Effects of neo-adjuvant chemotherapy for oesophago-gastric cancer on neuro-muscular gastric function

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Running title: Effects of chemotherapy on gastric contractility

Abbreviations: EFS, electrical field stimulation; AChE, acetylcholinesterase; nNOS, neuronal nitric oxide synthase

SUMMARY

Introduction : Delayed gastric emptying and ensuing troublesome symptoms are often reported after chemotherapy has ended. This study aims to characterise the effects of chemotherapy on gastric neuro-muscular function.

Methods : Patients undergoing elective surgery for oesophago-gastric cancer were recruited. Acetylcholinesterase, nNOS, ghrelin receptor and motilin expressions were studied in gastric sections from patients receiving no chemotherapy (n = 3) or oesophageal (n = 2) or gastric (n = 2) chemotherapy. A scoring system quantified staining intensity (0 - 3; no staining to strong). Stomach sections were separately suspended in tissue baths for electrical field stimulation (EFS) and exposure to erythromycin or carbachol; 3 patients had no chemotherapy; 4 completed cisplatin-based chemotherapy within 6 weeks prior to surgery.

Results: AChE expression was markedly decreased after chemotherapy (scores 2.3 ± 0.7, 0.5 ± 0.2 and 0 ± 0 in non-chemotherapy, oesophageal- and gastric-chemotherapy groups ($P<0.03$ each) respectively. Ghrelin receptor and motilin expression tended to increase (ghrelin: 0.7 ± 0.4 vs 2.0 ± 0.4 and 1.2 ± 0.2 respectively; *p=0.04* and *p=0.2*; motilin: 0.7 ± 0.5 vs 2.2 ± 0.5 and 2.0 ± 0.7; *p=0.06* and *p=0.16*). Responses to EFS and erythromycin were inconsistent in each group. Maximal contraction to carbachol was 3.7 ± 0.7g and 1.9 ± 0.8g (longitudinal muscle) and 3.4 ± 0.4g and 1.6 ± 0.6 (circular) in non-chemotherapy and chemotherapy tissues respectively ($p < 0.05$ each). **Conclusion :** Most notable was the loss of AChE and reduction in contractility to carbachol. The tendency for ghrelin receptors to increase suggests an attempt to upregulate compensating systems. Our study offers a mechanism by which chemotherapy markedly alters neuro-muscular gastric function and helps explain patients symptoms.

Keywords : Acetylcholinesterase, cholinergic, neuronal nitric oxide synthase, motilin, ghrelin, motility, stomach, chemotherapy, cisplatin, human

INTRODUCTION

Neoadjuvant chemotherapy for oesophageo-gastric cancers prior to surgery is now common practice following clinical trials that showed survival improvement (2002;Cunningham et al. 2006). However following completion of chemotherapy, these patients frequently continue to experience distressing symptoms resembling dyspepsia and attributed to a disorder in gastric emptying (Nelson, Walsh, & Sheehan 2002). Riezzo et al. (2005), suggested that gastric dysmotility-like symptoms (susceptibility to nausea, early satiety, post-prandial fullness) and tachygastria were common in patients seven days after completion of chemotherapy, during which emesis was well controlled. Such studies raise the possibility that the anti-cancer chemotherapy may somehow induce a gastric dysmotility which outlasts the treatment and which may lead to prolonged symptoms of dyspepsia. For this to occur, it is certainly not inconceivable that chemotherapy directly affects the integrity of the neuromuscular functions of the stomach. In rat gastro-duodenal muscle, for example, a reduction in the presence of the Ca^{2+}-calmodulin complex has been observed three days after a single treatment with cisplatin (Jarve & Aggarwal 1997).

To test the possibility that certain anti-cancer chemotherapy treatments may cause long-term damage to gastric function, we have looked at the effects of cisplatin-based treatments on the function and integrity of human stomach. For this purpose we examined the ability of human isolated stomach to contract in response to carbachol and other stimuli, following removal at surgery from patients who had undergone chemotherapy up to six weeks previously, compared to patients who had not

undergone chemotherapy. The integrity of the motor nerve system was also examined by immunohistochemical staining for acetylcholinesterase (AChE) and for the neuronal isoform of nitric oxide synthase (nNOS). In addition, we looked for changes in expression of the gastric motility stimulant motilin (Sanger 2008) and the receptors for ghrelin. The latter was included because of an association between ghrelin and changes in appetite and gastric motility (Inui et al. 2004) and also because of a previous study in rats, which showed that the ghrelin receptor may be up-regulated following administration of cisplatin (Malik et al. 2008).

METHODS

All patients enrolled were diagnosed with resectable oesophageo-gastric cancers. The study was approved by the local Coventry Research Ethics Committee and written informed consent was obtained from all patients.

Patients with gastric adenocarcinomas (n =2) received 3 courses of epirubucin ($50mgm^{-2}$), cisplatin ($60mgm^{-2}$) and capecitabine ($625mgm^{-2}$). Patients with oesophageal cancers (n = 2) received 2 courses of cisplatin ($80mgm^{-2}$) and 5-fluorouracil ($1Gm^{-2}$). Patients who did not undertake chemotherapy (n = 3) were physically frail, deemed at risk of severe side effects of chemotherapy or undergoing Whipple's procedure for pancreatobiliary tumours. All patients underwent surgical resection within a 2 week window of 4 to 6 weeks following completion of chemotherapy regime, based on local cancer pathway.

For the histological studies, paraffin-embedded samples were used for staining and immunohistochemistry. In the gastric tissue contractility experiments, gastric tissue was obtained from tumour free margins during surgery. Samples were then stored at $4^{o}C$ in Gey's balanced salt solution (Sigma, Gillingham, Dorset, UK) which was pre-

bubbled with 95% O_2/ 5% CO_2. These tissues were processed within 24 hours of collection (see below).

Immunohistochemistry and Histological Staining

Histological paraffin embedded tissue blocks showing normal tissue adjacent and distant from the tumour site were selected. 5μm thick sections were baked onto 3-aminopropyltriethoxy-silane coated slides. Specific antibodies were used to look for the expression of motilin, the ghrelin receptor, AChE and nNOS, in sections of gastric tissues taken from patients who did not have chemotherapy and in those receiving oesophageal-chemotherapy (cisplatin + 5-fluorouracil) or gastric chemotherapy (capecitabine).

Antigen retrieval was carried out by pressure cooking in Tris EDTA buffer pH 7.8 for 80 seconds. Vector Universal Elite ABC kit (Catalogue Number PK-6200; Burlingame, CA, USA) with a diaminobenzidine tetrahydrachloride visualisation agent (Vector ImPACT DAB substrate, Cat. No. SK-4105, Burlingame, CA, USA) was used for visualization of bound antibody. Optimised antibody dilutions were determined for motilin receptor (MBL International Corp, Woburn, MA, USA, Cat. No. LS-A134), ghrelin receptor (Chemicon, Billerica, MA, USA, Cat. No. AB9543), nNOS (Abcam, Cambridge, UK, Cat. No. ab40662) and AChE (Abcam, Cambridge, UK, Cat. No. ab2803). Haematoxylin was used as a counter stain, dehydrated, cleared and mounted. The intensity and distribution of peroxidase staining was examined and scored by a histopathologist who was blinded to the patient groups. For all procedures, a semi-quantitative scoring system for staining intensity was used (0 = no staining, 1 = weak, 2 = moderate, 3 = strong expression).

Gastric contractility

Specimens of stomach were placed in oxygenated (95% O_2, 5% CO_2) Gey's salt solution overnight. In some cases, on the following morning the tissues were placed in fresh Krebs solution, bubbled and then transported to the laboratory. On arrival the specimens were placed in fresh, oxygenated Krebs solution (containing in 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) which had been equilibrated with 5% CO_2 and 95% O_2. The mucosa was removed and strips (2-4 x 10-15 mm) were cut parallel to the longitudinal or the circular muscle. The strips (2-4 from each patient) were mounted in tissue baths (10 ml) containing Krebs solution at 37°C and gassed with 5% CO_2 in O_2. Tension was measured using Dynamometer UF1 force-displacement transducers (Pioden Control Ltd., UK). Data acquisition and analysis were performed using MP100 hardware and AcqKnowledge software (Biopac Systems Inc., USA). Tissues were initially suspended under 2-3g tension and allowed to equilibrate for at least 45 min during which time bath solutions were changed every 15 min. During this time, muscle tension stabilised at ~1 g.

At the end of the equilibration period, some longitudinal muscle strips were stimulated via two parallel platinum ring electrodes connected to a stimulator (STG2008, Scientifica Ltd, UK). The stimulation parameters were 50 V (~200 mA), 0.5 ms bipolar pulse duration, applied for 10 s, every 1 min. The frequency was changed every 3 min to produce a frequency-response curve, using frequencies of 1, 2, 5, 10, 15 and 20 Hz. After washing and in tissues not subjected to EFS, 10µM erythromycin was added for 15 min. As not all tissues responded to EFS or erythromycin, the data are expressed simply as the number of tissues which responded by contraction, or not. The tissues were washed and left for 30 min. For both longitudinal and circular muscle strips, a carbachol concentration-response curve

(1nM – 10 μM) was then constructed, with each additional concentration being added after a maximum response was observed to the previous, usually between 2-8 min). Changes in muscle tension (g) evoked by each carbachol concentration were expressed in grams.

Data analysis

All data are expressed as Means ± the Standard Error of the Mean (s.e.m.), or as Mean values with ranges; n values are numbers of patient donors. In the contractility studies, any differences in responses to carbachol between the two treatment groups were analysed using a two-way ANOVA with Bonferroni post-hoc test. Data from the immunohistochemistry study was analysed using the Student's paired t test.

Compounds

All drugs were freshly prepared before use. Erythromycin (Sigma, Gillingham, Dorset, UK) was dissolved in 100% ethanol, with subsequent dilution in distilled water. Carbachol (Sigma, UK) was dissolved in distilled water.

RESULTS

Histology and Immunohistochemistry

There were no clear differences in age for each of the patient groups. More males than females were recruited although the numbers are too small to observe meaningful differences (Table 1).

There was a marked decrease in expression of AChE (the scores were, respectively, 2.3 ± 0.7, 0.5 ± 0.2 and 0 ± 0 in non-chemotherapy vs oesophageal- and gastric-

chemotherapy groups (*p=0.03* and *p<0.001* respectively) - each compared with the non-chemotherapy tissues; Figures 1 and 2. By contrast, there was a tendency for ghrelin receptors and for motilin to increase, although this did not always achieve statistical significance (ghrelin receptor: 0.7 ± 0.4 in non-chemotherapy vs 2.0 ± 0.4 and 1.2 ± 0.2 in oesopheal- and gastric-chemotherapy groups respectively, *p=0.04* and *p=0.2*; motilin: 0.7 ± 0.5 vs 2.2 ± 0.5 and 2.0 ± 0.7, p=0.06 and *p=0.16*, respectively). There were no consistent changes in nNOS (1.2 ± 0.6 in non-chemotherapy vs 1.2 ± 0.6 and 0.5 ± 0.34 in oesophageal and gastric-chemotherapy respectively. *p=0.3* respectively).

Gastric contractility

Table 1 illustrates patient demographics. As before, there were more males than females. Of the four patients receiving chemotherapy, three had gastric-chemotherapy and one had oesophageal-chemotherapy. The patient numbers were too small for subgroup analysis and hence, were analysed together as a single chemotherapy group. Longitudinal muscle preparations from either group did not consistently contract in response to EFS or erythromycin. Contraction could be evoked by EFS in 2 of the 3 non-chemotherapy tissues (threshold 2-5 Hz) and in 1 of 4 chemotherapy tissues. Similarly, erythromycin 10 μM increased basal tone or contractility in some tissues, but with no obvious difference between chemotherapy and non-chemotherapy tissues (4 of 8 non-chemotherapy tissues responded, compared to 2 of 4 tissues from patients receiving chemotherapy).

Application of carbachol 1 nM – 10 μM caused a concentration-dependent contraction of both longitudinal and circular muscle tissues from both patient groups (Figure 3). However, there were clear differences between the groups of patients in terms of the

force of contraction evoked by carbachol. The maximal contraction produced by 10 μM carbachol in longitudinal muscle, for example, was 1.9 ± 0.8 g and 3.7 ± 0.7 g in chemotherapy and non-chemotherapy tissues respectively ($p<0.05$) and in circular muscle, these values were 1.6 ± 0.6 and 3.4 ± 0.4 g in chemotherapy and non-chemotherapy tissues ($p<0.05$).

DISCUSSION

Our preliminary results indicate, for the first time, that marked changes in the contractility and integrity of the human stomach are caused by cisplatin-based chemotherapy.

The absence of staining for AChE and the significant reduction in contractility to carbachol suggests that cisplatin profoundly influences gastric cholinergic function. These findings are consistent with the reported ability of cisplatin to affect cholinergic function in conscious rats (causing an increase in [^{3}H]-choline uptake into the cardiac region of the stomach), although not with accompanying change in gastric AChE staining, measured three days after treatment in the same study (Aggarwal et al. 1994). Differences in species, duration of chemotherapy and or length of time after chemotherapy are likely to contribute to the differences observed.

The reduction in cholinergic function was not clearly accompanied by a change in nNOS expression, suggesting that any sustained influence of cisplatin on enteric nerve function is not uniform throughout this nervous system. The effects of chemotherapy on the nNOS isoform has previously not been studied in humans, although in rats receiving cisplatin or taxol, reductions in expression of the inducible form of NOS expression are reported (Wang & Aggarwal 1997), along with increases in plasma nitric oxide (Nagahama et al. 2002). Conversely Jarve et al (5) showed no changes in

NOS localisation or intensity of staining five days after dosing with cisplatin in rats. Taken together, these studies suggest the possibility that NOS expression may be affected by cisplatin treatment but in our study, the changes are either too subtle to be detected using the present methods or perhaps changes occur immediately on treatment and recover thereafter.

The tendency for the expression of both ghrelin receptors and of motilin to be increased up to six weeks after cisplatin treatment does not, by itself, indicate that the functions of ghrelin and motilin are changed by chemotherapy. However, the observed trend is consistent with rodent studies. For example, Malik et al (2008) found that ghrelin receptor mRNA expression in rat stomach was increased two days after treatment with cisplatin, at a time when the cisplatin-induced gastric stasis was at its greatest; there were no changes in ghrelin mRNA expression. This up-regulation of ghrelin receptor expression suggested that ghrelin might be operating as part of a defence mechanism against the damage caused by cisplatin; this hypothesis is proposed because of the known ability of ghrelin to inhibit cisplatin-induced emesis, promote appetite and increase gastric emptying (Liu et al. 2006;Malik et al. 2008). However, the present findings, measured up to six weeks after discontinuation of treatment with cisplatin, suggest that if ghrelin were to play a similar defensive role in humans, any effects of ghrelin are likely to be overwhelmed by the more severe reduction in cholinergic function. A similar defensive role for motilin has not previously been proposed and although tempting to speculate that the gastric prokinetic activity of motilin would serve this purpose - the small numbers and lack of statistical significance limits our interpretation. Interestingly, Hursti et al. (2005) found that plasma levels of motilin were transiently decreased 4 days after a cisplatin-based treatment.

In summary, our study suggests that neo-adjuvant chemotherapy can cause marked and enduring damage to neuro-muscular gastric function. Most notable was the loss of AChE and reduction in contractility evoked by carbachol, providing a plausible explanation for some of the severe symptoms of gastric stasis reported by patients long after completion of chemotherapy. In addition, the tendency for ghrelin receptors to increase may be consistent with studies in animals exposed to cisplatin and suggests an attempt to up-regulate compensating systems. The consequences of these changes merits further assessment in the clinical setting.

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CONFLICTS OF INTEREST

The authors state no conflicts of interest. EZHS was funded by GSK.

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FIGURE LEGENDS

Figure 1.

Immunohistochemistry (IHC) scores for AChE comparing non-chemotherapy and oesophageal-chemotherapy (a) and gastric-chemotherapy (b) exposed tissues. Mean IHC scores were compared using the Student's paired t-test. $p<0.03$ when comparing AChE scores in non-chemotherapy and chemotherapy groups.

Figure 2.

Fig 2(a) Gastric section from a non-chemotherapy patient. Acetylcholinesterase staining showing positive (brown) cytoplasmic staining within ganglion cells (arrows). Fig (2b) Gastric section from a chemotherapy exposed patient. Despite an intensive search definite ganglion cells were not identified in this case, possibly because of degeneration from chemotherapy. The image shows a representative field illustrating negative staining in a neurovascular bundle. Original magnification x400.

Figure 3.

Concentration-response curves for carbachol-induced contraction of human isolated stomach. The contractions were compared using preparations obtained from patients treated with chemotherapy and from those who were not. * $p < 0.05$ (two way ANOVA with bonferroni post-hoc test) when the concentration-response curves were compared (chemo longitudinal muscle vs non-chemo longitudinal and chemo circular muscle vs non-chemo circular); n = 4 and 3, respectively, for both longitudinal and circular muscle.

Table 1.

Patient demographics in the immunohistochemistry and the contractility study.

Immunohistochemistry study			
	Non-chemotherapy	**Oesophageal-chemotherapy**	**Gastric-chemotherapy**
Female:Male ratio	1:5	1:5	2:4
Mean age	76	71	63
Contractility study			
	Non Chemotherapy	**Chemotherapy**	
Mean Age	**74**	**63**	
Female:Male ratio	**2:1**	**0:4**	