SMALL INTESTINE

The effect of faecal diversion on human ileum

Leith Williams, Matthew Armstrong, Paul Finan, Peter Sagar, Dermot Burke

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See end of article for
authors' affiliations

Correspondence to:
D Burke, Department of
Colorectal Surgery,
Clarendon Wing, Leeds
General Infirmary, Leeds LS1
3EX, UK;
d.burke@leeds.ac.uk

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Background: The use of a loop ileostomy is an effective method in protecting pelvic anastomoses. Its use has increased recently, although there is some debate as to the routine use of a stoma. Reversal of the ileostomy is associated with a significant morbidity, which may be related to impaired function of the bypassed distal limb of the ileum.

Aim: To investigate the changes that might occur in the distal limb after an interval of faecal diversion.

Methods: Full-thickness intestinal circular muscle (CM) strips were prepared from excised loop ileostomies taken at the time of closure. The study sample was from the distal limb and the control from the proximal limb. Contractile activity was measured using an organ bath set up to record isometric contraction after stimulation by acetylcholine (ACh). Histological sections were assessed for an index of villous atrophy, smooth muscle area, and nerve and vessel density. Analysis was with the Wilcoxon signed ranks test for paired data and the Mann-Whitney U test for unpaired data.

Results: Samples were acquired prospectively from 35 consecutive patients. The median time between formation and closure of ileostomy was 34 weeks. Significant reduction was observed in the strength of CM contraction, smooth muscle area and median villous index of the distal limb compared with the proximal limb.

Conclusion: Impaired intestinal function has been proposed as a contributory factor in the morbidity that may follow closure of loop ileostomy. Significant loss of contractility and smooth muscle strength and villous atrophy occur in the distal ileal limb after faecal diversion. Methods of preventing these changes should be considered.

A loop ileostomy is an effective method used for diverting the faecal stream to reduce the incidence, and protect against the sequelae, of sepsis in the event of an anastomotic leak. It is most commonly formed after restorative proctocolectomy for ulcerative colitis or low anterior resection for carcinoma of the rectum. Although the incidence of leakage is not reduced, the risk of re-operation and postoperative death is reduced if a stoma is raised. Although the need for surgery in these conditions is apparent, the requirement for faecal diversion continues to be a matter of debate because of the complications associated with the ileostomy.

The use of loop ileostomy has increased with the greater frequency and complexity of procedures performed and with the use of mechanical anastomotic devices.¹ This has led to a decline in the incidence of pelvic sepsis and anastomotic disruption after ileal pouch anal anastomosis, and has been recommended as a wise precaution.^{2–3} It is inconvenient, however, for the patient to have an ileostomy and a second operation for closure is necessary, with a morbidity of about 25%.^{4–5} Intestinal obstruction is the most common complication after loop ileostomy closure, with an incidence of up to 29%, and anastomotic leak with pelvic sepsis the second most common occurring in up to 19% of the patients.^{6–11} Patients with an ileal pouch often experience an increased frequency of passage of stool and a reduction in anal sphincter pressure, particularly in the first 3 months after closure of ileostomy.^{12–13}

Small intestinal motility and absorption are closely related to the structure of its mucosa and muscle, and structural changes may occur after ileostomy formation, which could contribute to the morbidity seen after closure. The distal limb may appear atrophied at the time of closure, and animal studies have documented atrophic changes within the defunctioned intestine.^{14–16} The pathophysiology of bypassed human ileum has, however, been rarely studied and structural changes that may occur in the bypassed distal limb of the ileum could influence these complications. A reduction in absorptive function and

motility of the bypassed limb has been demonstrated in patients with a diversion ileostomy.¹⁷ Motility may take up to 6 months to return to normal after closure of the ileostomy.¹⁸ Attempts to improve the absorptive function of the distal limb before ileostomy closure have met with limited success.^{19–21} A reduction in villous height and villous index (which are estimates of the absorptive surface area) has been shown in the bypassed ileum of rats, with no muscle atrophy after 10 weeks.¹⁴ A relative “functional” obstruction of the distal limb may occur following closure of an ileostomy. This has been proposed as a contributory factor in enterocutaneous fistula formation and could predispose to anastomotic leak.²²

The aim of this study was to investigate the changes that occur in the distal limb of the human ileum after an interval of faecal diversion.

PATIENTS AND METHODS

Patients scheduled to undergo closure of loop ileostomy were recruited into the study and gave fully informed written consent. Ethical approval was obtained from the local research ethics committee (Ref CA00/089). Full-thickness strips of the small intestine were obtained from the proximal and distal limbs of the excised ileostomy at the time of closure.

Isometric contractility

Initial work revealed that the optimum conditions for maximal contraction were a pre-tension of 2 g and a concentration of 5 mm of acetylcholine (ACh), with the tissue bathed in physiological saline (Tyrode solution) at 37°C.²³ Specimens were acquired fresh from the operating theatre and two full-thickness strips (10 mm long and 5 mm wide) were obtained

Abbreviations: ACh, acetylcholine; CD, crypt depth; CM, circular muscle; DAB, 3,3-diaminobenzidine; IQR, interquartile range; LM, longitudinal muscle; RT, resting tension; ST, stimulated tension; TMA, total muscle area; TMT, total mucosal thickness; VH, villous height; VI, index of villous atrophy

Figure 1 Screen shots of image analysis. Mucosa (A) ($\times 2.5$ objective): total mucosal thickness (TMT) and crypt depth (CD) are calculated by the computer from the green and blue lines, respectively. Muscle area (B) ($\times 2.5$ objective): circular muscle (CM) was highlighted in green different from the longitudinal muscle (LM) in yellow, and the two cross-sectional areas were measured. Vessel (C) and nerve (D) density ($\times 10$ objective): endothelium labelled with CD31 and nerve with PGP9.5. Vessels and nerves were highlighted in green separately, and the cross-sectional area was detected and measured automatically.

oriented along the axis of the circular muscle (CM), one each from the proximal and the distal limbs. The strips were weighed before being mounted in one of two 50 ml organ baths, which were set up in parallel, connected to the same reservoir of warmed water and oxygen supply. They were filled with aerated Tyrode solution, warmed to 37°C, with one end fixed to the base and the other attached by thread to a transducer. The strips were maintained under a resting pre-tension of 2 g and allowed to settle for 15 min, until they reached a steady state. The muscle strips were stimulated by instillation of ACh at a concentration of 5 mm into the organ bath. Mechanical activity of the muscle was recorded in analogue by an isometric transducer (Lectromed UF1, Jersey, UK) connected to a twin channel recorder (Lectromed Multi-trace 2, Jersey, UK). The stimulated tension (ST) and duration of contraction (T) as well as the basal resting tension (RT) generated were recorded. The proportion of CM with respect to the total specimen was calculated by image analysis, to provide a more accurate estimate of the contribution of CM alone to contraction. The values for ST and RT were then recalculated as a proportion of CM weight.

Histology

Excised ileostomy specimens were pinned onto corkboard to avoid distortion and fixed in 10% formalin solution.²⁴ Initial validation studies were performed to establish that sections were representative within the sample and within the paraffin wax block section. Samples for histological sections of approximately 10 mm length and 5 mm width were placed into cassettes, with the cross-sectional surface uppermost. The

cassetted samples were dehydrated through alcohol and xylene to paraffin wax using a standard 16-hour automated system (2LE Processor, Shandon Scientific, Cheshire, UK). They were then embedded in liquid paraffin wax (Tissue Tek III, Bayer Diagnostics, Basingstoke, UK) and cooled as solid blocks. Histological sections of 5 µm thickness were taken and H&E sections prepared.²⁴

Immunohistochemistry was used to identify blood vessels and neurones. The primary antibodies CD31 (monoclonal mouse anti-human (blood-vessel endothelium) clone JC70A M0823, Dako, Cambridge, UK) and PGP9.5 (monoclonal mouse anti-human (neuronal cell bodies and axons) clone 13C4 7863-0504 Biogenesis, Poole, UK) were chosen. The Sequenza immunostaining system (Shandon, Runcorn, UK) was used to ensure consistent immunolabelling and to prevent the section from dehydrating. Mounted sections were dewaxed in microwaved xylene and hydrated through xylene and graded alcohol to water. Endogenous peroxidase activity was blocked by treatment with 0.5% hydrogen peroxide in methanol and the slides washed with distilled water. Antigen retrieval, to expose antibody-binding sites masked during fixation, was carried out by heating in a pressure cooker at 103 kPa for 2 min. Non-specific antibody binding was blocked by incubation with 100 µl of 1:10 casein solution (Vector Laboratories, Burlingame, California, USA). Slides were subsequently incubated at room temperature with 100 µl of primary antibody (CD31 at 1:30 and PGP9.5 at 1:1000) diluted in 1:50 casein solution for 60 min. A two-stage EnVisionTM peroxidase technique (Dako) was used for secondary labelling.²⁵ Development was by modification of a double-staining technique, which involved sequential

Table 1 Summary of isometric contraction results calculated relative to weight of tissue

	Proximal (P) n = 26	Distal (D) n = 26	p Value	D:P ratio
Stimulated tension (mg/g)	3759 (1099–6813)	917 (403–2016)	<0.001	0.33 (0.20–0.51)
Duration of contraction (s)	99 (72–129)	66 (41–90)	<0.001	0.71 (0.47–0.85)
Resting tension (mg/g)	950 (428–1409)	278 (204–624)	<0.001	0.36 (0.17–0.59)

All values are expressed as median (interquartile range).

Table 2 Summary of isometric contraction results calculated relative to estimated weight of circular muscle

	Proximal (P) n = 15	Distal (D) n = 12	p Value	D:P ratio
Stimulated tension (mg/g of CM)	16933 (6040–33401)	5645 (2012–11054)	0.021	0.47 (0.11–0.60)
Resting tension (mg/g of CM)	6528 (1964–8213)	1579 (881–2353)	0.021	0.23 (0.22–0.31)

CM, circular muscle.

All values are expressed as median (interquartile range).

application of each primary antibody.²⁶ The 3,3-diaminobenzidine (DAB) chromogen was used for CD31 and the Vector SG chromogen for PGP9.5. Following application of the CD31 primary antibody, sections were incubated with two drops of EnVision polymer for 30 min and visualised with DAB. Casein solution was applied for a second time to further block non-specific antibody binding, before incubation with PGP9.5. EnVision polymer was applied in an identical manner, but visualised with the Vector SG substrate kit. Positive controls consisted of normal human appendix tissue and negative controls consisted of substitution of the antibodies with their dilutant in the following manner: (a) both CD31 and PGP9.5 primary antibodies; (b) CD31 alone; and (c) PGP9.5 alone. In all cases, there was a high level of specificity, with brown DAB specific to vessels, and blue Vector SG specific to nerves.

Image analysis

All microscopic sections were assigned computer-generated random number codes before analysis. Interpretation of each slide was thereby blinded as to its origin, whether distal or proximal. Digital images were captured onto a computer monitor using a Leica Q500IW image analysis system (Leica Imaging Systems, Cambridge, UK) and a dedicated software program for image processing (QWin Standard V 2.0, Leica Imaging Systems). Digital images were captured from H&E-stained sections with a $\times 2.5$ objective and from immunolabelled sections with a $\times 10$ objective. The analysis reference frames used were 3.3 mm^2 ($2224 \mu\text{m} \times 1485 \mu\text{m}$) for mucosa and muscle, $205\,995 \mu\text{m}^2$ ($558 \mu\text{m} \times 371 \mu\text{m}$) for vessels and $132\,311 \mu\text{m}^2$ ($534 \mu\text{m} \times 247 \mu\text{m}$) for nerves. Linear measurements of total mucosal thickness (TMT) and crypt depth (CD) were only performed in well-oriented biopsy specimens with a complete muscularis mucosae and no distortion of the villi. These were taken as the vertical distance from the muscularis mucosae; for TMT to the tip of the villus, and for CD to the dilation of the crypt at the base of the villus (fig 1).²⁷ The CM and longitudinal muscle (LM) were measured as cross-sectional areas relative to the reference frame area, which provided the most accurate estimate of muscle bulk in the sample. Each muscle layer was defined by freehand drawing and the area measured by a computer (fig 1). The reference frame was moved sequentially along the length of the histological section until it had all been sampled completely. The submucosal layer was assessed for vessel density as it contains the greatest proportion of blood vessels supplying the mucosa.²⁸ Nerve density was assessed in the myenteric plexus to determine the extent of the nerve supply to the muscularis propria. Vessels and nerves were highlighted in green separately and the cross-sectional area detected and measured automatically.

Statistical analysis

A power calculation was performed to determine the sample size for mucosal histology measurements. The first 10 full-thickness biopsy specimens (5 proximal and 5 distal) were assessed for a morphometric index of villous atrophy (VI). This analysis demonstrated approximately 20% atrophy in the distal

limb (0.300) compared with the proximal limb. (0.375). Therefore, to detect a 20% difference with 80% power, at a significance level of 0.05, 28 patients would be required. The median TMT and CD were calculated for each sample and the villous height (VH) was obtained from these ($\text{VH} = \text{TMT} - \text{CD}$), as well as the index of villous atrophy (VI) ($\text{VI} = \text{VH}/\text{TMT}$).²⁹ The median areas of CM and LM were calculated for each sample and the total muscle area (TMA) was obtained from these ($\text{TMA} = \text{CM} + \text{LM}$). The area of CD31 and PGP9.5 staining were calculated for each section to give median vessel and nerve density. These values (VI, VH, CD, TMT, CM, LM, TMA and vessel and nerve density) were then compared between the paired proximal and distal samples for each subject. Statistical analysis was carried out by a software package (SPSS V.11.0) using the Wilcoxon signed ranks test for paired data and the Mann–Whitney U test for unpaired data. A p value <0.05 was taken to indicate significance.

RESULTS

Resected ileostomy specimens were obtained from 35 patients (20 men, median age 56 years, interquartile range (IQR) 42–73 years), providing 35 paired samples (proximal and distal). Indications for loop ileostomy formation were faecal diversion after anterior resection for carcinoma ($n = 17$), ileal pouch-anal anastomosis for ulcerative colitis ($n = 14$), sigmoid colectomy for diverticular abscess ($n = 3$) and resection for endometriosis ($n = 1$). The median length of time between formation and closure of ileostomy was 34 (IQR 18–53) weeks. Intestinal obstruction occurred in four patients in the early postoperative period after reversal of loop ileostomy. All these cases were settled with conservative management.

Isometric contractility

Twenty-six pairs of CM strips (proximal and distal) were used for estimation of circular smooth muscle contractility. Table 1 summarises the results. The median values for stimulated tension, duration of contraction and resting tension were all significantly ($p < 0.001$) reduced in the distal limb samples compared with the proximal limb samples. This reduction was maintained when expressed relative to the estimated weight of

Figure 2 Index of villous atrophy. ♦, median; I, interquartile range.

Figure 3 Reduction in villous height seen in distal limb mucosa (right) compared with proximal limb mucosa (left), with similar crypt depth. H&E stain, magnification $\times 10$.

CM, although the relative reduction of distal to proximal contraction for ST was not as marked (table 2).

Of the 26 pairs of samples, histological slides of 19 samples were prepared. Of those, 15 proximal and 12 distal samples were suitable for estimation of CM proportion; the remainder were excluded as there was insufficient tissue present on the slide to measure the muscle area. The CM weight was estimated from the product of total tissue weight and percentage cross-sectional area of CM. There was no clear correlation between ST or RT and the estimated circular muscle weight.

Histology

Mucosal morphometry

Of the 70 biopsy samples, 68 (34 proximal, 34 distal) were suitably oriented for mucosal morphometric analysis. The two samples excluded from measurement had numerous Peyer's patches throughout the mucosal region. There was no significant difference in the number of frames counted between proximal (median 19, IQR 13–28) and distal samples (21, IQR 15–32; $p = 0.483$). TMT and VH were both significantly reduced in the distal limb samples (TMT 426, IQR 370–528 μm , VH 127, IQR 96–165 μm) compared with the proximal limb samples (TMT 655, IQR 563–762 μm , VH 327, IQR 270–399 μm ; $p < 0.001$). CD did not differ significantly between the proximal (342, IQR 246–410 μm) and distal limbs (297, IQR 234–413 μm ; $p = 0.521$), indicating that decreased TMT was solely due to decreased VH. The villous index was significantly lower in the distal limb (0.281, IQR 0.242–0.374) than in the proximal limb (0.477, IQR 0.422–0.560 $p < 0.001$) (figs 2 and 3).

Muscle morphometry

Of the 70 biopsy samples, one distal sample had no defined LM so the corresponding proximal sample was also discarded. A total of 68 samples (34 proximal and 34 distal) were used for

muscle morphometric analysis. The median number of frames measured for proximal and distal samples was three and two, respectively. Although this was a statistically significant difference, in practice, the frame count depended entirely on the overall size of the histological section. The median CM, LM and total muscle cross-sectional areas were all significantly decreased in the distal limb compared with the proximal limb ($p < 0.001$): CM–distal 0.808 (IQR 0.618–0.905) mm^2 per frame, proximal 1.302 (IQR 1.095–1.475) mm^2 per frame; LM–distal 0.666 (IQR 0.524–0.794) mm^2 per frame, proximal 1.091 (IQR 0.892–1.361) mm^2 per frame; total muscle–distal 1.471 (IQR 1.224–1.701) mm^2 per frame, proximal 2.526 (IQR 2.076–2.775) mm^2 per frame (fig 4 and 5). This reduction seemed to be uniform, as the proportion of CM and LM was 54% and 46% of the total muscle for both proximal and distal samples.

Vessel and nerve morphometry

All negative controls tissue sections were completely devoid of staining. All positive controls demonstrated intense, specific immunoreactivity. Positive CD31 and PGP9.5 immunolabelling was observed for all samples (fig 6), and all 70 sections were suitable for morphometric analysis. There was no significant difference in median vessel density within the submucosa between proximal and distal samples (5293, IQR 4475–6396 μm^2 per frame and 5045, IQR 3891–6712 μm^2 per frame, respectively; $p = 0.098$). Nor was there a significant difference in median nerve density in the myenteric plexus between proximal and distal samples (10 039, IQR 7424–14 729 μm^2 per frame and 8217, IQR 6607–11 683 μm^2 per frame, respectively; $p = 0.132$).

DISCUSSION

Our study has demonstrated a reduction in the strength and duration of stimulated circular smooth muscle contraction, with a reduced tone in the bypassed small intestine compared with the proximal. The parallel organ baths ensured that the ileal strips were maintained in identical environments and stimulated by identical concentrations of ACh. Variations in bathing solution and temperature were avoided because even slight increases in temperature, calcium and glucose concentrations can enhance contraction.³⁰ ACh-stimulated contractions were measured, as they were more reliable than spontaneous contractions, and occurred even in muscle strips that showed no spontaneous activity. The optimum pre-tension used for the study was set according to the contractile responses of proximal samples only. The measurements of the distal samples could, therefore, have been altered, as muscle that is not at its ideal resting length will not contract as efficiently. Smooth muscle does exhibit plasticity though, which means that tension can be variable at a given length, up to a certain point.³¹ Provided that the maximum length of the distal samples was not exceeded, the resting length used

Figure 4 Median smooth muscle cross-sectional area in the distal limb (right) compared with the proximal limb (left). ♦, median; I, interquartile range.

Figure 5 Reduction in both circular and longitudinal smooth muscle areas seen in the distal limb (right) compared with the proximal limb (left). H&E stain, magnification $\times 10$.

should be acceptable. There was also significant villous and smooth muscle atrophy in the defunctioned distal limb, but no difference in the vascularity or nerve density compared with the proximal intestine. Both the degree of vascularity within the submucosal layer of the ileum and the extent of nerve supply within the myenteric plexus of the ileal smooth muscle appeared unaltered by the loss of contact with the faecal stream. The mucosal and muscle atrophy seemed to occur despite these findings, although the action of vessels and nerves was not measured as part of the study.

It has been demonstrated that a well-constructed loop ileostomy has an efficiency of defunctioning that approaches 100%.³² Consequently, during the period of faecal diversion the distal ileum experiences a foreign environment, in which digested food in the form of chyme is absent from the lumen of the bowel. In comparison, the proximal ileum is exposed to a normal environment, in which chyme is moved along the intestine by peristalsis to allow digestion and subsequent absorption at the mucosal surface. Strong evidence from animal studies indicates that the villous and muscle atrophy observed in the distal limb is due in part to loss of contact with the faecal stream.^{33, 34} When luminal nutrients are excluded from the ileum, the mucosa becomes atrophic, with diminished function. This could be a direct effect of excluding luminal nutrition from the intestine, or an indirect effect as a result of decreased pancreaticobiliary secretions, which are thought to be trophic to the intestine.³⁵ A study of the rabbit ileum suggested that villous atrophy occurred mainly because one or more substances contained in the chyme were needed to maintain mucosal architecture.³⁶ These substances might be derived from foodstuff or could well be endogenous in origin (ie, enteroglucagon).

It is likely that the reduction in contractility, villous and muscle atrophy would contribute to impaired absorption and motility in the defunctioned segment. This has been demonstrated in patients who have undergone restorative proctocolectomy or

anterior resection.^{15, 16} Small-bowel obstruction, the most common complication after closure of ileostomy, could be explained, in part, by these corresponding histological and physiological findings. The incidence of small-bowel obstruction after ileostomy closure in this study was 12%, and the anastomotic leak rate 2%. The numbers were too small to draw meaningful conclusions when compared with the histological and physiological findings. A large number of patients would be required in any study designed to identify whether the atrophic changes are associated with individual postclosure complications. It is also not clear over what period of time the atrophic mucosal changes begin to occur. This was analysed in these patients, around a median of 34 weeks, but a significant difference was not found. To identify exactly when atrophic changes begin to occur after ileostomy formation, a prospective study is required. Samples of mucosa could be taken endoscopically at several time points after ileostomy formation, and if taken from further down the distal limb might exhibit a greater extent of villous atrophy. Short-chain fatty acids such as sodium acetate, sodium-propionate and sodium-butyrate act as nutritional substrates for colonic epithelial cells, and their direct mucosal contact has been shown to improve distal colitis.³⁷ Their absence may predispose to diversion colitis, either by a reduction in colonocyte metabolism or by an increase in bacteria. Epidermal growth factor has a significant effect on intestinal proliferation, specifically on increasing crypt cell proliferation and VH.³⁸ Distal limb irrigation with these compounds might conceivably improve function before closure of ileostomy.

A novel approach has been reported from Japan, where a U-shaped plastic connector was inserted into both limbs of the ileostomy.³⁹ The aims of this technique were to predict postclosure complications, using a simple reversible device and to reduce the frequency of defecation after closure. A custom-designed connector was inserted in a single patient, 12 weeks after ileostomy formation, until closure 3 months later. Stool frequency, stool consistency and pouch distension

Figure 6 Positive immunoreactivity for blood-vessel endothelium (left) and nerves (right). CD31 is labelled brown and PGP9.5 blue; magnification $\times 40$.

volume all improved after 2 months. The aims of the study were achieved, and although a single case was reported, the value of the technique seems obvious; a temporary closure can be achieved, with the ability to continue diversion without an operation. The only noted problem was mucous discharge from the ileostomy with the connector in place; however, the efficiency of the device approached 100%. Of all the attempts to perfuse the distal limb, this most closely achieves the normal physiological solution. Although a promising report, there have been no follow-up studies involving more subjects.

CONCLUSION

Impaired intestinal function has been proposed as a contributory factor in the morbidity that may follow closure of loop ileostomy. We have shown that significant loss of contractility, smooth muscle and villous atrophy occur in the distal ileal limb after faecal diversion. Methods of preventing these changes should be considered.

Authors' affiliations

Leith Williams, Matthew Armstrong, Paul Finan, Peter Sagar, Dermot Burke, Department of Colorectal Surgery, The General Infirmary at Leeds, Leeds, UK

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