Alterations of the small intestinal wall and motor function after repeated cisplatin in the rat.

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ABSTRACT

Background. Gastrointestinal adverse effects occurring during cancer chemotherapy are well known and feared; those persisting once treatment has finished are relatively unknown. We characterized the alterations occurring in the rat small intestine, after repeated treatment with cisplatin. **Methods**: Male Wistar rats received saline or cisplatin (2 mg kg^{-1} $week^{-1}$, for 5 weeks, ip). Gastric motor function was studied non-invasively throughout treatment (W1-W5) and one week after treatment finalization (W6). During W6, upper gastrointestinal motility was also invasively studied and small intestinal samples were collected for histopathological and molecular studies. Structural alterations of the small intestinal wall, mucosa, submucosa, muscle layers, and lymphocytic nodules were histologically studied. PAS staining and immunohistochemistry for Ki-67, chromogranin A and neuronal specific enolase (NSE) were used to detect secretory, proliferating, endocrine and neural cells, respectively. The expression of different markers in the *tunica muscularis* was analyzed by RT/qPCR. **Key results:** Repeated cisplatin induced motility alterations during and after treatment. After treatment (W6), the small intestinal wall showed histopathological alterations in most parameters measured, including a reduction in the thickness of circular and longitudinal muscle layers. Expression of c-KIT (for interstitial cells of Cajal, ICC), nNOS (for inhibitory motor neurons), pChAT and cChAT (for excitatory motor neurons) increased significantly (although both ChATs to a lesser extent). **Conclusions and inferences**: Repeated cisplatin induces relatively long-lasting gut dysmotility in the rat associated to important histopathological and molecular alterations of the small intestinal wall. In cancer survivors, the possible chemotherapy-induced

histopathological, molecular and functional intestinal sequelae should be evaluated.

KEYWORDS: cisplatin, gastrointestinal motility, histopathology, gene expression, chemotherapy-induced adverse effects.

KEY MESSAGES.

- Among other adverse effects, antineoplastic drugs induce gastrointestinal dysmotility whose underlying molecular and structural alterations in the gut wall have not been well characterized yet.
- In the rat, one week after a 5-cycle cisplatin, intestinal dysmotility was accompanied by histological damage, reduced thickness of submucosa and muscular layers, increased numbers of goblet cells, inflammatory nodules and enterochromaffin cells and, at the molecular level, a relatively higher expression of nNOS (a neuronal marker of inhibitory motor function).
- Gut dysmotility and different structural and molecular alterations in the gut wall occur after chemotherapy; these findings are clinically relevant in view of the increasing rates of cancer survivorship.

Cancer chemotherapy is associated with numerous and feared adverse effects including those affecting the gastrointestinal tract. Anorexia, nausea, emesis and/or diarrhea are typically encountered during treatment, but these effects are generally restricted to the day of administration and maybe a few more days after each cycle[1].

Chemotherapy-induced nausea and vomiting is a clinically significant adverse effect [1,2]. In the rat, which does not vomit, gastric stasis and pica (the intake of non-nutritive substances[3]), are used as indirect markers of nausea/emesis[4,5]. Like in humans, both markers were acutely triggered in the rat within the few hours after each cisplatin administration during a cyclic treatment, but these events were not apparent one week after treatment finalization[6,7]. Interestingly, some degree of basal, 'facilitated' pica developed throughout treatment and remained one week after[6-8]. This might constitute a defensive mechanism against the aggressive stimulus represented by repeated cisplatin in the rat[9]. 'Facilitated' pica could be due to the development of some relatively long-term alterations within the gut[6,8].

Other adverse effects induced by antitumoral drugs, like neurotoxicity, might be difficult to get rid of. Thus, peripheral neuropathy is frequently developed and affects not only sensory afferents, with the consequences of relatively persistent dysesthesia and pain symptoms[10,11], but also the autonomic nervous system, rising the possibility that the function of visceral organs might be consequently uncoupled[12]. Indeed, we described that repeated cisplatin in the rat induced the development of an enteric neuropathy affecting the myenteric plexus[13], which is the component of the enteric nervous system (ENS) responsible for intrinsically controlling gastrointestinal motility. Afterwards, Nurgali et al showed that chronic

oxaliplatin also induces an enteric neuropathy associated to colonic dysmotility in mice[14]. This enteric neuropathy induced by platinum antineoplastic drugs might contribute to the development of long-lasting gastrointestinal dysmotility[7,13,14] and maybe also to 'facilitated' pica[6-8].

The interstitial cells of Cajal (ICC) are in close association with both myenteric neurons and smooth muscle cells and play an essential role in gastrointestinal motor function, particularly in the coordination of contractile activity. Thus, ICC generate and propagate the so-called 'slow waves' (pacemaker activity), regulate ENS activity, and function as stretch mechanoreceptors in the *tunica muscularis*[15,16]. ICC, smooth muscle cells, and other components of the gut wall (enterocytes, enterochromaffin cells…) could also be altered by repeated cisplatin and contribute to alterations in motility and other gut functions.

Taking into account the increasing number of cancer survivors[17], the systematic analysis of the possible relatively long-term chemotherapy-induced alterations affecting the gastrointestinal tract constitutes an urgent need. Therefore, our aim is to characterize the structural, molecular and functional alterations occurring within the gastrointestinal tract after cyclic chemotherapy. In the present work, we show new data on the alterations induced by repeated cisplatin in the rat, one week after treatment finalization, on: small intestinal motility; general small intestinal wall structure, as well as histologically- and immunohistochemically-identified cell populations; and expression of markers pertinent to motor function of the *tunica muscularis* in the small intestine.

MATERIALS AND METHODS

The experiments were designed and performed in accordance with the European and Spanish legislation on care and use of experimental animals (2010/63/UE for animal experiments; Real Decreto 53/2013), and were approved by the Ethic Committee at Universidad Rey Juan Carlos (URJC).

Animals and treatment

Male Wistar rats (350-400 g) were obtained from the Veterinary Unit of URJC or Hospital General Universitario Gregorio Marañón (HGUGM), depending on the particular study, and housed (4 animals each cage) in standard transparent cages (60 cm x 40 cm x 20 cm), under environmentally controlled conditions (temperature = 20ºC; humidity = 60%), with a 12 h light/darkness cycles. Animals had free access to standard laboratory rat chow (Harlan Laboratories Inc.) and tap water.

Rats were acclimated for a week (W0) and thereafter received one intraperitoneal injection of cisplatin (2 $mgkg^{-1}$) or saline (0.9 % NaCl wv^{-1}, 1 $mLkg^{-1}$) once a week for 5 weeks (W1-W5). In order to prevent eventual nephrotoxicity induced by weekly cisplatin, 2 mL of saline were also injected subcutaneously just before intraperitoneal saline or cisplatin[18].

Throughout treatment (W1-W5) and one week after treatment finalization (W6), different experiments were performed as described below.

Gut motility experiments

Two different sets of experiments were performed in order to evaluate the alterations induced by cisplatin on gut motility.

First, radiographic techniques were applied at HGUGM in order to non-invasively analyze alterations in gastric motility induced by cisplatin[5]. For this, immediately after the first (acute effect) or last (chronic effect) drug injection, or one week after treatment finalization (W6, to evaluate the occurrence of residual effects), 2.5 mL of a suspension of barium sulfate (Barigraph ® AD, Juste SAQF, Madrid, Spain; 2gmL-1, tº= 22ºC) was administered per *os*. Plain facial radiographs of the gastrointestinal tract were obtained using a Siemens (Siremobil Compact L, Erlangen, Germany) digital X-Ray apparatus (60kV, 7mA) and captured with NPG Real DVD Studio II software. Exposure time was adjusted to 0.06 s. Immobilization of the rats in prone position was achieved by placing them inside adjustable hand-made transparent plastic tubes, so that they could not move. Habituation to the recording chamber prior to commencement of the study did not significantly alter gastrointestinal motility[5]. To further reduce stress, rats were released immediately after each shot (immobilization lasted for less than 2 min). X-rays were recorded at different times (immediately and 1, 2, 3 and 4h: T0-T4) after administration of the contrast medium. While X-ray shooting, the qualified investigator remained, behind a lead screen, at least 2 m away from the X-ray source.

Analysis of the radiographs was performed by a trained investigator blind to the drug administered. Alterations in gastric motility were semi-quantitatively determined from the images by assigning a compounded value to the stomach considering the following parameters: percentage of the stomach filled with

contrast (0-4); intensity of contrast (0-4); homogeneity of contrast (0-2); and sharpness of the stomach profile (0-2). Each of these parameters was scored and a sum (0-12 points) was made.

In the second set of motility studies, performed at the URJC, upper gastrointestinal transit was measured by invasive methods as previously described[13]. One week after treatment finalization (W6), rats were fasted for 24 h and received 1 mL of a 10% (wv^{-1}) charcoal suspension in a 5% (wv^{-1}) gum arabic solution via an orogastric cannula. After twenty minutes, the whole intestine was removed *en bloc*. Upper gastrointestinal transit was calculated as the ratio between the distance travelled by the charcoal meal and the total length of the small bowel. To avoid differences in this parameter related to technical issues, the same experienced researcher (GV) administered the charcoal and obtained the data in all groups of animals.

Histopathological analysis of the small intestine

During W6, 2-3 cm long samples were obtained from the terminal ileum (at least 10 cm oral to the ileocaecal junction) of 4-7 animals per experimental group, fixed in buffered 10% formalin and embedded in paraffin. Sections of 5 μm were stained with conventional hematoxylin-eosin (HE), Van Gieson´s stain, PAS or prepared for immunohistochemistry. They were studied under a Zeiss Axioskop 2 microscope equipped with the image analysis software package AxioVision 4.6 to calculate the morphometric parameters. The analysis was made by triplicate in 5-8 random fields measured in 20-40x objective microphotographs

per section and specimen. The experimenter was blind to the treatment received by the rat from which the sample under analysis was obtained.

Histological damage was evaluated in sections stained with HE using criteria adapted from Galeazzi et al.[19]. A numerical score of 0–9 was assigned to each section considering general loss of mucosal architecture (graded 0–3, absent to severe), extent of inflammatory cell infiltrate (graded 0–3, absent to transmural), crypt abscess formation (0–1, absent or present), goblet cell depletion (0–1, absent or present) and muscular layer thickness (0–1, normal to reduced). The number of damaged villi, inflammatory infiltrates per linear centimeter of intestine and thickness of both muscle layers were also measured. The number of goblet cells per villi was counted after PAS staining. Submucosa thickness was measured after staining with Van Gieson to detect collagen fibers.

For immunohistochemistry, samples were washed with phosphate buffered saline (PBS) with 0.05% Tween 20 (Calbiochem, Darmstadt, GER). Thereafter sections were incubated for 10 min in 3% (vol vol^{-1}) in hydrogen peroxide to inhibit endogenous peroxidase activity and blocked with 1% PBS-BSA (bovine serum albumin) or calf serum for 30 minutes to minimize nonspecific binding of the primary antibody. Pilot experiments performed to determine the optimal antibody dilution showed that some samples needed to be pretreated by boiling in 10mM citrate buffer for 30 min. Sections were then incubated overnight at 4°C with the following antibodies: monoclonal mouse anti-human chromogranin A (1:800; Thermo Scientific), to assess the number of enteroendocrine cells in epithelium per 30 villi; monoclonal mouse anti human Ki-67 (1:600; Novocastra, Newcastle, UK), as a proliferation marker[20]; and polyclonal rabbit anti-human neural specific enolase (NSE; 1:50; Sigma), to quantify neurons in myenteric

ganglia between the muscle layers. After incubation, samples were washed with PBS-Tween. The peroxidase-based kit Masvision (Master Diagnostica, Granada, Spain) was used as secondary antibody. Samples were counterstained with hematoxylin and coverslips mounted with Eukitt mounting media (O. Kindler GmbH & Co, Freiburg, Germany). To determine the level of non-specific staining, the preparations were incubated without the primary antibody.

Molecular analysis of the small intestine by Reverse Transcription (RT) and Quantitative PCR (qPCR).

The mRNA expression of different markers was evaluated in the *tunica muscularis* (containing both circular and longitudinal muscle layers as well as the myenteric plexus) of terminal ileum: NSE was used as a pan-neuronal marker[21]; neuronal nitric oxide synthase (nNOS) and choline acetyltransferase (two isoforms: common, cChAT; and peripheral, pChAT), were used as markers of myenteric neurons mainly involved in inhibitory and excitatory motor functions, respectively, with no significant overlapping in rat myenteric neurons[22,23]; calcitonin gene related peptide beta (CGRPβ), as a marker of intrinsic primary afferents in the myenteric plexus[24,25]; and the receptor tyrosine kinase c-KIT (CD117) as a marker of ICC[26,27].

Tissues were sampled from terminal ileum during W6 under sterile conditions. Mucosa and submucosa were carefully removed to isolate the *tunica muscularis*, which was frozen until processing. Total RNA was isolated using TRI Reagent® RNA Isolation Reagent (Sigma-Aldrich) according to the

manufacturer's protocol. 1 µg of total RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. Real time quantification of nNOS, cCHAT and pCHAT, CGRPβ, c-KIT, or NSE mRNA gene expression was assessed using the Power SYBR Green PCR Mastermix (Applied Biosystems). Samples were amplified with a precycling hold at 94 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 1 min, and annealing-extension at 60 °C for 15 sec. Primers used for nNOS, cCHAT, pCHAT, CGRPβ, c-KIT and NSE are listed in Table I. 18s ribosomal RNA (rRNA) gene was amplified as internal control using oligonucleotides listed in Table I. Quantitative Real-Time PCR (qPCR) was performed on the cDNA in triplicates using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The relative expression was calculated using the comparative Ct method and is expressed as $2^{-\Delta\Delta Ct}$ [28]. PCR efficiency was ~100%, estimated on standard curves using serial dilutions of cDNA mix and primer pairs for nNOS, cCHAT, pCHAT, CGRPβ, c-KIT, NSE and 18s rRNA.

Compounds and drugs

Barium sulfate (Barigraf® AD, Juste SAQF, Madrid, Spain) was suspended in tap water and continuously hand-stirred until administration. Cisplatin was purchased from Sigma-Aldrich (Spain) and was dissolved in saline (sonicated for about 15 min). Charcoal and gum arabic were purchased from Sigma-Aldrich (Spain) and dissolved in saline.

Statistical analysis

Data are presented as the mean values ± SEM. Differences were analyzed using Student´s t-test with Welch's correction where appropriate, or one- or two-way ANOVA followed by *post-hoc* Bonferroni multiple comparison test. Values of $P < 0.05$ were considered significantly different.

RESULTS

Just before the first cisplatin administration, body weight was 381 ± 10 and 394 ± 12 g in control and cisplatin-treated rats, respectively. At the end of the experiment (W6), body weight was 445 ± 13 g in saline-treated rats and 364 ± 8 g in cisplatin-treated animals. Thus, the percentage of body weight gain was 16.9 ± 1.3 and -7.1 ± 2 %, respectively ($p < 0.001$).

Gastrointestinal motility studies

The radiographic study was used to control for alterations in gastric motor function. Cisplatin did not significantly modify gastric motor function after acute administration, but delayed gastric emptying upon repeated administration (Fig. 1A-B). One week after treatment finalization (W6), no significant effect of cisplatin was detected in gastric emptying (Fig. 1C). At that time point (W6) we performed an invasive experiment to determine more precisely whether motility of the upper gastrointestinal tract was altered. In this experiment (Fig. 1D), using charcoal as a marker, upper gastrointestinal transit was significantly reduced in cisplatin- compared to saline-treated rats.

Morphological and histopathological analysis

The histological pattern in HE stained sections of the intestinal wall is shown in Fig. 2 (A-B). A general and significant damage was observed one week after repeated cisplatin (Fig. 2C). In fact, cisplatin evoked statistically significant structural changes in all regions of the intestinal wall. Thus, the loss of the normal mucosal architecture was particularly evident (Fig. 2D-F; P <0.01). Submucosa thickness (Fig 2G-I) significantly decreased in treated animals (P < 0.01). In the same way, muscle layer thickness also decreased with cisplatin treatment both in the circular and the longitudinal layers (P <0.05; Fig. 2J-L).

Repeated cisplatin increased the number of goblet cells in epithelium (P <0.01) as shown in fig. 3 (A-C). Inflammatory nodules were also larger (Fig. 3D-E) and their number per cm increased more than 2.5 times (P <0.01; Fig. 3F). Cell proliferation was not affected by cisplatin in epithelium as shown by immunostaining with anti-Ki-67 (Fig. 3G-I). However, there was intense cell proliferation in lymphoid tissue after cisplatin treatment (P <0.05; Fig. 3J-L). Also, chromogranin A-immunoreactive (enterochromaffin) cells in epithelia significantly increased their number with cisplatin treatment, (P <0.01; Fig. 4A-C).

Finally, the total number of neurons per ganglion, assessed by NSE immunostaining, did not significantly change with treatment (Fig. 4D-F).

Expression of markers of myenteric neurons and ICC

Regarding the myenteric neurons, we analyzed the expression of mRNA of the genes encoding nNOS (neurons involved in inhibitory motor activity), cChAT,

pChAT (neurons involved in excitatory motor activity) and CGRPβ (intrinsic primary afferents) normalized to NSE (pan-neuronal marker) (Fig. 5A). There was a significant increase in the expression of all these markers, except for CGRPβ, which did not significantly change. The increase was higher in the case of nNOS compared to either ChAT (approximately 4-fold vs 2-fold). In addition, cisplatin increased the mRNA expression of c-KIT (a marker for ICC) by 2-fold (Fig. 5B).

DISCUSSION

One week after a 5-cycle cisplatin treatment in the rat, when small intestinal (but not gastric) motility was significantly impaired, we observed profound alterations in the gut wall general structure, mucosal, submucosal and muscular layers, lymphocytic nodules and chromogranin A-immunoreactive cells, as well as in the expression of selected markers of cells from the *tunica muscularis*. Thus, chemotherapy-induced structural and molecular alterations in different components of the gut wall may underlie the relatively long-lasting sequelae of chemotherapy in intestinal function, including dysmotility.

Effects of repeated cisplatin on small intestinal wall general structure and mucosal components

We found a stage 4 mucositis, characterized by ulceration and inflammation of ileal mucosa[29]. Cisplatin causes profound gastrointestinal symptoms that in humans may occur even months to years after treatment finalization[30]. These symptoms may be related to structural damage of the mucosa (which other

antineoplastic drugs may also induce[31]), although most published data refer to acute doses[32,33]. Instead, we used lower doses, repeatedly administered, more adequate to mimic the clinical situation. In a previous report, in rats treated with cumulative doses of 5 and 15 mg kg^{-1}, we described a dose-dependent increase in the general histological damage[13]. Here we used an intermediate dose of cisplatin (cumulative dose 10 mg kg^{-1}), and found similar results[13] but as expected, intermediate in intensity.

Although repeated cisplatin reduced food intake in the following 24 h after each administration, food intake normalized thereafter until next injection, providing no significant differences in weekly food intake between groups[6-8]. Thus, animals were not subjected to a kind of "prolonged fasting" that might induce direct mucosal atrophy.

Ki-67 is widely used as a cell proliferation marker[34]. Whereas some antineoplastic drugs, like methotrexate, do not affect this early proliferation marker in the mucosa[35], cisplatin decreased proliferating cells in the few hours after its administration[32,36]. However, we did not detect any significant change in this parameter, suggesting that mucosal proliferation was recovered one week after the last cisplatin administration. In contrast, there was a significant increase in the size of inflammatory nodules and Ki-67-reactive cells proliferation. These likely increased the synthesis of proinflammatory cytokines, contributing to inflammation[29].

In addition to direct effects on the enterocytes and those related to the inflammatory response, cisplatin effects on the mucosa and the gut wall may be due to serotonin (5-hydroxyrtryptamine, 5-HT) release. After cisplatin treatment, an increase in 5-hydroxyindoleacetic acid was found in the urine of cancer

patients and rats, indicating increased 5-HT release[37-39]. Most chromogranin-A enteroendocrine cells are also enterochromaffin cells and produce the majority of 5-HT in the body[40]. Thus, the increase in chromogranin-A enteroendocrine cells induced by repeated cisplatin administration might be associated to an increase in 5-HT levels and release, as observed in the small intestine of guinea-pigs and ferrets[41,42]. Serotonin has pro-kinetic effects and plays a major role in the modulation of brain–gut communication[40]. Dysregulation of the gastrointestinal 5-HT signaling system plays a major role in gastrointestinal disorders, including chemotherapy-induced emesis, gastric distension and visceral pain[13,39,43-46], as well as in cytokine release. Interestingly, blocking 5-HT synthesis decreased inflammatory cytokine levels and produced less histological damage in models of acute and chronic colitis[47], whereas blocking 5-HT_3 receptors did not prevent damage but reduced impairment of intestinal fluid and electrolyte absorption in the rat[48].

The population of mucosal goblet cells significantly increased in cisplatin-treated rats. Mucin is involved in protection of the mucosa and the maintenance of normal intestinal flora[49]. Cavitated goblet cells (goblet cells which have released mucins through exocytosis) and Muc4 expression increased in the jejunum of rats treated with irinotecan or 5-FU[50,51], but mucin levels decreased in the rat jejunum and colon after 5-FU treatment[52]. The increased number of goblet cells reported here may be related to an over-production to protect the mucosa[50,51,53]. In addition, there is a paracrine relationship between enteroendocrine and goblet cells resulting in mucus secretion in response to situations like inflammation[54].

Regarding other components of the intestinal wall, we observed a decrease in the thickness of submucosa and muscular layers. This general decrease in bowel wall thickness caused by cisplatin could be responsible for the fragility found in the colon when it was mechanically stimulated with an intracolonic balloon, particularly when rats were treated with a cumulative dose of 15 mg kg^{-1} (data not shown).

Effects of repeated cisplatin on small intestinal motility and the motor components of the small intestinal wall

As expected, cisplatin induced gastric dysmotility in the few hours after administration but not one week after treatment finalization[5,7,55]. Gastric dysmotility in the few hours after cisplatin administration is mainly due to 5-HT release[39], and, in fact, 5-HT_3 antagonists, used as antiemetics in the clinic, prevented cisplatin-induced gastric dysmotility in the rat[56].

One week after the last cisplatin injection (when gastric emptying was as in saline-treated rats), a dose-dependent reduction of upper gastrointestinal transit was apparent (present results; [13]), suggesting that small intestinal motility was uncoupled. Although this might be due to muscle weakness associated to cisplatin-induced generalized toxicity (comprising both reduced body weight gain and muscle layer thinning), other factors might also contribute to muscle atrophy and dysfunction. Particularly, both ICC and myenteric neurons, could be altered by cyclic cisplatin, either in a direct or an indirect manner (i.e., through the release of 5-HT and other transmitters).

ICC integrate excitatory and inhibitory neurotransmission in the intestinal wall to control intestinal motility[57]. Repeated cisplatin doubled the expression of c-KIT, considered a marker for ICC in the small intestine[26]. Since c-KIT is highly expressed in mast cells[58,59], increased c-KIT expression could be explained by mast cell infiltration following cisplatin-induced damage. Interestingly, in situations of chronic inflammation such as a mouse model of post-infection irritable bowel syndrome, both proliferation of ICC and c-KIT expression were increased[60]. Alternative markers of ICC such as ANO-1 were also increased in murine models of tumor-induced cachexia[61] and diabetes[62,63], whereas ICC number decreased, suggesting increased ICC networking, since mast cells do not express ANO-1. Increased c-KIT mRNA levels have also been reported in a mouse model of ovarian cancer following cisplatin administration[64]. *In vitro* studies have shown that cisplatin induces changes in epigenetic marks (miRNAs, methylation, etc) and overexpression of transcription factors whose promoters are potentially regulated by c-KIT such as MITF or FOXO[65]. Further research is required to determine the mechanisms involved in cisplatin-induced increased c-KIT expression in our system.

Interestingly, increased ICC and c-KIT expression was associated to accelerated, not delayed, small intestinal transit[60]. Thus, changes in other components of motor function, particularly the myenteric plexus, might be more influential to produce delayed transit in our model. In the colon, both cisplatin in rats[13] and oxaliplatin in mice[14] produced long-lasting dysmotility, associated with the development of a neuropathy affecting the myenteric neurons, with a decrease in the general population of neurons, but a relative increase in the population of neurons immunoreactive to nNOS. In agreement with previous

results[13], the total number of neurons per ileal myenteric ganglion was not changed. Furthermore, as previously described for the colon using immunohistochemistry[13,14], the changes shown here in the molecular expression of markers characteristic of neurons involved in inhibitory (nNOS) and excitatory (pChAT, cChAT) myenteric motor function also suggest that cisplatin treatment unbalances small intestinal motility, towards a higher level of inhibition/relaxation. Other authors correlated increased nNOS with increased networks of ICC[66,67]. We report here changes in excitatory and inhibitory neuronal markers and thinning of muscular layers. Since ICC integrate excitatory and inhibitory neurotransmission with slow wave action potentials to control peristaltic motor activity, increased ICC markers such as c-KIT may represent a compensatory adaptation to ensure some degree of movement.

Finally, in addition to the possible direct toxic effects of cisplatin on the myenteric neurons[68], the imbalance in myenteric neurotransmission and reduced small intestinal transit could be related to the increased number of enteroendocrine cells reactive to chromogranin-A. As discussed above, most mucosal cells immunoreactive to chromogranin-A might synthetize 5-HT. In addition to facilitating inflammation and tissue damage[47], cyclic release of 5-HT might contribute to increase ICC markers expression, through the activation of 5-HT_{2B} receptors present on ICC plasma membrane[69]. On the other hand, chromogranin-A *per se* and its derived peptides, like vasostatin-1, may decrease spontaneous contractility of the circular muscular layer. These suppressive effects could be mediated by neural release of nitric oxide[70,71], which might be favored by the increased expression of nNOS (present results) and proportion of myenteric neurons immunoreactive to this marker[13,14].

Interestingly, patients suffering from chronic constipation have more chromogranin-A-reactive cells in the intestinal epithelium[72].

Concluding remarks

Different factors may contribute to long-lasting intestinal dysmotility after chemotherapy, including the development of an enteric neuropathy, alterations in ICC, and changes in components of the gut mucosa, like the enteroendocrine cells. Cancer chemotherapy-induced sequelae need to be better characterized if effective strategies for prevention and treatment are to be offered to the ever-increasing population of cancer survivors.

COMPETING INTERESTS

The authors declare that they have no competing interests

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CONTRIBUTIONS

JAU performed the histological analyses. CGJ and JMGM performed the molecular expression studies. GV and RA performed the functional studies. JAU, RA, CGJ and JMGM wrote the paper. RA designed the study. CGJ and MIMF provided essential intellectual input and obtained financial support.

Table I. RT/PCR primers:

Primer Name	***Sequence***
nNOS F	5′ ACCCCGTCCTTTGAATACCAG 3′
nNOS R	5′ GACGCTGTTGAATCGGACCTT 3′
cCHAT F	5′ AGGCCAGGACGGTCCTCT 3′
cCHAT R	5′ CTCGATCATGTCCAGGGAGTC 3′
pCHAT F	5′ GCACTTCCAAGACACCAATGAC 3′
pCHAT R	5′ ACACCGCAGGTGCCATCT 3′
CGRPβ F	5′ CTGAGGGTGCCCAGGTCTAG 3′
CGRPβ R	5′AGCCCCTTTCAGGTCAGCTT 3′
c-KIT F	5′ ATCCAGCCCCACACCCTGTT 3′
c-KIT R	5′ TGTAGGCAAGAACCATCACAATGA 3′
NSE F	5′ TGATGACCTGACGGTGACCA 3′
NSE R	5′ CAAACAGTTGCAGGCCTTCTC 3′
18s ribosomal Control F	5′ AGTCCCTGCCCTTTGTACACA 3′
18s ribosomal Control R	5′ GCCTCACTAAACCATCCAATCG 3′
F: Forward primer R: Reverse primer	

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FIGURE LEGENDS

Figure 1. Effect of repeated administration of cisplatin on GI motor function in the rat. A-C: Gastric motor function was evaluated by radiological methods (see text). Rats were injected intraperitoneally (ip) for 5 weeks with: saline (1 mL kg^{-1} $week^{-1}$; n=8) or cisplatin (2 mg kg^{-1} $week^{-1}$; n=8). Barium sulfate (2.5 mL, 2 g mL^{-1}) was intragastrically administered immediately after the first (A) or the last (B) administration of cisplatin or saline, or one week after treatment finalization (C). Data represent mean ± SEM. * $p<0.05$, ** $p<0.01$ vs saline (two-way ANOVA followed by *post-hoc* Bonferroni multiple comparison test). A', B', C': representative images of animals treated with saline or cisplatin 1 (T1) or 4 (T4) hours after contrast administration. Scale bar: 23 mm. D: Upper gastrointestinal transit was invasively evaluated using the charcoal method (see text for details) in rats injected for 5 weeks with: saline (1 mL kg^{-1} $week^{-1}$, ip, white bar, n=6) or cisplatin (2 mg kg^{-1} $week^{-1}$, black bar, n=5). Measurements were performed one week after treatment finalization, when gastric emptying evaluated radiographically was not significantly different between treatments. Data represent the mean ± SEM. **$p<0.01$ vs saline (Student's t-test).

Figure 2. Effect of repeated cisplatin treatment on the general structure of the rat small intestinal wall. Rats were injected intraperitoneally (ip) for 5 weeks with: saline (1 mL kg^{-1} $week^{-1}$) or cisplatin (2 mg kg^{-1} $week^{-1}$). Histological samples embedded in paraffin sections were obtained one week after the last administration. Left (A, D, G, J): tissue samples from saline-treated animals. Center (B, E, H, K): tissue samples from cisplatin-treated animals. Right (C, F, I, L): quantitative analyses. Bars show mean values ± SEM for control (white) and cisplatin-treated animals (red). Each group consisted of 4-7 rats. *P <0.05, **P

<0.01 *vs.* saline; (one-way ANOVA followed by Bonferroni or Student's by t-test). A-B: General view of the small intestine wall showing its different layers (HE-staining). C: Histological damage. D-E: Epithelial villi architecture (HE-staining). F: Damaged villi per 30 counted. G-H: Van Gieson´s staining of submucosa (red). I: Submucosa thickness (μm). J-K: Circular and longitudinal muscle layers (HE). L: Muscle layer thickness (μm) of the circular and longitudinal layers. Bar 100 μm, except A-B, 400 μm.

Figure 3. Effect of repeated cisplatin treatment on secretory and proliferating cells of the rat small intestinal wall. Rats were injected intraperitoneally (ip) for 5 weeks with: saline (1 mL kg^{-1} $week^{-1}$) or cisplatin (2 mg kg^{-1} $week^{-1}$). Histological samples embedded in paraffin sections were obtained one week after the last administration. Left (A, D, G, J): tissue from saline-treated animals. Center (B, E, H, K): tissue samples from cisplatin-treated animals. Right (C, F, I, L): quantitative analyses. Bars show mean values ± SEM for control (white) and cisplatin-treated animals (red). Each group consisted of 4 to 7 rats. ***P* <0.01 *vs.* saline; (Student's t-test). A-B: Goblet cells in the intestinal mucosa (PAS stain). C: Percentage of PAS positive cells. D-E: Lymphocytic nodules under the epithelium (HE). F: Number of nodules per linear cm. G-H: Cells entering mitosis in the mucosa (Ki-67 antibody). I: Percentage of Ki-67 positive cells per villi. J-K: Cells entering mitosis in the core of lymphocytic nodules (Ki-67). L: Percentage of nodule area stained with Ki-67 antibody. Bar 100 μm.

Figure 4. Effect of repeated cisplatin on endocrine and neural cells of the rat small intestinal wall. Rats were injected intraperitoneally (ip) for 5 weeks with: saline (1 mL kg^{-1} $week^{-1}$) or cisplatin (2 mg kg^{-1} $week^{-1}$). Histological

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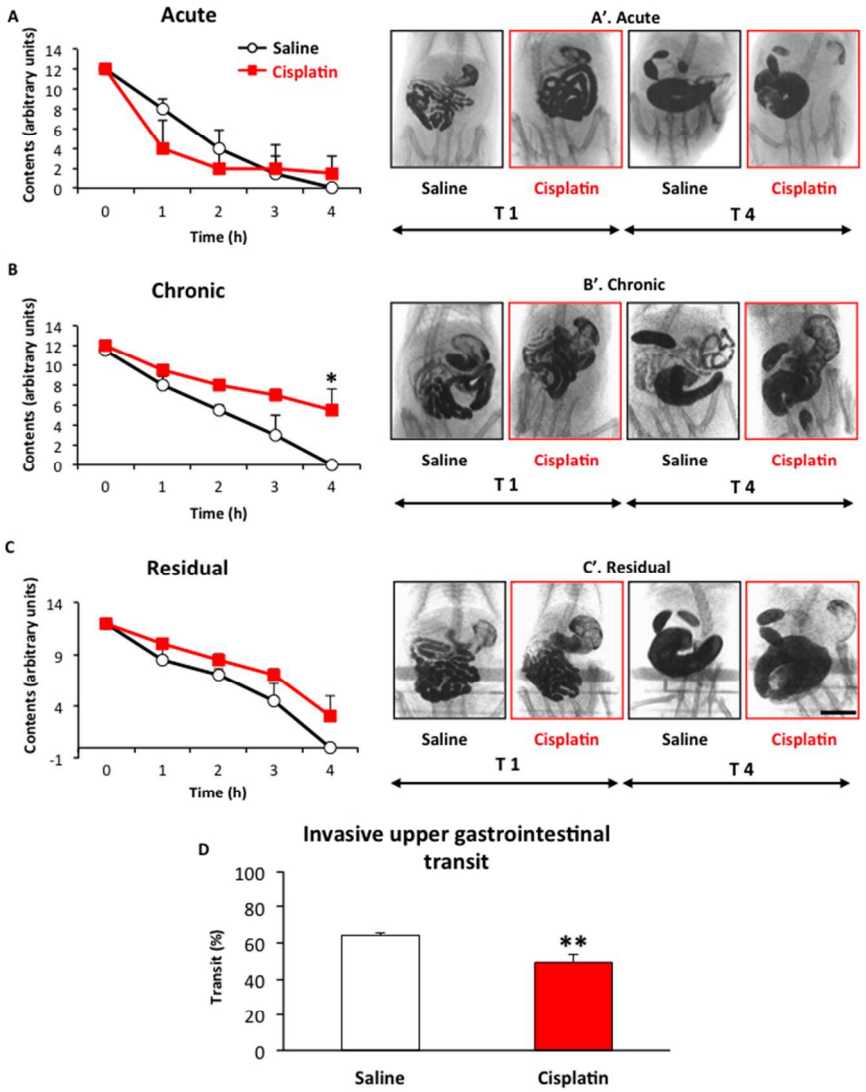
Figure 5. Gene expression of neuronal and ICC markers in the rat small intestinal *tunica muscularis*. Reverse transcription coupled to quantitative PCR using specific primers to NSE, nNOS, pChAt, cChAt and CGRPβ (in A) and c-KIT (in B), and mRNA from rat small intestinal *tunica muscularis*. Samples were obtained one week after treatment finalization from rats injected with saline (1 mL kg^{-1} $week^{-1}$, ip) or cisplatin (2 mg kg^{-1} $week^{-1}$, ip) once a week for 5 weeks. Values normalized to the endogenous control (18S) are referred as fold-induction over saline-treated animals. In A, nNOS, pChAt, cChAt and CGRPβ expression were normalized to NSE expression, used as a marker for the general neuronal population. **P <0.01, ***P <0.001 *vs*. saline (Student's t-test). n≥4, each marker.



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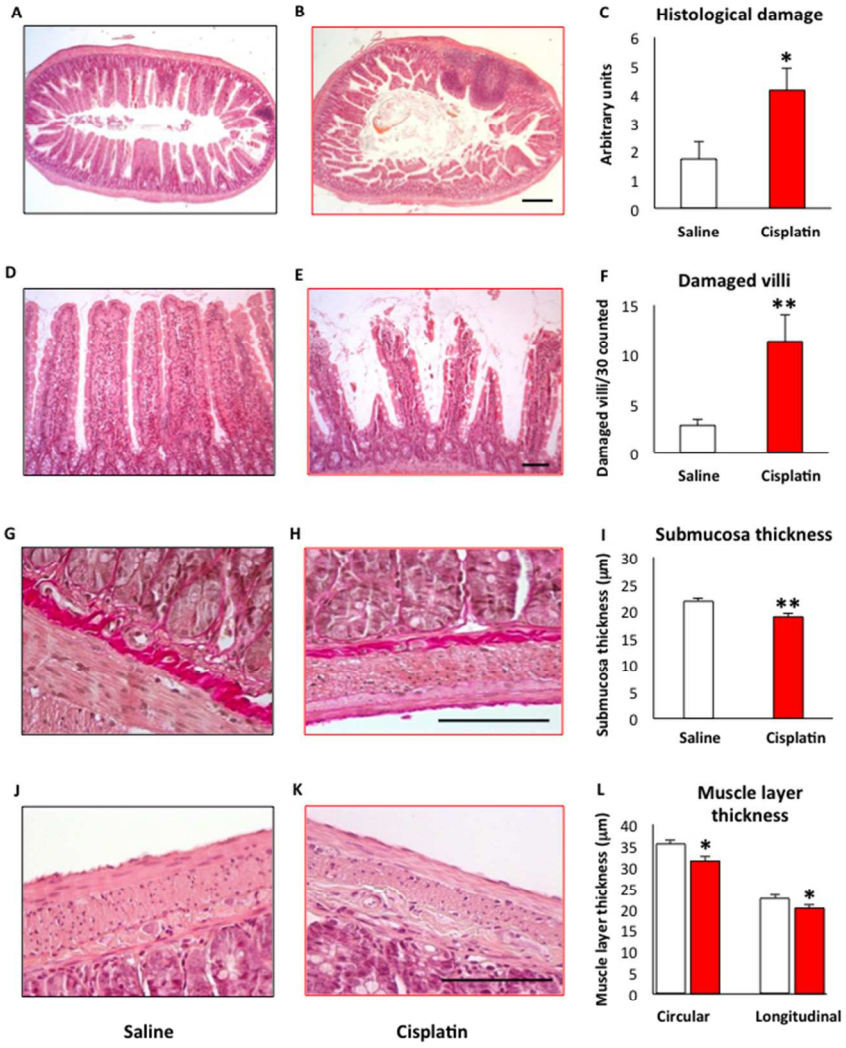

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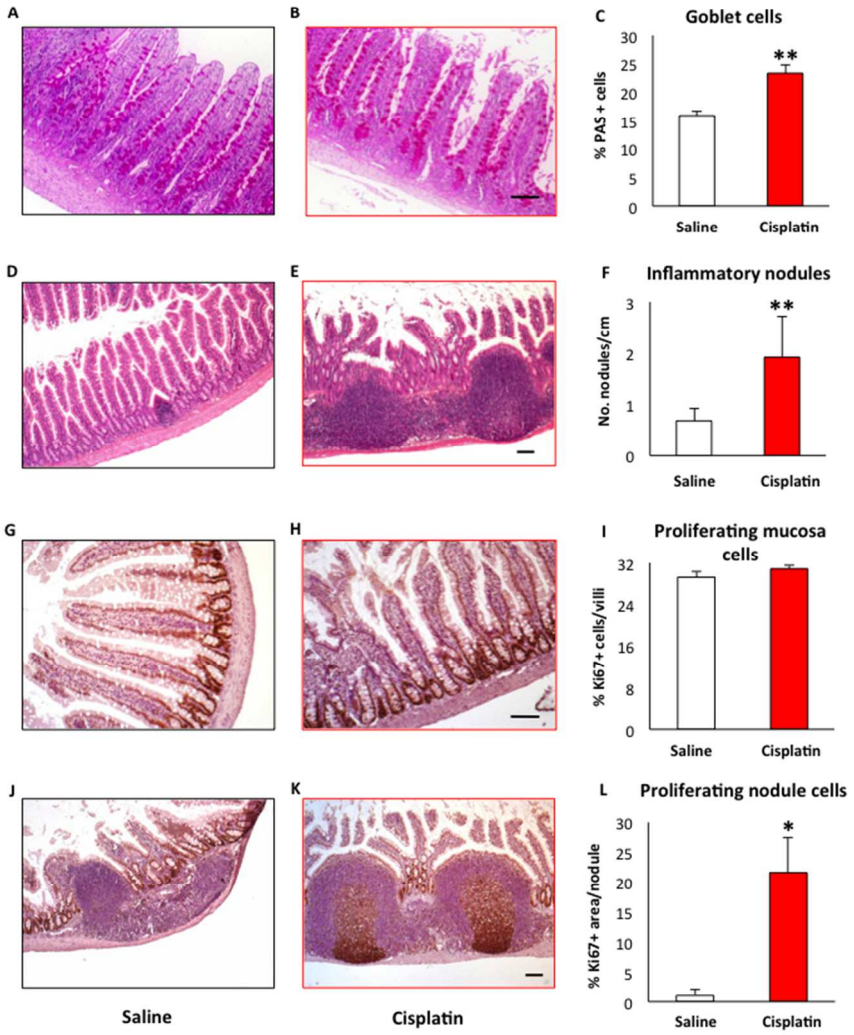

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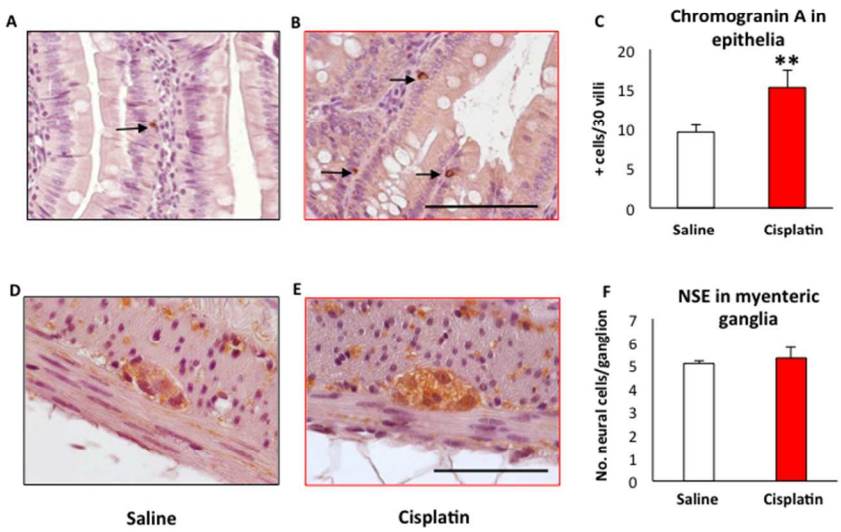

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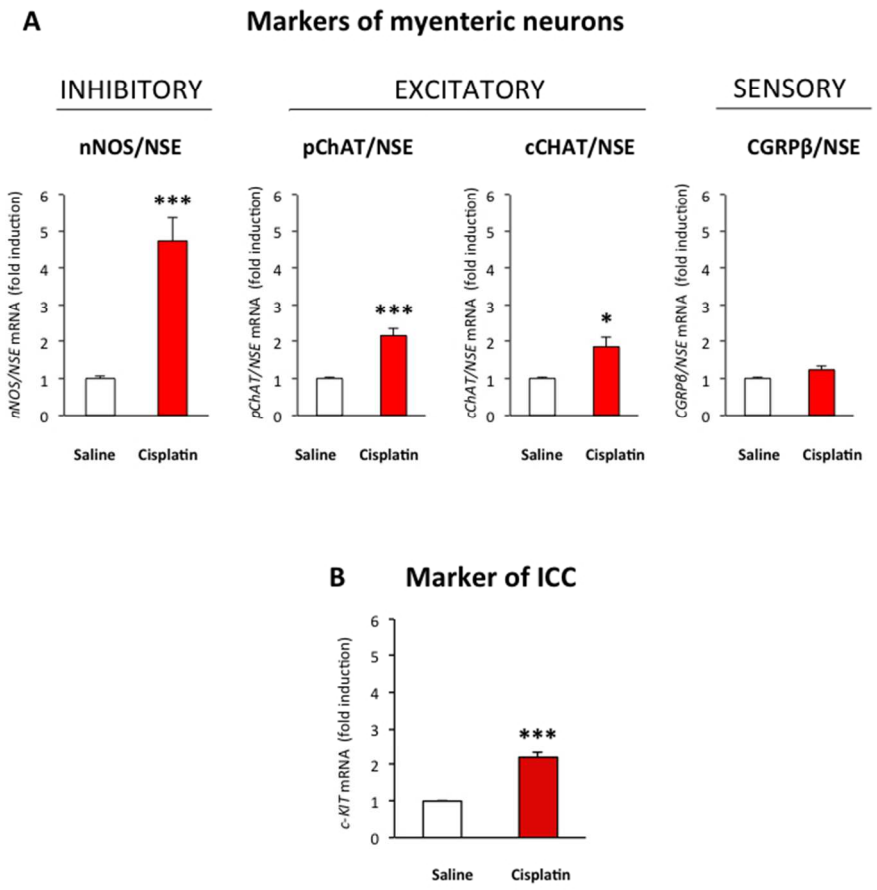

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