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Note: The following files were submitted by the author for peer review, but cannot be converted to PDF. You must view these files (e.g. movies) online.	
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PRECLINICAL EVALUATION OF THE EFFECTS ON THE GASTROINTESTINAL TRACT OF THE ANTINEOPLASTIC DRUG VINCRISTINE REPEATEDLY ADMINISTERED TO RATS

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Running Title: Gastrointestinal effects of vincristine.

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ABSTRACT

Background: Vincristine is a commonly used chemotherapeutic agent. It is associated with undesirable digestive side effects. However, the impact of vincristine on gastrointestinal structure and motility or its long-term effects have not been deeply studied in animal models. This could be useful in order to develop therapeutic or preventive strategies for cancer patients. The aim of this study was to analyze such effects.

Methods: Rats received saline or vincristine (0.1 mg kg^{-1}, ip) daily for 10 days. Evaluations were performed during treatment and 2-6 weeks after. Somatic mechano-sensitivity was assessed using von Frey hairs. Gastrointestinal motor function was studied by means of radiographic still images and colonic propulsion of fecal pellets using fluoroscopy videos. Histological assessment of the gut morphology and immunohistochemistry for HuC/D and nNOS were performed in whole-mount myenteric plexus preparations.

Key Results: Peripheral sensitivity was increased in animals treated with vincristine and did not subside 2 weeks after treatment finalization. Vincristine treatment inhibited gastrointestinal motility although this was recovered to normal values with time. Damage in the digestive wall after vincristine treatment was greater in the ileum than in the colon. Villi shortening (in ileum) and large inflammatory nodules still remained 2 weeks after treatment finalization. Finally, the proportion of nNOS-immunoreactive neurons was increased with vincristine and continued to be increased 2 weeks after treatment finalization.

Conclusions & Inferences: Vincristine alters gastrointestinal motility, peripheral sensitivity and mucosal architecture. Vincristine-induced neuropathy (somatic and enteric), intestinal mucosa damage and inflammatory infiltrations are relatively long lasting.

Key Words: vincristine, gastrointestinal motility, fluoroscopy, immunohistochemistry, neuropathy.

KEY POINTS

- Vincristine is a common chemotherapeutic agent but its impact on gastrointestinal structure and motility or its long-term effects have not been deeply studied.
- Rats were treated with 2-cycle vincristine and studied just after treatment or several weeks afterwards. Vincristine altered somatic peripheral sensitivity, gastrointestinal motility and mucosal architecture.
- Some effects of vincristine disappeared soon after treatment, but peripheral neuropathy and alterations in myenteric neurons did not. Chemotherapy-induced long lasting effects should be more closely monitored.

INTRODUCTION

Vincristine, formulated as vincristine sulfate, is a vinca alkaloid used as an antineoplastic agent since 1960s. It is one of the most commonly used chemotherapeutic agents because of its lack of significant bone–marrow suppression activity[1]. Similarly, it is also used in the treatment of idiopathic and thrombotic thrombocytopenic purpura. Vincristine works by binding to tubulin heterodimers, causing microtubule depolymerization, inhibition of cell cycle progression and apoptosis in cells undergoing mitosis[1-3].

Vincristine is associated with undesirable side effects, which constitute a major hurdle that compromises its efficacy. Vincristine-induced dose-limiting neurotoxicity is especially significant. It particularly alters sensorimotor peripheral nerves due to microtubule dysfunction affecting axoplasmic vesicular traffic. Peripheral neuropathy may affect not only somatic sensory afferents, but also the autonomic nervous system, raising the possibility that the function of visceral organs could be consequently compromised. Thus, common manifestations include symmetric loss of deep tendon reflexes, paresthesias, urinary retention, cranial nerve palsies and profound muscle weakness with subsequent muscle atrophy[2]. Similarly, vincristine treatment is associated with digestive symptoms like constipation that can affect up to 57% of patients[2,4]. Damage to the myenteric plexus by vincristine could be implicated in gastrointestinal hypomotility[5-7]. Patients treated with vincristine can also develop symptoms indicating dysmotility of the upper gastrointestinal tract, including anorexia and nausea or even extreme symptoms such as paralytic ileus. In fact, paralytic ileus occurs in up to 32% of vincristine-treated patients[8]. However, the impact and mechanisms of vincristine on gastrointestinal structure and motility have not been

deeply studied in experimental animals. This is a very important subject, particularly to develop therapeutic or preventive strategies for cancer patients with increasing survival rates.

Therefore, our aim was to characterize the structural and functional alterations occurring within the gastrointestinal tract during and after cyclic chemotherapy with vincristine in the rat.

MATERIALS AND METHODS

The *in vivo* experiments were performed in accordance with the European and Spanish legislation on care and use of experimental animals (EU Directive 2010/63/EU for animal experiments; R.D. 53/2013). The experiments were designed to minimize the number of animals used and were approved by the Ethics Committees at both Universidad Rey Juan Carlos (URJC) and Hospital General Universitario Gregorio Marañón (HGUGM).

Animals and protocol

Male Wistar rats were used in two studies. In the first study (Fig. 1A), rats (275-300 g, n=20) were obtained from the Veterinary Unit of URJC. For the fluoroscopy study (Fig. 1B), rats (188-276 g, n=16) were obtained from the Veterinary Unit of HGUGM (Madrid, Spain).

Animals were housed in groups (3-4/cage) in standard transparent cages under environmentally controlled standard conditions, with a 12 hr light/12 hr dark cycle. They

were fed *ad libitum* (Harlan Laboratories Inc.) and had free access to sterile tap water.

Rats received 2 cycles of 5 daily intraperitoneal (i.p.) injections (Monday to Friday, 2 consecutive weeks) of saline (2 mL kg^{-1}) or vincristine (0.1 mg kg^{-1}) (see Fig. 1 for a general overview of the experimental protocol).

Body weight of the animals (both studies, Fig. 1A and 1B), as well as the intake of drinking water and of freely available food (first study, Fig. 1A) were recorded at least once a week throughout the study.

Assessment of peripheral neuropathy

Mechanical sensitivity was assessed by measuring the withdrawal threshold to calibrated von Frey hairs[9] (Bioseb Instruments, USA). The test was performed before, right after and 1 and 2 weeks after treatment finalization. Each stimulus was applied approximately for 1 second with an interstimulus interval of approximately 3 seconds. A significant decrease in von Frey hair threshold evoked by mechanical stimulus was defined as presence of mechanical allodynia. Animals were habituated to the test environment 2 days before the experiment.

Gastrointestinal motor function

Radiographic study

The first study (Fig. 1A) was the evaluation of gastrointestinal motor function by means of radiographic techniques[10]. Three radiographic sessions were performed: after the 1st administration, after the 10th administration (week 2), and 2 weeks after treatment finalization (week 4). In each session, 2.5 mL of barium sulfate (Barigraph ® AD, Juste SAQF,

Madrid, Spain; 2g mL^{-1}) in tap water was administered *per os*. A CS2100 (Carestream Dental, Spain) digital X-ray apparatus (60 kV, 7 mA) was used, and X-rays were recorded on Carestream Dental T-MAT G/RA film. Films were developed using a Kodak X-omat 2000 automatic processor. Exposure time was adjusted to 20-60 ms and focus distance was fixed to 50 ± 1 cm. Rats were immobilized inside plastic tubes and no anesthesia was used, in order to avoid interference with gastrointestinal motility. X-rays were recorded at different times (immediately and 1, 2, 4, 6 and 8 h) after barium. A trained investigator blind to the drug administered performed the analysis. Alterations in gut motility were semi quantitatively determined by assigning a compounded value to each region of the gastrointestinal tract, including: percentage of the gastrointestinal region filled with contrast (0-4); intensity of contrast (0-4); homogeneity of contrast (0-2); and sharpness of the gastrointestinal region profile (0-2). Each of these parameters was scored and a sum (0-12 points) was made. The X-ray images were used to analyze alterations of stomach size and caecum with the aid of an image analysis system (Image J 1.38 for Windows, NIH, USA, free software: http://rsb.info.nih.gov/ij/).

Fluoroscopic study

Colonic propulsion of fecal pellets was evaluated by fluoroscopy in a different set of animals. Three sessions of fluoroscopy were performed (Fig. 1B): first, after the 10^{th} drug administration; second, 3 weeks after the last administration (week 5; for technical reasons, it was not possible to perform the second session at the same time-point as in the radiographic study, week 4); third, 6 weeks after the last administration (week 8).

For these experiments, 2 loads of barium sulfate (1.5 mL) were administered *per os*: the first one 21.5 h (approximately) and the second one 1.5 h before fluoroscopy. Thus, at the time of fluoroscopy, the first load of barium was generally found as a weak staining of the caecum and fecal pellets, and the second load of barium strongly stained the stomach and part of the small intestine (but not the caecum). This procedure allows the recording of motor function of all gastrointestinal regions in just one fluoroscopy session per time-point.

Fluoroscopy was performed as previously described[11], with slight modifications. Plain facial radiographs and fluoroscopic videos of the gastrointestinal tract were obtained using a Digital X-Ray apparatus (Siemens, Siremobil Compact L, Erlangen, Germany; 60kV, 7mA) and captured with Elgato Video Capture software. Exposure time was 60 ms for X-ray shots, and 120 s for fluoroscopic recordings. As in the radiographic study, rats did not receive anesthesia, and were immobilized inside the plastic tubes. An X-ray was taken immediately after the second load of barium to confirm staining of both proximal (stronger) and distal (weaker) parts of the gastrointestinal tract. Fluoroscopic videos were recorded at 25 frames s^{-1}, 1.5 h after the second load of barium, when several loops of the small intestine had filled with contrast but before the caecum had been reached by this second load of barium (this time-point was determined as adequate based on pilot experiments and previous studies[11]). When fluoroscopy was performed after the 10^{th} vincristine administration, the drug (or saline) was injected 30 min before the recording.

Analysis of the fluoroscopic recordings

In the present study, we analyzed the movement of the fecal pellets stained by the first load of barium. Therefore, rats not showing any staining in the caecum or fecal pellets in the X-

ray performed after the second barium load were not used. Fluoroscopic videos in which movement of the rat did not allow for an analysis of at least 80 seconds were also discarded. Only videos displaying fecal pellets in the colon were further analyzed. Each fluoroscopic recording was broken down into 120 frames with the *Quick Time Pro* program (Apple Inc, California, United States) and analyzed as follows:

1. The number of fecal pellets observed along the colon during the fluoroscopic video was recorded; their diameter was measured with *Image J*.

2. For each bolus within the colon, the propulsion speed (PS) was measured as the distance travelled per time unit from its proximal position towards the anus.

3. A spatiotemporal map (STM) was created with Excel program to represent the progress of fecal pellets within the descending colon and rectum, using a binary code to represent the position of each fecal pellet along the colorectum: 0=absent; 1=present. Staining of fecal pellets was generally too faint and their contrast too low against the background (due to the interference of the soft tissue and bones) to use available computerized tools to build diameter maps, but it was possible to follow their movement along the colon by eye, using a constant reference point for each pellet (distal end, proximal end or middle point, depending on the particular pellet), and manually represent their position on the STM (see Supporting information for further details about STM creation and examples of videos).

4. Fecal pellets as shown in the STM were categorized in 2 groups. Group 1: pellets that moved 0, 1 or 2 cm. Group 2: pellets that moved 3 or more cm. Fecal pellets initially positioned 1 or 2 cm proximal to the anus were included in the fast category if they exited the colon in less than 60 s (half the duration of the movies); otherwise, they were included in

the slow category. Fecal pellets of each category were evaluated in each STM and percentages were obtained according to the treatment received by the corresponding rat and the time-point evaluated.

Histopathological analysis

Animals were analyzed at 2 different time-points: after 10 days of vincristine administration (week 2) and 2 weeks after vincristine treatment finalization (week 4).

Samples (2-3 cm long) were obtained from fundus and fore stomach, terminal ileum and distal colon of 4-8 animals per experimental group, fixed in buffered 10% formalin and embedded in paraffin. Sections of 5 µm were stained with conventional hematoxylin-eosin (H/E), Van Gieson´s staining and PAS staining. 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 analyzed sample was obtained.

Histological damage was evaluated in ileal sections stained with H/E using criteria adapted from Galeazzi et al.[12]. 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 length of villi and the thickness of both muscle layers were also

measured. The number of goblet cells per villi was counted after PAS staining. Submucosa thickness was also measured after Van Gieson's staining to detect collagen fibers.

Histological damage was also evaluated in colonic sections stained with H/E using a semi-quantitative score system[13] in which the following features were graded: damage of epithelium (0-3 normal to severe destruction), inflammatory cell infiltration (0-4 absent to severe), separation of muscular layer (0-2 normal to severe), and goblet cell depletion (0-4 no depletion to complete depletion). The total score for histological damage was the average of the different scores.

Whole-mount preparations

Conventional methods for immunohistochemistry[14,15] were also applied to longitudinal muscle-myenteric plexus whole-mount preparations to evaluate the effects of vincristine on the myenteric plexus. A 2-cm length sample was obtained from the distal colon of each rat, placed in saline and rapidly stretched and pinned flat on a Sylgard-coated dish (VWR, Barcelona, Spain). After conventional fixation and clearing, mucosa, submucosa and circular muscle were removed. Each preparation was incubated (36 h at room temperature) with a mixture of both the pan-neuronal marker HuC/D (mouse biotin-conjugated anti-HuC/D, 1:500; Molecular Probes, A21272, Invitrogen, Barcelona, Spain), and sheep anti-nNOS (neuronal nitric oxide synthase, 1:500; Chemicon, AB1529; Milipore, Madrid, Spain). After washing with PBS, tissues were exposed for at least 3 h at room temperature to a mixture of streptavidin-AlexaFluor 488 (1:500, Molecular Probes, S11223), and donkey anti-sheep-RRX (1:500, Jackson, 713-295-003). The preparations were observed under a fluorescent microscope (Nikon Eclipse TE2000-U; Nikon, Barcelona, Spain). In the study, 7-8 whole-mount preparations of the myenteric plexus from different animals were used. The

preparations were analyzed by randomly capturing 3 to 5 10x objective microscopic fields using a DXM1200 camera (Nikon, Barcelona, Spain). *Image J* was used for the analysis of the images. The proportions of neurochemically identified populations were then expressed relative to the number of HuC/D immunoreactive neurons.

Compounds and drugs

Barium sulfate (Barigraf®AD, Juste SAQF,Madrid, Spain) was suspended in tap water. Vincristine was purchased from Abcam (UK) 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 were appropriate, or one- or 2-way ANOVA followed by *post-hoc* Bonferroni multiple comparison test. Fisher's exact test was used to analyze differences due to treatment with vincristine in the movement of fecal pellets in the STM. Values of $p<0.05$ were considered significantly different.

RESULTS

General health parameters: body weight, food and water intakes

Before treatment, body weight was similar for both experimental groups. Antineoplastic drug significantly reduced this parameter. Two weeks after chemotherapy discontinuation (sequelae), body weight was markedly recovered but still remained significantly lower than animals treated with saline (Fig. 2A).

Solid food intake was similar at the start of treatment in both experimental groups. Treatment with vincristine significantly reduced food intake as compared to control animals. Two weeks after treatment finalization (early sequelae), solid food intake was again similar in both experimental groups (Fig. 2B).

Water consumption was similar in both experimental groups all along the experiment (Fig 2C).

Peripheral neuropathy

Before treatment, mechanical sensitivity threshold was found to be approximately between 20 and 30 g in all animals. Just after vincristine treatment was ended, sensitivity to mechanical stimulation with the Von Frey filaments was increased as suggested by a significant decrease in the mechanical sensitivity threshold needed for paw withdrawal (Fig. 2D). Mechanical sensitivity did not recover to control values 1 or 2 weeks after treatment finalization, suggesting that mechanical allodynia (as a sign of peripheral neuropathy) associated with vincristine treatment is relatively long-lasting in the rat.

Radiographic study

In saline-treated rats, during the first radiographic session, gastric emptying was progressive and only a low amount of barium was still visible in the stomach 4-6 h after its intragastric administration (Fig. 3A). Barium content reached its maximum in the small intestine in just one hour and this part of the gut was practically empty by 6 h (Fig. 3B). Barium started to stain the caecum and the colorectum 2 and 4 h after intragastric administration, respectively. Both organs filled progressively until the end of the study (Fig. 3C-D). When this experiment was performed immediately after the 10^{th} administration (week 2) and 2 weeks

after treatment finalization (week 4), similar curves were obtained for saline-treated rats (not shown).

Compared with control, vincristine delayed gastric emptying both after the 1st and the 10th administrations, although the effect was intensified by repeated treatment and differences were statistically significant at 2, 4, 6 and 8 h after drug, whereas a statistically significant difference was found only at 6 h after the 1st vincristine administration (Fig. 3A). In the small and large intestine (Fig. 3B-D), the 1st vincristine administration did not induce any significant effect, whereas the 10th vincristine administration delayed the emptying phase of the small intestine and the filling phase of both caecum and colorectum. Two weeks after treatment finalization, no significant difference in any of the motility curves was found for any of the gastrointestinal regions compared to saline-treated rats (Fig. 3A-D).

In the morphometric study of stomach and caecum, significant differences in organ size between control and vincristine-treated rats were found after the 10th drug administration (Fig. 3E-G), but not after the 1st administration or 2 weeks after treatment finalization (not shown).

Fluoroscopy study

Saline-treated rats subjected to 3 sessions of 120 seconds showed a normal increase in their body weight throughout the duration of the whole experiment. Vincristine reduced body weight gain during treatment, but thereafter body weight gain resumed. In fact, the difference in body weight between both groups of rats was only significant at the end of treatment and for the first 2 weeks after treatment finalization (Fig. 4A).

In table I (supplementary information) are shown the basic features of the recorded movies. Of a total of 45 movies, only 5 were discarded due to rat movements preventing a segment of at least 80 s to be analyzed. Of the remaining, most were useful for the analyses for their whole duration and only 3 had a useful segment between 80 and 119 s. Of the 40 rats included in the analyses, 35 had fecal pellets in their distal colon at the time of recording and were used for building STM. The number of fecal pellets within the distal colon was variable but differences were not statistically significant.

The diameter of the fecal pellets was about 5-5.5 mm for control rats at the 3 different time-points evaluated. In vincristine-treated animals, fecal pellets were slightly but significantly narrower after the 10^{th} administration of the drug, but in the 2 remaining fluoroscopy sessions no significant differences were found in fecal pellet diameter between treatments (Fig. 4B).

Natural fecal pellet propulsion within the colon was altered by vincristine, as shown in the representative STMs (Fig. 4C). The analysis of the propulsion speed (PS) of fecal pellets (Fig. 4D), and the distribution of fecal pellets as “slow” (moving 0, 1, or 2 cm) or “fast” (moving 3 or more cm) was according to their behavior in the maps (Fig. 4E).

PS for control animals decreased throughout time, but the difference was not statistically significant ($p=0.0823$, One-Way ANOVA). At the end of treatment, the mean PS of the pellets from saline-treated rats was 2.85 ± 0.75 cm/min, whereas in rats treated with vincristine this parameter was significantly lower, of only 0.55 ± 0.11 cm/min (Fig. 4D). Accordingly, the proportion of “slow” fecal pellets was higher in vincristine-treated animals, compared to control rats, and the difference was almost statistically significant ($p=0.0505$) (Fig. 4E).

During the second fluoroscopy session (3 weeks after treatment finalization, week 5), mean PS of fecal pellets in vincristine-treated animals was not significantly different from that in saline-treated rats (which was slightly lower than in the previous session, the difference not being statistically significant) (Fig. 4D). The percentage of "slow" pellets was now lower in vincristine-treated rats than in control animals, although the difference did not reach statistical significance (p=0.1367) (Fig. 4E).

Finally, during the third fluoroscopy session (6 weeks after treatment finalization, week 8), no significant effects of vincristine were apparent for PS or distribution of fecal pellets compared to saline-treated rats (Fig. 4D-E).

Histopathological analysis

The histopathological analysis of the samples from fundus and fore stomach did not show any remarkable changes (not shown).

General damage was observed in the ileum wall after chronic vincristine treatment (Fig. 5A and C), when compared with saline-treated animals (Fig. 5B). Thus, inflammatory cell infiltration in the lamina propria and submucosa, and some damage in the epithelial cells of the mucosal layer were found in vincristine-treated animals. Two weeks after treatment finalization, histological damage was reduced to similar values to the saline-treated controls (Fig. 5D).

There were some structural changes in some components of the intestinal wall. Chronic vincristine caused statistically significant ($p<0.05$) villi shortening that still remained 2 weeks after vincristine treatment finalization (Fig 6A). In contrast, no remarkable changes were found in the number of goblet cells in epithelium after sample evaluation (data not shown).

Submucosal thickness increased in vincristine-treated animals in a statistically significant manner (Fig. 6B, $p<0.05$). Similarly, muscle layer thickness increased with vincristine treatment both in the longitudinal ($p<0.05$) and the circular layers (although this difference was not statistically significant, $p=0.11$). The normal thickness of all these layers was recovered in samples obtained 2 weeks after treatment finalization (Figs. 6C and 6D).

The histological analysis of colonic samples revealed some damage ($p=0.069$) after chronic vincristine (Fig. 7A, C) compared with saline treatment (Fig 7A, B), with presence of extended inflammatory cell infiltration and occasional large lymphocytic nodules. Some damage was observed on the surface epithelial cells of the mucosa (Fig. 7C). Two weeks after treatment finalization, the damage to the epithelial cells decreased, but prominent inflammatory nodules could still be observed (Fig. 7D). Thus, the difference between vincristine-treated and control animals at this time-point was similar to the previous time-point ($p=0.067$) (Fig. 7D).

Myenteric plexus analysis in whole-mount preparations

Compared to saline-treated animals, the proportion of nNOS-IR neurons significantly increased in vincristine-treated animals. Two weeks after vincristine treatment finalization (early sequelae), the proportion of nNOS-IR cells was still higher in these animals than in saline-treated ones (Fig. 8).

DISCUSSION

In the present study, we have evaluated the effects of vincristine treatment on general health parameters, and on the gastrointestinal motor function and structure.

Chronic vincristine administration significantly reduced body weight gain, which may be explained by the concomitant reduction in food consumption. This is a well-known effect of antineoplastic drugs after acute or chronic treatment[7,15-20]. Chronic vincristine also induced peripheral neuropathy, which is in agreement with previous reports from experimental animals[20]. Furthermore, this sign persisted 2 weeks after treatment finalization, suggesting that this neurotoxicity is relatively long lasting. In fact, neuropathic pain followed by mixed sensory-motor neuropathy is a well-known adverse effect of vincristine that often limits its clinical use and persists even after stopping its administration[21-23].

Thus, the dose and pattern of administration of vincristine chosen for the present study induced some adverse effects typically encountered in vincristine-treated patients. Gastrointestinal adverse effects occurring once chemotherapy treatment has finished are relatively unknown. In a previous study we evaluated the effects of single doses of vincristine on general gastrointestinal motor function. However, the high doses used in that study may affect survival and do not allow for the study of chemotherapy-induced long-term sequelae[24]. Here we show, for the first time by radiographic means (both X-rays and fluoroscopy), the effects of the repeated administration of a lower dose, which better mimics the clinical situation.

Regarding gastric motor function, vincristine slightly reduced gastric emptying after the first administration, but this effect was aggravated with its repeated administration. It has been reported that vincristine-induced gastric hypomotility is not an early event[6,25] and 24-48 h may be necessary to see significant effects[24]. However, the cumulative doses of the drug also

contributed to its increased effect seen after the last administration. Interestingly, Mitolo-Chieppa et al.[26] described a progressive inhibition of the rat gastric vagus nerve activity with increasing vincristine doses, and vagus nerve activation mediates gastric emptying[27]. Whatever the case may be, aggravation of gastric dysmotility with repeated administration of antineoplastic drugs has also been described in rats treated with cisplatin[28] and 5-fluorouracil[29]. Interestingly, stomachs of animals chronically treated with vincristine were not distended, whereas distension was typically encountered after treatment with cisplatin[28]. The level of gastric distension produced could be related to the different emetogenic potential of these two drugs: whereas cisplatin is highly emetogenic, vincristine is not[30]. Accordingly, whereas cisplatin induces pica in the rat (an indirect marker of nausea in non-vomiting species[31]), vincristine does not[32]. Thus, gastric distension (and not simply delayed gastric emptying) might be considered an additional indirect marker of nausea in the rat.

The small and large intestines are common sites for drug adverse effects to occur[33]. Thus, we evaluated the intestinal motility, the persistence of the effects encountered during treatment and if they were accompanied by structural alterations in the gut wall, particularly in the myenteric neuronal population that has been shown to be chronically altered by other antineoplastic drugs[34,35].

Regarding the small intestine, motility was affected by the last vincristine administration (but no differences with control animals were observed after the first dose or 2 weeks after treatment finalization). Although the delayed emptying phase observed in the motility curve could be due, at least partly, to the delayed gastric emptying, direct effects of vincristine on the small intestine have also been reported to occur, including altered myoelectric activity,

increased tone and spasmogenic actions[36,37]. Our previous work using acute vincristine administration also indicated that the small intestine might be directly affected by vincristine[24]. Interestingly, only slight histological damage was present after the last administration of the drug, and normal histology of the small intestinal wall was completely recovered 2 weeks after. Regarding particular parameters of the ileal architecture, villi were shortened, whereas the thickness of submucosa and smooth muscle layers increased. Similar damage in the distinct structures of the mucosa, mainly in the villi, has been previously described for other chemotherapy compounds after acute[29,38] and repeated treatment[34,35], reflecting the action of chemotherapy on cell division and inflammation. In contrast, repeated cisplatin decreased the width of submucosa and muscle layers whereas vincristine increased it. When the histological study was performed 2 weeks after the last administration, only the length of villi was altered, whereas probably related to the recovery of normal motility in the small intestine, the thickness of both muscular layers (and submucosa), were similar to control animals. As far as we know, this is the first time that a complete histological analysis on the acute and long-term effects of vincristine has been performed. More research is needed to determine the mechanisms involved.

After the last vincristine administration, delayed emptying of the small intestine affected also filling of the caecum, which was significantly delayed. Similarly, the motility curve for the colorectum was delayed. We performed an additional study in which we could directly observe colonic propulsion of fecal pellets within the colorectum using fluoroscopy and barium contrast. Thus, after the last administration of vincristine, the propulsion speed and the proportion of fast fecal pellets were significantly reduced, compared to control animals. Three (early sequelae) and six (late sequelae) weeks afterwards, these parameters increased and were not significantly different from saline-treated animals. Our results are in

accordance with the reports of constipation and paralytic ileus associated to treatment with vinca alkaloids described in humans and animals[8,24,39-46]. In addition, similar to our results, Peixoto Junior et al[7] detected vincristine-induced fecal retention, with recovery of fecal output after finalization of treatment.

Interestingly, at time-points when caecum of vincristine-treated animals was full of contrast, this organ showed a smaller size, suggesting its contents were probably less hydrated. Furthermore, vincristine treatment reduced the mean diameter of colonic fecal pellets, as seen in our fluoroscopy experiment. Dryness of intestinal contents may be a cause but also a consequence of constipation. Less hydrated fecal pellets could be smaller, leading to reduced mechanical stimulation of the gut wall[47] and pellet retention. On the other hand, the longer fecal pellets stay in the gut the dryer they may get, aggravating the problem. Interestingly, changes in aquaporin expression have been related to diarrhea or opioid-induced constipation[48,49]. To our knowledge, whether this mechanism is also involved in vincristine-induced constipation is not known.

The histological analysis of the colon performed after the last vincristine administration revealed some damage to the mucosa layer and lymphocyte infiltration. Although it persisted for 2 weeks after treatment finalization, the contribution of this damage to motility alterations was probably negligible. In contrast, gastrointestinal motility changes might be due to autonomic dysfunction[6] caused by drug-induced neurotoxicity[5,50], including direct effects on ion conductance[51]. The presence of neuropathic pain associated with chronic vincristine treatment could potentially enhance adrenergic activity and delay GI transit. However, disruption of the normal propulsive activity of the gastrointestinal tract reverted soon after treatment interruption[7,36,44], in contrast with sensory neuropathy that persisted,

as also shown here. We observed an increase in the proportion of myenteric neurons immunoreactive to nNOS, which are mainly involved in inhibitory motor circuits. Changes in the enteric nervous system have been suggested as a potential underlying mechanism for abnormal colonic motor function and constipation[53]. Recently, several reports have described chemotherapy-induced alterations in upper GI transit and/or colonic motor activity associated with changes in the myenteric plexus, suggesting that chemotherapy might trigger the development of an enteric neuropathy. For example, 5-fluorouracil induced diarrhea and accelerated transit at short times after treatment[29,53], but repeated administration of this drug was associated with delayed GI transit, inhibition of colonic contractions and myenteric neuronal loss[35]. Also, repeated cisplatin and oxaliplatin reduced intestinal motor activity in rats and mice, respectively, and induced changes in glial cell populations[54], as well as a disbalance in the expression of markers in the myenteric plexus, favoring inhibitory pathways[15,34,54,55]. Interestingly, the altered immunoreactivity found here in the myenteric plexus did not recover 2 weeks after treatment discontinuation. Persistent changes in the myenteric plexus may predispose to altered motility in response to pathological stimuli or may induce compensatory effects. In fact, we observed a tendency in the proportion of fast endogenous pellets to be increased, as an early sequel of treatment. Six weeks after treatment finalization, colonic propulsion and distribution of fecal pellets as slow and fast was normalized.

Finally, patients at risk of suffering vincristine-induced constipation are currently treated with laxatives, but these are not always useful and alternative treatments are needed[46]. We have recently shown that endocannabinoid release and activation of CB1 cannabinoid receptor might be involved in vincristine-induced inhibition of intestinal motility, at least

after a single high dose[24]. More research is needed to determine if similar mechanisms are involved in the effects exerted by vincristine repeatedly administered.

CONCLUDING REMARKS

Some of the effects produced by vincristine repeatedly administered in rats are described. Whereas reduction in general gastrointestinal motility and colonic propulsion of endogenous fecal pellets seem to recover relatively soon after treatment, vincristine-induced neuropathy (somatic and enteric), intestinal mucosa damage and inflammatory infiltrations are relatively long lasting. Sequelae of chemotherapy should be more closely monitored.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

RA designed the study. Parameters on general health were obtained by EH and VLM. SDR, RG, AELP and RA performed the functional experiments on gastrointestinal motor functions. Experiments on peripheral neuropathy and myenteric neuronal counts were performed by GV. LLG and JAU performed the histological studies. LLG, SDR, JAU and RA wrote the manuscript. KN and MIMF contributed essential intellectual input. MIMF contributed financial support. All authors reviewed and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Table I. Basic features of movies used for the analyses. **N':** Number of animals included in the study per experimental group and time point**. A (%):** Number and % of movies that were used for further analyses (movies in which rat movements did not allow for the analyses to be performed for longer than 80 s were excluded; their duration is shown in italics, second raw in each cell). **D**: Mean duration of movie segments used for further analyses (obtained from the movies included in A, given in seconds). Most recordings were used for their whole duration (120 s); duration of shorter useful movie segments is shown in italics. **B (%):** Number and % of analyzed movies (obtained from A) in which animals had stained fecal pellets in their colon at the time of recording and from which spatiotemporal maps (STM) where built. **MFP**: Mean number of stained fecal pellets per STM. Rats were injected intraperitoneally (ip) with: saline (0.5 ml) or vincristine (**VC**), (0.1 mg kg^{-1}), in 2 daily cycles of 5 administrations each, separated by 2 days. Fluoroscopy was performed after the 10^{th} ip drug administration (1^{st} fluoroscopy session: week 2, VCx10) as well as 3 (2^{nd} fluoroscopy session: week 5, seq) and 6 weeks after treatment finalization (3^{rd} fluoroscopy session: week 8, seq). For each session, rats were gavaged a load of contrast medium (barium sulfate, 2 g ml^{-1}, 1.5 ml) at least 20 hours before, so that stained fecal pellets could be found within the colon at the time of recording.

Figure S1: Representative spatiotemporal maps built from fluoroscopic recordings. Rats were gavaged two loads of barium contrast: the first one, 20-22 h before recording, so that

fecal pellets could be seen faintly stained at the time of recording; the second one, 1.5 h before recording, so that the stomach and the small intestine were stained but caecum was not reached by this load. Fluoroscopic recordings were 120 s long. In this study we only analysed fecal pellet movement within the colon. For this, spatiotemporal maps (STM) were built in Excel after transforming the fluoroscopic recordings in series of images at 1 frame per s. See main text and legend for Fig. 4 for details on STM construction. Two representative examples are shown corresponding to videos S1 (left) and S2 (right), respectively. In each example, the STM and two frames (30 s, highlighted in red; and 60 s, highligthed in blue) are shown. Arrows indicate the position of one fecal pellet in each frame. S1 corresponds to a rat treated with saline (control). S2 corresponds to a rat treated with vincristine. Drugs were administered in two cycles of 5 administrations per day separated by two days. Recordings were obtained 30 min after the last administration. Only one very faintly stained fecal pellet was present in S1, whereas 5 well stained fecal pellets could be followed in S2. S: stomach. SI: small intestine. C: caecum. Scale bar: 4 cm.

FIGURE LEGENDS

Figure 1. Experimental protocol. Rats were injected intraperitoneally (ip) with saline (2 ml kg^{-1}) or vincristine (0.1 mg kg^{-1}) in 2 daily cycles of 5 administrations each, separated by 2 days. **A**: general parameters such as body weight and food and water intake were regularly recorded; X-Rays were performed after the first vincristine (VC) administration on week 1 (acute effect), just after the last VC administration on week 2 (chronic effect) and 2 weeks after treatment finalization, on week 4 (early sequelae), in order to evaluate VC short- and

relatively long-term effects on general gastrointestinal motor function (see text for details). To evaluate the presence of peripheral neuropathy, mechanical sensitivity was assessed by measuring the withdrawal threshold of the hindpaws to calibrated von Frey hairs. The test was performed before (week 0), after (week 2) and one (week 3) and 2 weeks (week 4) after treatment finalization. Gastrointestinal samples for histopathological analysis were taken right after finishing VC treatment (week 2) and 2 weeks after treatment finalization (week 4). At the same time-points, additional samples from colon were processed as whole-mount preparations (WMP) to evaluate differences in myenteric neuronal populations. **B**: body weight was regularly monitored; fluoroscopy recordings were performed in three different sessions (see text for details), the first one was right after the 10^{th} VC administration, the second one was 3 weeks after that administration (week 5), and the third one was 6 weeks after it (week 8).

Figure 2. Effect of vincristine (VC) on general health parameters in the rat: body weight, food and water intake, and mechanical sensitivity. Body weight (A), food (B) and water (C) intakes, and mechanical sensitivity to calibrated Von Frey's hairs (D) are shown. Rats were injected intraperitoneally (ip) with: saline (2 ml kg^{-1}, dotted line and white bars) or vincristine (0.1 mg kg^{-1}, red line and red/black bars) in 2 daily cycles of 5 administrations each, separated by 2 days. Recordings were performed before (week 0), during (weeks 1-2, red bars) and after treatment (weeks 3-4: black bars). Lines and bars show mean values ± SEM. *p<0.05, ***p<0.001 *vs* saline (control group) (one-way ANOVA followed by Bonferroni *post-hoc* test).

Figure 3. Effect of repeated vincristine (VC) on general gastrointestinal motor function in the rat. Gastrointestinal motor function was evaluated by radiological methods (see text) in:

(A) stomach (gastric emptying); (B) small intestine; (C) caecum and (D) colorectum. Rats were injected intraperitoneally (ip) with: saline (2 ml kg^{-1}) or vincristine (0.1 mg kg^{-1}) in 2 daily cycles of 5 administrations each, separated by 2 days. Radiographic sessions were performed after the 1st (VCx1) and 10th (VCx10, week 2) ip administrations, and 2 weeks after the end of treatment (VC seq, week 4). In each session, barium sulfate (2.5 ml, 2 g ml^{-1}) was intragastrically administered and X-rays were obtained 0–8 h after contrast. Morphometric analysis of the temporal changes in the size of the (E) stomach and the (F) caecum were made for both groups of treated rats after the 10th administration using the image processor Image J. (G): Representative images of animals treated with saline (upper raw, dotted black line border) or vincristine (bottom raw, solid red line border), after the 10th ip administration, at 0, 1, 4 and 8 h after contrast. Data represent mean ± SEM. N=7-8, each group. *p<0.05, **p <0.01, ***p <0.001*vs* saline; # p<0.05, ## p<0.01, ### p<0.001 *vs* VCx1; $ p<0.05, $ p<0.01, $ p< 0.001 *vs* VCx10 (2-way ANOVA followed by *post-hoc* Bonferroni multiple comparison test). Scale bar = 3 cm.

Figure 4. **Effect of repeated vincristine (VC) on colonic motility measured by *in vivo* fluoroscopy recordings in the rat.** Rats were injected intraperitoneally (ip) with: saline (0.5 ml; n=8; dotted line and white bars) or vincristine (VC, 0.1 mg kg^{-1}; n=8, red line and red/black bars) in 2 daily cycles of 5 administrations each, separated by 2 days. Fluoroscopy was performed after the 10th ip administration (1st fluoroscopy session: VCx10, week 2, red bars), as well as 3 (2nd fluoroscopy session: week 5 seq, black bars) and 6 weeks after treatment finalization (3rd fluoroscopy session: week 8 seq, black bars). For each session, rats were gavaged a load of contrast medium (barium sulfate, 2 g ml^{-1}, 1.5 ml) at least 20 hours before, so that stained fecal pellets could be found within the colon at the time of recording.

(A): Body weight gain throughout the experiment. Lines represent mean ± SEM. *p<0.05, **p<0.01 *vs* saline (2-way ANOVA followed by post-*hoc* Bonferroni multiple comparison test). (B): Diameter of fecal pellets found within the distal colon at the time of recording. Diameter of fecal pellets was evaluated using *Image J* program (see text). Bars represent mean ± SEM. *p<0.05 *vs* saline (Student's *t* test). (C): Representative spatiotemporal maps (STM) for saline- and VC-treated rats (one for each treatment) at the different fluoroscopy sessions. In each map, the horizontal axis represents distance along the colon (left to right = proximal to distal; only the last 7.27 cm proximal to the anus were evaluated, each horizontal square represents a 10^{th} of this distance) whereas the vertical axis represents time of recording (top to bottom = 0 to 120 s, each vertical square represents 3 s). Instant position of fecal pellets is represented as black squares in each cell of the STM; movement is shown as a horizontal change of the fecal pellet position. Grey rectangles represent parts of the recordings in which fecal pellets could not be seen due to overlapping of other gastrointestinal structures. (D): Propulsion speed of fecal pellets. Bars represent mean ± SEM. **p<0.01 *vs* saline (2-way ANOVA followed by post-*hoc* Bonferroni multiple comparison test); # p<0.05 *vs* vincristine after 10^{th} administration (one-way ANOVA for treatment). (E): Pie charts showing the distribution of fecal pellets with "slow" (0, 1 or 2 cm, black) and "fast" (≥ 3 cm, white) movement as shown by the STM during the 3 fluoroscopy sessions. The results of the statistical analysis are shown at the right for each fluoroscopy session (Fisher's exact test).

Figure 5. Effect of vincristine treatment on the general structure of the rat small intestinal wall. Rats received 2 cycles of 5 daily intraperitoneal (ip) injections (2 consecutive weeks) of saline (2 ml kg^{-1}) or vincristine (0.1 mg kg^{-1}). Histological damage of Ileum was evaluated

after vincristine treatment (week 2) and 2 weeks after its end (week 4). Ileal sections were embedded in paraffin and stained with H/E. (A): Histological damage was evaluated using criteria adapted from Galeazzi et al.[12], which gives a maximum value of 9 points (dotted horizontal bar). Each group consisted of 4-8 rats. Bars represent mean ± SEM. *p<0.05 *vs* saline (Student's *t* test). Results from saline-treated controls are represented in white, those from animals sacrificed right after vincristine treatment (chronic) in red, and those from animals sacrificed 2 weeks after vincristine treatment finalization (sequelae) in black. (B): Photomicrograph of an ileal sample stained with H/E, from a control rat. (C): Photomicrograph of an ileal sample, stained with H/E, from a vincristine-treated rat, right after treatment finalization (chronic, week 2). (D): Photomicrograph of an ileal sample, stained with H/E, from a vincristine-treated rat, 2 weeks after vincristine treatment finalization (sequelae, week 4). Scale bar: 100 µm.

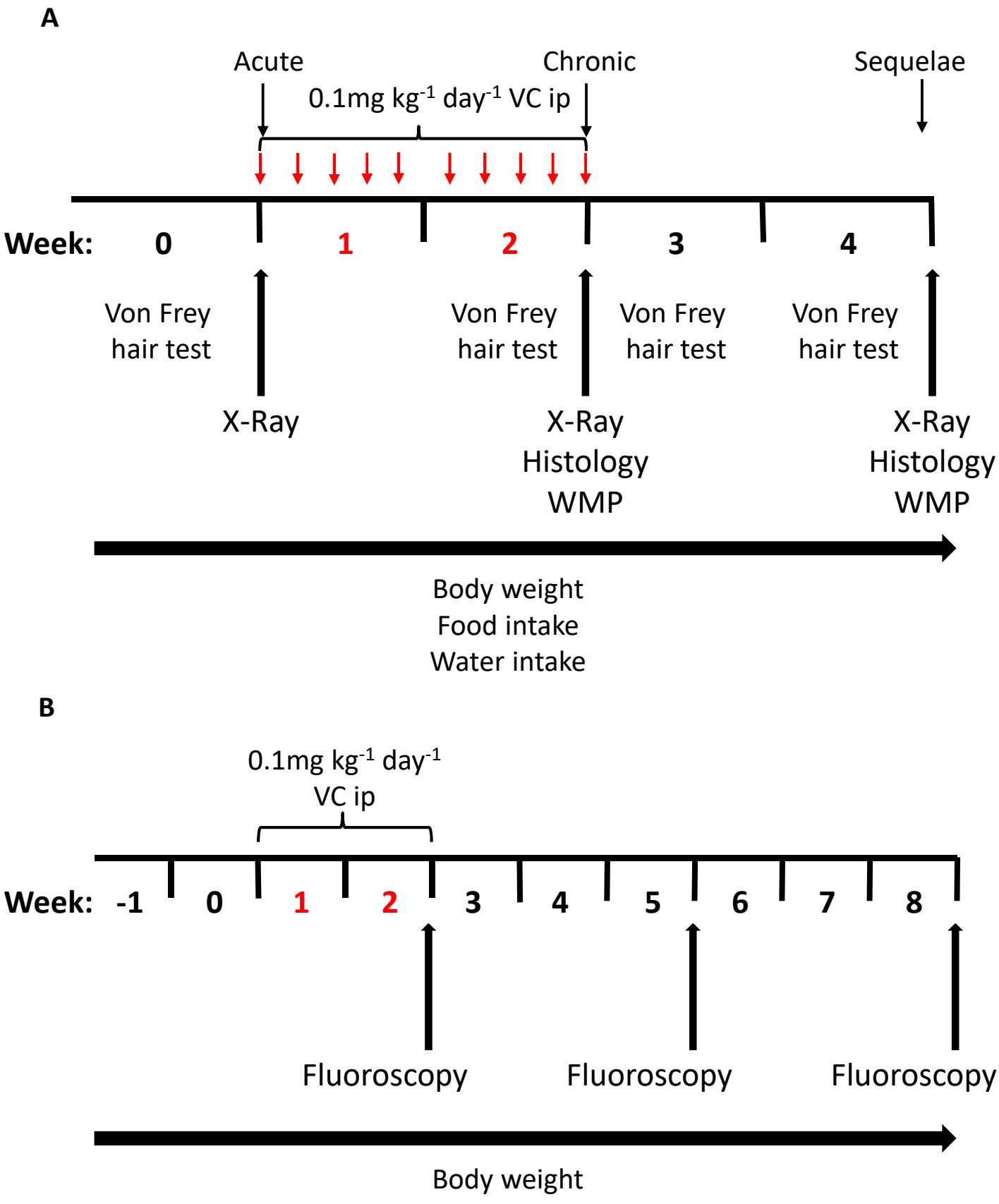
Figure 6. Effect of vincristine treatment on particular parameters of small intestinal wall structure. Rats received 2 cycles of 5 daily intraperitoneal (i.p.) injections (2 consecutive weeks) of saline (2 ml kg^{-1}) or vincristine (0.1 mg kg-1). Each group consisted of 4-8 rats. Bars represent mean ± SEM. *p<0.05 *vs* saline (Student's *t* test). Results from saline controls are represented in white, those from vincristine-treated rats, right after treatment finalization (week 2, chronic) in red, and those from vincristine-treated rats, 2 weeks after treatment finalization (week 4, sequelae) in black. (A): Villi length evaluated on ileal sections embedded in paraffin and stained with H/E. (B): Submucosa thickness evaluated on ileal sections embedded in paraffin and stained with Van Gieson. (C): Inner circular muscle layer thickness evaluated on ileal sections embedded in paraffin and stained with H/E. (D): Outer

longitudinal muscle layer thickness evaluated on ileal sections embedded in paraffin and stained with H/E.

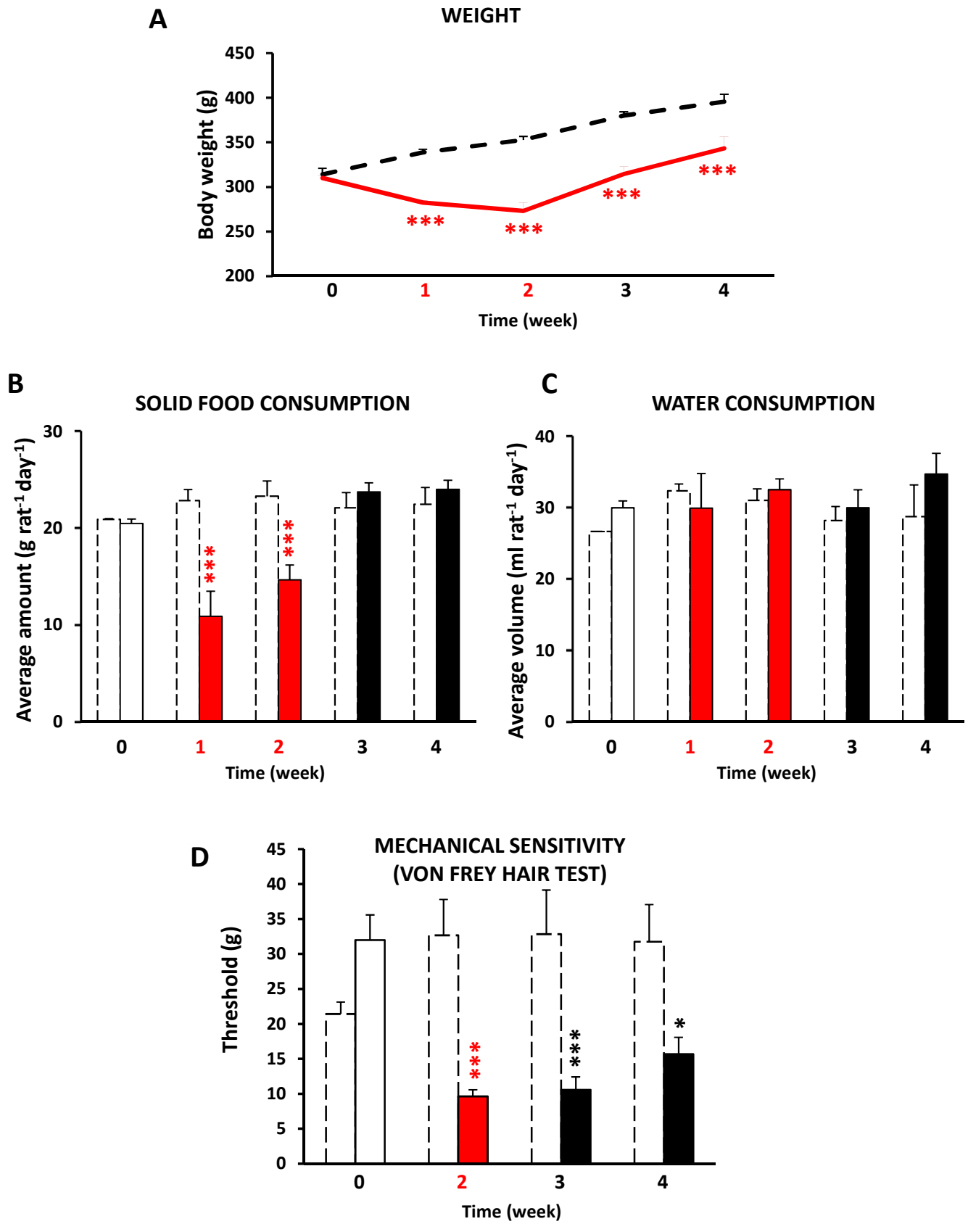
Figure 7: Effect of vincristine treatment on the general structure of the rat large intestinal wall. Rats received 2 cycles of 5 daily intraperitoneal (i.p.) injections (2 consecutive weeks) of saline (2 ml kg^{-1}) or vincristine (0.1 mg kg^{-1}). Histological damage of the colon was evaluated after vincristine treatment (week 2) and 2 weeks after its end (week 4). Colonic sections were embedded in paraffin and stained with H/E. (A): Histological damage was evaluated using criteria adapted from Saccani et al.[13], which gives a maximum value of 13 points (dotted horizontal bar). Each group consisted of 4-8 rats. Bars represent mean ± SEM. P value *vs* saline (Student's *t* test). Results from saline-treated controls are represented in white, those from animals sacrificed right after vincristine treatment (chronic) in red, and those from animals sacrificed 2 weeks after vincristine treatment finalization (sequelae) in black. (B): Photomicrograph of a colonic sample stained with H/E, from a control rat. (C): Photomicrograph of a colonic sample, stained with H/E, from a vincristine-treated rat, right after treatment finalization (chronic, week 2). (D): Photomicrograph of a colonic sample, stained with H/E, from a vincristine-treated rat, 2 weeks after vincristine treatment finalization (sequelae, week 4). Scale bar: 200 µm.

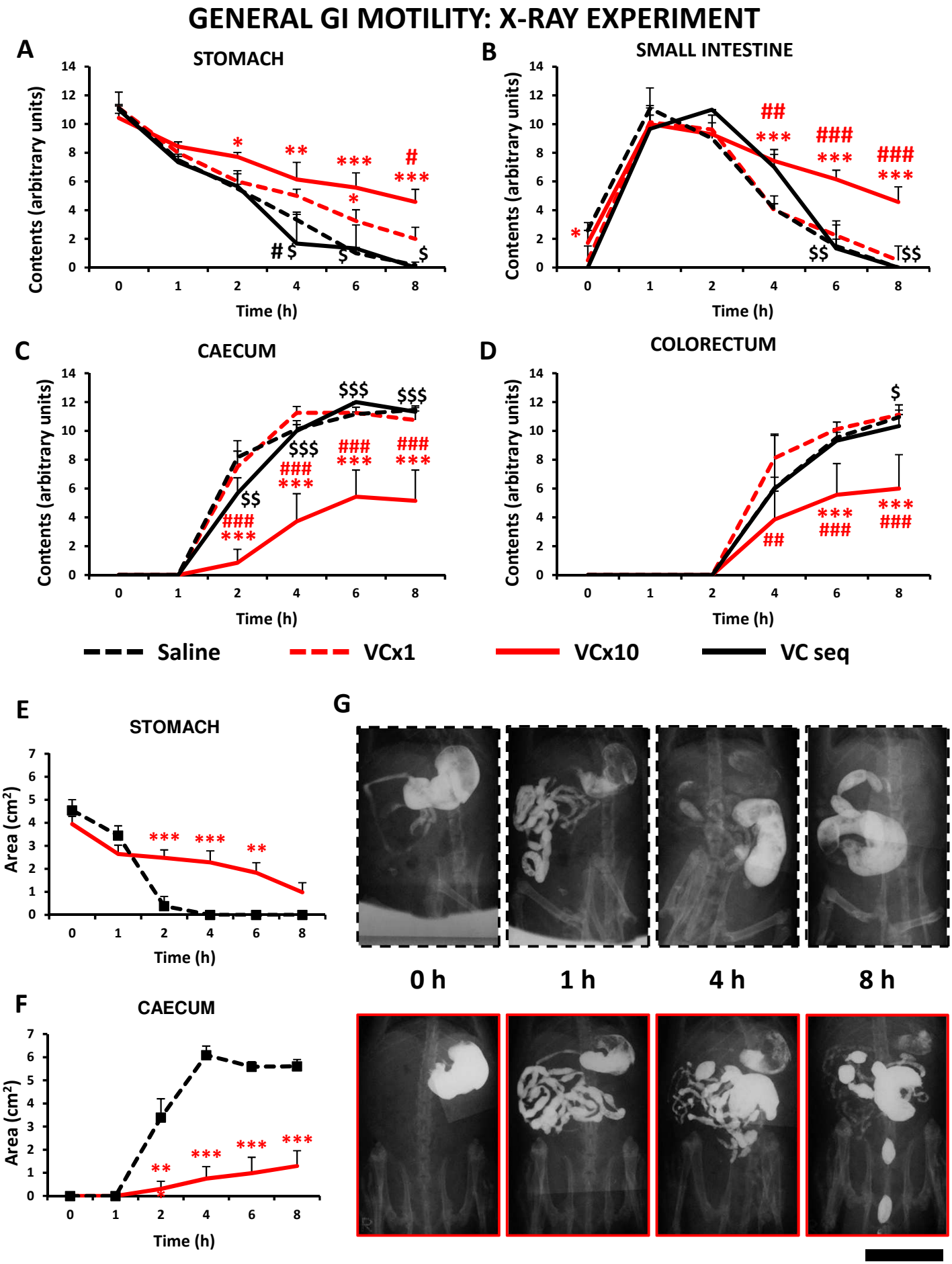
Figure 8: Effect of vincristine treatment on the populations of myenteric neurons immunoreactive to nNOS in the rat distal colon. Rats received 2 cycles of 5 daily intraperitoneal (i.p.) injections (consecutive weeks) of saline (2 ml kg^{-1}) or vincristine (0.1 mg kg^{-1}). The proportions of myenteric neurons immunoreactive to neuronal nitric oxide synthase (nNOS-IR) relative to the general neuronal population (IR to HuC/D) were analyzed in whole-mount preparations from the distal colon of rats after vincristine treatment (week

2) and 2 weeks after its end (week 4). A: Quantitative analysis. Bars represent the mean ± SEM. *p<0.05, **p<0.01 *vs* saline (Student's t test). Results from saline-treated controls are represented in white, those from animals sacrificed right after vincristine treatment (chronic) in red, and those from animals sacrificed 2 weeks after vincristine treatment finalization in black (sequelae). B: Photomicrographs of a whole-mount preparation immunolabelled for HuC/D and nNOS from a control rat. C: Photomicrographs of a whole-mount preparation, immunolabelled for HuC/D and nNOS from a vincristine-treated rat, right after treatment finalization (chronic, week 2). D: Photomicrographs of a whole-mount preparation, immunolabelled for HuC/D and nNOS from a vincristine-treated rat, 2 weeks after vincristine treatment finalization (early sequelae, week 4). Scale bar: 20 µm.

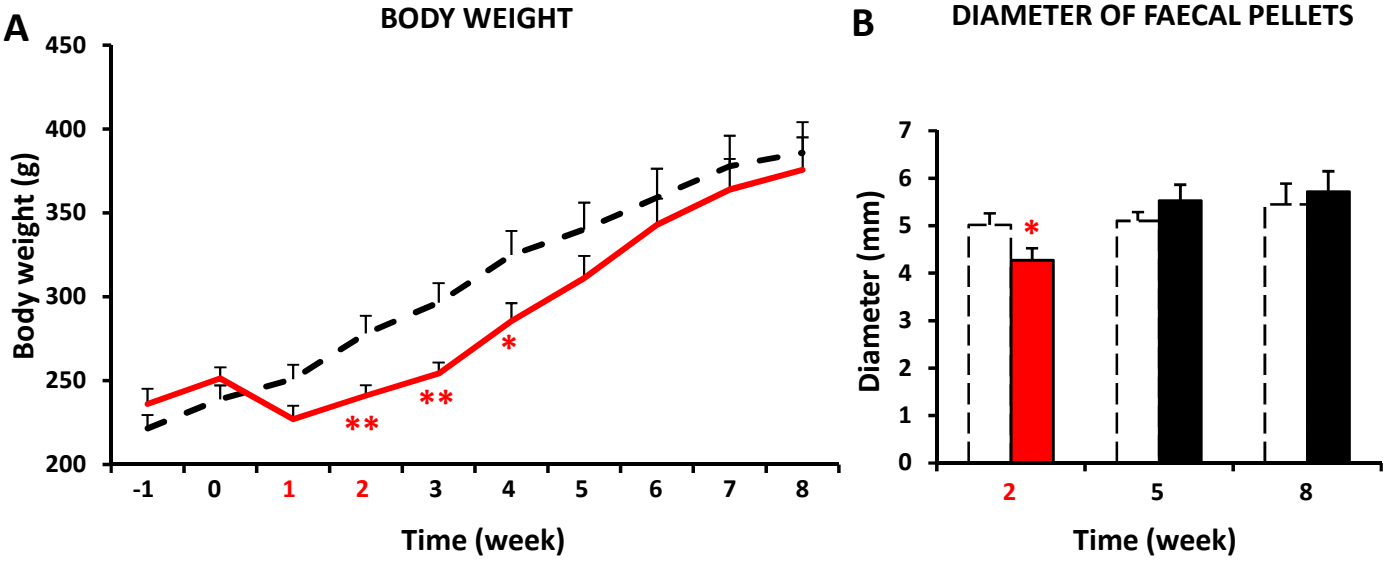

EXPERIMENTAL PROTOCOL
A
Acute
Chronic
Sequelae
0.1mg kg-1 day-1 VC ip
Week: 0 1 2 3 4
Von Frey hair test
Von Frey hair test
Von Frey hair test
Von Frey hair test
X-Ray
X-Ray Histology WMP
X-Ray Histology WMP
Body weight
Food intake
Water intake
B
0.1mg kg-1 day-1 VC ip
Week: -1 0 1 2 3 4 5 6 7 8
Fluoroscopy
Fluoroscopy
Fluoroscopy
Body weight

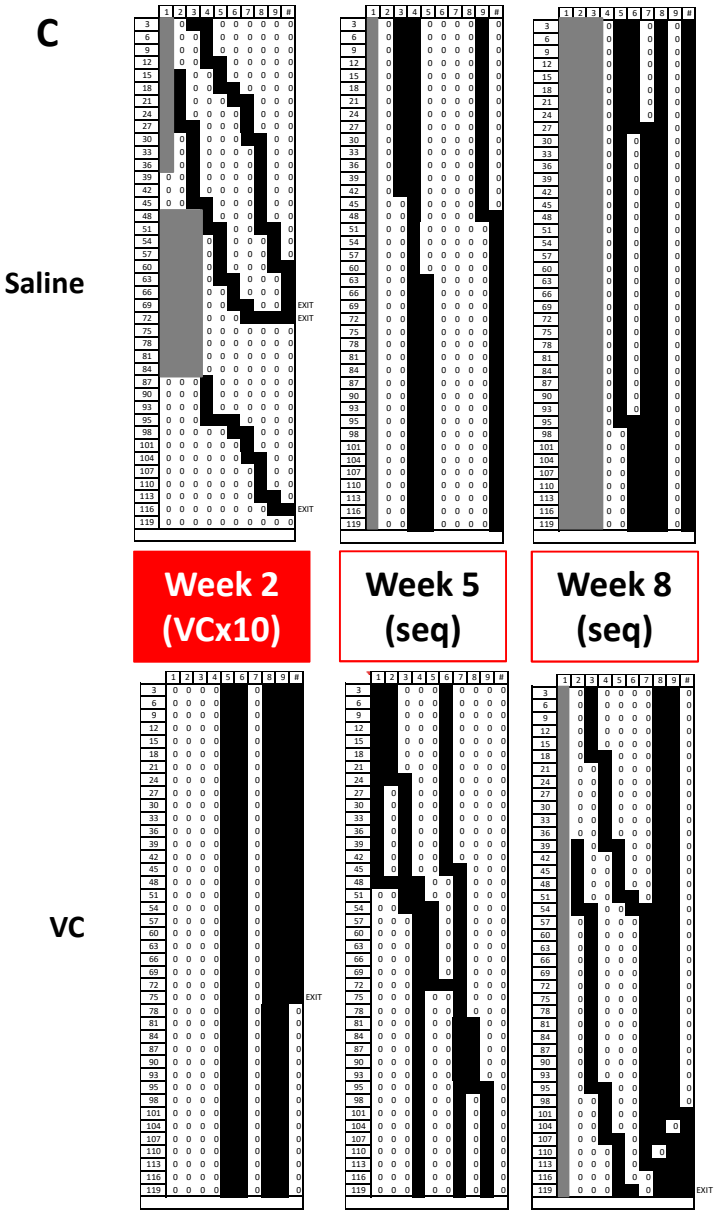
GENERAL HEALTH PARAMETES

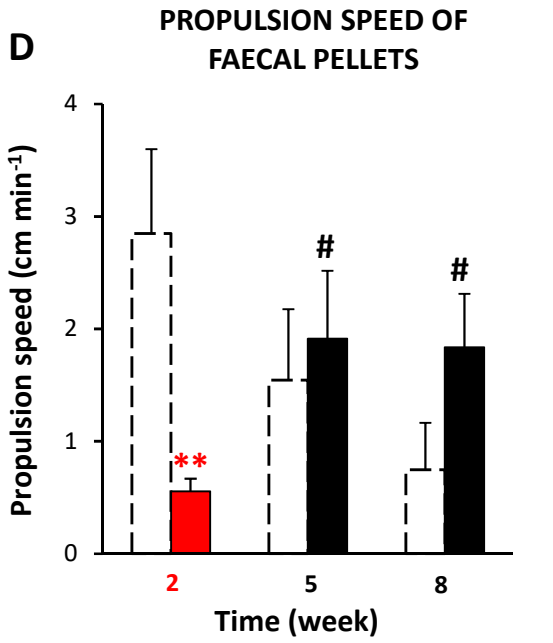


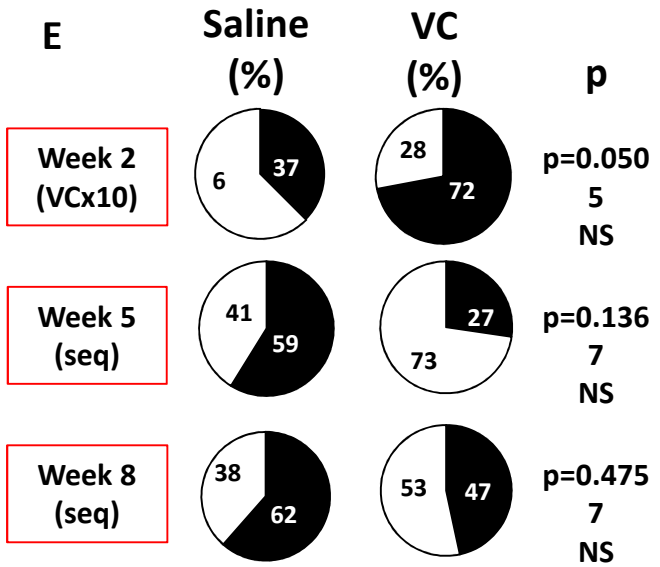

GENERAL GI MOTILITY: X-RAY EXPERIMENT
A
STOMACH
B
SMALL INTESTINE
C
CAECUM
D
COLORECTUM
Contents (arbitrary units)
Time (h)
Saline
VCx1
VCx10
VC seq
E
STOMACH
F
CAECUM
Area (cm²)
G
0 h
1 h
4 h
8 h

COLONIC MOTILITY: FLUOROSCOPY EXPERIMENT

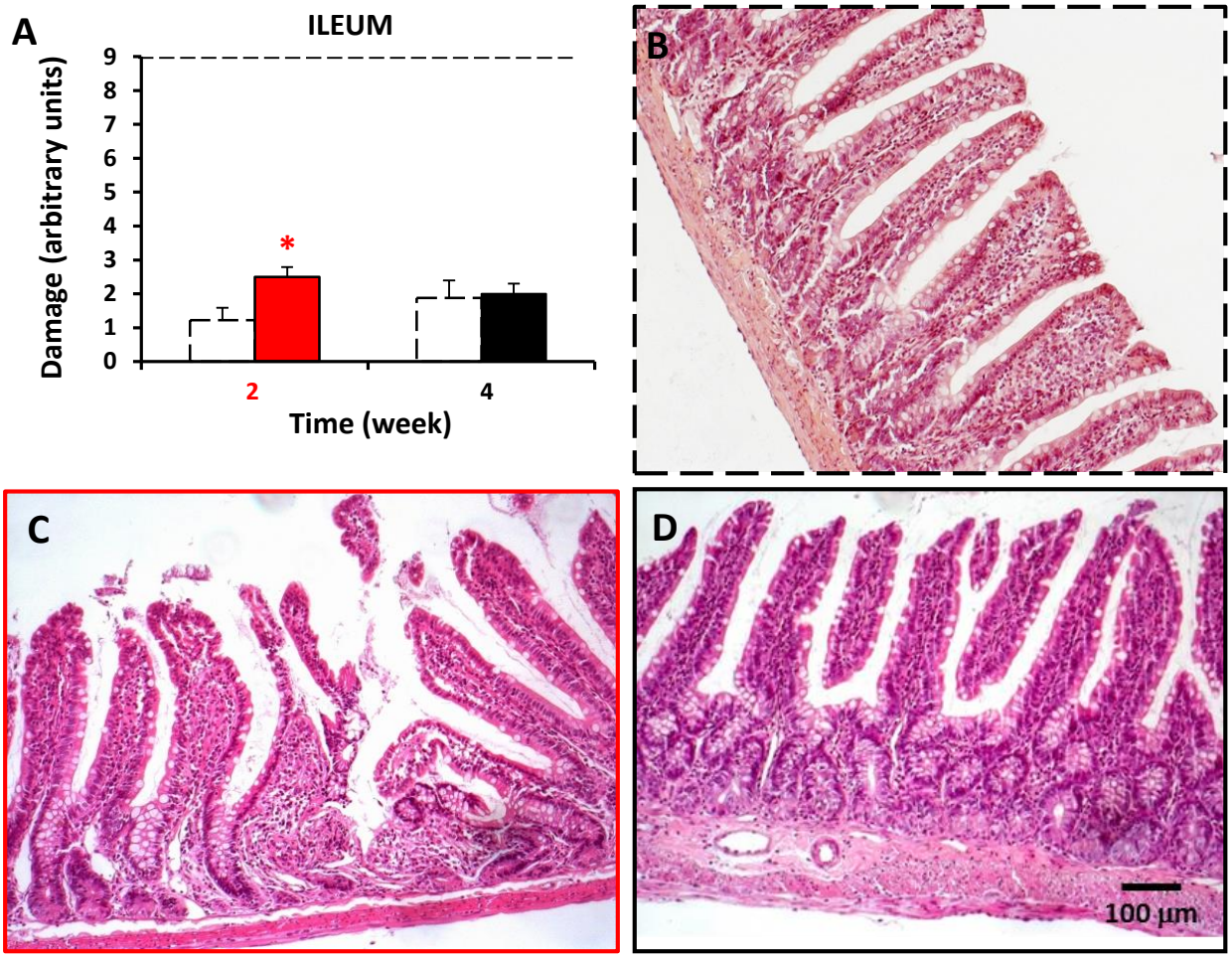




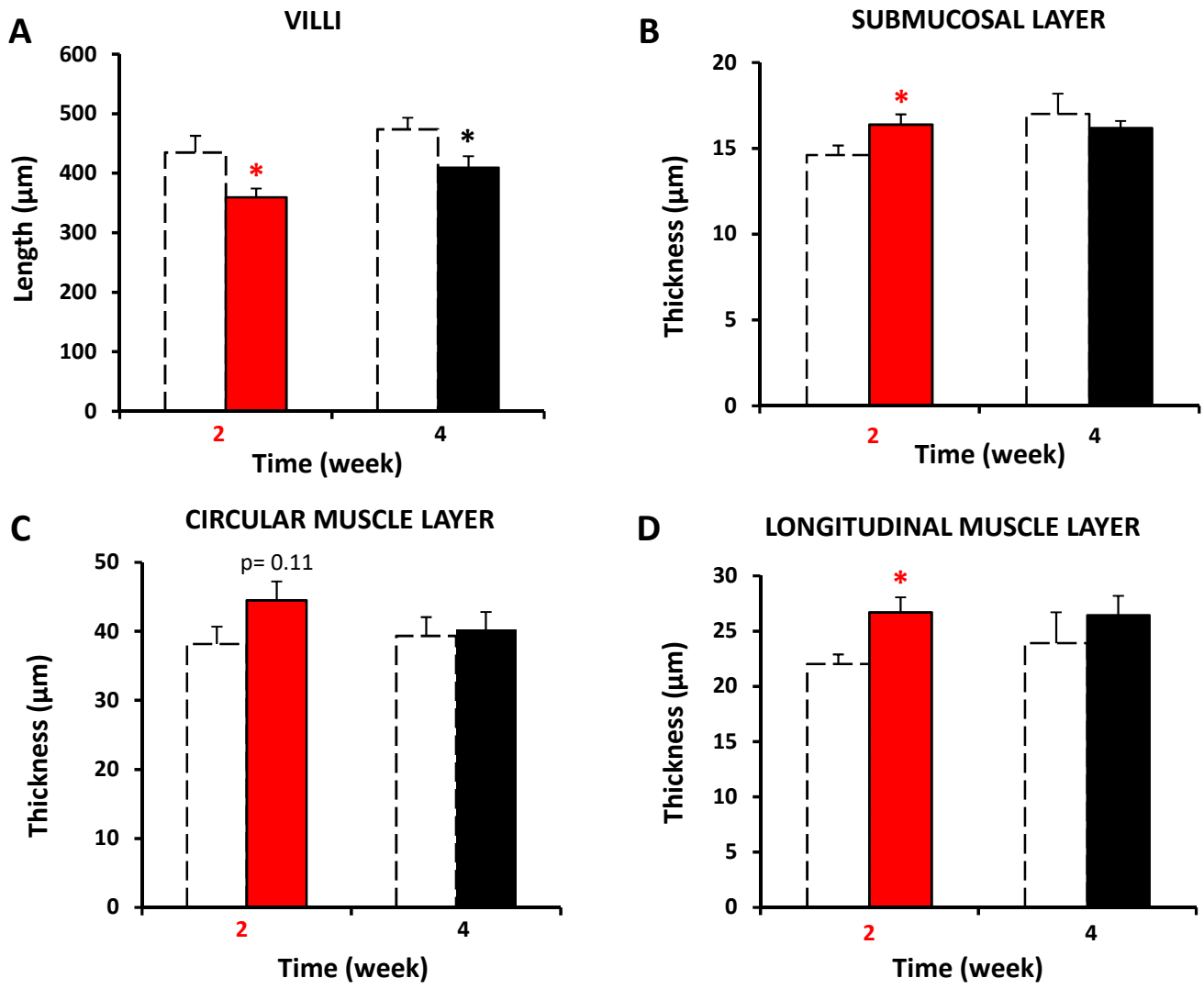




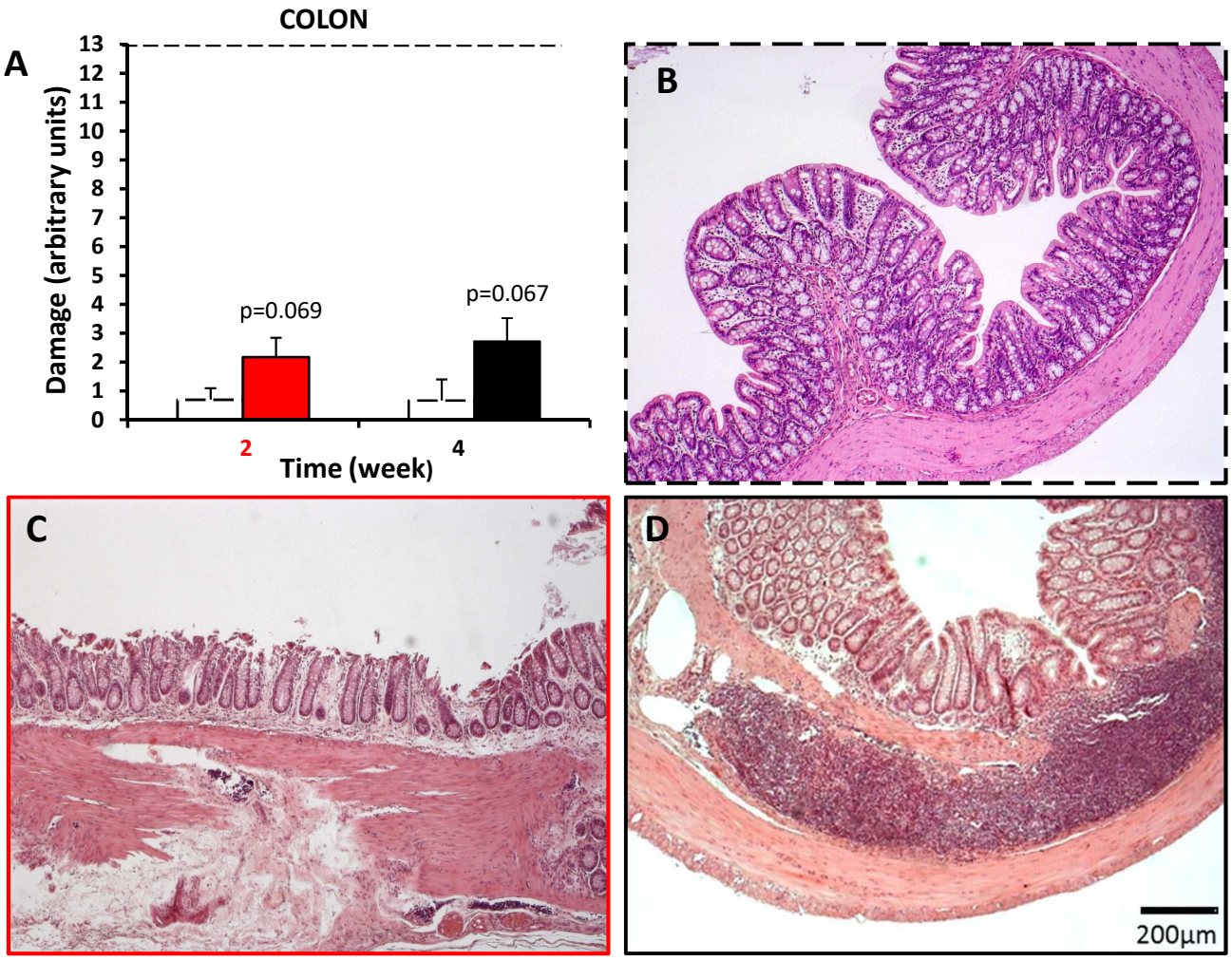
HISTOLOGICAL DAMAGE: ILEUM



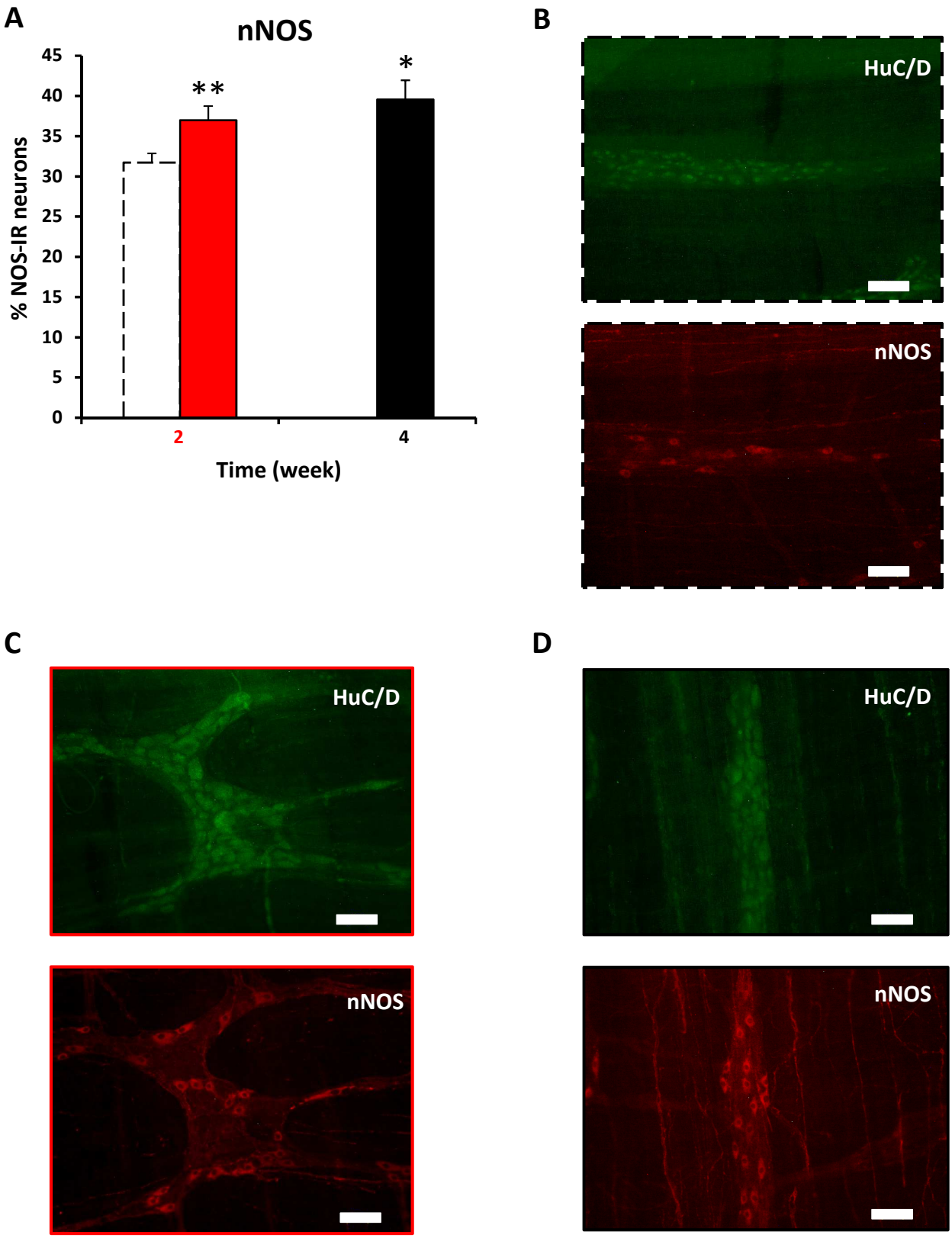
OTHER PARAMETERS AFFECTING ILEUM ARCHITECTURE



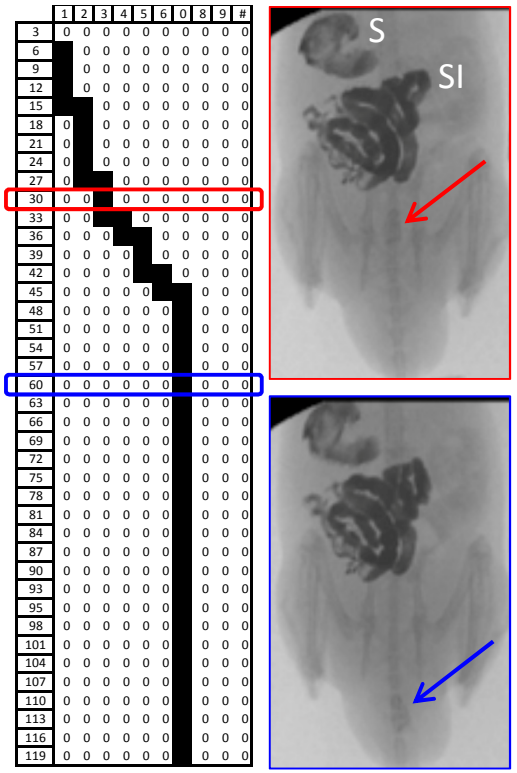
HISTOLOGICAL DAMAGE: COLON



WHOLE-MOUNT PREPARATIONS: COLON



S1-Not well stained



S2-Well stained

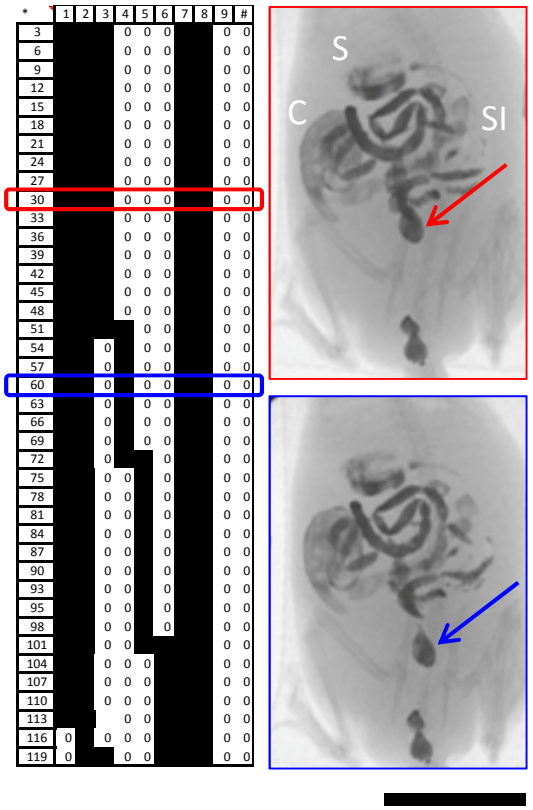


TABLE I. Basic features of movies used for the analyses.

		N'	A (%)	D (s)	B (%)	MFP
1st FLUOROSCOPY SESSION (week 2, VCx10)	**Saline**	8	7 (87.5) *1: 75 s*	120±0	6 (85.7)	2.43±0.57
	VC	6	6 (100)	113±7 *1: 80 s*	6 (100)	4.33±0.67
2nd FLUOROSCOPY SESSION (week 5, seq)	**Saline**	8	7 (87.5) *1: 0 s*	117±3 *1: 116 s; 1: 100 s*	7 (100)	2.43±0.32
	VC	7	5 (71.4) *2: 0 s*	120±0	5 (100)	2.20±0,47
3rd FLUOROSCOPY SESSION (week 8, seq)	**Saline**	8	7 (87.5) *1: 0 s*	120±0	6 (85.7)	2.17±0.66
	VC	8	8 (100)	120±0	5 (62.5)	3.40±0.97

N': Number of animals included in the study per experimental group and time point. **A (%):** Number and % of movies that were used for further analyses (movies in which rat movements did not allow for the analyses to be performed for longer than 80 s were excluded; their duration is shown in italics, second raw in each cell). **D**: Mean duration of movie segments used for further analyses (obtained from the movies included in A, given in seconds). Most recordings were used for their whole duration (120 s); duration of shorter useful movie segments is shown in italics. **B (%):** Number and % of analyzed movies (obtained from A) in which animals had stained fecal pellets in their colon at the time of recording and from which spatiotemporal maps (STM) where built. **MFP**: Mean number of stained fecal pellets per STM. Rats were injected intraperitoneally (ip) with: saline (0.5 ml) or vincristine (**VC**), (0.1 mg kg^{-1}), in 2 daily cycles of 5 administrations each, separated by 2 days. Fluoroscopy was performed after the 10^{th} ip drug administration (1^{st} fluoroscopy session: week 2, VCx10) as well as 3 (2^{nd} fluoroscopy session: week 5, seq) and 6 weeks after treatment finalization (3^{rd} fluoroscopy session: week 8, seq). For each session, rats were gavaged a load of contrast medium (barium sulfate, 2 g ml^{-1}, 1.5 ml) at least 20 hours before, so that stained fecal pellets could be found within the colon at the time of recording.