconstructive surgery and intestinal rehabilitation in the management of short bowel syndrome]. Orv Hetil. 2020;161:243-251. Hungarian. **IF 0.54**

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Response to a commentary related to the subject of the thesis

Marei MM, **Urbán D**, Cserni T. Response to commentary to 'Mucosectomy disrupting the enteric nervous system causes contraction and shrinkage of gastrointestinal flaps: potential implications for augmentation cystoplastsy'. J Pediatr Urol. 2020;16:29-30.

1. INTRODUCTION

Mucosectomy, the dissection and removal of the intestinal mucosa is technically feasible by conventional and minimally invasive surgical techniques. Ex-vivo mucosectomy is performed by researchers and pathologists as well to explore and study the function and structure of the enteric nervous system (ENS) (Cserni T et al. 2007, Cserni T et al. 2009). Endoscopic submucosal dissection (ESD) has become increasingly popular in recent times. Using natural body orifices, this approach can eliminate the peri-operative burden of open surgeries without compromising the oncological outcome (Ahmad et al. 2021, Carmichael et al. 2020). Although ESD is well tolerated, several studies have reported strictures or stenosis requiring steroid treatment and multiple balloon dilatations (Park et al. 2020, Ohara et al. 2016, Iizuka et al. 2010).

Mucosectomy plays significant roles in reconstructive urology as well. Parts of the digestive system, especially ileum and colon are routinely used for bladder augmentation as donor organs. The presence of the intestinal mucosa in the augmented bladder is however, associated with several complications Therefore, intensive research attention has been focused on “mucus free” bladder augmentation. Composite flaps with cultured urothelium coverage after mucosectomy seemed to be viable and promising approach (Turner et al. 2010). Similar experimental attempts were made to increase the absorptive surface in severe short bowel syndrome assuming the colon will remain functional after mucosectomy (Avansino et al. 2006, Tait et al. 1994, Sugimoto et al. 2021). However, despite the promising experimental results the findings cannot be translated into clinical practice because contraction of the intestinal flaps remained a major concern.

In this context it is essential to understand the impact of mucosectomy on the intramural microcirculation, we hypothesized that removal of the mucosa/submucosa may disrupt the intramural vascular plexuses compromising the perfusion of the remaining sero-muscular wall.

Similarly, the consequence of the disruption of the ENS in this scenario is less understood, Our hypothesis is that mucosectomy disrupts the ENS, which may cause contraction in the involved segments. This possibility has not been explored before in real-life or clinically relevant experimental conditions.

1.1 Stricture after mucosectomy in clinical practice

1.1.1 ESD and strictures in the GI tract

Endoscopic excision of large mucosal-submucosal lesions in esophagus is possible, however rate of complications i.e. stricture is increasing with the size of excision from 2% to 88% (Espinel et al. 2015, Spadaccini et al. 2021, Alzoubaidi et al. 2016). Approx. 66%-88% of patients with higher than 75% circumferential resection developed esophageal stricture (Park et al. 2020, Ohara et al. 2016). At present multiple endoscopic balloon dilatation and systematic or local steroid therapy is available for treatment (Park et al. 2020).

Only a few reports are available about post-ESD stricture in stomach (Iizuka et al. 2010, Tsunada et al. 2008). All cases were reported where the resected ratio was at least 75% or higher of the whole circumference of the lumen (Iizuka et al. 2010). Significant stenosis was localised on pre-pylorus, antrum and cardia (Ohara et al. 2014, Iizuka et al. 2010).

In terms of complications the rectum seems to be the “most forgiving” space, because strictures were not reported after circumferential resection of under 90% (Ohara et al. 2014, Kantsevoy et al. 2017). However, in case of resection over 90% of the circumference, stricture was registered in 43.8% of patients. In case of total circumferential resection, the complication rate rises further (71.4%) (Ohara et al. 2014, Kantsevoy et al. 2017).

The difference in ESD limitation and complications may be explained with the different functional features of esophagus, stomach and rectum (Ohara et al. 2014). For example, rectum has larger lumen than the esophagus, in addition it holds the stool before defecation, which generates a constant pressure on the wall and it keeps the rectum dilated (Ohara et al. 2014).

1.1.2 Short bowel syndrome (SBS)

In SBS cases intact colon is usually available, but the change of the colonic mucosa is does not help in absorption of nutrients. Therefore creation of composite intestine from the colon with transplanted cultured small bowel mucosa or stem cells is the focus of present GI research (Tait el al. 1994, Sugimoto et al. 2021). The colonic mucosa is removed with surgical or chemical mucosectomy and epithelial organoid containing ileal stem cells is implanted. The neo-ileal mucosa demonstrates villus structures and crypts, and contains enterocytes, goblet cells, enteroendocrine cells, and Paneth cells (Tait el al. 1994, Sugimoto et al. 2021). Moreover, in human organoids sodium-dependent bile acid uptake activity and restored lymphovascular system was detected. (Sugimoto et al. 2021). During mucosectomy it is crucial to remove the

residual colonic mucosa and preserve the viability of submucosal layer. The ambiguous consequences of surgical mucosectomy were emphasized, a heterogeneous surface with residual native mucosa and deep debridement-induced scar formation was demonstrated (Avansino et al. 2006). Chemical mucosectomy were found to be effective experimental means to remove the mucosal layer, however this approach is still far from being safe alternative in clinical practice (Avansino et al. 2006).

To achieve the final goal "to turn the colon to small bowel" it is not enough to transplant the small bowel mucosa into it. The colonic segment should remain functional after mucosectomy. Contraction and/or motility problems in the composite bowel would lead to ultimate failure to treat SBS.

1.1.3 Reconstructive urology - bladder augmentation

At present, enterocystoplasty is the most favoured method for augmentation, while other procedures, such as auto-augmentation of the detrusor muscle, are less common. Stomach and colon can be used for bladder enlargement, but the preferred organ is the ileum (Jednak et al. 2014).

The presence of intestinal mucosa is associated with significant complications as increased infection rate due to mucus production, stone formation, absorption of electrolytes from urine and the long-term risk of adenocarcinoma (Kropp et al. 2007). Bladder augmentation with de-mucosalised (mucosectomised) intestine or stomach has been under research since the early 1950s.

The results of the experiments had been suggested that the shrinkage of the flaps is possibly multifactorial (Salle et al. 2000). Researchers are now attempting to eliminate the chemical irritation caused by urine on the mucosectomised raw surface of the flaps using urothelium, but despite the use of postoperative splints, the shrinkage of flaps remains a significant problem (Turner et al. 2011, Zhang et al. 2012, Hidas et al. 2015).

1.2 Mucosectomy and intramural circulation of the intestine

1.2.1 Anatomy of the intramural blood supply of small intestine and colon

To understand the consequences and boundaries of the mucosectomy / ESD the knowledge about the intramural circulation of the intestine is essential. The intramural vessels

of the intestine are organised in subserosal, submucosal intermuscular and mucosal interconnected plexuses (Kachlik et al. 2010).

The subserosal vascular plexus: Structure of intramural circulation commences with vasa recta (VR), which are branched from *arteria marginalis* or arcades and they run toward to the serosa at straight (Kachlik et al. 2010). The VR feed external muscle layer (Kachlik et al. 2010).

The intermuscular plexus: recurrent muscular branches of the VR turn back from the submucosal plexus to the muscle layers and they compose the intermuscular plexus. Recurrent branches serve as the main arterial source, however subserosal plexus also send off branches, but their role is insignificant in human (Kachlik et al. 2010).

Since the submucosal plexus has such a crucial role in arterial supplementation of muscle wall it is likely that destruction of this layer (removal of submucosa or ESD) may result in significant ischemia of the muscularis propria. Whether the direct branches from the subserosal plexus already labelled "insignificant" could replace them remains a question. No study measured the effect of the mucosectomy (ESD) on the microcirculation of the remaining intestine.

The submucosal plexus: The long VR penetrate the muscle layer and create the submucous plexus in the loose connective tissue. Mucosal plexus consists of branches from submucosal plexus (Kachlik et al. 2010). Gallavan et al. (1980) observed the main role of submucosal plexus *in vivo* dog models, where the arterial pO_2, pCO_2, and pH were measured during prandial phase (Gallavan et al. 1980). The results indicated that the mucosal layer via this plexus obtains approx. 75% of the whole intramural blood flow, while muscle layer receives only 25% (Gallavan et al. 1980, Granger et al. 2015).

The mucosal plexus: The formation of mucosal arterioles shows difference between the colon and small intestine, this diversity originated from their physiological function.

1.2.3 The correlation between ischemia and fibrosis, stricture in the GI system

There is colloquial evidence for close relationship between ischemia, fibrosis and contraction (Cheng et al. 1994, Shavell et al. 2009, Strowitzki et al. 2019, Lim et al. 2015). An example for systemic hypoxia induced intestinal stricture is necrotising enterocolitis (NEC) in premature neonates, where the link between NEC and intestinal stricture is well established (Phad et al. 2014). Almost 30 % of NEC patients develop intestinal stricture in the long run (MSD Manual Professional 2020). Posttraumatic ischemic stenosis of the small bowel is rare condition, but a good example for local ischemia. After blunt trauma mesenteric defect causes ischemia of the corresponding bowel and result in stenotic, fibrotised and strictured bowel segment (Lee-Elliott et al. 2002, Lien et al. 1984, Bryner et al. 1980). Another proof for local

ischemia is when the superior rectal artery is divided below Sudeck's point during sigmoidectomy, this has been reported to cause ischemic stricture (Yamazaki et al. 1997).

1.3 In-vivo imaging of tissue microcirculation

There are several techniques available for study tissue microcirculation in-vivo in real time. Intact microvascular flow is recognized as a primary factor in the healing of GI anastomoses; there is therefore a high demand for reliable, easy-to-use intra- or postoperative diagnosis. Not surprisingly, a wide range of clinically available methods have been developed and tested with variable success.

Orthogonal polarisation spectral (OPS) imaging was used by the first generation of handheld microscopes, which were able to directly visualise human microcirculation without contrast materials (Groner et al. 1999). It is still appropriate for intraoperative measurement, but image stabilisation is difficult, because movments may distort the results (Jansen et al. 2018). Sidestream dark field imaging (SDF) illuminates the tissue with green LED light with higher resolution, but it still requires direct contact with the tissue (Bertschy et al. 2000). Incident-dark field illumination (IDF) was the next generation of handheld devices; it was completely digital and showed a significantly higher rate of total vessel density (van Elteren et al. 2015). Moreover, it has a shortened pulse time, a digital stabiliser and reduced weight. In brief, OPS, SDF and IDF can all measure the important parameters of microvascular flow, including total vessel density, the proportion of perfused vessels and the flow heterogeneity index, as recommended by a roundtable consensus meeting on this subject (De Backer et al. 2007).

In Study II Cytocam Braedius was applied, which is the successor of the OPS and based on IDF technology. This device is able to describe tissue microcirculation with quantitative parameters, however the original software of Cytocam could not calculate red blood cell velocity (RBCV), therefore we had to develop a new software for this purpose. The CapScan is an image analysing software, which is capable of exportation of analysing metrics of capillary. In the viewer, it is able to stop, or analyse the video per frame. In the software image stabilizer algorithm is integrated.

Usage of Cytocam is relatively straightforward, but direct connection with tissue sample is required, moreover the hand oscillation and compression generated artefacts decrease the efficiency of analysing. To solve this issue special device holder was used. Another

disadvantage of this instrument that there is no laparoscopic version, thus clinical application during minimally invasive surgery is not resolved.

1.4 Mucosectomy and the ENS

1.4.1 The ENS

An intact ENS is essential to maintain the intestinal integrity and function. ENS is an organised mesh of ganglionated plexuses. The two main parts of ENS are the myenteric and submucosal plexuses (Furness et al. 2012). The motoneurons primarily located in the myenteric plexus innervates muscularis propria thereby it is responsible for peristaltic movement. The fine balance of the excitatory and inhibitory neural circuits responsible for normal muscle tone and motility does not exists if the ENS is not fully developed and intact are missing. Chronic higher anorectal pressure is associated with increased AchE-positivity is a diagnostic histological feature of HD (Furness et al. 2012).

Submucosal plexus mostly has sensory function, however there is a significant structural difference in the ENS of small rodents and large mammals. While in motor neurons of small rodent are located only in the myenteric plexus, in larger mammals the submucosal plexus also contains nitrergic inhibitory motor neurons in inner submucosal plexus, which are responsible for muscle relaxation (Furness et al. 2012, Wiley 2007). This may explain why researchers did not see flap contraction after mucosectomy in rodents with sero-muscular bladder augmentation while others observed this in large animals like dogs (Oesch et al. 1988, Salle et al. 2000, Salle et al. 1997, Cheng et al. 1994).

Since the ENS is an interconnected structure and its integrity is important to maintain muscle tone and in large mammals submucosal plexus clearly contains motoneurons it is highly likely that mucosectomy, ESD or removal of submucosal layer will destroy these structures, however this has not been demonstrated.

2. MAIN GOALS

Our goal was to study and understand the effect of surgical mucosectomy on the intestine to explain the background and origin of the major complication, stricture and contraction seen with the mucosectomised flaps.

1. We aimed to assess the effect of surgical mucosectomy on the microcirculation of the colon and the ileum in the same experimental setting and monitor intramural microcirculation for possible recovery beyond the known warm ischemia time.

2. We aimed to evaluate the effect of mucosectomy on the dimension of the intestinal flaps and assess the changes in the anatomy of the ENS.

3. MATERIAL AND METHODS

3.1 Experimental animals and anaesthesia in Study I and II.

13 female Vietnamese minipigs (weighing 45±8 kg) were used (n=5 in Study I, n=8 in Study II). The animal experiments were performed according to EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and carried out in strict adherence to the NIH guidelines for the use of experimental animals. The experiments were approved by the National Scientific Ethical Committee on Animal Experimentation, with the license number V/1637/2013 and V/148/2013.

The animals were kept under conventional circumstances, in standard cages. They were fed with commercially available mixed food, fasted 24 hrs before surgery and always had free access to water. Anaesthesia was induced with an intramuscular injection of a mixture of ketamine (20 mg/kg) and xylazine (2 mg/kg) and maintained with a continuous infusion of propofol (2%; 50 µl/kg/min i.v.), via a cannulated ear vein. Endotracheal tube was inserted, and the animals were ventilated mechanically, with a volume-controlled ventilator. The tidal volume was set at 8-9 ml/kg, and the respiratory rate was adjusted to maintain the end-tidal carbon dioxide pressure (EtCO2) to 35-45 mmHg. Norocarp S (carprofen; 4 mg/kg) and normal saline infusion were administered via the ear vein catheter. Heart rate, O_2 saturation (pulse oxymetry), $EtCO_2$ (capnometry) and body temperature were continuously monitored perioperatively.

3.2 Protocol in Study I

3.2.1 Surgical procedure

First, the ileal and colonic segments were isolated and detubularised along the anti-mesenteric line. The bowel was kept warm with 0.9% saline solution. The detubularised bowel strips were placed on wet gauze. The mesenteric pedicle was not clamped. The width and the microcirculation were recorded on the serosal and mucosal surfaces. Mucosectomy was then performed. In the sero-musculo-submucosal group (Group A), only the mucosa was scraped off with the back of a forceps from the bowel at the level of the mucosal lamina propria. Surgical loops with 2.5 X magnification were used to make sure that no mucosal islands are left behind. In the seromuscular group (Group B), the mucosa and the submucosa were peeled off the seromuscular layer, as one layer and in one piece. Injection of saline into the submucosa was

not necessary. The microcirculation was rerecorded with IDF side stream videomicroscopy (Cytoscan Braedius, The Netherlands), in each group, after the mucosectomy procedure, on the serosal and the raw surface, as well, at 0, 5, 15, 30, 60, 90, 120, 180 minutes.

3.2.2 Quantifying microcirculation

Microcirculatory videos were performed with IDF-imaging device (Cytocam, Braedius medical, Huizen, Netherlands), in accordance with international standard recommendations (De Backer et al. 2007). The camera is fully digital and contains a high-resolution sensor, with a pixel size of 1.4 micron. Video files were saved directly to the Braedius CytoCam HDD in AVI format. Optical magnification of 4x was used, to provide a 1.55 x 1.16 mm field of view.

The Microvascular flow index is a semi-quantitative and summarized score, which is the mean value of four quadrant measurements. Categorical values are given based on predominant flow (no flow = 0, intermittent = 1, sluggish = 2, continuous = 3). Red blood cell velocity (RBCV) being a quantitative marker, which was measured using the CapScan software, for this purpose, programmed by our team. RBCV is presented in micrometer/second (μm/s).

3.3 Protocol in Study II

3.3.1 Surgical procedure

In each animal, 3 five-cm-long ileum and colonic segments were isolated and detubularized along the mesenteric line, one of those three segments represented the control group, and one represented each of the experimental groups (Group I and II). Similarly, three 5x5 cm large segments were created in each animal from the stomach at the greater curvature preserving the right gastroepiploic artery. The small intestine was kept warm with 0.9% saline solution. The detubularized bowel and stomach flaps were placed on wet gauze and the width was measured with linear ruler under no tension.

The mucosectomy was performed in 2 groups (a segment from each animal was allocated to each group). In Group I (sero-musculo-submucosal flap group), only the mucosa was scraped off with the back of the forceps from all segments at the level of lamina propria mucosae. In Group II (sero-muscular flap group), the submucosa was separated from the sero-muscular layer at the edge of the flap with fine forceps and peeled away from the sero-muscular layer in one piece. The mucosectomised flaps of both groups than were placed on wet gauze and measured again with a linear ruler with no tension.

3.3.2 Histological procedures

3.3.2.1 Haematoxylin and eosin (HE) and immunohistochemistry staining

In Group I. histology samples were taken from the sero-musculo-submucosal flaps. In Group II. the specimens were taken from the sero-muscular layer and the mucosa-submucosa layer which was peeled away. After fixation in 4% formalin, the specimens were embedded in paraffin and staining was done with conventional HE and neurofilament (NF) immunohistochemistry. For standard NADPH-diaphorase (NADPH-d) and acetylcholine esterase (AchE) enzyme histochemistry, 1x1 cm specimens were excised from the mucosa-submucosal layer, which was pealed way from the sero-muscular layer. The whole mount preparations were incubated, rinsed and mounted according to the official institutional instructions. The slides were examined with an Olympus BX 63 light microscope. The animals were sacrificed after the procedure. The specimens were compared and contrasted to each other.

3.4 Statistical analysis

3.4.1 Study I

Data analysis was performed with a statistical software package (Sigmaplot 13.0.0/2017 for Windows by Systat Software Inc., Jandel Scientific, Erkrath, Germany, 2017). Normality of data distribution was analysed with the Shapiro–Wilk test. The Friedman ANOVA on ranks was applied within groups. Time-dependent differences from the baseline for each group were assessed with Dunn's method. Differences of extra- and intraluminal side within the groups (Group A-I, Group B-I, Group A-C and Group B-C) were analysed with the Mann-Whitney test. Median values and 75th and 25th percentiles are provided in the figures; $p<0.05$ were considered significant.

3.4.2 Study II

Data analysis was performed with a statistical software (Sigmaplot 13.0.0/2017 for Windows by Systat Software Inc.). To find the differences between the sero-musculo-submucosal, the sero-muscular and the control group, we used Saphiro-Wilk normality test, one and two-way ANOVA. P values < 0.05 was considered significant.

4. RESULTS

4.1 Study I

Both the MFI and RBCV showed an abrupt reduction of microcirculation on both surfaces of the remaining intestinal flap of the ileum and the colon. Slightly better values were seen on the colon (Group A-C), but even these values remain far below the preoperative (control) results. The values on the raw surface of the bowel flaps was slightly higher than that measured on the serosal surface. Some recovery of the microcirculation was noted after 60-90 minutes, but this remained significantly lower than the preoperative control values.

4.2 Study II

There was no significant difference in the original size of the segments from the stomach, ileum and colon, measured across the different animals (n=5). The stomach contracted to a lesser extent of its original width, 92.82 ± 7.86% in Group (I) and 82.24 ± 6.96% in Group (II). The ileum contracted to 81.68 ± 4.25% in Group (I) and to 72.675 ± 5.36% in Group (II). The flap shrinkage was most significant in the colon, down to 83.89 ± 15.73% in Group (I) and to 57.13 ± 11.51% in Group (II). One-way ANOVA test showed a significant difference ($p < 0.05$) for each segment between Group (I) and (II), comparing the stomach to the ileum and the ileum to the colon.

4.2.1 HE staining

The same features were observed consistently in all flaps. In Group (I), small mucosal remnants were observed on the top of the muscularis mucosae. In Group (II), the submucosal layer was completely missing.

4.2.2 Neurofilament immunohistochemistry

In Group (I), all plexuses, the superficial and deep submucosal and the myenteric plexuses were intact in all flaps. In Group (II), irrespective of whether it was ileum, colon or stomach, only the myenteric plexus could be observed intact. In any individual slide, only 1-2 submucosal ganglia were occasionally found loosely attached to the sero-muscular flap and the vast majority of the deep and superficial ganglia were missing. These ganglia could be observed in the removed submucosa-mucosal layer.

4.2.3 Whole-mount preparation and AchE and NADPH-d histochemistry

The full plexus of submucosal ganglia was found on the surface of the submucosal layer that was previously facing the muscle layers. Cholinergic neurons stained brown while nitrergic neurons stained blue in the submucosal ganglia.

5. DISCUSSION

5.1 Experimental Study I

In a previous study we had demonstrated that mucosectomy results in an abrupt gross reduction of the capillary blood flow in the ileum (Cerveillone et al 2016). The colon was not studied, and the length of the ischemic period was not recorded. However, every organ tolerates warm ischemia for a certain time period. The warm ischemia time of ileum was found to be up to 45 minutes in rats (Illyés et al. 1992), and 2 h in dogs (Robinson et al. 1974). Light microscopy showed loss of crypt epithel, congestion and bleeding in lamina propria, neutrophil infiltration, wavy myocytes and focal necrosis in both muscular layers after 3 hours of ischemia and 8 h of reperfusion. Three hours of total ischemia of the small bowel followed by reperfusion was considered to be the upper limit for viability in porcine mesenteric ischemia model (Robinson et al. 1974). In humans the warm ischemia cut off time of the ileum for microvascular transplantation is considered between 1-2 hrs (Chen et al. 2013).

In the present study we demonstrated that the microcirculation does not recover within the warm ischemia time of the ileum and colon after mucosectomy. Only minimal residual circulation was detected after mucosectomy. There was a degree of recovery in the RBCV, however both remained far below the control (preoperative) values. The partial re-establishment of circulation was seen after 60-90 minutes, sufficient to prevent acute necrosis. This is in accordance with our earlier observation where the flaps used for augmentation remained viable, even though they contracted (Cervellione et al. 2017). When the muscularis propria and the muscularis mucosae are damaged, peristalsis and the movement of the villi will be lost. Regenerated scar tissue might not uphold sufficient peristalsis, and may lead to later stricture (Horgan et al. 1992).

With the advancement of ESD, it has become clear that extensive removal of mucosa-submucosa (above 75% of the circumference) results in stricture in esophagus, stomach or colorectum) (Park et al. 2020, Ohara et al. 2016). In our experiment the removal of mucosa/submucosa corresponds to this situation, the stricture seen after excessive ESD could be in accordance with our finding. In this sense, our study first provides a possible link between mucosectomy and ischemia and explains stricture after excessive ESD-induced mucosectomy.

Our results provide the first clear evidence that surgical mucosectomy in both ileum and colon induces ischemia beyond the warm ischemia time. This will inevitably result in flap fibrosis and contraction. Vascularisation is key to the long-term success of all bladder augmentations and must be the focus if we are to achieve mucus free composite flaps. We did

not observe any significant difference between the 2 mucosectomy processes (Group A and B). One would expect better results in Group A, with sero-musculo-submucosal flaps, where the submucosus vascular plexus is not destroyed. This is however not the case according to our findings. The microcirculation of the sero-mucular-submucosal flaps also dropped significantly and only partially recovered showing no significantly better values than the seromuscular flaps. This is in accordance with our experience in the earlier study where we found sero-mucular-submucosal flaps contracted after augmentation (Cervellione et al. 2017, Urbán et al. 2020). Furthermore, it is known that due to mucosal regrowth sero-muscular-submucosal flaps may not be ideal to eliminate mucus from augmented bladder.

In addition, mucosectomy causes damage to the enteric nervous system which may compromise results (Urbán et al. 2020). Mucus free bladder augmentation using intestinal segment is an ideal goal but remains difficult to achieve. Surgical removal of the mucosa result in severe prolonged ischemia as well as damage to the enteric nervous system both in the ileum and the colon with resultant fibrosis and contraction of these flaps.

5.2 Experimental Study II

Augmentation with de-mucosalised intestinal segment is still not fully reliable and did not gain widespread popularity. The main concern is the mechanism of flap shrinkage, which is still poorly understood. This study provided evidence that mucosectomy has a significant immediate effect on the dimensions of the GI segments, irrespective of whether it is a stomach, ileum or colon. Our results also offer an explanation to why shrinkage of the mucosectomised flaps is unavoidable in large animals, while in previous studies on smaller mammals in which motor neurons are only located in the myenteric plexus, this phenomenon has not been observed.

During ENS development, the neuroblasts, which migrate from the myenteric plexus act as a bridge of signals between the musculosa and the mucosa of the gut (Puri et al. 2008). Submucosal neurons connect to each other, to the mucosa and to the myenteric plexus, so that disruption of this fine network at any level detrimentally affects the physiology of motility and vascularity (Román et al. 2004, Brandt et al. 1996, Foxx-Orenstein et al. 1996).

This understanding is in keeping with our observations that flap shrinkage occurred in both of our studied groups (I) and (II), albeit to a lesser degree when only the mucosa was scrapped in group (I), whilst it occurred more significantly when the submucosal plexus was fully disrupted in group (II). The flap shrinkage that we recorded was an instant phenomenon

after mucosectomy. This can be explained with the disrupted nerve fibres connecting submucous and myenteric ganglia. In that situation, the inhibitory motoneurons located in the submucosa cannot suppress the myenteric cholinergic motoneurons, which being un-inhibited would induce an increased muscle tone and contraction of the muscle fibres.

The fine balance of the excitatory and inhibitory neural circuits responsible for normal muscle tone and motility does not exists if the ENS is not fully developed and intact. Similarly, decreased peptidergic and nitrergic innervation (inhibitory motoneurons) has been hypothesised as an aetiology for intrinsic pelviureteric junction obstruction due to failure to relax to stimuli (urine bolus), and it is at least suspected as an aetiology of hypertrophic pyloric stenosis (Vanderwinden et al. 1992, Knerr et al. 2001, Wang et al. 1995).

Zhang et al (2011) found that botulinum toxin infiltration of the mucosectomised gastric flaps is able to prevent flap contraction (shrinkage). A clear explanation as to how and why did this happen was not given by the authors, but this clearly supports our hypothesis. It is plausible that non-specific paralysis of the imbalanced nerve circuits of the stomach after mucosectomy resulted in muscle relaxation i.e. prevented the shrinkage of the flaps. Other drugs, like local anaesthetics, nitric oxide (NO) gas, NO synthase (nNOS) and botulinum toxin can prevent contractions, however we did not pursue testing this because the accurate administration and dosage of these drugs would have complicated the study as confounding factors and most likely would have had a temporary effect of variable and unknown duration.

There is a significant difference between the different organs in the extent of flap shrinkage. It affected the stomach lesser and the colon greater. We can only speculate that this is affected by the anatomy and spatial pattern of the muscle layers of these organs. The more developed the individual layers (circular, oblique and longitudinal), the more likely they can oppose sliding and hold-on to one-another thus oppose contraction and shrinkage.

the flap shrinkage was significantly more expressed in Group (II) versus Group (I) due to the proven more disruption of the ENS in Group (II). However, it is highly likely the bowel segment will still behave like one with HD i.e. will not be able to relax, therefore we can expect less compliance once augmentation is done with mucosectomy. Our main message is that; this should be considered when planning an augmentation using a de-mucosalised flap. It is noteworthy that, augmentation with sero-musculo-submucosal intestinal flaps does not result in a totally mucus-free bladder, as the mucosa will regrow (Dewan et al. 1997, Jednak et al. 2000, Fraser et al. 2004). Despite that we were very meticulous and used surgical loops with

2.5x magnification during the mucosectomy, we have seen mucosal island on the flaps after HE staining.

In our previous study, we found that significant disruption of the intrinsic blood supply and microcirculation of the flaps may result in long term ischemia and fibrosis (Cerveillone et al. 2017). By highlighting this, together with our current observation of damaged ENS and acute shrinkage, we aim to contribute to the discussion pertinent to microvascular and neuronal aspects of experimental efforts to remove or replace the mucosal coverage of the gastro-intestinal flaps used for augmentation.

The limitations of our study include the fact that we did not monitor the shrinkage in the long term, however we did not observe an improvement during the 2-hour duration of the experiment in each pig. In our previous animal study, where microcirculation of the mucosectomised ileal flaps was assessed before and after mucosectomy and the flaps were used for augmentation in a reverse fashion, we were able to demonstrate the long-term shrinkage of both types of these flaps (Cerveillone et al 2017).

6. SUMMARY OF THE NEW RESULTS AND CONCLUSIONS

1. Surgical mucosectomy results in an immediate and severe cessation of the microcirculation within the intestinal wall and the microvascular blood flow is not recovering within the warm ischemia time. No significant difference was found between the ileum and colon in this respect. This finding may offer explanation for the stricture seen after mucosectomy.
2. Surgical mucosectomy disrupts the ENS of the intestine resulting in immediate shrinkage. No significant difference was found between the stomach, the ileum and the colon, the damaged reflex circuits of the intestine may provide another explanation for shrinkage seen after mucosectomy.

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