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ONCOLOGY AND GENETICS DOCTORAL SCHOOL

UNIVERSITY OF SIENA

Ph.D. Thesis

EFFECTS OF NEOADJUVANT TREATMENT FOR RECTAL CANCER ON ANORECTAL PHYSIOLOGY: FROM THE LABORATORY TO THE PATIENT

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Thesis suitable for the title of "Doctor Europeus"

Academic Year 2011 – 2012

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I thank prof. Alessandra Renieri and prof. Francesco Cetta (University of Siena) for their constant support and supervision during these years of study and research.

Prof. Alison Brading
and myself in Oxford

It is with immense love that I remember and thank the late prof. Alison F. Brading (University Department of Pharmacology, University of Oxford). I will always remember her enthusiasm for research and life. This thesis is mostly the result of the work done in her lab.

I thank Prof. Neil J. Mortensen (Department of Colorectal Surgery, University of Oxford) for the clinical supervision to this work.

Finally, I would like to thank my family and my friends for their invaluable support.

Oxford University Hospitals NHS
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Friday, 16 November 2012

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TO WHOM IT MAY CONCERN

Report on the PhD Thesis
of
Bruno Lorenzi

"Effects of neoadjuvant treatment for rectal cancer on anorectal physiology: from the laboratory to the patient"

This is a well written and beautifully illustrated thesis. There are two main sections, the first on the action of the Interstitial Cells of Cajal on the anal sphincter, and the second on the effects of neoadjuvant chemo-radiotherapy on sphincter function.

The background to the work in each section is well described. The experiments were carried out in a world class laboratory using well established organ bath techniques and imunocytochemistry and human material from the internal anal sphincter was collected from 10 patients. Classical pharmacological methods were used to study the effects of the interstitial cells on myogenic tone, and the added influence of the tyrosine kinase inhibitor imatinib. Myogenic tone is excited by the ICC's which in turn may be inhibited by imatinib. These are novel findings.

Whilst radiotherapy is now widely used for the treatment of rectal cancer its effect on the anal sphincter is poorly understood. In this set of experiments, organ bath studies demonstrated a clear impairment of internal anal sphincter function and the effect seems to be on both ICC's and muscle cells. These are novel findings and help explain the clinical experience of patients who have impaired sphincter function after radiotherapy.

I can unreservedly support the work and presentation in this thesis for a PhD in the University of Siena.

Professor Neil Mortensen
MB ChB, MA, MD, FRCS Eng, hon FRCPS Glas, hon FRCS Edin
Professor of Colorectal Surgery

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To:
Professor Neil Mortensen
Department of Colorectal Surgery
University of Oxford, UK

Siena, 18th November 2012

Dear Professor Mortensen,

I have really appreciated your comments on my work, and I would like to thank you very much for your review of my Thesis.

It was a great experience working with you and the late prof. Alison Brading at the University of Oxford.

I look forward to expanding my research with clinical studies in the near future.

With best wishes,

Bruno Lorenzi

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12th November 2012

To whom it may concern:

Report on the PhD Thesis of

Bruno Lorenzi

Title

EFFECTS OF NEOADJUVANT TREATMENT FOR RECTAL CANCER ON ANORECTAL PHYSIOLOGY: FROM THE LABORATORY TO THE PATIENT

The present thesis is structured in 2 main sections and spanning 82 pages, including front page, Ph.D. Dissertation Board, Abstract and References.
Each section is composed by: Introduction, Materials and Methods, Results and Discussion. There is a final chapter (Conclusion) that summarize the work done.
The number and the quality of the figures is adequate.

In the first section, the Author analyze the role of Interstitial Cells of Cajal in the human internal anal sphincter.

There is a concise introduction of the physiology and pharmacology of the internal anal sphincter as well as a short chapter on the role of the Interstitial Cells of Cajal in the gastrointestinal tract.
The properties of these cells have been extensively studied in the last few years, and the candidate could have expanded this chapter slightly more.
Materials and methods, as well as Results are clear and complete, and have led to the conclusion that the Interstitial Cells of Cajal have a peculiar distribution in the internal anal sphincter associated to a possible specific function. They seem involved in the maintenance of the tone of the internal anal sphincter. This is a considerable finding, that could lead to clinical applications in the near future.
Although these experiments appear to be solid and have been performed by a group with great expertise in this method, the number of patients studied was relatively small and additional experiments are needed in the future.

The Author subsequently analyzed the effects of chemoradiotherapy on the funtion of the internal anal sphincter. This work resulted in a recent peer reviewed manuscript.
The Introduction of this second section is concise. Patients and methods, and Results are again appropriate.
In the Discussion, the Author presents a comprehensive review of the relevant literature and subsequently analyze his results.
In summary, the Author present an in vitro controlled trial comparing the contractility of internal sphincter muscle in patients that have received chemoradiation and patients who have not. The hypothesis is that CRT impacts IAS function. He found that significantly affects the IAS. This is novel and very interesting work with high impact, considering the considerable number of patients receiving treatment for rectal cancer.

Again the size of the sample is relatively small, but we should consider the difficulties of obtaining human tissue for laboratory research.

The last chapter with the Conclusion is helpful, but it could have bring more personal thoughts. A short abstract is present at the end just before the References.

In conclusion, This is a very well written thesis on a very important clinical problem.

The quality of the work is very good and fulfils the conditions that are required to allow Dr Bruno Lorenzi to take his PhD examination.

Dr Francisco CARDENAS CAUQUI

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To:
Dr. Francisco Cardenas Cauqui
Department of General Surgery
Hospital Juan Grande, Jerez, Spain

Siena, 18th November 2012

Dear Dr Cardenas Cauqui,

Thank you very much for your comments on my Thesis.

I am aware of the relatively small number of the patients studied, due to the obvious difficulties in obtaining human tissue. Nevertheless, the experiments were carried out in a renowned laboratory using well-established techniques allowing the group to obtain highly reliable data.

I look forward to expanding these experiments, and plan clinical studies in the near future.

With best wishes,

Bruno Lorenzi

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Section 1

ROLE OF INTERSTITIAL CELLS OF CAJAL IN THE HUMAN INTERNAL ANAL SPHINCTER

INTRODUCTION

Physiology and pharmacology of the internal anal sphincter

Anal continence is a complex mechanism of interrelating factors. The anal sphincters play a major role in maintaining the anal continence creating a high-pressure zone in the anal canal. In particular, the internal anal sphincter comprises an inner ring of smooth muscle that is under an involuntary state of chornic contraction. This is due both to intrinsic myogenic properties and external innervation. Experimental studies have clarified the mechanisms involved in IAS function.[1]

The autonomic nervous system supply to the internal anal sphincter comprises a sympathetic input from the hypogastric plexus and parasympathetic supply from the first, second and third sacral nerves via the pelvic plexus.

Although sphincter function is modified by these sympathetic and parasympathetic nerves, it now well known that an intramural nervous system controls the motility of the gastrointestinal tract, and plays a relevant role also in the IAS.

The internal anal sphincter generates a zone of high pressure in the resting state and is responsible for the majority of overall resting anal tone.[2] It has now been established that this is due to both intrinsic myogenic activity and extrinsic adrenergic innervation.

The effects of adrenergic agonists are well-documented. *In vitro* studies have shown that contractions were mediated via α-receptors and relaxations via β-receptors.

Nicotine caused relaxation of internal anal sphincter strips, and acetylcholine has an inhibitory effect via muscarinic receptors.

Electrical field stimulation (EFS) of internal anal sphincter strips resulted in relaxation of the muscle. These relaxations are abolished by tetrodotoxin, indicating that they are nerve mediated. The addition of guanethidine and atropine to block adrenergic and cholinergic effects had no effect on the response to electrical field stimulation, indicating that this relaxation was mediated by non-adrenergic non-cholinergic nerves.

Rattan first suggested that nitric oxide was the main inhibitory neurotransmitter of the IAS.[3]

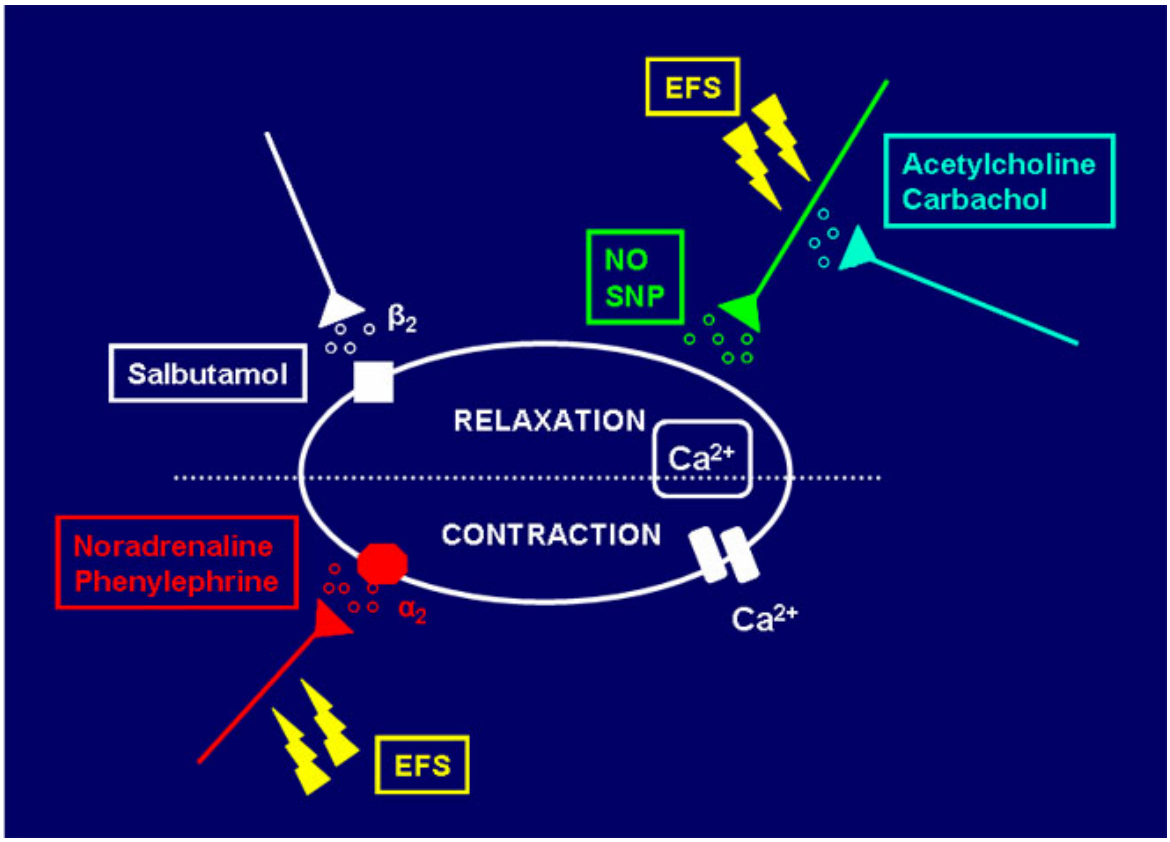
Furtheremore, relaxations induced by EFS were inhibited by the addition of L-N^G-nitro-arginine, an inhibitor of nitric oxide synthase, the enzyme

responsible for the production of nitric oxide. The effect of enzyme inhibition was countered by the addition of the nitric oxide precursor, l-arginine.[4]

O'Kelly *et al*. have demonstrated similar effects of nitric oxide and electrical field stimulation in the presence of nitric oxide synthase inhibitors and l-arginine in human internal anal sphincter tissue. In addition, muscarinic receptors have been found on nitric oxide-releasing nerves.[5]

There is now evidence that nitric oxide is the main inhibitory neurotransmitter in the IAS.

Contraction and relaxation of the IAS is dependent upon cytosolic levels of calcium. *In vitro* experiments have demonstrated that tone and spontaneous activity of internal anal sphincter strips are dependent upon extracellular calcium and flux across the cell membrane through l-type calcium channels (the principal calcium channel in smooth muscle). However, agonist-induced contractions were dependent mainly on calcium release from intracellular stores. Relaxation is effected by mechanisms causing a decrease in cytosolic calcium. [6]



EFS, electrical field stimulation; NO, nitric oxide; SNP, sodium nitroprusside, Ca^{2+}, calcium; α_2, α_2-adrenergic receptor; β_2, β_2-adrenergic receptor.

Figure 1

Schematic diagram of an internal anal sphincter smooth muscle cell showing the principal contractile and relaxatory receptors, transmitter and other pharmacological agents

Role of the Interstitial Cells of Cajal in the gastrointestinal system

The interstitial cells of Cajal (ICC) are specialised pacemaker cells that regulate the spontaneous electrical activity of the smooth muscles in the gastrointestinal tract. These cells form cellular networks interconnecting via gap junctions into the smooth muscle and on the borders of myenteric and submucosal plexuses. They generate and propagate spontaneous slow waves, and mediate transmission between enteric neurones and muscle cells.[7,8]
Defects in or absence of ICC result in disorders in the gastrointestinal motility.[9-12]

ICC express the proto-oncogene c-kit,[13] and, recently have been described in animal and human internal anal sphincter (IAS), using antibodies to the c-kit receptor.[14-16]

The internal anal sphincter is a specialized smooth muscle that exhibits specific features and plays a major contribution to maintenance of anal continence generating a continuous tone. Therefore, it is unlikely that ICC play an identical role in the IAS to that played in the rest of the gut.
So far, the function of ICC in the IAS has only been initially investigated in two studies using transgenic mice.[17,18] The role of ICC in human IAS is therefore still unclear.

It has been shown that the pacemaker activity of ICC involves intracellular Ca^{++} release via IP3-related stores and Ca^{++} activated channels;[19] however, it is difficult to investigate the function of ICC using drugs that interfere with these mechanisms as they will also interfere with the myocytes.

Imatinib mesylate (Glivec®; Novartis Pharmaceuticals) is a selective inhibitor of the c-kit tyrosine kinase and the oncogene Bcr-Abl, and has USA Food and Drug Administration approval for the treatment of Philadelphia chromosome-positive chronic myeloid leukaemia (CML) and c-kit positive gastrointestinal stromal tumours (GIST).[13,20] Because the myocytes do not express the c-kit receptors and signaling via the receptor tyrosine kinase gene product, kit, is essential for development of the phenotype and electrical rhythmicity of ICC, drugs interfering with this receptor are able to selectively inhibit the ICC.[21]

We investigated the role of the ICC in the IAS examining the effects of the c-kit tyrosine kinase inhibitor imatinib mesylate (Glivec®) on the physiological properties of human IAS strips.

PATIENTS AND METHODS

Patients

Samples were obtained from 10 patients (6 men; age range, 32-82 years; mean 59 years, median 58 years) undergoing APR or proctectomy.

Studies on human anal sphincter tissue were approved by the Ethics Committee of the University of Oxford and informed consent was obtained from all patients.

Immunohistochemistry

Immunohistochemistry was assessed using unfixed 1 x 1 cm specimens of IAS, embedded in OCT Embedding matrix (R.A. Lamb, Eastbourne, UK) and snap-frozen in liquid nitrogen. The specimens were stored at -70º C until required. Cryostat sections (10 μm) were cut and thaw-mounted onto glass slides pretreated with Vectabond^{TM} reagent (Vector Laboratories, Peterborough, UK) and air-dried for 30-60 minutes. Sections were fixed with acetone for 10 minutes at room temperature, and then incubated overnight at room temperature in a moisture chamber with the primary antiserum, a rabbit polyclonal antibody CD117 for c-kit receptor (Santa Cruz Biotechnology, Santa Cruz, USA), diluted 1:50 with phosphate buffered saline (PBS, Oxoid

Ltd, Basingstoke, UK) and 0.5 % bovine serum albumin (BSA, Sigma-Aldrich Company Ltd, Poole, UK). The next day, slides were washed three times with PBS and then incubated with the secondary antibody, a peroxidase conjugated swine anti-rabbit antibody (DAKO Ltd, Cambridge, UK), diluted 1:50 with PBS with 0.5% bovine serum albumin, for 30 minutes at room temperature in a moisture chamber. Slides were washed three times with PBS. The slides were incubated with a tertiary antibody a peroxidase conjugated rabbit anti-swine antibody (DAKO Ltd, Cambridge, UK), diluted 1:50 with PBS with 0.5% bovine serum albumin, for 30 minutes at room temperature in a moisture chamber. Slides were washed again three times with PBS. Subsequently, slides were incubated in substrate solution made with Sigmafast^{TM}3'3-Diaminodenzidine tablets (Sigma-Aldrich Company Ltd, Poole, UK) for 5 minutes at room temperature, and then washed with PBS. Sections were then counterstained in filtered Harris's Hematoxylin (VWR International, Lutterworth, UK)) for 15 seconds, dehydrated in alcohol, cleared in Histoclear (R.A. Lamb, Eastbourne, UK) and then overslipped before mounting in Histomount (R.A. Lamb, Eastbourne, UK). Tissue areas were photographed at high magnification (x400).

Organ bath studies

Tissue was transferred immediately to Krebs' solution at 4°C, equilibrated with 97 percent oxygen and 3 percent carbon dioxide and maintained at pH of 7.4 ± 0.05. Krebs' solution contained 120 mM NaCl, 5.9 mM KCl, 15.4 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 11.5 mM glucose.

Using a dissecting microscope, the epithelium of the anal canal was removed together with the submucosa. Strips of IAS were dissected following the direction of the muscle bundles and measured approximately 1 x 1 x 8 mm (weight range 2 to 10 mg). Strips were tied at each end with fine (5/0) silk ligatures, mounted for isometric tension recording in small organ baths (capacity, 0.2 ml) between two platinum ring electrodes 1 cm apart and continuously superfused with Krebs' solution (37°C) at a flow rate of approximately 1 ml/minute.[23] The apparatus allowed 6 strips to be studied simultaneously (Figure 2 and 3).

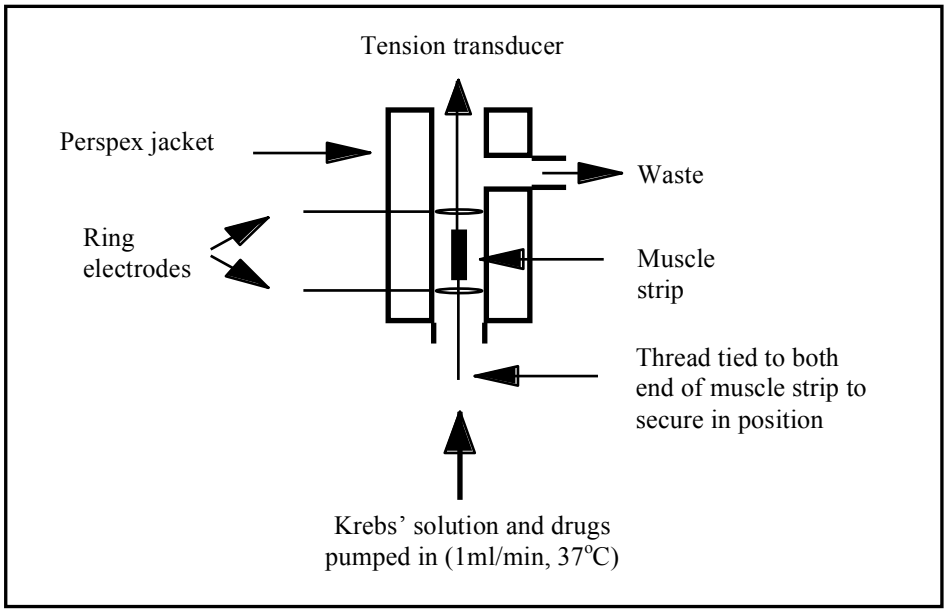


Figure 2

Schematic representation of the organ bath used for the *in vitro* study of the physiological and pharmacological properties of IAS strips.

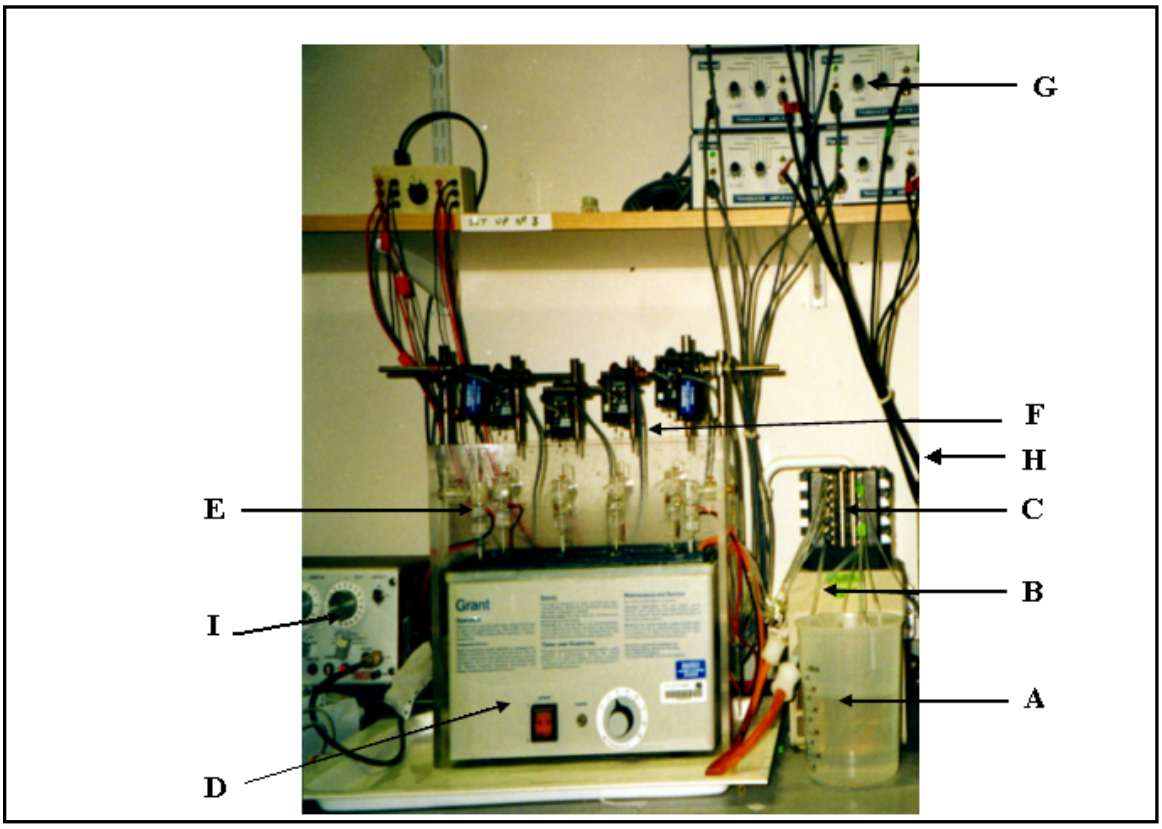


Figure 3

The system for the *in vitro* study of IAS strips: (**A**) Krebs' solution; (**B**) silicon tubes; (**C**) peristaltic pump; (**D**) heating system; (**E**) organ baths; (**F**) transducers; (**G**) amplifiers; (**H**) connection to MacLab data acquisition system; (**I**) eletrical stimulator.

Each strip was initially loaded with 1 g of tension and allowed to equilibrate for at least 90 minutes. The tension generated by the smooth muscle strips was measured by Pioden™ dynamometer UF1 transducers (Pioden™ Controls, Canterbury, UK), amplified (Harvard Transducer/Amplifier, Harvard Apparatus, Edenbridge, UK) and recorded using the Chart v3.6 and MacLab™ Data Acquisition System (AD Instruments, Sidney, Australia).

Electrical field stimulation (EFS) was delivered by a Grass S48 Stimulator™ (Grass Instruments, Quincy, MA, USA) and applied with parameters established after preliminary experiments (1 s train pulses, 0.5 ms duration, 10 V voltage and frequency varying from 1 to 100 Hz). A minimum of 5 minutes recovery between stimulations ensured no diminution of response.

The drugs used were phenylephrine (at a concentration of 10 μM) (Sigma Chemical Co., Poole, UK), carbachol (10 μM) (Sigma Chemical Co., Poole, UK) and imatinib mesylate (concentration from 1 μM to 50 μM) (Glivec®, kindly provided by Novartis Pharmaceuticals, Basel, Switzerland). All drugs were dissolved in Krebs' solution. Phenylephrine and carbachol were applied with intervening 10 minutes washout periods. Strips were allowed to equilibrate for 30 minutes after each application of imatinib mesylate.

At the conclusion of the experiment, strips were superfused with calcium-free Krebs' solution ($CaCl_2$ replaced isosmotically with $MgCl_2$, and 0.5 mM ethylene glycol-bis (2-aminoethyl)-N,N,N',N'-tetraacetic acid (Sigma

Chemical Co., Poole, UK)). The tension present in strips perfused with calcium-free solution was regarded as passive tension, and subtracted from observed tension to give active tension. A maximum of six strips were used from each patient.

Statistical Analysis

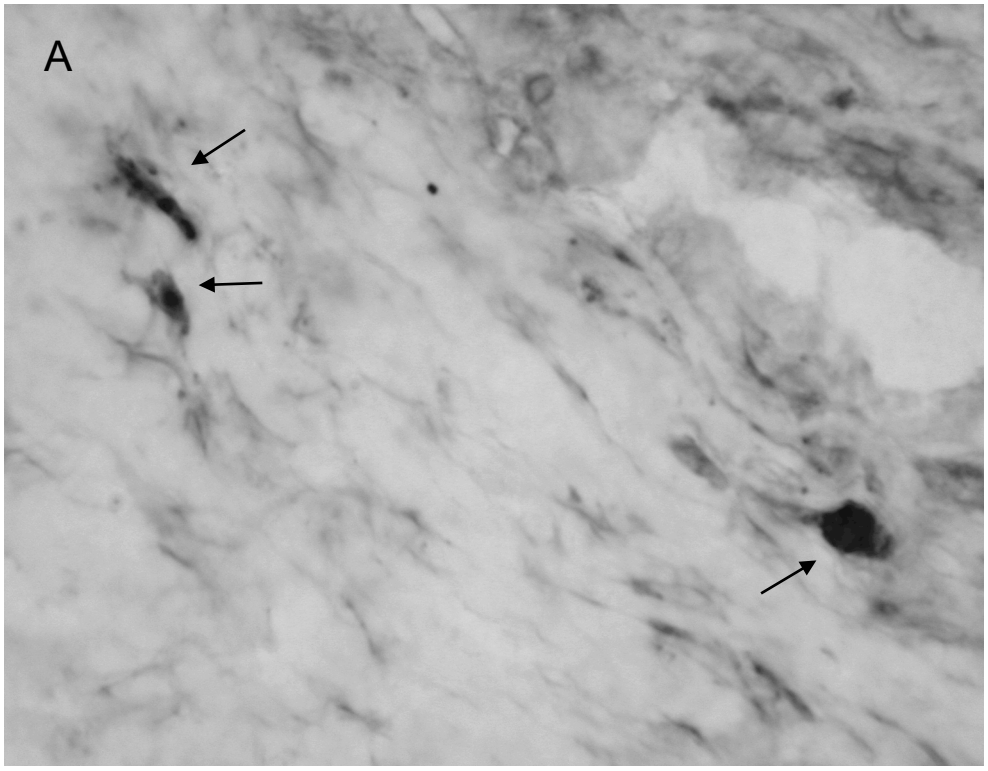
The results are expressed as mean (± standard error of the mean). The number of replicates are expressed as n = *x* (*y*), where *x* is the total number of strips and *y* is the number of patients. Data were fitted using Microsoft Excel® and values subsequently analyzed using SPSS® 15.0 for Windows. Statistical analysis was performed by one-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni's multiple comparison *post hoc* analysis. *P* values less than 0.05 were considered significant.

RESULTS

Immunohistochemistry

At histologic examination, c-kit positive cells were identified on the boundares of IAS bundles. They were typically fusiform with branching processes and stained orange with anti c-kit antibody (Figure 4). ICC were morphologically distinct from the surrounding smooth muscle cells. Muscle cells had a typical spindle shape, were generally larger and more homogeneous than ICC. Moreover, they showed no dendritic processes and immunoreaction to c-kit antibody.

No immunoreactivity was observed in negative controls, in which the primary antibody was omitted.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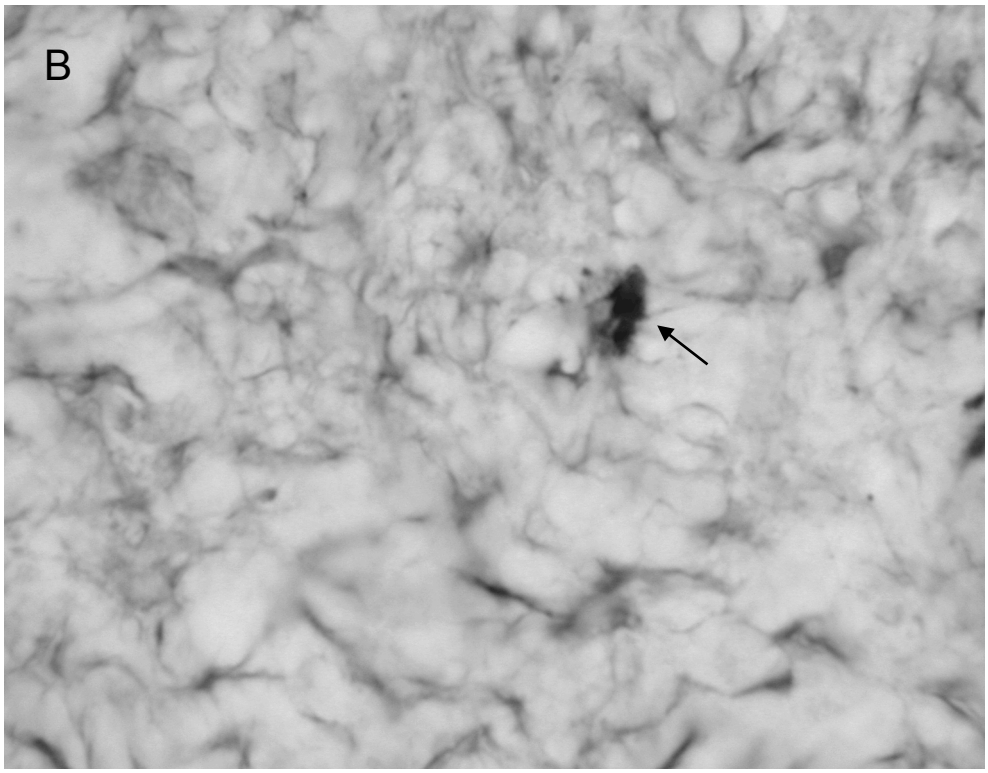
B

Figure 4

Interstitial cells of Cajal labelled with anti-*c-kit* antibody *(arrows)* in the human internal anal sphincter. Cells appear elongated showing dentritic processes (**A**) and forming cellular network (**B**). (c-kit immunostain counterstained with hematoxylin, original magnification x 400).

Organ bath studies

Tone and spontaneous activity

After 90 minutes equilibration, IAS strips developed both intrinsic tone and spontaneous activity. In absence of drugs, the tone generated was 147.7 ± 33.0 mg/mg tissue (n= 60(10)). Administration of imatinib mesylate caused a dose-dependent reduction in tone of IAS strips (120.1 ± 24.0 mg/mg tissue at 1 μM, *P*=0.91; 97.5 ± 19.4 mg/mg tissue at 5 μM, *P*=0.017; 76.5 ± 15.9 mg/mg tissue at 10 μM, *P*=0.005; 51.2 ± 11.2 mg/mg tissue at 50 μM, *P*=0.003) (Figure 5).

Spontaneous activity was characterised by intermittent low-amplitude contractions and calculated as the number of contractions ≥ 0.1 % of basal tone during an interval period of 10 minutes. In absence of drugs, IAS strips developed 135.6 ± 4.6 contractions (n=60(10). Imatinib mesylate significantly reduced the number of contractions at concentration from 5 to 50 μM (122.6 ± 4.1 at 1 μM, *P*=0.336; 110.7 ± 3.2 at 5 μM, *P*=0.005; 111.6 ± 2.7 at 10 μM, *P*=0.001; 109.3 ± 4.7 at 50μM, *P*=0.002) (Figure 6).

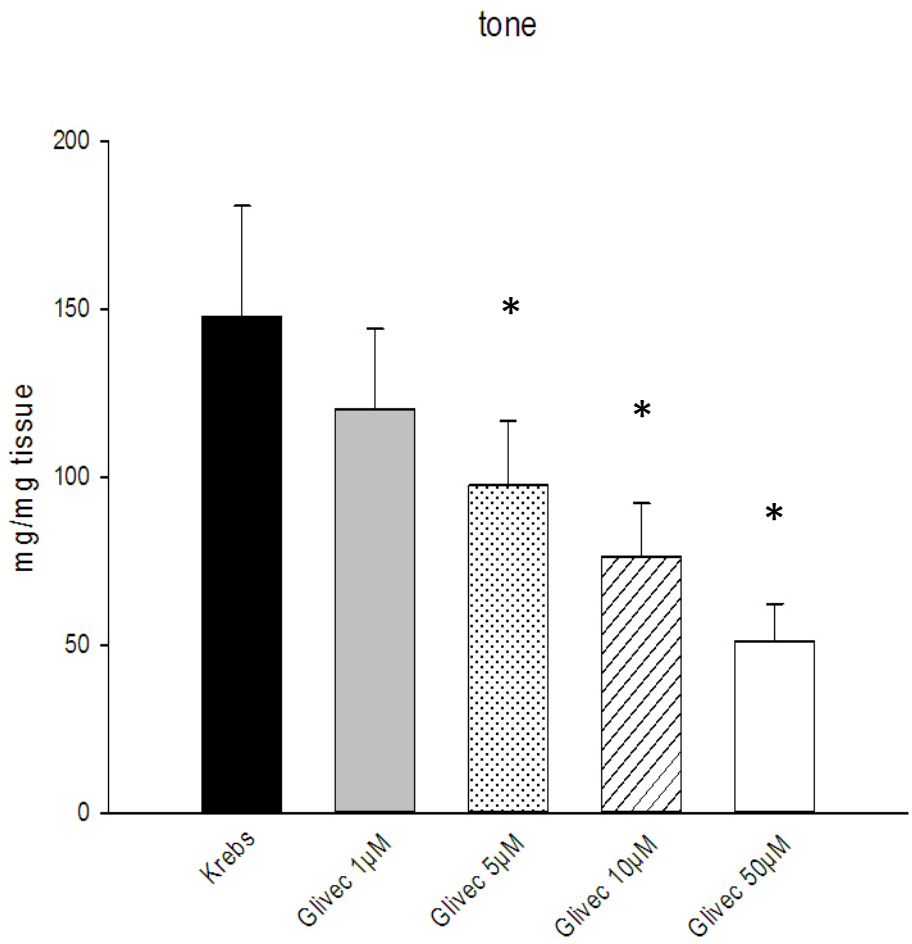

Figure 5

Mean (± standard error of the mean) tone generated by strips of internal anal sphincter after 90 minutes equilibration in absence of drugs and in response to increasing dose of imatinib mesylate (1 to 50 µM) (*P<0.05) (n=60(10).

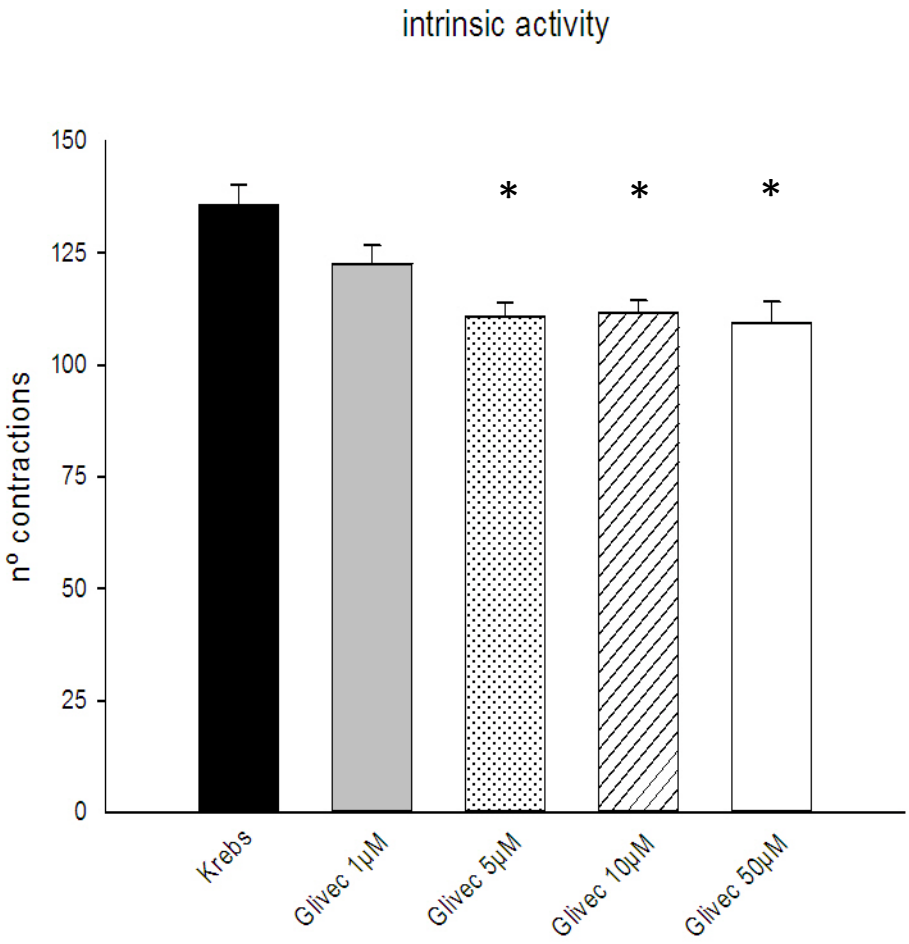

Figure 6

Mean (± standard error of the mean) number of contractions ≥ 0.1 % of basal tone minutes generated by strips of internal anal sphincter during an interval period of 10 in absence of drugs and in response to increasing dose of imatinib mesylate (1 to 50 µM) (*P<0.05) (n=60(10).

Responses to electrical field stimulation

EFS produced an initial relaxation followed by a contraction. The relaxation response predominated at low stimulation frequencies, whereas the contractile element was more evident at higher frequencies. In preliminary experiments, both relaxation and contraction was blocked by 3 μM TTX, thus confirming that the response was nerve-mediated.

The responses were analysed considering the fact that the amount of relaxation and contraction is dependent on background basal tone. Therefore, they were calculated as a percentage of the tone expressed at each concentration of imatinib mesylate (from 0 – absence of drug – to 50 μM) after equilibration to offset this effect. Subsequently, in case of relaxation, the level of tone during perfusion with calcium free Krebs' was considered as maximal relaxation; whereas the amount of contraction was calculated as a percentage increase of tone from baseline tone.

Relaxation responses to EFS of IAS strips (n=60(10) were not significantly different in absence of drugs and after administration of imatinib mesylate (*P*=0.107 at 1 Hz, *P*=0.441 at 5 Hz, *P*=0.422 at 10Hz, *P*=0.421 at 25 Hz, *P*=0.277 at 50 Hz and *P*=0.375 at 100 Hz) (Figure 7). Maximal relaxation was seen at 100 Hz, when EFS produced 52.6 ± 8.3 percent of maximum relaxation in absence of imatinb mesylate, 46.6 ± 3.8 percent at 1 μM, 50.0 ±

4.3 percent at 5 µM, 56.7 ± 5.3 percent at 10 µM, 54.8 ± 6.2 percent at 50 µM.

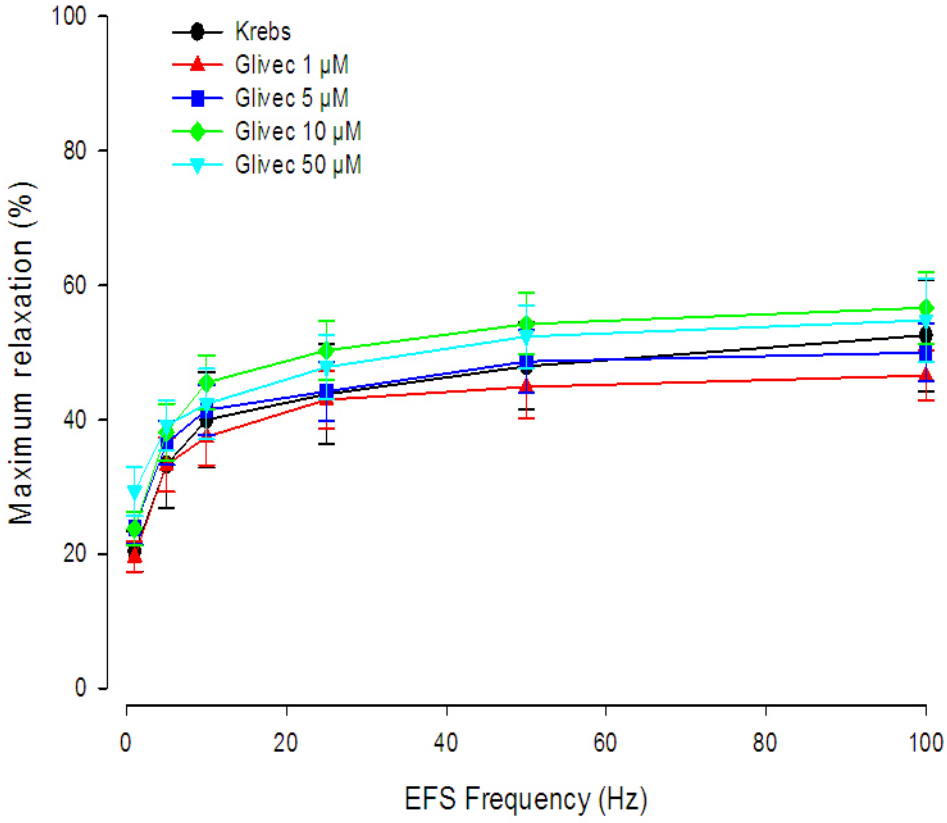

Figure 7

Mean (± standard error of the mean) percentage of maximal relaxation of internal anal sphincter strips in absence of drugs and in response to increasing dose of imatinib mesylate (1 to 50 µM) (n=60(10).

No significant differences were also seen in contractile responses in absence of drugs and after administration of imatinib mesylate (n=60(10) (*P*=0.276 at 1 Hz, *P*=0.379 at 5 Hz, *P*=0.264 at 10Hz, *P*=0.182 at 25 Hz, *P*=0.153 at 50 Hz and *P*=0.114 at 100 Hz) (Figure 8). Maximal contraction was observed at 100 Hz (15.4 ± 1.8 percent of maximum relaxation in absence of imatinb mesylate, 14.4 ± 1.6 percent at 1. μM, 17.8 ± 2.3 percent at 5 μM, 19.5 ± 1.8 percent at 10 μM, 16.7 ± 2.3 percent at 50. μM).

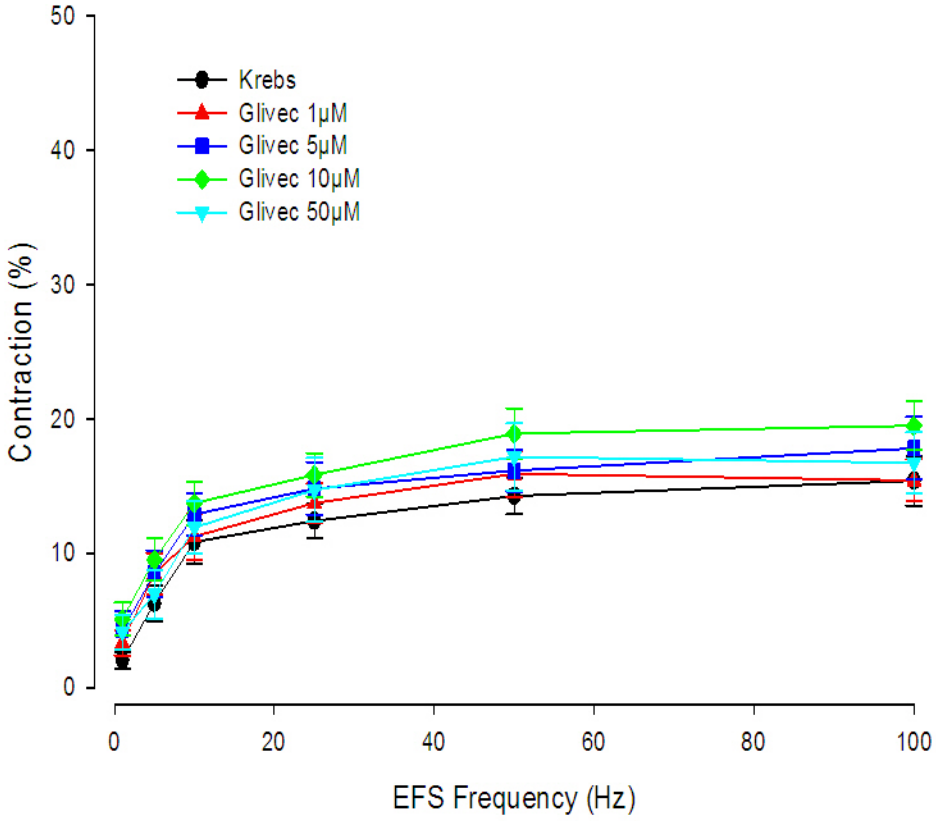

Figure 8

Mean (± standard error of the mean) percentage of contraction of internal anal sphincter strips in absence of drugs and in response to increasing dose of imatinib mesylate (1 to 50 µM) (n=60(10).

Responses to chemical stimulations (phenylephrine and carbachol)

IAS strips contracted in response to the α-adrenergic agonist phenylephrine (10 μM). There was no significant differences in the contractile response to phenylephrine in absence (n=60(10), 54.4 ± 6.6 percent) or after administration of imatinib mesylate (n=60(10), 53.9 ± 6.6 percent at 1 μM, 54.9 ± 6.1 percent at 5 μM, 58.0 ± 5.9 percent at10 μM, 56.2 ± 7.4 percent at50 μM; *P*=0.699) (Figure 9).

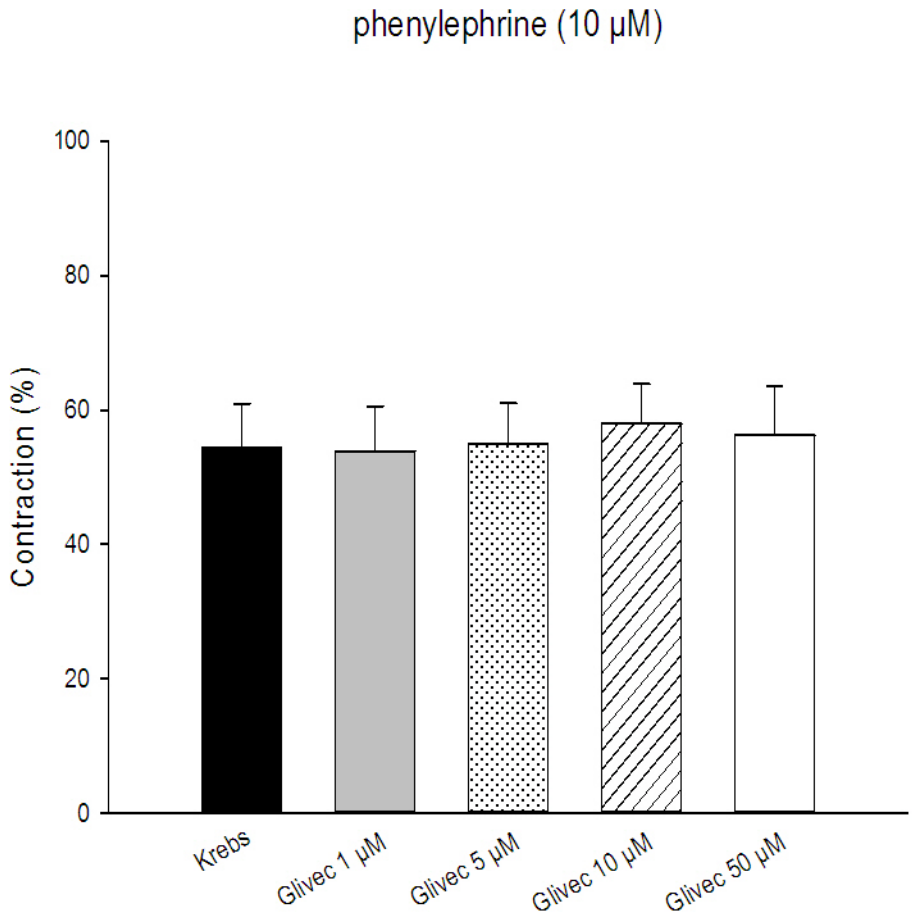

Figure 9

Mean (± standard error of the mean) percentage of contraction of internal anal sphincter strips in response to 10 µM phenylephrine in absence or after administration of imatinib mesylate (n=60(10).

In contrast, IAS strips relaxed in response to carbachol (10 μM), a muscarinic cholinergic agonist. Carbachol-induced relaxation responses were not significantly different in absence (n=60(10), 46.9 ± 6.4 percent of maximum relaxation) or after administration of imatinib mesylate (n=60(10), 47.3 ± 6.1 percent at 1 μM, 46.6 ± 3.5 percent at 5 μM, 51.6 ± 3.5 percent at 10 μM, 52.6 ± 3.6 percent at 50 μM; *P*=0.405) (Figure 10).

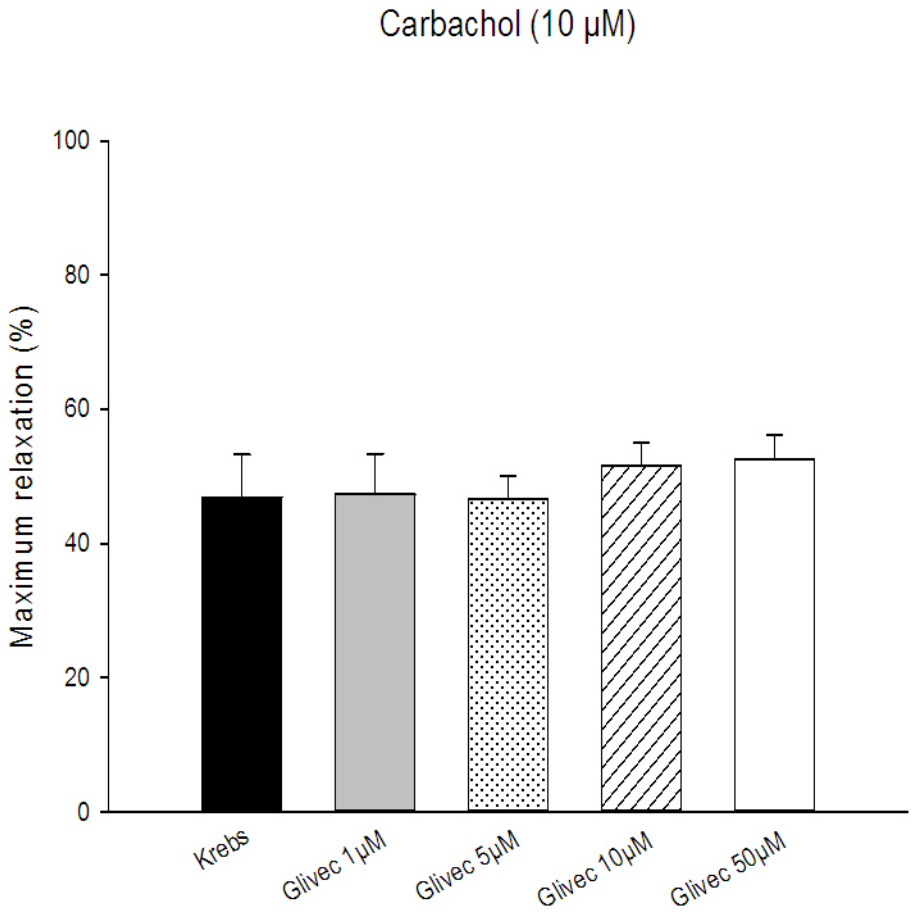

Figure 10

Mean (± standard error of the mean) percentage of contraction of internal anal sphincter strips in response to 10 µM carbachol in absence or after administration of imatinib mesylate (n=60(10).

DISCUSSION

Recent studies have identified ICC on the boundary of smooth bundles in human IAS. [14-16] However, the function of these cells in the IAS has as yet not been clearly defined, and, to our knowledge, this is the first study that has examined the role of ICC in human IAS.

The function of ICC in the gastrointestinal tract has been widely investigated. They occur in dense networks electrically interconnected via gap junctions and function as pacemakers generating spontaneous slow waves and signalling to the muscle cells to contract. In addition, they are in connection with a large number of enteric neurones and seem to mediate neurotransmission to muscle cells.[7,8] Absence or defects in ICC may be responsible to motility disorders of the gastrointestinal tract, such as Hirschsprung's disease, gastroparesis and functional intestinal obstruction with associated voiding dysfunction.[9-12]

The physiological characteristics of the IAS are extremely different from those of the gut.[1-6] The IAS is made of specialized smooth muscle cells that display sustained tone, essential for maintenance of faecal continence, whereas gastrointestinal smooth muscles cells generate phasic contractions,

necessary for peristalsis and food progression. Therefore, it is unlikely that ICC have similar function in the IAS to the rest of the gastrointestinal tract.

In addition, the distribution of the ICC in IAS is different. At microscopic examination, we found that they are present on the bundles of the IAS, but do not form the same dense networks seen in the gut. Similarly, Hagger et al. showed that ICC have a lower density in IAS compared to rectum.[14]

The rationale for the use of imatinib mesylate to investigate the properties of the ICC came from the fact that the myocytes do not express the c-kit receptors whereas signaling via the receptor tyrosine kinase gene product, kit, is essential for development of the phenotype and electrical rhythmicity of ICC.[21]

Previous studies have used imatinib mesylate to investigate the function of the ICC in other organs and tissues.[23-26] They showed that imatinib mesylate affects the electrical and mechanical activity of ICC at concentrations that have no effects on the action potential shape or the ion channels of the myocytes, and support the hypothesis that imatinib mesylate can selectively inhibit the ICC.[23] Imatinib mesylate is in clinical use for patients with CML and GIST.[20]

In our study, imatinib mesylate reduced the tone of IAS strips at concentration from 5 to 50 μM. This finding is extremely interesting. Until now, the factors

responsible for the sustained tone of the IAS have not been completely understood, and the tone simply labeled as myogenic. Our results suggest that ICC may contribute to maintain the excitability of myocyes and consequently sustain the tone of IAS.

A similar mechanism has been also hypothesized in the urethral sphincter, that exhibits very similar characteristics to the IAS.[27,28] Both sphincters maintain sustained tone, essential to continence, which is enhanced by sympathetic activity and inhibited via nitrergic pathway during micturition or defecation. In particular, it has been hypothized that ICC induce periodic depolarization of the smooth-muscle cells allowing sufficient entry of Ca2+ to maintain sustained tone of the sphincter, and to contribute to continence.[28]
In the IAS, therefore, the ICC may not act as a coordinated pacemaker electrical network as they do in the rest of the gastrointestinal tract, but they may be responsible for increase excitability of the muscle cells in order to maintain the basal tone.

Studies from mice with mutations leading to defects in the development of ICC populations showed that without pacemakers, the coordination of smooth-muscle contractions in the gastrointestinal tract is lost.[28]

Interestingly, Terauchi *et al*. have observed that transgenic W/W^v mice lacking muscular ICC exhibit lower IAS basal tone, suggesting that they may be involved in augmenting and sustaining the basal tone of the IAS.[29]

De Lorjin *et al.* have also studied the manometric properties of transgenic w/w mice, and, despite they observed no significant difference in the basal tone compared to controls, they described a more irregular pressure pattern with twitch contractions.[30]

Moreover, Sivarao *et al.* studied the properties of the lower oesophageal sphincter (LOS) in transgenic mice.[31] The LOS maintains a basal tone and relaxes with swallowing that is caused by activation of inhibitory nitrergic nerves, and has many similarities with the IAS. It is therefore interesting the fact that transgenic w/w mice showed hypothensive lower oesophageal sphincters.

Interestingly, strips exhibited less spontaneous activity after administration of Glivec. This finding also support our hypothesis that ICC may be responsible for increase excitability of the muscle cells in IAS, and the diminished spontaneous activity of the strips after administration of Imatinib Mesylate may reflect an impaired function of ICC under these conditions.

Responses to EFS, as well as to phenylephrine and carbachol, were not affected by the administration of Glivec in our experiments. It has been

clearly demonstrated that relaxation and contraction induced by EFS, phenylephrine and carbachol are mediated by nitrergic and adrenergic pathways in the IAS.[1] Our results therefore suggest that ICC may not play a relevant role in mediating the nitrergic and adrenergic innervation in the IAS, in contrast to what has been postulated in other tissues.

In addition, our data correlate with the study of De Lorjin et al, that showed that electrical stimulation of the IAS of KIT w/w mice relaxed to the same extent as those from controls, whilst blockade of the NO biosynthesis significantly reduced these responses.[30]

A similar conclusion was suggested by Sivarao et al, who demonstrated that relaxations of the LOS in response to swallowing or vagal stimulation was not significantly affected in KIT w/w mice, and ICC did not play a role in nitrergic transmission.[31]

In conclusion, our study confirmed that Glivec selectively inhibit the ICC at concentrations that do not affect the myocytes, and could represent an important molecule to investigate the function of the ICC in the human body. We observed that ICC may play an important role in maintaining the basal tone and modulating the electrical activity of the IAS, whilst they may not be involved in regulating the nitrergic and adrenergic transmission. Further studies are necessary to expand our results and characterize the physiologic properties of the ICC in the IAS, however, these cells, with their peculiar

characteristics, are sufficiently different from smooth muscle cells, and may represent a target for the development of drugs capable to inhibit the c-kit receptors or modulating their activity, and provide a new approach to anorectal pathologies.

Section 2

EFFECTS OF NEOADJUVANT CHEMORADIOTHERAPY ON INTERNAL ANAL SPHINCTER FUNCTION

INTRODUCTION

Treatment of rectal cancer and anal sphincters injury

It is well known that sphincter preserving surgery with total mesorectal excision (TME) for rectal cancer can result in anorectal functional disorders and incontinence.[32-34] Neoadjuvant chemoradiotherapy (CRT), introduced with valuable results in terms of reduced locoregional recurrence rate and improved survival, may also impair the functional outcome of these patients.[35] Both short- or long-term course of radiotherapy have been associated with this adverse effect and concurrent chemotherapy may add adjunctive damage.

The evidence of the anorectal damage caused by neoadjuvant treatment has been so far indirect, based on clinical and instrumental differences between patients submitted to surgery alone or neoadjuvant CRT before surgery.
The radiation damage of the anorectum is considered an important factor causing anal incontinence in rectal cancer patients after CRT and a direct radiation injury of the sphincters has been postulated when the anal canal is included in the field of irradiation.[36-42]

Clinical studies have evaluated the effects of radiotherapy on anorectal function indicating that continence disorders are significantly increased

compared to patients treated by surgery alone. However, specific symptoms caused by radiation injury may be difficult to differentiate inside the complex syndrome of altered bowel habits and faecal incontinence following rectal surgery.

Few studies have reported the results morphological and functional investigations, such as anorectal physiology tests and endoanal ultrasonography, in patients treated for rectal cancer.

The results of anorectal manometry after radiotherapy for rectal cancer have been considered unchanged or minimally altered in two studies.[43,44] Several other studies have shown that irradiated patients have more clinical symptoms of anal incontinence and lower pressures on anorectal manometry in comparison to patients treated with surgery alone.[45-50] Radiotherapy for other pelvic malignancies (with higher radiation doses) caused reduction of both mean resting and squeeze pressures and confirmed that pelvic irradiation is a major contributing factor to anal sphincter dysfunction.[51-58]

There is a lack of studies dedicated to investigate the morphological alterations of anal sphincters after CRT. Few studies have evaluated limited features, such as sphincter thickness of the anal sphincter, or described structural defects (i.e. scarring) that are not precisely scored in order to provide a quantitative evaluation of the damage of the sphincter.[48]

Finally, pathological features are limited to two main reports on rectal and internal anal sphincter damage, respectively after radiotherapy for prostate and rectal cancer.[46,59] Both studies reported direct evidence of nervous injury of the myoenteric plexus. Muscle fibres of the rectal wall appeared hypertrophied after radiotherapy. Histological features of the internal anal sphincter after CRT have shown increased collagen deposition and hypertrophy of the myenteric plexus in irradiated sphincters of patients undergoing abdominoperineal resection (APR), which might be responsible for the reduced elasticity and responsiveness of IAS after irradiation.

Subsequent data are taken from a personal study recently published on a peer-reviewed Journal:

Lorenzi B, Brading AF, Martellucci J, Cetta F, Mortensen NJ. Short-term effects of neoadjuvant chemoradiotherapy on internal anal sphincter function: a human in vitro study. Dis Colon Rectum. 2012 Apr;55(4):465-72.

PATIENTS AND METHODS

Samples were obtained from patients undergoing APR or proctectomy. Six patients (4 men; age range, 63-80 years; mean 70 years, median 69 years) who had locally advanced rectal cancer and received preoperative CRT composed the study group. All these patients completed the proposed neoadjuvant protocol. Five patients (2 men; age range, 31-52 years; mean 40 years, median 34 years) treated by surgery alone formed the control group. This included 4 patients with ulcerative colitis, who had refused pouch formation, and underwent proctectomy with intersphincteric anorectal excision, and one patient with ulcerative colitis who had previously submitted to restorative proctocolectomy and underwent pouch excision and intersphinteric anal resection. Final histology showed no evidence of features of Crohn's disease or indeterminate colitis and no anal involvement in all patients included in the control group.

Patients affected by anal diseases (*i.e.* perianal Crohn's disease) or had previous anal surgery were excluded from the study. Of the 5 female patients included in the study, only two (one in each group) had vaginal delivery. None of them required operative birth, and none had obstetric injury post-partum.

In both groups, tissue was collected in the distal part of the anal canal, away from the tumour in patients treated for rectal cancer. The study was focus on the IAS properties. Experiments on external anal sphincter (EAS) were not performed.

Studies on human anal sphincter were approved by the Ethics Committee of the University of Oxford and informed consent was obtained from all patients.

Chemoradiotherapy protocol

All patients who underwent neoadjuvant treatment received 50 Gy irradiation in 25 fractions administered over a five-week period (2 Gy/fraction). A three-field technique was used. The anal canal was included in all patients within the field of irradiation. The superior field border included the body of S1 and the lower limit was at minimum 1 cm below the inferior margin of the ischial tuberosity in all cases. Typically, the lateral borders were 1 cm lateral to the bony pelvis, and the posterior limit extended 1 cm beyond the anterior margin of the sacrum. A continuous intravenous infusion of 5-fluorouracil (5-FU) at the dose of 1 g/m^2 on day 1-4 was co-administered to four patients in weeks 1 and 5 of radiation treatment. Because of age, one patient received a reduced dose of 5-FU (750 mg/m^2 on day 1-4 in weeks 1 and 5 of radiotherapy) and

one had only radiotherapy. Four patients underwent laparoscopic-assisted creation of a stoma before adjuvant treatment. Surgery was performed at approximately six to eight weeks after completion of CRT.

Organ bath studies

Specimens were obtained immediately after surgical excision. Fresh tissue (including part of the IAS) was collected from the distal part of the anal canal under the supervision of a pathologist, and transferred immediately to Krebs' solution at 4°C, equilibrated with 97 percent oxygen and 3 percent carbon dioxide and maintained at pH of 7.4 ± 0.05. Krebs' solution contained 120 mM NaCl, 5.9 mM KCl, 15.4 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 11.5 mM glucose.

With the aid of a dissecting microscope, the epithelium of the anal canal was removed together with the submucosa and the fibres of the EAS, when present. Strips of IAS were dissected following the direction of the muscle bundles and measured approximately 1 x 1 x 8 mm (weight range 2 to 10 mg). Strips were tied at each end with fine (5/0) silk ligatures, mounted for isometric tension recording in small organ baths (capacity, 0.2 ml) between two platinum ring electrodes 1 cm apart and continuously superfused with Krebs' solution (37°C) at a flow rate of approximately 1 ml/minute.[22] The

apparatus allowed 6 strips to be studied simultaneously. The entire process from the surgical excision to the beginning of the experiment lasted approximately 30 minutes.

Each strip was initially loaded with 1 g of tension and allowed to equilibrate for at least 90 minutes. The tension generated by the smooth muscle strips was measured by Pioden dynamometer UF1 transducers (Pioden Controls, Canterbury, UK), amplified (Harvard Transducer/Amplifier, Harvard Apparatus, Edenbridge, UK) and recorded using the Chart v3.6 and MacLab Data Acquisition System (AD Instruments, Sidney, Australia).

Electrical field stimulation (EFS) was delivered by a Grass S48 Stimulator (Grass Instruments, Quincy, MA, USA) and applied with parameters established after preliminary experiments (1 s train pulses, 0.5 ms duration, 10 V voltage and frequency varying from 1 to 100 Hz). A minimum of 5 minutes recovery between stimulations ensured no diminution of the response.

The drugs used were phenylephrine (at a concentration of 10 μM), carbachol (10 μM), Nω-nitro-L-arginine (L-NO Arg, 10 μM), sodium nitroprusside (SNP, concentration from 1 nM to 10 μM) and tetrodotoxin (TTX, 3 μM) (Sigma Chemical Co., Poole, UK); they were chosen on the basis of previous similar studies, and doses were tested in preliminary experiments. Drugs were

dissolved in Krebs' solution and applied with intervening 10 minutes washout periods.

At the conclusion of the experiment, strips were superfused with calcium-free Krebs' solution ($CaCl_2$ replaced isosmotically with $MgCl_2$, and additional 0.5 mM ethylene glycol-bis (2-aminoethyl)-N,N,N',N'-tetraacetic acid (Sigma Chemical Co., Poole, UK)). The tension present in strips perfused with calcium-free solution was regarded as passive tension, and subtracted from observed tension to give active tension. A maximum of six strips were used from each patient.

Statistical Analysis

The responses of IAS strips were evaluated in a blinded fashion and the results are expressed as mean (± standard error of the mean). The number of replicates are expressed as n = *x* (*y*), where *x* is the total number of strips and *y* is the number of patients. Data were fitted using Microsoft Excel and values subsequently analyzed using SPSS 15.0 for Windows. Statistical analysis was performed by Student's *t*-test for unpaired samples, and *P* values less than 0.05 were considered significant.

RESULTS

Tone and spontaneous activity

After 90 minutes equilibration, IAS strips developed both myogenic tone and spontaneous activity (Figure 11 A, B).

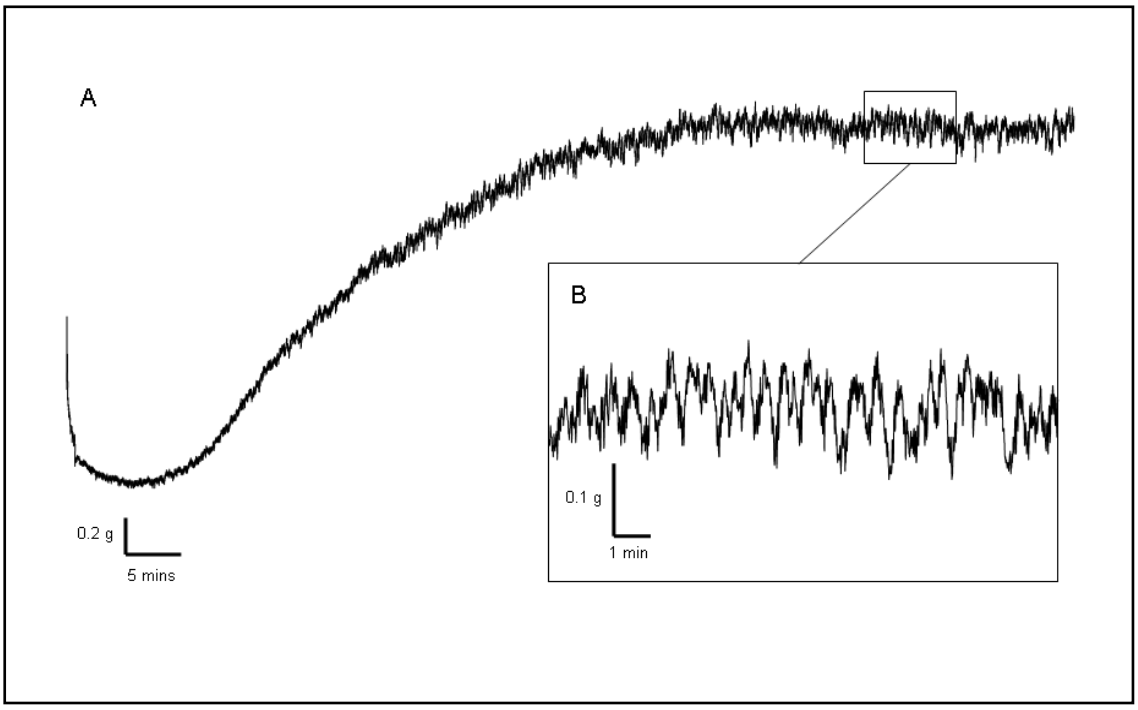

Figure 11

A. Representative trace showing the equilibration of strips from internal anal sphincter. Strips, initially loaded with 1 g of tension and allowed to equilibrate for 90 minutes, developed myogenic tone and spontaneous activity. **B.** Spontaneous activity was characterised by intermittent low-amplitude contractions (mins=minutes).

In CRT strips, the tone generated was 133.1 ± 16.6 mg/mg tissue (n=36(6)) compared to 198.8 ± 37.3 mg/mg tissue in controls (n=30(5)) (*P*=0.118).

Spontaneous activity, characterised by intermittent low-amplitude contractions superimposed on the basal tone, was calculated as the number of contractions ≥ 0.1 % of basal tone during an interval period of 10 minutes and was less in CRT strips (97.6 ± 3.3 contractions, n=36(6)) compared to control strips (139.4 ± 10.8, n=30(5), *P*=0.001).

Responses to electrical field stimulation

EFS produced an initial relaxation followed by a contraction. The relaxation response predominated at low stimulation frequencies (when the contraction was almost absent), whereas the contractile element was more marked at higher frequencies (Figure 12A). At 50 and 100 Hz, a faster onset contraction preceding relaxation was also seen in some cases. In preliminary experiments, both relaxation and contraction was blocked by 3 μM TTX, thus confirming that the response was nerve-mediated.

The responses were analysed taking into account the probability that the amount of relaxation and contraction is dependent on background developed tone. Therefore, they were calculated as a percentage to offset this effect. In

case of relaxation, the level of tone during perfusion with calcium free Krebs' was considered as maximal relaxation; whereas the amount of contraction was calculated as a percentage increase of tone from baseline tone after equilibration.

Relaxation responses to EFS of CRT strips (n=36(6)) were significantly reduced compared to control strips (n=30(5)) at all frequencies of stimulation except 10 Hz (*P*=0.008 at 1 Hz, *P*=0.035 at 5 Hz, *P*=0.096 at 10Hz, *P*=0.033 at 25 Hz, *P*=0.009 at 50 Hz and *P*=0.003 at 100 Hz) (Figure 12B).

Maximal relaxation was seen at 50 Hz, when EFS produced 25.6 ± 4.9 percent of maximum relaxation in the CRT group compared to 47.0 ± 6.2 percent in control strips.

L-NO Arg (10 μM), a nitric oxide (NO) synthase inhibitor, significantly reduced the relaxation response in CRT (n=36(6)) (*P*=0.008 at 1 Hz, *P*=0.012 at 5 Hz, *P*=0.005 at 10 Hz, *P*=0.001 at 25, 50 and 100 Hz) and control strips (n=30(5)) (*P*=0.007 at 1 Hz, *P*=0.000 at 5, 10, 25, 50 and 100 Hz) and indicated that this was nitrergic in nature (Figure 12B, significant *P* values are not shown in the Figure). Under these conditions, there were no significant differences between the two groups.

Contractile responses were significantly reduced in treated strips (n=36(6)) compared to controls (n=30(5)) at stimulation frequencies higher than 10 Hz

(*P*=0.546 at 1 Hz, *P*=0.143 at 5 Hz, *P*=0.045 at 10 Hz, *P*=0.003 at 25 Hz, *P*=0.007 at 50 Hz and *P*=0.002 at 100 Hz) (Figure 12C). Maximal contraction was observed at 100 Hz (5.4 ± 0.8 percent of tone increase in CRT strips compared to 21.1 ± 4.5 percent in control strips).

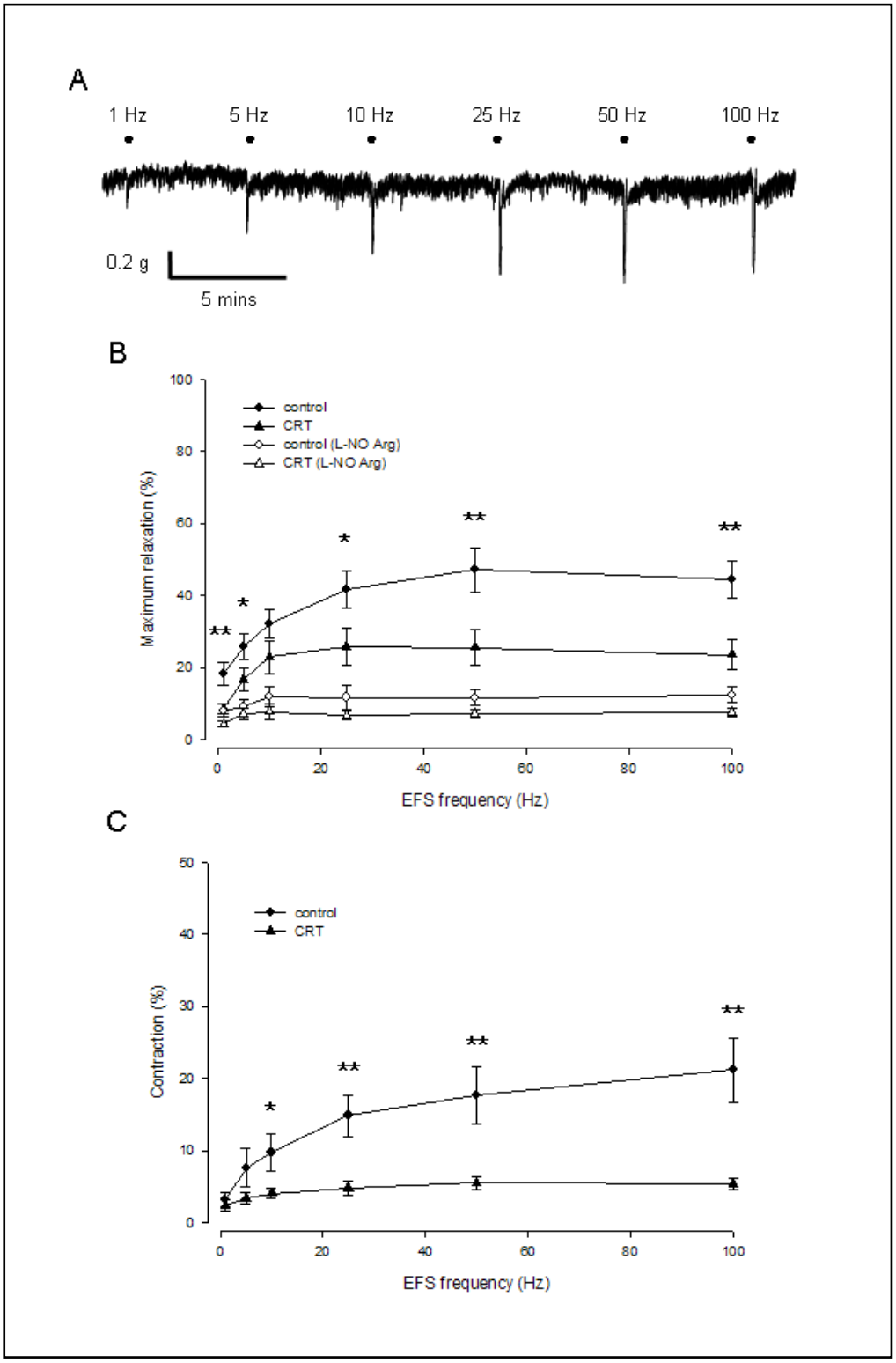
A
1 Hz
5 Hz
10 Hz
25 Hz
50 Hz
100 Hz
0.2 g
5 mins
B
control
CRT
control (L-NO Arg)
CRT (L-NO Arg)
Maximum relaxation (%)
EFS frequency (Hz)
C
control
CRT
Contraction (%)
EFS frequency (Hz)

Figure 12

A. Representative trace showing the response of internal anal sphincter strips to electrical field stimulation (EFS) in the absence of drugs (1 s train pulses, 0.5 ms duration, 10 V voltage and frequency varying from 1 to 100 Hz). (mins=minutes). **B.** Mean (± standard error of the mean) percentage of maximal relaxation of internal anal sphincter strips from patients treated with chemoradiotherapy (CRT) (n=36(6)) and controls (n=30(5)) in response of increasing frequencies of EFS. (*P<0.05 and **P<0.01 between CRT and control strips in absence of drugs). Responses after addition of 10 μM Nω-nitro-L-arginine (L-NO Arg) are also shown. L-NO Arg significantly reduced relaxation responses in both groups (significant P values are not shown), however there were no differences between them under these conditions. **C.** Mean (± standard error of the mean) percentage of contraction of internal anal sphincter strips from CRT (n=36(6)) and control strips (n=30(5)) in response of increasing frequencies of EFS. (*P<0.05 and **P<0.01).

Responses to chemical stimulation

IAS strips contracted in response to phenylephrine (10 μM), an α-adrenergic agonist (Figure 13A). There was a significant reduction in the contractile response to phenylephrine between CRT (n=36(6), 33.1 ± 6.2 percent of tone increase) and control strips (n=30(5), 62.7 ± 10.5 percent, *P*=0.021) (Figure 13B).

In contrast, IAS strips relaxed in response to carbachol (10 μM), a muscarinic cholinergic agonist (Figure 13C). Carbachol-induced relaxation was 32.0 ± 2.5 percent of maximum relaxation in CRT group (n=36(6)) compared to 49.2 ± 6.2 percent in controls (n=30(5), *P*=0.018) (Figure 13D).

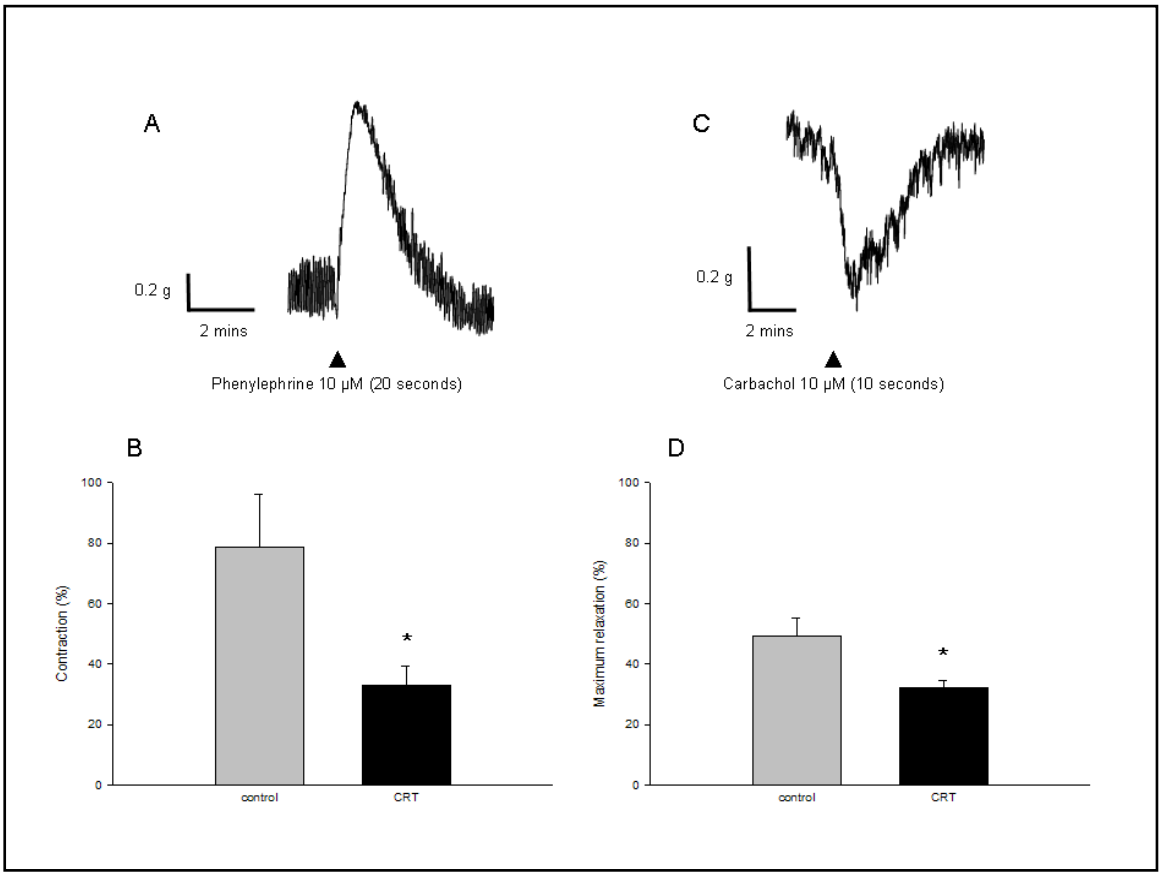

Figure 13

A. Representative trace showing the response of internal anal sphincter strips to application of 10 µM phenylephrine (mins=minutes). **B.** Mean (± standard error of the mean) percentage of contraction of internal anal sphincter strips from patients submitted to chemoradiotherapy (CRT) (n=36(6)) and controls (n=30(5)) in response to 10 µM phenylephrine. (*P<0.05). **C.** Representative trace showing the effects of 10 µM carbachol on internal anal sphincter strips (mins=minutes). **D.** Mean (± standard error of the mean) percentage of maximal relaxation of internal anal sphincter strips from CRT (n=36(6)) and control strips (n=30(5)) in response of 10 µM carbachol. (*P<0.05).

SNP, an NO donor, caused a dose-dependent reduction in tone of IAS strips of both groups (Figure 14A). There were no significant differences in concentration-response curves between treated (n=36(6)) and control strips (n=30(5)) (P=0.181 at 1 nM, P=0.800 at 10 nM, P=0.287 at 100 nM, P=0.937 at 1 μM and P=0.986 at 10 μM) (Figure 14B).

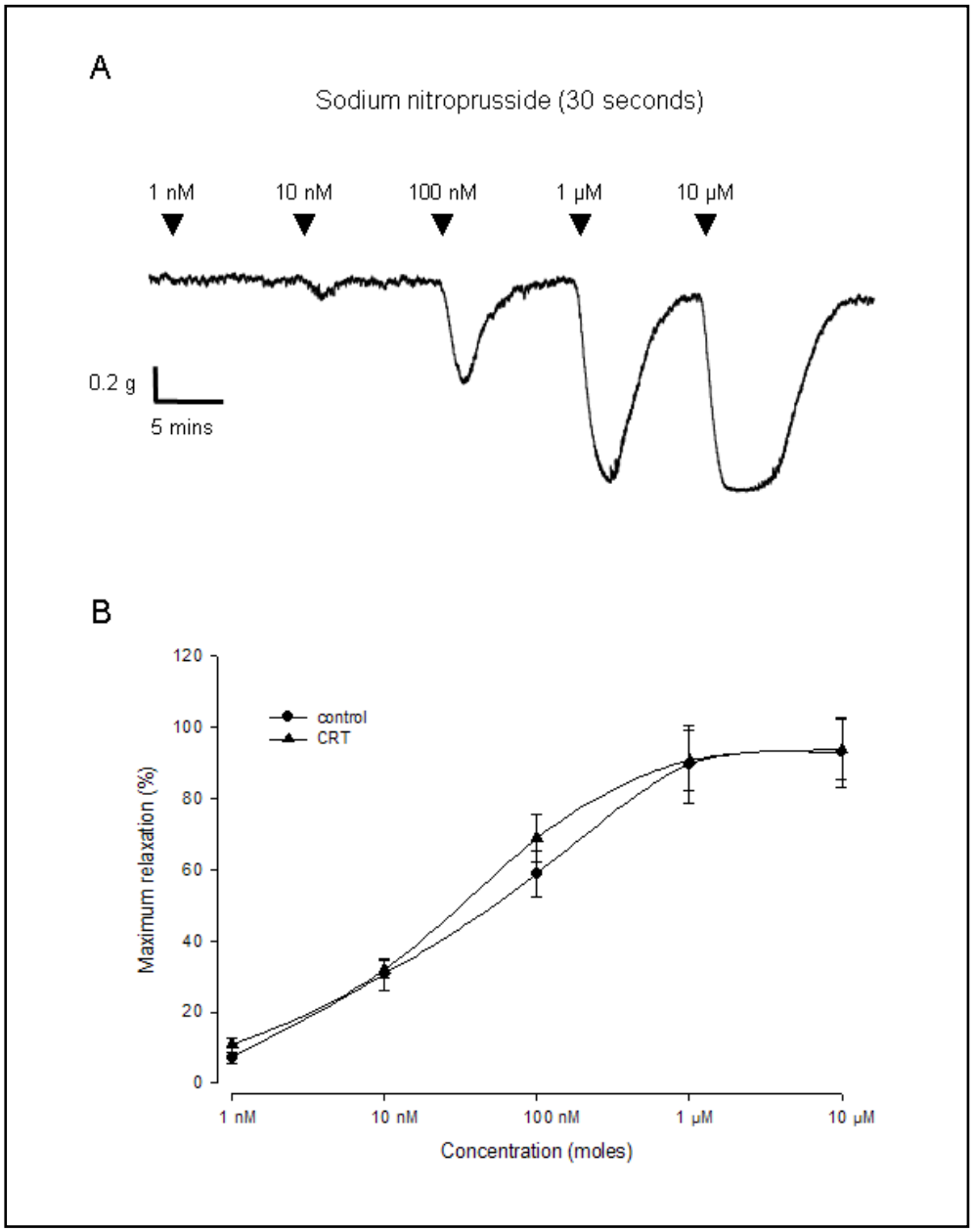
A
Sodium nitroprusside (30 seconds)
1 nM
10 nM
100 nM
1 µM
10 µM
0.2 g
5 mins
B
Maximum relaxation (%)
120
100
80
60
40
20
0
control
CRT
1 nM
10 nM
100 nM
1 µM
10 µM
Concentration (moles)

Figure 14

A. Characteristic trace showing the response of internal anal sphincter strips to application of increasing doses of sodium nitroprusside (1nM to 10μM) (mins=minutes). **B.** Concentration-response curves for sodium nitroprusside (1nM to 10μM) of internal anal sphincter strips from patients treated with chemoradiotherapy (CRT) (n=36(6)) and controls (n=30(5)). Values are mean (± standard error of the mean). There were no significant differences.

DISCUSSION

Several studies using questionnaires, clinical examination and anorectal physiological testing in patients treated either for rectal cancer or for other malignancies, have suggested that pelvic irradiation impairs anorectal function.[37-58]

To date, however, the evidence for the functional effects of pelvic irradiation on anal sphincters has been indirect and incomplete, based only on clinical and manometric alterations. The majority of the studies present in the literature lack uniformity and are a mixture of prospective and retrospective work, various target organs, pre- and post-operative treatments, different radiation delivery techniques and doses received by anal sphincters, various surgical procedures (with or without TME, pouch, etc.), variable extent of resection and length of rectum remnant, and short- and long-term results. In addition, some series have failed to demonstrate a significant impairment of sphincter function after radiotherapy.[43-44]

It is therefore difficult to interpret the currently available data and, in particular, as it has long been known that surgery alone adversely affects anorectal function of patients treated for rectal cancer, it is arduous to quantify to which extent the damage is caused respectively by surgery or pelvic irradiation.[36, 39, 49]

It is also important to consider that the mechanism of anal continence is complex and involves many structures and pathways, and each of them could be affected by CRT.

Consequently, the identification of the specific damage caused by each factor may offer physiological and pathological mechanisms to prevent and/or treat its dysfunction.

da Silva *et al*.[59] have documented progressive fibrosis of the smooth muscle and hypertrophy of the myenteric plexus in irradiated IAS of patients that had undergone APR compared to controls. Our study is the first to provide a physiological basis for CRT-induced impairment of IAS function in patients treated for rectal cancer.

In our experiments, the myogenic tone developed by strips from IAS of irradiated patients was not significantly different to that from controls. An explanation of this finding is difficult, because many factors are probably responsible for myogenic tone in the IAS and are not yet completely understood.[6, 60] Additional investigations will in future be necessary to elucidate the underlying mechanisms governing the myogenic tone in IAS strips, and, subsequently, to compare any differences in this phenomenon.

Strips from IAS of patients submitted to CRT exhibited less spontaneous activity than those from controls. The diminished spontaneous activity of the strips of the CRT group may simply reflect diffuse damage of the sphincter, characterised by increased muscle fibrosis and collagen deposition as previously described in sections of irradiated IAS.[59]. However, this cannot be the only explanation. Intracellular recording techniques have shown that sinusoidal membrane depolarizations are associated with the phasic muscle contraction of the cat IAS, and specialised pacemaking cells, the interstitial cells of Cajal (ICC), have recently been identified in human IAS.[14-16] These cells could be responsible for generating electrical waves and controlling the spontaneous activity in the IAS as they do in many other tissues, and damage to them induced by CRT could also explain our finding.[61]

EFS-induced relaxation was also significantly reduced in patients treated with CRT compared to controls at all frequencies of stimulation except 10 Hz.

To investigate whether this was related to a direct damage to intrinsic nerves of the myenteric plexus, or secondary to muscle injury, we tested the IAS strips responses after application of an inhibitor of NO synthase (L-NO Arg) and an exogenous NO donor (SNP).

The addition of L-NO Arg significantly reduced the relaxant responses of IAS strips in both groups confirming that the relaxation was NO-mediated. No

differences between strips of the two groups were observed under these conditions.

SNP mimicked the effect of EFS and caused a dose-dependent reduction in tone of IAS strips. There were no significant differences in concentration-response curves between control and treated strips. These results, despite obtained in a relatively small sample, indicate that CRT has more striking effects on intrinsic nerves, which seem to be more susceptible to radiation-induced injury than the smooth muscle itself.

A correlation with the histological findings was not available in our work; however, da Silva *et al.*[59] found nerve alterations more evident than fibrotic changes in the slides of IAS of irradiated patients.

Furthermore, the relaxation induced by carbachol (10 μM) was reduced in strips from irradiated IAS compared to those from controls. It has been demonstrated that carbachol, a cholinergic agonist, produces relaxation of IAS via muscarinic receptors present on nitrergic nerves.[1] Therefore, this also supports the theory that damage to intrinsic nerves is caused by pelvic irradiation; however, there may also be a direct injury of the muscarinic receptor.

Typically, EFS-evoked relaxation was followed by a slower onset contraction, particularly evident at high frequencies. We found that the contractile element

of the EFS response was also reduced in IAS strips of irradiated patients. Previous studies have shown that this contraction can be blocked by the addition of guanethidine, demonstrating that is mediated via adrenergic nerves.[62] Therefore, CRT could be responsible for an injury to the sympathetic nerves responsible for this contraction. Alternatively, the effects of CRT may affect a subgroup of nerves regulating sympathetic activity or directly damage the smooth muscle.

Interestingly, a significant reduction in the contractile response of strips from patients submitted to CRT was also observed after application of phenylephrine (10 μM). It is well documented that adrenergic agonists evoke a contraction of IAS.[1] Thus, this suggests that the damage induced by CRT is not selective to the intrinsic nerves, but also affects the muscular component of the IAS.

The effect of CRT on EAS were not examined in this study because of the difficulty of finding EAS to use as controls and of studying the EAS together with the pudendal nerves. In addition, the EAS seems to be more resistant to radiation-induced injury than IAS, probably because the somatic nerve axons and striated muscle are relatively resistant to radiation.[37, 46, 63] Nevertheless, the data present in the literature are contradictory and some studies have

shown a decrease of anal squeeze pressures in irradiated patients compared to controls.[41, 48, 50, 52, 53, 57]

It is also noteworthy that five of the six patients who underwent adjuvant treatment received a combination of radio- and chemotherapy, and chemotherapy may have played a part in the injury.

In this study, the control group was mainly composed of young patients. Therefore, a detrimental effect related to age on the function of IAS of CRT patients can not be excluded.[64] However, age did not influence anorectal function in the series of healthy adults published by Rao *et al*.[65]

It is important to highlight that we collected the specimens at the time of surgery, few weeks after neoadjuvant treatment, and, thus, we have evaluated the short-term effects of CRT on IAS. However, studies with long-term follow-up have shown that manometric pressures remain low and symptoms are permanent in many patients when the anal sphincters are irradiated.[40, 41, 48, 52, 54, 57]

Furthermore, this was an experimental *in vitro* work on the effects of CRT on IAS function in a relatively small number of patients, due to the difficulty of finding human tissue for laboratory research. Further *in vivo* studies are

required to strengthen these results and explore the damage induced by CRT to different structures involved in the mechanism of anal continence.

In conclusion, this study clearly shows that CRT adversely affects the function of IAS in patients treated for rectal cancer and provides a physiological basis to the clinical and histological alterations described previously. Taking in consideration our results and the current available data in the literature, we can advice the exclusion of the anal canal from the field of irradiation when this is oncologically safe. The intrinsic nerves seem to be more susceptible to radiation injury than smooth muscle, although a muscular damage also occurs. Further studies are required to clarify the molecular mechanisms of this phenomenon.

CONCLUSIONS

Various experimental and clinical studies have investigated the pharmacological properties of the internal anal sphincter (IAS). These researches have elucidated the mechanisms of the intrinsic and extrinsic pathways that regulate IAS motility.

This study develops new insights on IAS physiology analyzing the electrical activity of smooth muscle cells. Specialised pacemaker cells, the interstitial cells of Cajal (ICC), expressing the proto-oncogene c-kit, has been shown to regulate the spontaneous activity of the smooth muscles in the gastrointestinal tract. Recently, ICC have been described in human anal sphincters; however, their role in this specialised tissue is still unknown. The effects of the c-kit tyrosine kinase inhibitor Imatinib Mesylate (Glivec®) on IAS human strips have been examined in order to investigate the function of the ICC in the IAS. The application of Glivec® at concentration higher than 5×10^{-6} M significantly reduced the tone and the spontaneous activity of the strips. No statistical significant differences were observed in the responses to EFS, carbachol, and phenylephrine. These results suggest that ICC play a role in modulating the tone and regulating the spontaneous activity of the IAS.

The identification of ICC in the IAS revealed a cell population, involved in anal sphincter motility, that may provide a foundation for new approaches to preclinical and clinical research.

Neo-adjuvant chemoradiotherapy has been shown to reduce reduce the risk of recurrence and improve survival of patients with high risk rectal cancer and is currently recommended in their treatment. However, several reports have suggested that chemoradiotherapy adversely affects anorectal function.

Investigations on functional changes of the IAS after chemoradiotherapy were performed on IAS strips from patients undergoing abdominoperineal resection or proctectomy. Five patients were treated by surgery alone, and six received pre-operative radiotherapy.

IAS strips were mounted in organ baths and monitored the responses to electrical field stimulation (EFS) and different drugs. Significant differences, between irradiated strips *vs* controls, were observed in the spontaneous activity of the IAS and in the responses to EFS ($p<0.01$), Nω-nitro-L-arginine ($p<0.01$), carbachol ($p<0.05$) and phenylephrine ($p<0.05$). These results suggest that adjuvant chemoradiotherapy impair IAS function contributing to worsen anal continence. Intrinsic nerves seem to be more susceptible to muscle cells and the reduction of the spontaneous activity of irradiated IAS strips may suggest a damage of ICC.

ABSTRACT

The role of the interstitial cells of Cajal (ICC) in the internal anal sphincter (IAS) is unknwon. These cells express the proto-oncogene c-kit, and their function was evaluated using the c-kit tyrosine kinase inhibitor Imatinib Mesylate. The tone and the spontaneous activity of IAS were significantly reduced. No significant differences were observed in the responses to EFS, carbachol and phenylephrine. The ICC may play a role in modulating the tone and the spontaneous activity of the IAS.

Chemoradiotherapy has been shown to reduce the risk of recurrence and improve survival in patients with locally advanced rectal cancer. An increasing number of patients have been receiving neoadjuvant treatment before surgery. However, there is evidence that chemoradiotherapy adversely affects anorectal function.

The functional changes of the IAS after chemoradiotherapy on strips from patients undergoing pelvic surgery were studied. Strips were mounted in organ baths and responses to electrical field stimulation (EFS) and drugs were monitored. Patients treated by surgery alone formed the control group. We found significant differences in the responses to EFS, Nω-nitro-L-arginine, carbachol and phenylephrine. Chemoradiotherapy impaired anal continence damaging the IAS function.

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