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Article

Involvement of MT_2 receptors in protective effects of melatonin against cisplatin-induced gastrointestinal damage in mice

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Melatonin (MLT) reportedly reduces side effects associated with certain antineoplastic agents. Accordingly, we investigated the effect of MLT on cisplatin (CP)-induced gastric emptying (GE) delay. Mice were intraperitoneally pretreated with vehicle (ethanol 5%; control group), MLT (5, 10, or 20 mg/kg), or N-acetylcysteine (NAC; 150 mg/kg), followed by CP treatment (5 mg/kg). Pharmacological modulation was analyzed using relevant receptor antagonists (luzindole: non-selective MT_1/MT_2 antagonist; 5 mg/kg or 4-P-PDOT: selective MT_2 antagonist; 4 mg/kg) before treatment with MLT plus CP. All treatments were performed once daily for three days. GE was assessed using phenol red. Gut morphology was examined using scanning electron microscopy and optical microscopy. Compared with the control, CP decreased GE. Pretreatment with NAC and MLT (5 and 10 mg/kg) did not prevent CP-induced gastric dysmotility; however, pretreatment with 20 mg/kg MLT prevented this effect. In addition, luzindole and 4-P-PDOT suppressed MLT-mediated gastroprotection against cytotoxic effects of CP. CP caused degeneration of the gut mucosa, which was attenuated by MLT treatment. Thus, 20 mg/kg MLT prevented the GE delay and decreased CP-induced adverse effects on the gut mucosa. In addition, the gastroprotective activity was mediated via the MT_2 receptor.

Keywords: Gastric emptying. Gut motility. Gastrointestinal. Chemotherapy.

INTRODUCTION

Cisplatin (*cis*-diamminedichloroplatinum II; CP) is one of the most effective and widely employed drugs for treating adult and pediatric cancers, including testicular, ovarian, bladder, breast, head, neck, and gastric cancers (Babu *et al*., 2019; Kim *et al*., 2019). However, treatment with CP has been associated with the development of serious adverse effects, impacting the kidney, heart, liver, reproductive, nervous, and gastrointestinal (GI) systems (Vera *et al*., 2011; Viana-Cardoso *et al*., 2011; Madhu, Reddy, Reddy, 2015).

Notably, CP-induced GI dysfunctions, such as gastroparesis, gastric distension, mucositis, diarrhea, and delayed gastric emptying (GE), are caused by peripheral neuropathy and/or local tissue damage (Cabezos *et al*., 2010; Vera *et al*., 2011; Viana-Cardoso *et al*., 2011; Pini *et al*., 2016; Babu *et al*., 2019), triggered by nuclear and mitochondrial DNA platination, along with the increased production of reactive oxygen species (ROS) and decreased activity of antioxidant enzymes (Ko *et al*., 2016; Shahid, Farooqui, Khan, 2018). Therefore, treatment with antioxidant agents can ameliorate CP-mediated toxic effects on the GI tract (Ko *et al*., 2016; Riyaz *et al*., 2017).

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N-acetylcysteine (NAC) contains a sulfhydryl group that acts as a source of cysteine for glutathione (GSH) synthesis, affording long-term antioxidant protection. NAC is administered to patients at relatively high levels, exhibiting an excellent toxicity profile, and is currently used in clinical practice (Monti *et al.*, 2017). In addition, NAC possesses anti-inflammatory effects (Anand *et al.*, 2015; Abdel-Wahab, Moussa, 2019). Furthermore, NAC can potentiate the effects of antineoplastic agents by decreasing the growth of tumor cells, in addition to mitigating the adverse effects of chemotherapy (Anand *et al.*, 2015; Muldoon *et al.*, 2015). Melatonin (*N*-acetyl-5-methoxytryptamine; MLT) is another efficient antioxidant agent that reduces toxic reactions associated with drugs and other processes known to generate free radicals (Reiter *et al.*, 2016; Farhood *et al.*, 2019).

MLT, a hormone primarily synthesized in the pineal gland, regulates circadian rhythms, immune responses, and reproductive functions (Tamura *et al.*, 2013; Reiter *et al.*, 2016). Additionally, MLT can be synthesized in various peripheral organs, including bone marrow, blood cells, skin, and the GI tract (Singh, Jadhav, 2014). It is well-established that GI enterochromaffin cells can secrete MLT, presenting a concentration that surpasses the blood (10- to 100-fold) and pineal gland (approximately 400-fold). Accordingly, it can be suggested that MLT may also play an important role in the GI system (Konturek *et al.*, 2007).

MLT, a lipophilic compound, diffuses rapidly across biological membranes to act on the muscular mucosa or GI myenteric plexus (Chen *et al.*, 2011; Söderquist, Hellström, Cunningham, 2015), promoting the regulation of GI motility, epithelial protection, epithelial permeability, vascular function, and entero-pancreatic endocrine crosstalk to impact metabolic control (Thor *et al.*, 2007; Söderquist, Hellström, Cunningham, 2015). In addition, MLT maintains gut regional blood circulation, increases bicarbonate release from the gastric mucosa, decreases neutrophil infiltration, and reduces the release of inflammatory cytokines induced by deleterious stimuli on the GI tract, for example, drugs (Flemström, Sjöblom, 2005; Shahrokhi *et al.*, 2016). These MLT effects are possibly mediated via interactions with membrane receptors type 1 (MT_1) and/or type 2 (MT_2), known to be present in the GI tract (Thor *et al.*, 2007; Söderquist, Hellström, Cunningham, 2015). However, the effects of MLT on CP-induced GI toxicity, as well as the underlying mechanisms, remain unclear.

Given that MLT modulates chemotherapy-induced toxicity, improves chemotherapeutic efficacy (Lu *et al.*, 2019), and can be employed as a complementary treatment to enhance the quality of life and survival of patients undergoing chemotherapy (Ginzac *et al.*, 2020), we aimed to investigate the effect of MLT on GI motility in an experimental mouse model of CP-induced dysautonomia. Additionally, we examined the role of MT_1 and/or MT_2 receptors in mediating the potential effects of MLT on gastroparesis.

MATERIAL AND METHODS

Animals

The present study was conducted in accordance with the guidelines of the Ethics Committee on Animal Use at the Federal University of São Francisco Valley (protocol number 0022/220515). Adult female Swiss mice (n=58), 8-week-old and weighing 30–45 g, were housed in an air-conditioned atmosphere at 25°C with alternating 12 h/12 h light-dark cycles. Animals were maintained on a diet of standard pellets (Presence Rats and Mice® - Agribands Purina do Brasil Ltda) and water *ad libitum*.

Experimental Study

Phase I – Effect of different melatonin doses on cisplatin-induced GE delay

Mice (n=30) were randomly divided into six experimental groups (five animals per group) and treated for three days as follows: the first group, i.e., the control group, was administered an intraperitoneal injection (i.p.) of the vehicle (ethanol 5% in NaCl 0.15 M, 0.15 mL/mouse), once daily, over three days (grouped termed "S"). The second group received CP (5 mg/kg body weight, i.p.; Libbs Farmacêutica Ltda, São Paulo, Brazil) 30 min after vehicle administration, once daily, for three days (total of 15 mg/kg CP). Mice in the third, fourth, and fifth groups were treated with MLT (Sigma Aldrich Chemical

Co., St. Louis, MO, USA) at 5, 10, or 20 mg/kg body weight (i.p.), respectively, once daily; then, mice were administered CP (5 mg/kg body weight, i.p.) 30 min after the MLT injection, once daily for three days (total of 15 mg/kg CP). Finally, the positive control (sixth group) was administered the antioxidant NAC (150 mg/kg body weight; i.p.) before CP, replacing MT pretreatment.

In the present study, the CP dose employed was as reported by Viana-Cardoso *et al.* (2011) and Shin *et al.* (2018); this dose sufficiently induced GE delay by dysautonomia. The dose of MLT was based on previous studies indicating its protective effects (Kasimay *et al.*, 2005; Konturek *et al.*, 2007; Barberino *et al.*, 2017; Kim *et al.*, 2019). The NAC dose utilized was described by Anand *et al.* (2015) and Barberino *et al.* (2017).

Evaluation of gastric emptying of the liquid test meal

GE was measured in mice subjected to an 18 h fasting period, with free access to water for up to 2 h prior to experimentation. Then, all mice were fed a liquid test meal (0.3 mL phenol red, 0.5 mg/mL in 5% glucose) 24 h after the last injection. Over 10 min intervals, mice were sacrificed by cervical dislocation, and their stomach contents were emptied and measured. Briefly, after laparotomy, the gut was quickly ligated and divided into two consecutive segments: the stomach and small intestine. The volume of each segment was calculated by flooding it within a graduated cylinder containing 20 mL of NaOH (0.1 N). After homogenization of each segment, proteins were precipitated using 200 µL of 20% trichloroacetic acid. After centrifugation, 600 µL of the supernatant was added to 800 µL of 0.5 N NaOH. Samples were spectrophotometrically measured at 560 nm to construct dilution curves by plotting dye concentrations against optical densities. The amount of dye emptied by the stomach was expressed as a percentage (Silva *et al.*, 2015).

Gut morphology examined by scanning electron microscopy (SEM)

For assessing gut morphology using SEM, another set of mice (n=9) was randomly divided into three experimental groups (three animals per group) and treated once daily for three days with vehicle (ethanol 5% in NaCl 0.15 M; control group: S), CP (5 mg/kg body weight), or 20 mg/kg MLT plus CP (which produced the best results), as described previously. Twenty-four hours after the last injection, mice were sacrificed to assess gut morphology, as described by Lu *et al.* (2017). Briefly, 2 cm distal to the pylorus, 1 cm duodenal sections were harvested and washed with 0.1 M phosphate buffer and phosphate-buffered saline, fixed in 3% glutaraldehyde and 1.5% paraformaldehyde, 0.1 M phosphate-buffered saline, pH 7.4. Then, sections were sequentially fixed in a 1% magnesium tetroxide solution, dehydrated in a graded series of ethanol, and stored in a vacuum desiccator for 10 h. The samples were metalized with gold for 120 s in the Quorum Q15OR ES metallizer, forming a 50 nm metallic layer. We employed a scanning electron microscope (Tescan, Vega 3XM) to obtain SEM micrographs, with a 16 mm working distance, standard amplitudes, and accelerating voltage of 5 kV in the secondary electron detection mode.

Gut morphology examined by optical microscopy

Twenty-four hours after the last injection, 1- to 2-cm duodenal samples were harvested (at least 2 cm distal to the pylorus) from three animals per group (S, CP, or 20 mg/kg MLT plus CP), fixed in 10% buffered formalin (Dinâmica, São Paulo, Brazil), dehydrated in increasing concentrations of ethanol (Dinâmica), and clarified in xylene (Dinâmica). After paraffin embedding (Dinâmica), the fragments were cut into 5-µm thick serial sections, mounted on glass slides, and stained with hematoxylin and eosin (HE; Vetec, São Paulo, Brazil) to examine morphological changes under a light microscope (Nikon, Tokyo, Japan) (Uranga *et al.*, 2017).

Phase II – Effect of administering melatonin receptor 1 and 2 (MT_1 and MT_2) antagonists before melatonin treatment on cisplatin-induced GE delay

We next evaluated the possible mechanism of the protective effect of MLT on CP-induced GE delay. Accordingly, pharmacological modulation via MT_1 and

MT_2 receptors was analyzed by administering MT_1 and/or MT_2 antagonists. In this experiment, the first, second, and third groups were control (S), CP, and MLT 20 mg/kg plus CP, which afforded the best results. All groups were described in phase I. For the fourth and fifth groups, mice (n=10; five animals per group) were pretreated with luzindole (LZD), a non-selective MT_1/MT_2 antagonist (5 mg/kg body weight, i.p.; Sigma Aldrich Chemical Co.) or 4-phenyl-2-propionamidotetralin (4-P-PDOT), a selective MT_2 antagonist (4 mg/kg body weight, i.p.; Sigma Aldrich Chemical Co.), respectively. MLT (20 mg/kg body weight, i.p.) was administered after 15 min. Then, 30 min after the MLT injection, mice received CP (5 mg/kg body weight, i.p.). The treatment was performed once daily for three days. The doses of LZD and 4-P-PDOT were as reported by Chen *et al.* (2014).

As described in phase I, mice were sacrificed for GE assessment 24 h after the last injection, as described previously (Silva *et al.*, 2015).

Statistical analysis

Data are presented as the mean ± standard error for each group. One-way analysis of variance (ANOVA) was performed for multi-group comparisons, followed by Tukey's test. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad, San Diego, CA, USA).

RESULTS AND DISCUSSION

Rapidly proliferating cells increase the susceptibility of the GI tract to CP-mediated cytotoxic effects. The most common CP-associated clinical GI symptoms include nausea, emesis, anorexia, diarrhea, delayed gastric motility, mucositis, gut malabsorption, and barrier impairment (Shahid, Farooqui, Khan, 2018). These symptoms are initiated in 4-8 h, typically peaking on day 4 after starting chemotherapy (Viana-Cardoso *et al.*, 2011; Guo *et al.*, 2019). In addition, some symptoms may persist even after completion of the therapeutic regimen (Stojanovska, Sakkal, Nurgali, 2015; Liu *et al.*, 2019). Thus, in experimental studies, CP reportedly induces dose-related gastric distention and gastric stasis in rodents (Cabezos *et al.*, 2010; Pini *et al.*, 2016; Liu *et al.*, 2019). Considering that gastric motility reduces within 2-4 days of CP treatment in animals (Viana-Cardoso *et al.*, 2011; Guo *et al.*, 2019), we evaluated the GE of liquids in mice 3 days after CP treatment and examined the possible gastroprotective effect of MLT.

In the present study, Figure 1 shows that CP treatment decreased ($P < 0.05$) GE of the liquid meal (29.5 ± 2.6%) when compared with the control group (56.4 ± 3.1%). Consistent with our findings, Nam *et al.* (2016) and Shin *et al.* (2018) have reported CP-induced delayed GE of phenol red solution in rodents. Studies have suggested that CP stimulates the excessive release of 5-hydroxytryptamine (5-HT) from enterochromaffin cells in the GI tract, which induces peripheral 5-HT receptors on the vagal afferent fibers and enteric nerves. This stimulation can cause relaxation of the stomach, possibly leading to delayed GE (Ando *et al.*, 2012). Based on our findings, MLT pretreatment at 5 and 10 mg/kg failed to prevent ($P > 0.05$) CP-induced GE delay (26.4 ± 2.1% and 29.1 ± 3.7%, respectively); however, pretreatment with 20 mg/kg MLT prevented this effect ($P < 0.05$) (49.9% ± 3.7%). Interestingly, treatment with NAC (positive control) did not prevent ($P > 0.05$) CP-induced GE delay (27.5 ± 3.0%; Figure 1).

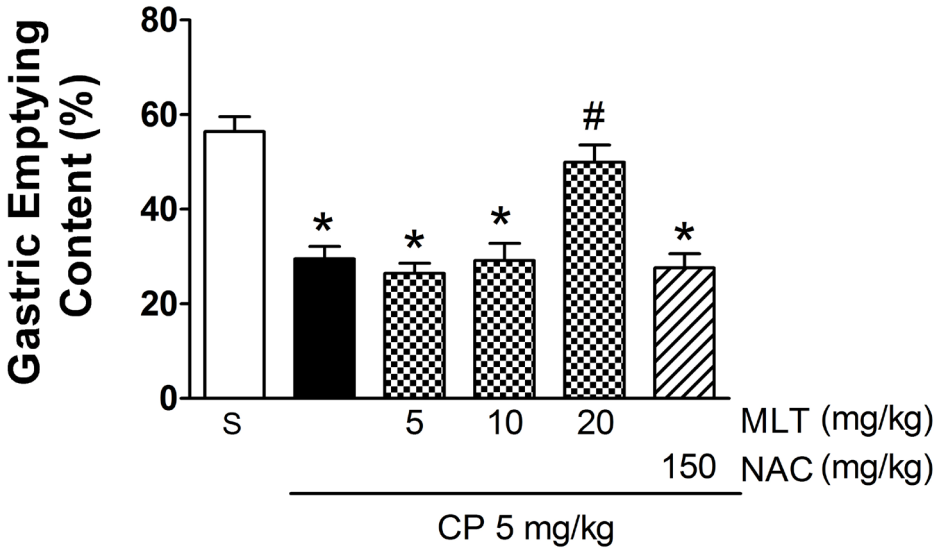

FIGURE 1 - Effects of pretreatment with melatonin (MLT) or N-acetylcysteine (NAC) on the percentage of gastric dye emptied, as induced by cisplatin (CP) in mice. $P < 0.05$, *compared with the control group (S), #compared with the group treated only with cisplatin (ANOVA followed by Tukey test).

To the best of our knowledge, this is the first study to demonstrate the protective role of MLT against the inhibitory action of CP on GE in mice. MLT is a small lipophilic compound that easily diffuses across cell membranes and rapidly accesses different tissues, allowing paracrine and endocrine actions (Söderquist, Hellström, Cunningham, 2015). *In vivo* animal studies have revealed that MLT exerts both excitatory and inhibitory effects on gut motility depending on the dose employed (Kasimay *et al.*, 2005; Thor *et al.*, 2007; Chen *et al.*, 2011; Carrascal *et al.*, 2018). In the present study, a pharmacological dose of MLT (20 mg/kg) prevented CP-induced gastroparesis. Some reports have highlighted the protective effects of MLT on drug-induced GI motility disorders and gut mucosal lesions (Zhu *et al.*, 2018). In the case of neurodegenerative processes, such as those induced by chemotherapy, the protective effect of MLT has been examined in numerous experimental systems generating oxidative stress and inflammatory processes, both directly or indirectly (Carrascal *et al.*, 2018; Wongprayoon, Govitrapong, 2020). This possible CP-induced neurodegeneration may be related to an increase in gastric volume (Cabezos *et al.*, 2010; Pini *et al.*, 2016). In the present study, on opening the abdomen, gastric distension in CP-treated mice (Figure $2B_1$) appeared more notable when compared with control (Figure $2A_1$) or MLT-treated mice (Figure $2C_1$). The vagus nerve has important functions in the GE of liquid, and a previous report has indicated that the antioxidant and gastroprotective effects of MLT were mediated by the vagus nerve (Shahrokhi *et al.*, 2016). Thus, we cannot exclude the possibility that MLT mediated vagus nerve neuroprotection in the present study.

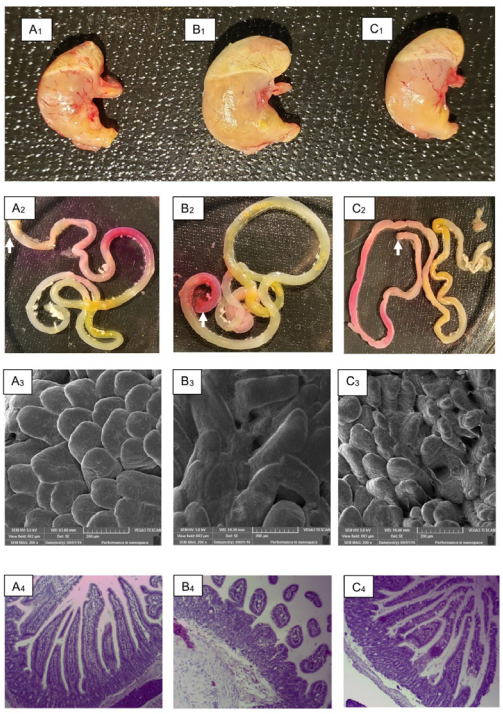

FIGURE 2 - Effects of melatonin (MLT) on cisplatin (CP)-induced gastrointestinal damage in mice. Gross anatomy of mice stomachs after excision from the abdomen (section 1), upper intestinal dye transit (section 2), scanning electron microscopy (SEM) images acquired at 10 kV (section 3), and qualitative optical microscopy (100×, section 4) showing the luminal surface of the duodenum. Box A_{1-4}: control group, animals treated with vehicle (ethanol 5% in NaCl 0.15 M); Box B_{1-4}: Cisplatin group, animals treated with 5 mg/kg CP; BOX C_{1-4}: melatonin group, animals pretreated with 20 mg/Kg MLT plus 5 mg/kg CP. White arrow: Proximal duodenum.

Numerous studies have reported improvements in oxidative status and attenuation of morphological changes following the antioxidant activity of NAC on CP-induced toxicity in various tissues (testis; Anand *et al.*, 2015; ovary; Barberino *et al.*, 2017; brain; Abdel-Wahab, Moussa, 2019). However, in the present study, 150 mg/kg NAC did not influence CP-induced gastroparesis. In other experimental models simulating gastric damage in rats, NAC afforded protective effects at a higher dose (500 mg/kg) (Hegab, Abd-Ellatif, Sadek, 2018), which can be evaluated in future assays for CP-induced gastroparesis in mice.

Herein, we used phenol red as a marker to observe GI dye propulsion. We noted that upper intestinal transit (Figure 2A2) was reduced in the control group after CP treatment (Figure $2B_2$). However, this reduced gut dye transit was restored in 20 mg/kg MLT-treated mice ($2C_2$). Considering that CP-induced GE delay can result in injuries in the gut wall general structure, as well as in mucosal, submucosal, and muscular layers (Uranga *et al.*, 2017), we determined the efficiency of MLT in protecting the gut mucosa using SEM to provide real-time visualization of the surface of gut structures (Lu *et al.*, 2017) and the histological pattern in HE-stained duodenal wall sections. As shown in Figures $2A_3$ and $2A_4$, no changes in the duodenal structural morphology were observed in vehicle-treated animals (control group). However, following CP treatment, the intestinal structural villi showed significant damage, with eruptions and atrophied villi (Figure $2B_3$), further augmented by histological alterations (e.g., shortening and fragmentation of villi and deepening of the crypts), as observed in Figure $2B_4$. In CP-treated mice, structural examination showed a significant decrease in mucosa thickness and epithelium height, which could be attributed to the loss of cell rows in the spinous layer, given the antimitotic properties of CP on proliferating spinous cells (Konturek *et al.*, 2007; Shahid, Farooqui, Khan, 2018; Kim *et al.*, 2019). In addition, studies have revealed that CP (doses $\geq$ 6 mg/kg body weight) can induce distortion of glandular mucosa architecture, crypt ablation or abscess formation, intense inflammatory cell infiltration, degeneration, density reduction, or diminished villi height, accompanied by acute necrosis and apoptosis of the intestinal epithelial cells (Shahid, Farooqui, Khan, 2018), thus reducing fluid and electrolyte absorption from the gut. Therefore, the prevention of small intestine injury is a critical therapeutic strategy during CP therapy. Accordingly, Figures $2C_3$ and $2C_4$ present that pretreatment with MLT 20 mg/kg attenuated CP-induced duodenal morphological injury, demonstrating that MLT maintains the gut tissue integrity, despite the use of an antineoplastic agent with well-known destructive effects on the GI mucosa (Pandit *et al.*, 2015; Shahid, Farooqui, Khan, 2018). Laboratory and clinical studies indicate that MLT can prevent or treat pathological conditions such as esophageal and gastric ulcers, irritable bowel syndrome (IBS), constipation-predominant IBS, diarrhea-predominant IBS, Crohn's disease, ulcerative colitis, and necrotizing enterocolitis

(Brzozowska *et al.*, 2002; Konturek *et al.*, 2013; Bang, Yang, Baik, 2019).

Furthermore, it has been established that MLT exerts complex actions in the GI tract by binding and activating two distinct receptor types: membrane receptors MT_1 and MT_2 (Thor *et al.*, 2007; Söderquist, Hellström, Cunningham, 2015). MLT receptor-dependent protective effects have recently been reported in various animal models, including protection against myocardial injury (Han *et al.*, 2019), brain injury (Wongprayoon, Govitrapong, 2020), and protection against CP-induced ovarian damage (Barberino *et al.*, 2017). However, the participation of MLT receptors in CP-induced gastroparesis needs to be comprehensively elucidated.

To determine whether the gastroprotection afforded by MLT is mediated via membrane MT_1 and/or MT_2 receptors, the influence of LZD (an antagonist of both MT_1 and MT_2) and 4-P-PDOT (a selective MT_2 antagonist) on CP-induced GE delay was examined. Our data revealed that the protective effect of MLT on CP-induced GE delay was suppressed ($P < 0.05$) by LZD and 4-P-PDOT pretreatment (Figure 3), indirectly indicating that the gastroprotective effect of MLT against CP-mediated effects might be mediated via MT_2 receptors.

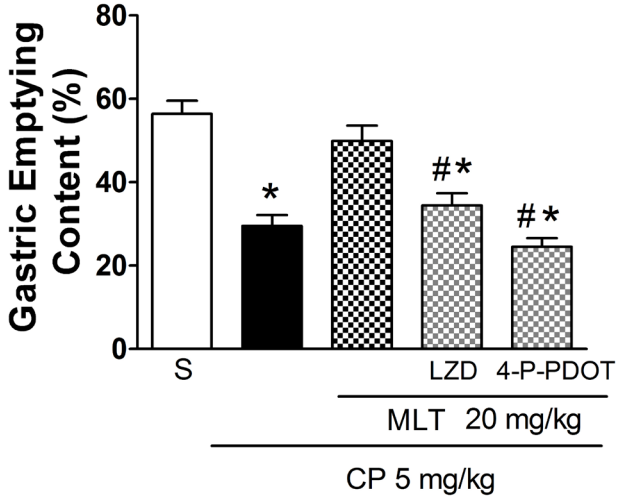

FIGURE 3 - Effects of melatonin (MLT) on the percentage of gastric dye emptied, as induced by cisplatin (CP) in mice pretreated with luzindole (LZD, 5 mg/kg) or 4-phenyl-2-propionamidotetralin (4-P-PDOT, 4 mg/kg). $P < 0.05$, *compared with the control group (S), #compared with the group treated only with MLT (ANOVA followed by Tukey test).

Detection of MT_2 receptors in the gut muscle layers provides strong evidence that these receptors play a role in regulating GI motility (Stebelová *et al.*, 2010). MT_2 receptors can interact with multiple signal transduction pathways, including phosphoinositol production, inhibition of adenylate cyclase, and inhibition of the soluble guanylate cyclase pathway, which are directly involved in smooth muscle contraction (Stebelová *et al.*, 2010; Chen *et al.*, 2011; Söderquist, Hellström, Cunningham, 2015). In addition, MT_2 receptors may be involved in the overall enhancement of gastric microcirculation, probably mediated by nitric oxide (NO), known to exert gastroprotective and ulcer healing actions (Brzozowska *et al.*, 2002; Konturek *et al.*, 2013). Thus, given that MLT receptors are targets for therapeutic management of GI disorders such as gastroesophageal reflux disease and IBS (Siah, Wong, Ho, 2014; Bang, Yang, Baik, 2019), our results reinforce the use of MLT as complementary therapy during chemotherapy and highlight a promising therapeutic target (MT_2 receptor), which could be used to promote the development of gastroprotective agents. Considering that MLT can also act on 5-HT and cholecystokinin B (CCK2) receptors involved in motility and permeability of the GI tract (Sjöblom, Flemström, 2001; Ando *et al.*, 2014), future studies should attempt to elucidate the role of neurohumoral pathways in the protective effects of MLT against CP-induced gut injury.

In conclusion, pretreatment with 20 mg/kg MLT prevented GE delay and preserved the morphology of the gut mucosa during CP treatment, demonstrating the contribution of MLT in maintaining gastrointestinal functionality during CP therapy. In addition, the use of MLT receptor antagonists confirms that at least MT_2 receptors mediate MLT action against CP-induced GI damage. Thus, clarifying the actions of MLT and its receptors in the GI tract could establish the therapeutic potential, which should be explored for developing novel gastroprotective strategies/agents. However, further studies need to be conducted to assess the activity of the association between CP and MLT against the growth of tumor cells.

CONFLICT OF INTEREST

All authors declare no competing interests.

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