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The impact of long-course chemoradiotherapy on the myenteric plexus, neuromuscular functions and responses to prokinetic drugs in the human rectum

Victor W. S. Kung[1] | John Broad[1] | Raj Makwana[1] | Alexandra Palmer[1] | Nicholas Baidoo[1,2] | Sarah Epton[1,3] | Shezan Elahi[1] | Joanne Chin-Aleong[4] | Mohamed Thaha[1,3] | Charles H. Knowles[1,3] | Gareth J. Sanger[1]

[1]Faculty of Medicine and Dentistry, Blizard Institute, Queen Mary University of London, London, UK

[2]University of Westminster, School of Life Sciences, London, UK

[3]Department of Colorectal Surgery, Barts Health NHS Trust, The Royal London Hospital, London, UK

[4]Department of Pathology, Barts Health NHS Trust, The Royal London Hospital, London, UK

Correspondence
Gareth J. Sanger.
Email: g.sanger@qmul.ac.uk

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Abstract

Background & Aims: The long-term effects of chemoradiotherapy on human rectum are poorly understood. The aims were to investigate changes in inflammatory status, myenteric neuron numbers/phenotype, neuromuscular functions and prokinetic drug efficacy.

Methods: Macroscopically normal proximal-to-mid rectum was obtained from 21 patients undergoing surgery for bowel cancer, 98 days (range: 63–350) after concurrent capecitabine and pelvic radiotherapy, and 19 patients without chemoradiotherapy. Inflammatory status was measured by H&E, CD45 staining and qPCR. Myenteric neurons were examined by immunohistochemistry. Neuromuscular functions and drug efficacy were studied using exogenous agents and electrical field stimulation (EFS) to activate intrinsic nerves.

Results: Inflammation was not detected. Numbers of myenteric ganglia/neurons were unchanged (11.7 ± 2.4 vs. 10.3 ± 2.2 neurons/mm myenteric plexus with/without chemoradiotherapy) as were the numbers of cholinergic/nitrergic neurons. EFS stimulated cholinergic and nitrergic neurons so the contractile response of the muscle was the sum of both but dominated by cholinergic (causing contraction) or less often, nitrergic activity (relaxation), followed, after termination of EFS, by neuronally mediated contraction. Inhibition of nitric oxide synthase (by L-NAME 300 μM) more clearly defined EFS-evoked contractions. The 5-HT_4 agonist prucalopride 10 μM and the cholinesterase inhibitor donepezil 1 μM, respectively increased and greatly increased the composite contractile response to EFS (measured as 'area-under-the curve') and the contractions isolated by L-NAME (respectively, by 22 ± 14% and 334 ± 87%; n = 11/8). After chemoradiotherapy, nitrergic-mediated muscle relaxations occurred more often during EFS (in 29.8 ± 6.1% preparations vs. 12.6 ± 5.1% without chemoradiotherapy, n = 21/18).

Victor W. S. Kung and John Broad are equal first authors.

With L-NAME, the ability of prucalopride to facilitate EFS-evoked contraction was lost and that of donepezil approximately halved (contractions increased by 132 ± 36%; $n = 8$).

Conclusions: Several months after chemoradiotherapy, the rectum was not inflamed and myenteric neuron numbers/phenotype unchanged. However, nitrergic activity was increased relative to cholinergic activity, and prokinetic-like drug activity was lost or greatly reduced. Thus, chemoradiotherapy causes long-term changes in neuromuscular functions and markedly reduces the efficacy of drugs for treating constipation.

KEYWORDS
adenocarcinoma, bowel, CRC, donepezil, inflammation, lower anterior resection syndrome, myenteric plexus, neoadjuvant, prucalopride

INTRODUCTION

Lower anterior resection syndrome, or bowel dysfunction following mesorectal excision, includes symptoms such as difficult evacuation, incontinence, urgency, constipation, and diarrhoea.[1] This may be caused by pelvic nerve damage, but several studies have also shown that chemoradiation is a risk factor for the development of more severe symptoms.[2] For example, more than 4 years after mesorectal excision, radiation-treated patients were more likely to experience increased bowel movements and incontinence with reduced quality-of-life compared with those treated by surgery alone.[3]

In animals, cisplatin-based chemotherapy or 5-fluorouracil can cause enteric/sensory neuropathy.[4] In human large bowel, studies with different regimens of chemoradiotherapy have generated variable outcomes, including reduced muscle contractility, increased myenteric neuronal density, muscle fibrosis,[5,6] hyper-excitability of myenteric neurons with altered nerve cell body morphology,[7] and reduced sensory processing.[8] None of the studies investigated the effects on the efficacy of drugs acting within the enteric nervous system (ENS) to alleviate diarrhoea or constipation.

There is a clear need to better understand the consequences of chemoradiotherapy on human bowel functions during recovery and in the longer-term.[1,3,9,10] Here, we investigated the effects of long-course capecitabine (metabolised to 5-fluorouracil) together with pelvic radiotherapy on rectum removed several months after completing chemoradiotherapy. We looked for inflammation, myenteric neuropathy, and changes in neuromuscular functions—often characterised in different gastrointestinal (GI) regions without prior chemoradiotherapy,[5] but rarely in human rectum. We also looked for changes in the efficacy of drugs acting via the ENS. These were the 5-HT_4 agonist prucalopride[11] (used to treat constipation, facilitating enteric cholinergic and nitrergic functions[12,13]) and donepezil, a selective inhibitor of acetylcholinesterase (facilitating acetylcholine availability), used to treat severe intestinal dysmotility such as pseudo-obstruction, alone or with a 5-HT_4 agonist,[14,15] and shown to alleviate constipation.[16] The results did not reveal enteric neuropathy but demonstrated changes in neuromuscular functions and efficacy of drugs acting via the ENS.

Key summary

Summarise the established knowledge on this subject

- Lower Anterior Resection Syndrome may be caused by pelvic nerve damage, but chemoradiation is a risk factor for development of more severe symptoms.
- Long-term effects of chemoradiotherapy on functions of the human rectum are poorly understood.
- Long-term effects of chemoradiotyerapy on efficacy of drugs to alleviate diarrhoea or constipation have not been studied.

What are the significant and/or new findings of this study?

- In rectum removed 9–26 weeks after concurrent capecitabine and pelvic radiotherapy, there was no evidence of inflammation or changes in myenteric ganglia, neuron numbers, or phenotype.
- In rectum from patients naïve to chemoradiotherapy, electrical field stimulation caused muscle contraction (cholinergic) and less often, relaxation (nitrergic). Contractions were increased by prucalopride and greatly increased by donepezil, reflecting prokinetic-like activity and use to treat constipation or severe intestinal dysmotility.
- Chemoradiotherapy resulted in a long-term increase in nitrergic activity relative to cholinergic activity, potentially reducing rectum muscle functions, and markedly reducing prokinetic drug efficacy, affecting subsequent treatment strategies.

PATIENTS AND METHODS

Patients

The study was approved by the East London ethics committee (REC 10/H0703/71). Informed written consent was obtained from all patients undergoing anterior resection for non-obstructed colorectal cancer. One group was treated by surgery ($n = 19$). Another patient received 25 fractions of 45 Gy radiation to the rectum with concurrent capecitabine (825mcg/m^2, 2 × daily) over ~5 weeks, then surgery 98 days (range: 63–350) later ($n = 21$).

Inflammation

Sections from paraffin blocks were stained with haematoxylin and eosin (H&E) and significant inflammation noted (by observation of crypt architecture, distortion and atrophy, lamina propria cellularity, basal plasmacytosis, active inflammation, granulomata, mucin depletion, crypt abscesses, cryptitis and ulceration).

Levels of mRNA were quantified for pro-inflammatory (IL6, TNFα) and anti-inflammatory (IL13) cytokines, following extraction of RNA from muscle, stored at −80°C in RNAlater (Sigma Aldrich), using TRIzol and Direct-zol RNA Miniprep Kit (Zymo Research). SYBR Green reagents (Thermo Fisher) were used for qPCR, with primers at 40 μM (Supporting Information S1).

Localisation of CD45 antibody (Hoffmann-La Roche, U.K; cat.760–4279), a pan-lymphocyte marker (including B and T cells)[17] was investigated in mucosa-free rectum unexposed (5 female, 3 male) and exposed (5 female, 5 male) to chemotherapy. For each sample, two alternate 4μm-thick sections were counterstained (haematoxylin) and digitally scanned (Ventana system). Bins (362 × 362 μm) selected at random were analysed using Image-J (https://imagej.nih.gov/ij/). Regions of interest (ROI) were Longitudinal muscle (LM), and Circular muscle (CM) adjacent to the myenteric plexus and mucosa. For each patient, 48 bins were analysed (16/each ROI) across multiple sections (total area 6.3 mm^2). For each ROI, CD45-positive cells (haematoxylin-stained nuclei) were counted independently by two observers 'blinded' to patient identity using FIJI software.[18]

Myenteric neuronal quantification

Full thickness tissues, ~1 × 1 cm, were fixed and embedded in paraffin blocks before cutting 4 μm transverse sections.[19] Sections were stained with anti-human neuronal protein C/D (anti-HuC/D; Life technologies 1600899), anti-choline acetyltransferase (anti-ChAT; Millipore AB144P) and anti-neuronal nitric oxide synthase (anti-nNOS; Abcam ab72428). In blocks from each patient, multiple sections were used, at least 12 μm apart, so at least 10 mm myenteric plexus was assessed for each stain (usually more). Following antigen retrieval and visualisation (depending on antibody, using DAKO EnVision™ FLEX + or Vectastain Universal elite visualisation, with Dako Autostainer Instruments), cell nuclei were counter-stained with haematoxylin. Stained slides were scanned and digitally visualised for neuronal quantification,[19] independently by two observers 'blinded' to patient identity. After drawing a line along the myenteric plexus at low magnification (10–20×; freehand draw in NDP.view2 Viewing software: U12388-01 Hamamatsu), numbers of ganglia/mm myenteric plexus were determined in sections stained with anti-HuC/D (~40 × magnification). Numbers of cell bodies within ganglia/mm myenteric plexus were quantified using sections stained with anti-HuC/D (60-80 × magnification). Sections stained with anti-ChAT and anti-nNOS were used to count myenteric neuron subclasses.

Neuromuscular functions

Macroscopically normal, full thickness, proximal-to-mid rectum, ~5 cm from the tumour, were received in Krebs solution (mM: NaCl 121.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 5.6; equilibrated with 5/95% CO_2/O_2) at room temperature within 1.5 h following removal at surgery. The mucosa/submucosal plexus was removed and multiple strips (~4 × 15 mm) were cut parallel to the CM. These were used immediately or after overnight storage in fresh Krebs solution at 4°C. Previous studies found no differences in responses to EFS between tissues treated this way.[19,20]

Muscle strips (3–16/patient) were suspended between two parallel platinum ring electrodes in tissue baths containing Krebs solution (34°C, 5/95% CO_2/O_2, changed every 15 min).[15,19] These were stretched (20 mN tension) and isometric tension changes were recorded (Biopac Systems Inc.). After 60 min, EFS was delivered every minute for 10s at 5 Hz (maximum-effective voltage [50 V; c.200 mA], 0.5 ms bipolar pulses) until responses stabilised (at least 120 min). After 30 min, drugs were applied non-cumulatively and wet weights of muscle strips were obtained after all experiments.

EFS evokes contraction or relaxation during stimulation, usually followed by an 'after-contraction' on termination of EFS. These were measured as 'area under the curve' (AUC) for the total response to EFS, and drug-induced changes were expressed as a percentage of the mean of three pre-drug responses. Donepezil and prucalopride were investigated using single concentrations per strip.

Contractions were usually evoked during EFS, although relaxations were sometimes observed even among different strips from the same rectum (caused by small differences in muscle orientation, tone and/or innervation[19]). Thus, to investigate the effects of chemotherapy, responses to EFS were examined in several strips from each rectum. The numbers which relaxed or contracted during EFS, and contracted after EFS, were expressed as the percentage total for that patient. Subsequently, the number of patients in whom

relaxation occurred during EFS in at least 20% or 40% of the strips studied was determined.

The overall ability of the muscle to contract and relax was measured as the response to single concentrations of, respectively, carbachol (CCH) and sodium nitroprusside (SNP). Non-cumulative concentration-response curves, measured as tension changes in millinewtons/gram wet weight (mN/g) of tissue, were plotted using a 3-parameter agonist-response curve fitting (GraphPad Prism 5.0; GraphPad Software) with EC_{50} and E_{max}'s determined from the fitted curve.

$$\text{Effect of drug} = \text{Baseline} + \frac{(E_{max} - \text{Baseline})}{1 + 10^{(\text{LogEC}_{50} + \text{Log[Drug]})}}$$

Drugs were freshly prepared before use. Tetrodotoxin (TTX; Tocris Bioscience, UK) was dissolved in H_2O to 1 mM. Atropine (Sigma-Aldrich, UK), carbachol (Sigma), donepezil-HCl (Molekula, UK) and prucalopride succinate (Shire-Movetis, Belgium) were dissolved in H_2O to 10 mM. L-NAME (Nω-nitro-L-arginine methyl ester hydrochloride) and sodium nitroprusside (Sigma) were dissolved in H_2O to 100 mM.

Statistical analysis

Data are expressed as medians and interquartile range (examining the response characteristics in patient groups) or means ± S.E.M. where indicated, and n-values denote number of patients. Differences between medians were analysed using Mann-Whitney *U*-test for 2 groups of unpaired observations. One sample *t*-test analysed % changes in magnitude of AUC for EFS following exposure to drugs, changes in response (mN/g)/g to SNP, and differences in CD45 quantification. *T*-tests analysed differences in responses to drugs between tissues unexposed or exposed to chemoradiotherapy. Two-way ANOVA with Sidak's multiple comparison post-tests analysed differences in CCH-evoked contractions between patient groups.

When counting the number of cell bodies, inter-rater differences (95% CI) were analysed using one sample Student's *t*-tests and proportional biases investigated using Bland-Altman plots. For comparisons, normality testing (D'Agostino & Pearson) was performed and neurons/mm length analysed for differences in means using ANOVAs with Sidak's multiple comparisons test. $p < 0.05$ represented statistical significance.

RESULTS

Treatment groups

Forty patients aged 51–76 years, undergoing surgery for colorectal cancer, consented to provide surgical rectum tissue. One group underwent surgery without chemoradiotherapy. A second patient received long-course neo-adjuvant radiotherapy with concurrent capecitabine before surgery. Ages of patients in each group were similar and there was a tendency for males to be more common among patients receiving chemoradiotherapy (Table 1; Supporting Information S2).

Inflammation

On H&E staining, only a minority of tissues unexposed or exposed to chemoradiotherapy before surgery showed low-grade inflammation, limited to the mucosa (data not shown, $n = 5$ each group). In the rectum without mucosa, unexposed, or exposed to chemoradiotherapy, there were no differences in the expression of mRNA for IL6, IL13 or TNFα relative to GAPDH (respectively, $p = 0.32$, 0.09, 0.68; $n = 5$, each group). For example, the relative quantification values for TNFα relative to GAPDH were, respectively, 0.7 ± 0.1 ($n = 16$) and 0.6 ± 0.1 ($n = 20$). Finally, the numbers of CD45-positive cells (Figure 1) were similar between ROIs in tissues unexposed or exposed to chemoradiotherapy ($n = 5$ each): deep circular muscle (respectively, 0.5 ± 0.2 and 0.8 ± 0.3 cells, $p = 0.53$), CM near myenteric plexus (0.9 ± 0.3 and 0.3 ± 0.1 cells, $p = 0.13$) and LM (1.0 ± 0.5 and 0.5 ± 0.0 cells, $p = 0.52$).

Myenteric neurons

In tissue from 5 chemoradiotherapy-naïve patients and 5 receiving chemoradiotherapy (7 females, 3 males; 67 (31–77) years), a mean of 47 mm myenteric plexus/antibody/patient was sampled from 75

TABLE 1 Summary of patients donating tissue for the study.

Treatment group	N (strips)	Age (years)	Comparison with surgery only	Gender (male: female)
Surgery only	19 (187)	69 (61–76)	–	1:1.4
Capecitabine and pelvic radiotherapy followed by surgery	21 (221)	65 (51–71)	$p = 0.12$	2:1

Note: Patients received surgery or long course pelvic radiotherapy with concurrent capecitabine (825 mcg/m^2) twice daily, over ~5 weeks, followed by surgery 98 (mean value) days later.

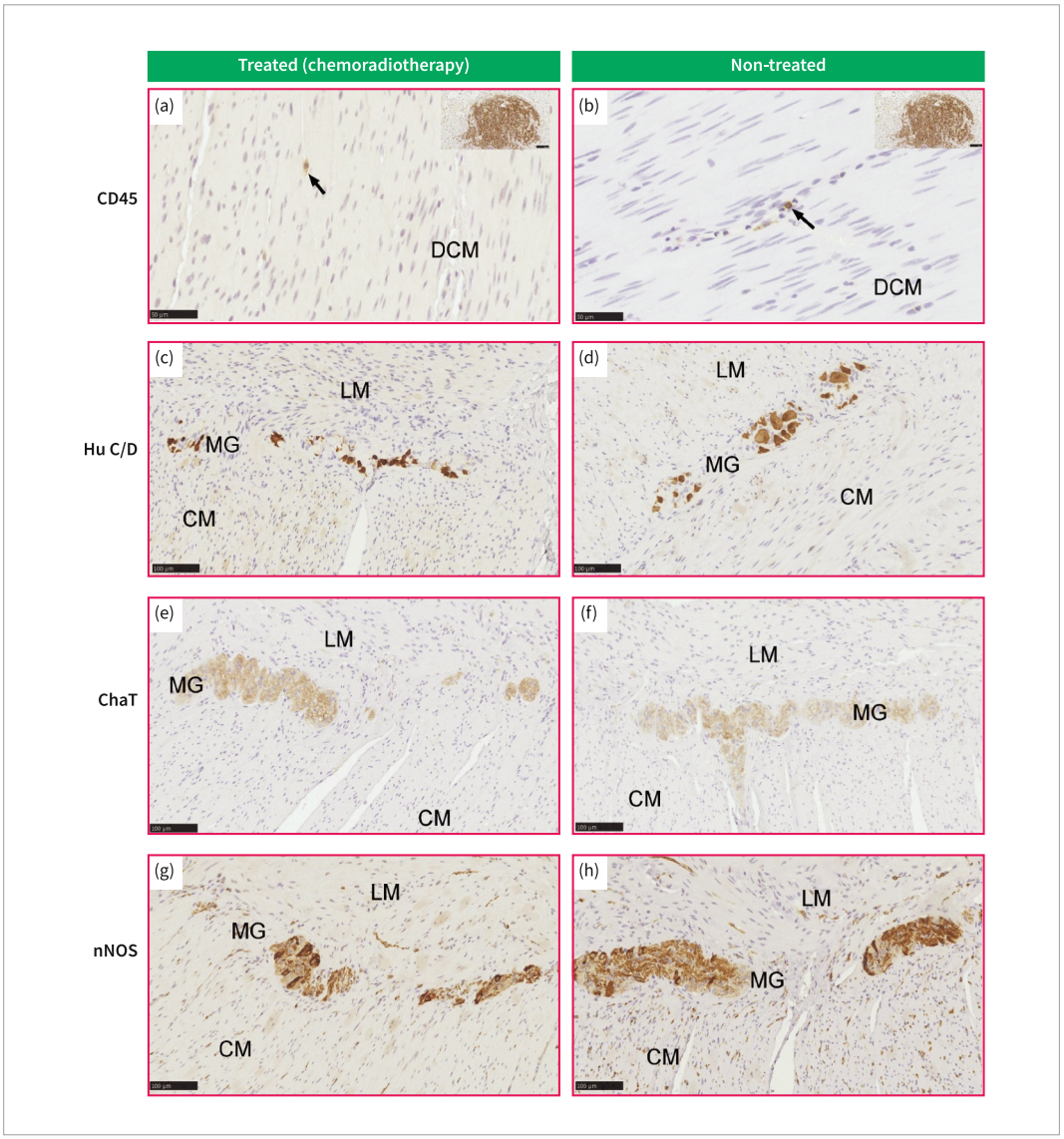

FIGURE 1 Representative expressions of CD45 and myenteric neuronal markers in treated (with chemoradiotherapy) and non-treated mucosa-free rectal tissues. Formalin fixed, paraffin embedded mucosa -free rectal samples were stained using an immunohistochemical method. Immunoreactivity (IR) was visualised with 3,3′diaminobenzidine tetrahydrochloride (DAB; brown) and nuclei stained with haematoxylin (Black). (a, b) Assessed inflammatory status with a pan-lymphocytic marker CD45 (arrow) in patient samples. Positive controls were run for quality assurance (inset in a, b images, scale bar 100 μm). Myenteric neurons were examined using antibodies for (c, d) human neuronal protein C/D (HuC/D), (e, f) choline acetyltransferase (ChAT) and (g, h) neuronal nitric oxide synthase (nNOS). Quantification of all immunoreactive cells (i.e. CD45, Hu C/D, ChAT and nNOS) showed no differences in protein expression levels between treated ($n = 5$) and non-treated ($n = 5$) samples. All images were taken under identical conditions. Black scale bar: (a, b) 50 μm; (c–h) 100 μm. CM, circular muscle; DCM, deep circular muscle; LM, longitudinal muscle; MG, myenteric ganglion.

sections. Both observers counted 9922 neurons stained with anti-HuC/D, 2020 for anti-ChAT, and 5398 for anti-nNOS, uninfluenced by inter-observer differences (Figure 1; Supporting Information S3). Tissues unexposed or exposed to chemoradiotherapy showed no differences ($p > 0.6$) in numbers of ganglia (respectively 1.4 ± 0.3 and 1.6 ± 0.2 ganglia/mm myenteric plexus), neurons (11.7 ± 2.4 and 10.3 ± 2.2 cells/mm myenteric plexus), or cell bodies expressing ChAT (2.2 ± 0.5 and 2.5 ± 0.4 cells/mm myenteric plexus) or neuronal nitric oxide synthase (6.3 ± 1.2 and 5.6 ± 1.1 cells/mm myenteric plexus); $n = 5$ each.

A pilot study,[13] examining colonic myenteric plexus at unknown times after different regimens of chemoradiotherapy (one received 5-fluorouracil) found increased translocation of HuC/D staining from the cytoplasm into its nucleus, and increased soma size of nNOS-immunoreactive neurons. In the present study, nuclear translocation of HuC/D staining (Figure 1) was not observed (nuclear staining without or with chemoradiotherapy, respectively, 34.7 ± 4.9% and 32.9 ± 3.4% of total HuC/D staining, $p = 0.76$) nor changes in the soma size of nNOS-immunoreactive cell bodies (respectively, 294 ± 30 μm^2 and 256 ± 17 μm^2, $p = 0.30$); $n = 5$ each group.

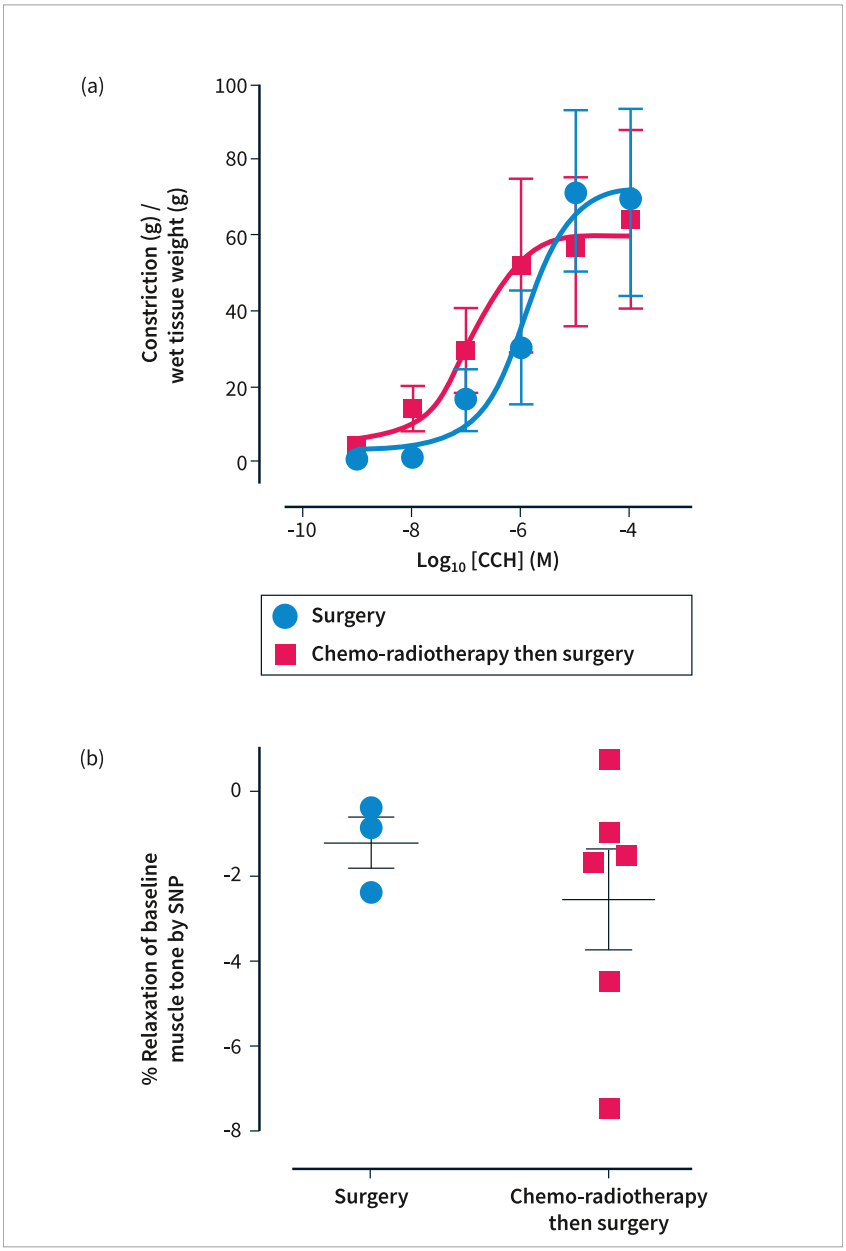

FIGURE 2 Contraction and relaxation evoked by carbachol (CCH, (a)) or sodium nitroprusside (SNP, (b)) in macroscopically normal human rectum muscle from patients treated for cancer by surgery alone or by chemoradiotherapy prior to surgery. The latter group of patients received long course pelvic radiotherapy with concurrent capecitabine (825 mcg/m2) twice daily, over ~5 weeks, followed by surgery 98 (mean value) days later. In each experiment, single concentrations were applied to different strips, with at least 15 min contact time. Data are expressed as the mean ± S.E.M. muscle tension generated during contraction or relaxation, per gram wet weight of tissue and analysed using a 3 parameter concentration response curve in GraphPad Prism; $N = 7$ (CCh comparison) and 6 (SNP) each.

Functional studies

CCH (0.001–100 μM; pEC_{50} 6 ± 0.4; $n = 6$) evoked muscle contraction (Figure 2a) with high but variable maximal tension (E_{max} 734 ± 123 mN/g). SNP (100 μM) evoked small muscle relaxations (Figure 2b). Chemoradiotherapy had no effects on CCH-induced contractions (pEC_{50} and E_{max} respectively, 7 ± 0.6 and 602 ± 105 mN/g; $n = 7$; $p = 0.76$; vs. chemoradiotherapy-naive, $p > 0.38$ each concentration; Figure 2a). Relaxation to SNP was unchanged versus chemoradiotherapy-naive ($p = 0.48$; Figure 2b).

In rectum naïve to chemoradiotherapy, most preparations contracted during and after termination of EFS; the maximum tensions developed were, respectively, 2.3 ± 0.9 and 13.4 ± 1.6 AUC/g ($n = 8$). These were prevented by TTX 1 μM (Table 2). Atropine 1 μM prevented contractions during EFS and reduced but did not prevent after-contractions (Figure 3a; Table 2). L-NAME 300 μM prevented

TABLE 2 Pharmacology of responses to EFS in macroscopically normal circular muscle of human proximal-to-mid rectum removed for bowel cancer without or with prior chemoradiotherapy.

	Surgery			Chemoradiotherapy then surgery		
Treatment	Contraction during EFS	After-contraction	N	Contraction during EFS	After-contraction	N
Tetrodotoxin 1 μM	Abolished	Abolished	3	Abolished	Abolished	4
Atropine 1 μM	Prevented	Decreased by donor 1: 43.1%, donor 2: 31.4%, donor 3: 18.1%	3	Prevented (n = 2), revealing muscle relaxation (n = 1)	Decreased by donor 1: 13.6%, donor 2: 90.3%, donor 3: 49.4%	3
L-NAME 300 μM	Prevented any relaxation which sometimes occurred during EFS (n = 5) and facilitated contractile amplitude by 181.6 ± 31.8% (n = 11)		16	Prevented any relaxation which sometimes occurred during EFS (n = 8) and facilitated contractile amplitude by 262.0 ± 44.9% (n = 10)		18

Note: The latter group of patients received long course pelvic radiotherapy with concurrent capecitabine (825 mcg/m^2) twice daily, over ~5 weeks, followed by surgery 98 (mean value) days later. There was no difference in the effect of L-NAME on 5 Hz electrical field stimulation (EFS) in human rectum circular muscle compared with patients treated with surgery alone (p = 0.3). Data are mean ± SEM.

Abbreviation: N, number of donors.

any relaxation during EFS, changing to contraction, and increased contraction amplitudes (Figure 3b, Table 2). In the rectum exposed to chemoradiotherapy, EFS-evoked responses were also prevented by TTX and modulated by atropine and L-NAME (Table 2).

In both groups of patients, the occurrence of contractions or relaxations during EFS sometimes varied among different muscle strips from the same tissue, so 11 (8–13) strips/rectum were studied. In chemoradiotherapy-naive tissues, most contracted during EFS (usually followed by after-contraction) but a small number relaxed (in 20% or 40% of strips in, respectively, 4 and 1 of 19 patients). In tissues from chemoradiotherapy-treated patients, relaxations during EFS were observed in 40% of the strips studied in 7 of 21 patients. Overall, the % incidence of relaxation during EFS in all muscle strips from all tissues was greater after chemoradiotherapy (chemoradiotherapy-naive: 12.6 ± 5.1%, n = 18 tissues in which relaxations were observed; surgery after chemoradiotherapy: 29.8 ± 6.1%, n = 21, p = 0.03; Figure 4), whereas the incidence of after-contractions and their magnitude (respectively, 60 (23–108) and 54 (21–127) mN/g, n = 12 and 18) were unchanged (p > 0.8 each). There was no apparent correlation between tissues responding to EFS by relaxation and patient's age or gender, and data were uninfluenced by times between surgery and completion of chemoradiotherapy (Supporting Information S4).

Effects of prucalopride and donepezil

In the chemoradiotherapy-naive rectum, prucalopride 10 μM, caused a small increase in overall EFS-evoked contractile activity (AUC increased 17.0 ± 5.9%; n = 14; p = 0.004) but had no consistent effect in the presence of L-NAME 300 μM (which by itself, increased the contraction amplitude; see above) (n = 11; p = 0.15; Figure 5). Donepezil 1 μM caused a large increase in the amplitude of contractions evoked by EFS during the absence (by 273 ± 71%; n = 8) and presence of L-NAME 300 μM (295 ± 99%; n = 10; p = 0.006 each, Figure 5).

After chemoradiotherapy, prucalopride 10 μM had no consistent ability to change the response to EFS in the absence (n = 13; p = 0.29) or presence (n = 11; p = 0.99) of L-NAME 300 μM (Figure 5c,d). Donepezil 1 μM caused a large increase in the amplitude of EFS-evoked responses during the absence of L-NAME 300 μM (by 295 ± 99%; p = 0.015; n = 10). However, in the presence of L-NAME, the excitatory activity of donepezil was greatly reduced (132 ± 36%; n = 8; p = 0.008 vs. no L-NAME; p = 0.05 vs. no chemoradiotherapy plus L-NAME; Figure 5e,f).

DISCUSSION

Patients receiving capecitabine and radiotherapy were found not to have rectal enteric neuropathy. This is consistent with other reports after chemoradiotherapy (including 5-fluorouracil),[4,9] and with a generally low incidence of neuropathy after 5-fluorouracil or capecitabine (e.g.,[10,21]). Thus, there were no changes in the numbers or phenotype of myenteric nerve cell bodies. Swelling of nNOS-immunoreactive nerve cell bodies was not observed and there was no apparent translocation of HuC/D-immunoreactive protein from the cytoplasm into the nucleus.

Instead, persistent changes in the balance between myenteric cholinergic and nitrergic activity were observed, together with a marked reduction in the ability of donepezil to increase cholinergic function. These changes were not related to persistent inflammation. Thus, several months after chemoradiotherapy, there were no marked changes in H&E staining, CD45-positive cells, or mRNA for IL6, IL13 or TNFα. More cytokines could have been assessed (e.g., IL8, IL-1ß), but nevertheless, when combined, the results suggest resolution of any inflammation following treatment.

Before investigating the effects of chemoradiotherapy on neuromuscular functions, it was necessary to characterise EFS-evoked responses. These were neuronally mediated (prevented by the voltage-gated sodium channel blocker TTX). During EFS, cholinergic-mediated muscle contraction was attenuated by

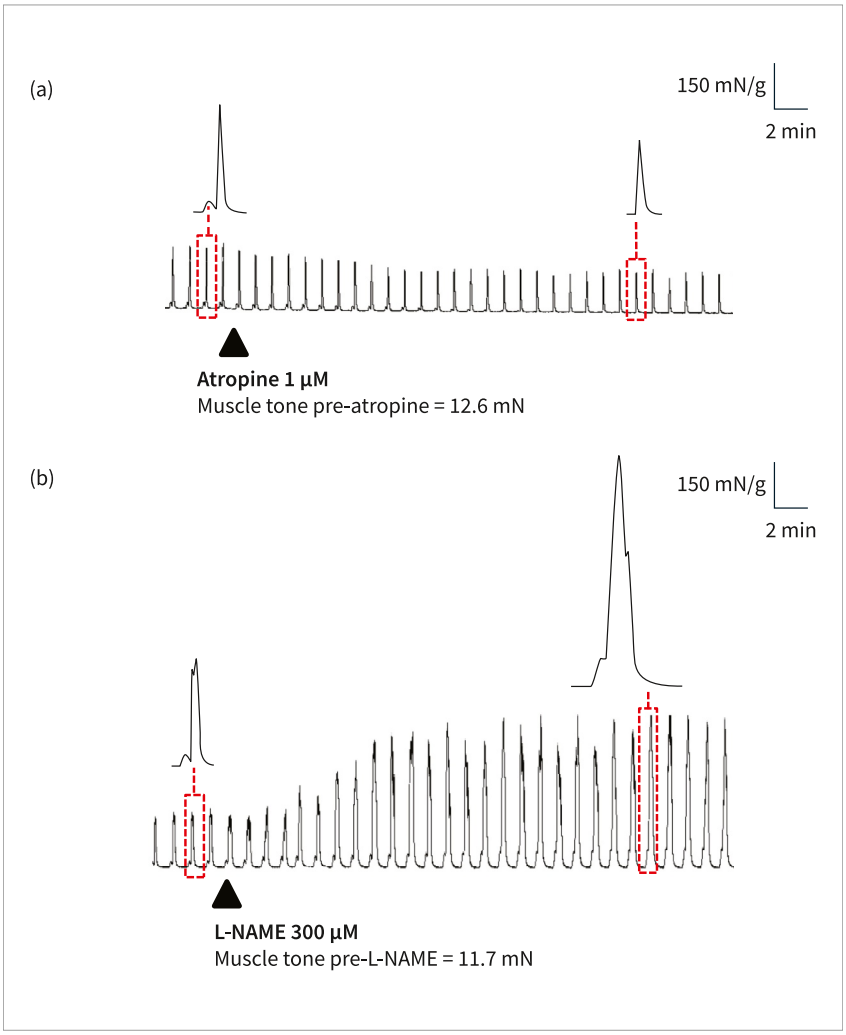

FIGURE 3 Responses to electrical field stimulation (EFS) in macroscopically normal human rectum circular muscle from patients treated for cancer by surgery alone. In (a, b) EFS was applied at 5 Hz (for 10 s at 5 Hz, 50 V, 0.5 ms bipolar pulse duration, every minute) and representative traces illustrate the phenotype of responses. (a) Shows inhibition by atropine 1 μM of contractions evoked during EFS with reduction in amplitude of after-contraction. (b) Shows facilitation by L-NAME 300 μM of contractions evoked during and after termination of EFS.

simultaneous activation of inhibitory nitrergic neurons (previously shown in pre-contracted mid-rectum[22]), and on terminating EFS, a partially cholinergic-mediated after-contraction occurred. In the human descending colon this non-cholinergic component is tachykinergic.[19] Also in human colon, similar frequencies of EFS activate cholinergic and nitrergic inhibitory activity, in a broadly similar manner, with some region-dependence.[15,19] By contrast, nitrergic-mediated muscle relaxation was the dominant response to EFS in the distal rectum and internal anal sphincter, often followed by after-contraction (e.g.,[23,24]). These region-dependent differences exist without variations in myenteric neurochemical coding.[25] Perhaps they reflect a greater need of the descending/sigmoid colon and proximal rectum to maintain muscle tone and propulsive movements, compared with the internal anal sphincter and to a lesser extent, distal rectum, in favour of nitrergic-mediated muscle relaxation.[26]

Chemoradiotherapy changed the balance between cholinergic and nitrergic activity in favour of nitrergic. Thus, there was a small increase in the number of tissues relaxing during EFS instead of contracting. No changes were observed in the ability of the muscle to contract or relax in response to carbachol or SNP, suggesting that chemoradiotherapy directly affected neuronal functions. In other studies, responses of human internal anal sphincter to carbachol and EFS (nitrergic-mediated relaxations) were reduced six-to-eight weeks after radiotherapy and 5-fluorouracil.[6] In human sigmoid colon capecitabine and radiotherapy increased sensitivity to contractions by CCH, histamine and EFS in males, not females.[27] Following pelvic irradiation and 5-fluorouracil,[5] there was a trend toward increased fibrosis and nerve density in the internal anal sphincter. Finally, in the colon exposed to different chemotherapeutics sometime before surgery,[7] myenteric S-type neurons were hyperexcitable. The

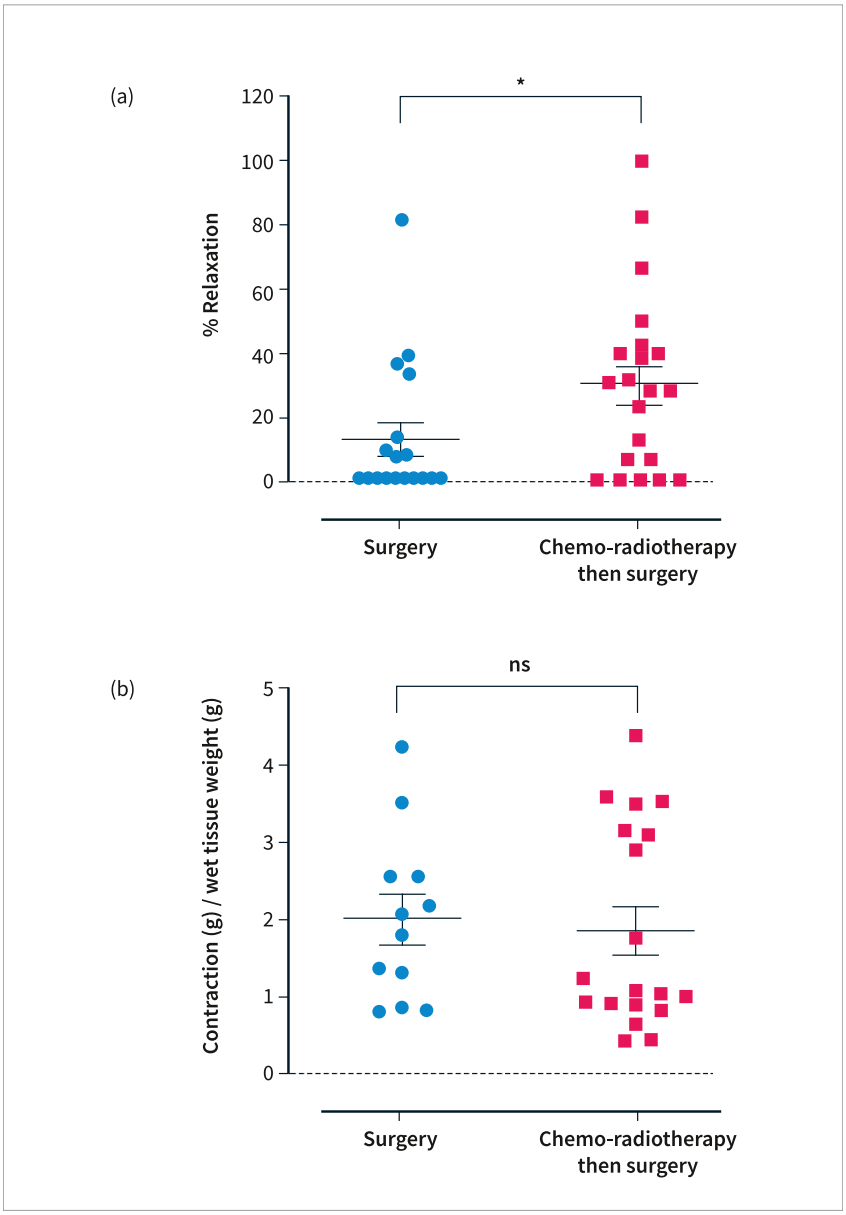

FIGURE 4 Increased occurrence of muscle relaxation in response to 5 Hz electrical field stimulation (EFS) in human rectum circular muscle from patients receiving long course neoadjuvant radiotherapy with concurrent capecitabine prior to surgery compared with patients receiving surgery alone. Data for the group with surgery alone was obtained using 187 muscle strips from 18 patients. Data for the group with chemoradiotherapy and surgery were obtained using 221 muscle strips from 21 patients. For each patient, the % of strips which relaxed in response to EFS was calculated following application to 11 (8–13) muscle strips, for 10 s at 5 Hz, 50 V (c. 200 mA), 0.5 ms bipolar pulse duration, every minute. (a) Shows the overall difference in % relaxations between the two groups; $^{*}p = 0.03$. (b) Shows a comparison of the magnitude of the response to EFS/g tissue (wet weight) for those strips which contracted in response to EFS. There was no significant difference in the magnitude of the response between the surgery alone group ($n = 12$) and the chemoradiotherapy with surgery group ($n = 19$).

differences between these studies may reflect differences in GI regions and chemoradiotherapeutics.

In the rectum of patients not exposed to chemoradiotherapy, prucalopride (10 μM; maximum-effective, selectively active concentration in human colon[11,15]) caused a small increase in overall response to EFS. This was lost when the EFS-evoked contractile response was isolated and made larger by L-NAME. By contrast, prucalopride facilitated cholinergic activity in similar experiments with human colon (e.g.,[13,15,28]). In other studies with human rectum, 5-HT_4 mRNA[29] and low levels of 5-HT_4 protein were apparent on muscle, not myenteric plexus.[30] In another study, contractions of the human rectum were increased by prucalopride,[28] although the use of *taenia coli* suggests sigmoid colon. Finally, in healthy volunteers, prucalopride did not affect anorectal functions at a dose which increased colonic transit.[31] Together, these observations suggest region-differences in the ability of prucalopride to stimulate lower bowel motility.

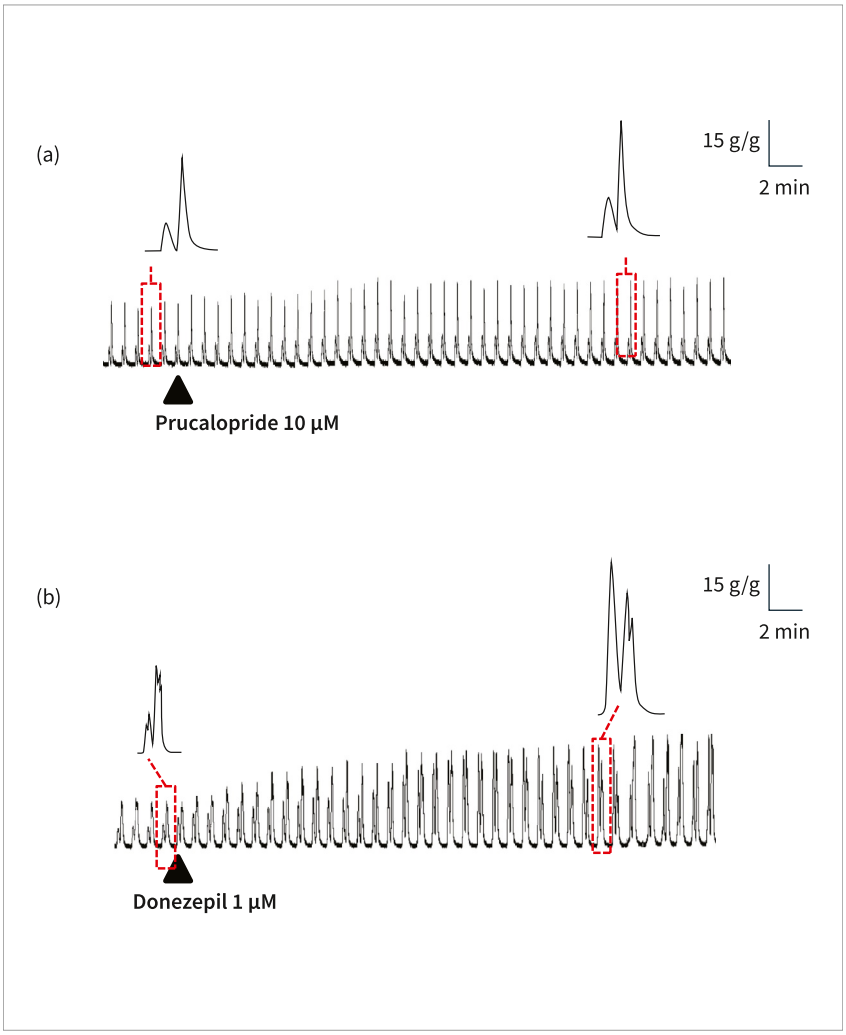

FIGURE 5 Facilitation by prucalopride and donepezil of the area under the curve (AUC) for the response to electrical field stimulation (EFS) in human isolated rectum circular muscle. Tissue was obtained from patients treated with long course neoadjuvant radiotherapy with concurrent capecitabine prior to surgery compared with patients treated with surgery alone. (a, b) are representative examples illustrating the effects of prucalopride (a) and donepezil (b) on responses to EFS. (c–f) show the changes in EFS-evoked AUC amplitude caused by prucalopride (c, d) and donepezil (e, f) for each tissue studied, together with the median and interquartile range, in the absence (c and e) and presence (d and f) of L-NAME 300 µM. EFS (50 V, 0.5 ms bipolar pulse duration, 5 Hz) was given for 10 s every 1 min. *N* values (patients) are located underneath each plot. ǂ $p < 0.05$, ǂǂ $p < 0.01$ analysing the effects of each drug compared to the baseline; *$p < 0.05$ comparing the effects between patients treated by surgery alone or with chemoradiotherapy then surgery.

Severe constipation or megacolon may be treated with the cholinesterase inhibitors neostigmine or pyridostigmine.[32] Their side-effects (including hypotension) argue for better-tolerated inhibitors, such as donepezil, alone[9] or together with prucalopride, especially for the elderly. In the present study donepezil strongly facilitated cholinergically mediated contractions of the rectum, similar to human colon (where efficacy was substantially greater than for prucalopride[15]).

Following chemoradiotherapy, the small excitatory activity of prucalopride was lost, and the excitatory activity of donepezil was approximately halved. The latter is consistent with reduced cholinesterase activity in human stomach following platinum-based therapy.[33] Thus, together with the increased nitrergic (relative to cholinergic) activity following chemoradiotherapy (see above), these findings suggest damaged cholinergic function. They also suggest that following chemoradiotherapy, the efficacy of other drugs on the rectum and other areas of the bowel must now be investigated.

Finally, an important limitation is that although the ages of patients in both groups were similar, the numbers used to examine the effects of EFS ($n = 19, 21$) are too small to investigate any effects of ageing within the present data.[34] In the human colon, reduced myenteric cholinergic function decreases the 'reserve capacity' of the ENS, so when other degenerative events occur (e.g., chemoradiotherapy), symptoms such as constipation become more likely.[19]

AUTHOR CONTRIBUTIONS

Victor W. S. Kung, John Broad, Raj Makwana, Sarah Epton, Alexandra Palmer, and Nicholas Baidoo conducted the experiments and/or analysed the data. Mohamed Thaha, Charles H. Knowles, Sarah Epton, and

Joanne Chin-Aleong, performed the surgery and/or facilitated the collection of human tissues. Gareth J. Sanger obtained the funding. Victor W. S. Kung, Raj Makwana, Alexandra Palmer, and Gareth J. Sanger wrote the manuscript. All authors critically revised the manuscript.

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CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Victor W. S. Kung https://orcid.org/0000-0002-9166-4181
John Broad https://orcid.org/0000-0002-1641-7076
Raj Makwana https://orcid.org/0000-0003-2175-3530
Alexandra Palmer https://orcid.org/0000-0002-4786-4960
Nicholas Baidoo https://orcid.org/0000-0001-7017-2287
Shezan Elahi https://orcid.org/0000-0002-9408-8917
Mohamed Thaha https://orcid.org/0000-0002-5043-6107
Charles H. Knowles https://orcid.org/0000-0001-9854-6754
Gareth J. Sanger https://orcid.org/0000-0002-4231-1945

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