



The role of melatonin in reducing chemotherapy-induced jejunal toxicity in a mouse-model for hepatocellular carcinoma

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ABSTRACT – Background: While chemotherapy is a key component of cancer treatment, it often damages healthy gastrointestinal cells, resulting in toxicity marked by symptoms such as diarrhea, pain, and impaired nutrient absorption. The damage disrupts the gut mucosal barrier integrity and epithelial function, compromising the patient well-being and treatment continuity. These side effects limit chemotherapy's effectiveness and highlight the need for protective strategies targeting the gut. This study investigates the potential protective effect of melatonin in mitigating chemotherapy-induced gastrointestinal toxicity.

Methods: This study investigated the effects of melatonin on doxorubicin treatments in the jejunal segment on male SV129 mice with chemically induced hepatocellular carcinoma. Jejunal tissue samples were collected and analyzed using various staining techniques, including hematoxylin and eosin, Alcian blue, and Sirius red to evaluate the impact of melatonin treatment.

Results: Melatonin in combination with doxorubicin promoted intestinal recovery, as evidenced by longer villi and increased crypt depth. Goblet cell numbers increased significantly with the combined treatment. Chemotherapy did not induce any significant differences in the parameters examined.

Conclusion: The study optimized the Swiss roll technique for detailed intestinal analysis. Histological analysis showed modest changes in jejunal villus length and crypt depth, likely due to tissue collection timing. Findings align with previous studies highlighting the role of goblet cells in intestinal repair. Our study demonstrated the need for future research on intestinal regeneration dynamics and timing of sampling.

KEYWORDS – Chemotherapeutic-induced gastrointestinal toxicity – Jejunum – Melatonin – Doxorubicin – mice

ABBREVIATION LIST

- GI: gastrointestinal
- HCC: hepatocellular carcinoma
- TA cells: transit-amplifying cells
- SSCs: somatic stem cells
- IECs: intestinal epithelial cells
- CID: chemotherapy-induced diarrhea
- CIM: chemotherapy-induced mucositis
- DEN: diethylnitrosamine
- DOX: doxorubicin
- MEL: melatonin
- TRP: tryptophan
- ROS: reactive oxygen species

INTRODUCTION

Chemotherapy remains the primary adjuvant treatment for most tumors. By 2040, the global number of new cancer cases is expected to reach 26 million, of which 15 million individuals will require chemotherapy (M. Chen *et al.*, 2025;

Wilson *et al.*, 2019). However, current chemotherapeutic agents do not distinguish between fast-growing cancer cells and rapidly dividing healthy cells, such as the epithelial cells lining the gastrointestinal (GI) tract. As a result, the effectiveness of chemotherapy is often accompanied by detrimental side effects, including chemotherapy-induced gastrointestinal toxicity (Dahlgren *et al.*, 2021; Sougiannis *et al.*, 2021). It is estimated that approximately 60%–100% of patients receiving high-dose chemotherapy will experience GI toxicities as a side effect (Akbarali *et al.*, 2022).

GI toxicity includes a range of symptoms, such as mucositis, diarrhea, constipation, pain, nausea, and loss of appetite. In severe cases, it can progress to dehydration, electrolyte imbalances, and malnutrition, significantly compromising the quality of life and patient safety (Sougiannis *et al.*, 2021). The severity of GI toxicity is influenced by patient-specific factors such as age, gender,

ethnicity, type of cancer and other underlying systemic diseases (Sougiannis *et al.*, 2021). At a cellular level, chemotherapeutic agents cause direct damage to intestinal epithelial cells, resulting in superficial necrosis and disruption of the homeostatic balance between villus and crypt cells within the intestinal epithelium (Chen *et al.*, 2025). This epithelial damage impairs intestinal function, disturbing the balance between absorption and secretion. (Chen *et al.*, 2025). GI toxicity may also compromise the overall effectiveness of chemotherapy by causing treatment delays, dose reductions, or even unexpected termination of treatment (Sougiannis *et al.*, 2021). Therefore, patients may experience longer hospital stays, resulting in higher costs for the public hospital system (Sougiannis *et al.*, 2021). This highlights the urgent need to develop protective agents during cancer treatment (Tooley *et al.*, 2009).

This project investigates whether melatonin can protect the jejunum from doxorubicin-induced toxicity in a mouse model for HCC.

1. The small intestine

The small intestine plays a crucial role in digestion and nutrient uptake (Walter F. Boron, 2016). Its single-layered epithelium forms numerous finger-like shapes called villi, which extend into the intestinal lumen to maximize the surface area for nutrient absorption (Jasper, 2025). Furthermore, enterocytes, the epithelial cells lining the intestine, have brush border microvilli on their apical surface, which significantly expand the surface area exposed to the lumen (Crawley *et al.*, 2014). Between the villi are the crypts of Lieberkühn, which are tubular invaginations housing stem cells, Paneth cells, and transit-amplifying (TA) cells (Jasper, 2025). As these cells migrate upward, they differentiate and mature into various specialized cell types (Jasper, 2025), see Figure 1.

The small intestine has an exceptional regenerative capacity (renewed every four to five days) and contains a strong population of somatic stem cells (SSCs) (Sougiannis *et al.*, 2021). Stem cells in the crypt actively divide to self-renewal or give rise to

progenitor cells. These proliferate to the TA zone before differentiating into specialized epithelial cells (Won *et al.*, 2022). This rapid regeneration of the intestinal epithelial cells (IECs) also explains the GI tract's vulnerability to antineoplastic drugs that target cell growth and division (Dahlgren *et al.*, 2021).

Enterocytes mediate the uptake of nutrients and water while also secreting antimicrobial peptides (Miron & Cristea, 2012). Goblet cells produce mucins that create a mucus layer on the surface of the intestinal epithelium. With a thickness range from 300 to 700 μm , the mucosal barrier serves as the first line of innate defense in the GI tract, with selective permeability that enables the passage of small particles and nutrients (Duangnumsaeng *et al.*, 2021; M. Zhang & Wu, 2020). Other epithelial cell types include enteroendocrine cells, which regulate gut hormone secretion. This group includes enterochromaffin cells, which are the main producers of 5-hydroxytryptamine (5-HT, also known as serotonin) and will be discussed later as a key source of melatonin production (Davidson *et al.*, 2022). Tuft cells detect and help defend against parasitic helminths via a chemosensing pathway (McGinty *et al.*, 2020). Paneth and microfold cells are found only in the small intestine and contribute to host defense. Paneth cells, located at the crypt base, secrete antimicrobial peptides (Akbarali *et al.*, 2022).

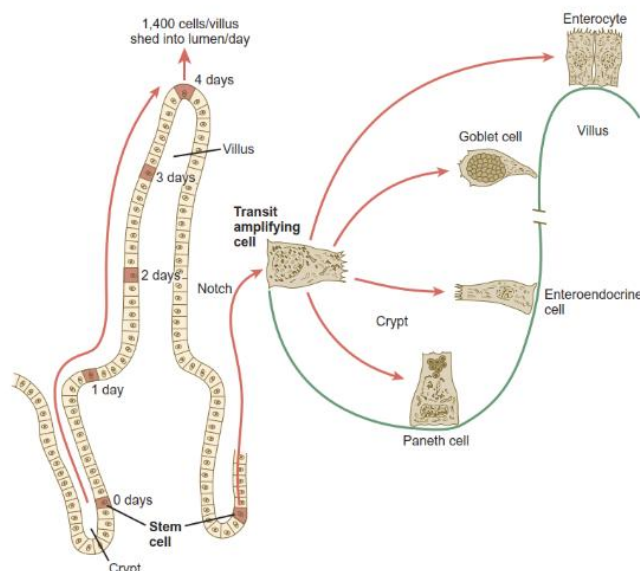


Figure 1: Crypt and villi of the small intestine. Intestinal epithelial stem cells are present at the base of the crypts and give rise to TA cells, which differentiate and mature into various cell types as they migrate towards the tip of the villi (entirely renewed every four to five days, see red line). Differentiated cell-types are Paneth cells, enteroendocrine cells, goblet cells and enterocytes on specific positions within the intestinal epithelium (see green line). (Carlson BM: 2014 Human Embryology and Developmental Biology 5/e, Elsevier Saunders, Philadelphia)

2. Chemotherapy-induced gastrointestinal toxicity

Chemotherapy-induced GI toxicity is a frequent complication of anti-neoplastic drugs, occurring due to the drugs' non-specific targeting of rapidly proliferating cells (Tooley *et al.*, 2009). It typically manifests in two main forms. The first is chemotherapy-induced diarrhea (CID), which impairs fluids and electrolytes retention, potentially causing dehydration, malnutrition and renal or cardiac dysfunction (Akbarali *et al.*, 2022). Villus atrophy reduces absorption, causing fluid retention and osmotic shifts that worsen diarrhea (Akbarali *et al.*, 2022). CID is widely considered a by-product of intestinal mucositis, characterized by mucosal injury involving inflammation and ulceration (Akbarali *et al.*, 2022). The second manifestation is chemotherapy-induced mucositis (CIM), which is characterized by inflammation of the mucous membranes lining the digestive tract, resulting in structural, functional, and immune-related variations (Sougiannis *et al.*, 2021). It is described as a five-phase sequence (Figure 2). The

first phase is the initiation phase. It is triggered by the production of reactive oxygen species (ROS) and it directly contributes to DNA damage. This damage impacts the innate immune system and stimulates the production of secondary messenger cascades. Both pathways lead to an increased presence of macrophages in the intestinal epithelium (Sougiannis *et al.*, 2021; Akbarali *et al.*, 2022). The second phase is called the upregulation and secondary messenger generation phase. This stage is marked by a significant increase in secondary messengers, including Wnt/ β -catenin, p53, caspase-1/3. A particularly well-studied secondary messenger, NF- κ B, activates pro-inflammatory cytokines such as TNF- α , IL-6, IL-1, IL-18, and IL-33 (Sougiannis *et al.*, 2021; Akbarali *et al.*, 2022). By the third phase signal amplification occurs, leading to a strong inflammatory response due to elevated cytokine levels. Moreover, TNF- α , initially activated by NF- κ B, further stimulates NF- κ B, amplifying the inflammatory process. These processes drive mucosal inflammation and an increased rate of apoptosis in epithelial cells (Sougiannis *et al.*, 2021; Akbarali *et al.*, 2022).

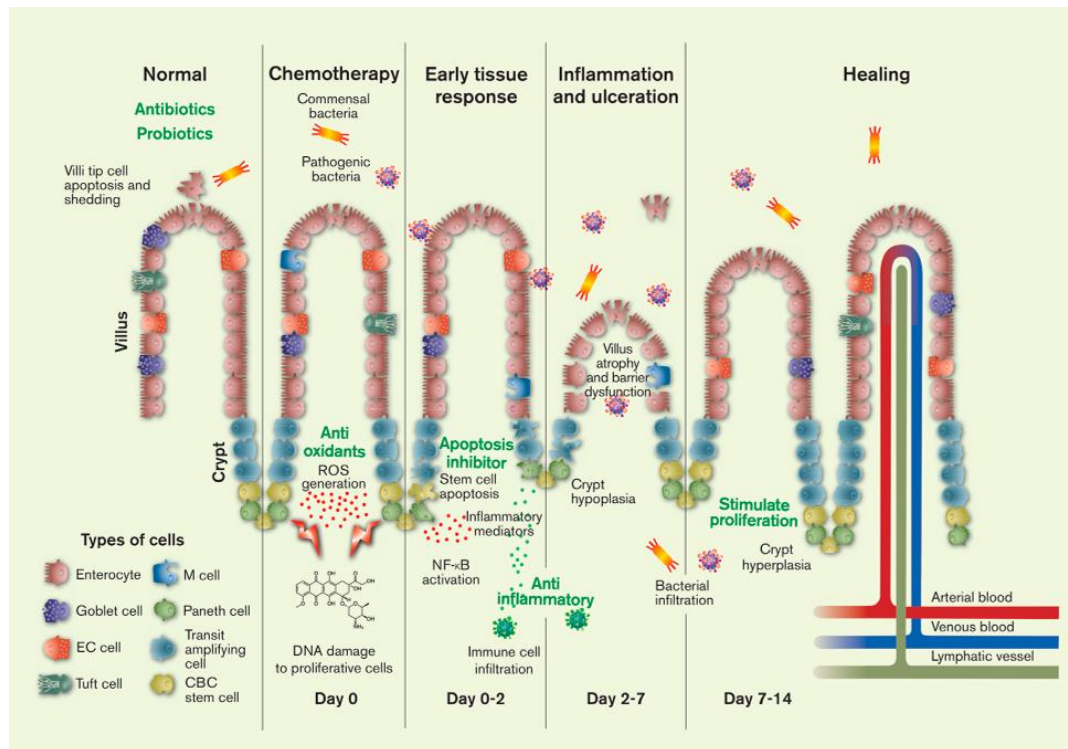


Figure 2: The timeline of CIM. It is primarily related to the effect of cytostatic on stem cells in the proliferation zone of the crypts. Injury of the DNA of the cells initiates generation of ROS and inflammation mediators, leading to inflammation, ulceration, villus and crypt atrophy. This figure shows after two weeks the histology of the intestine is restored in humans (one week for rodents). The green texts show potential targets for CIM intervention. The figure also shows the different mature cell types of the intestine and the lymphatic, venous and arterial vessels (Dahlgren *et al.*, 2021).

As a result of extensive apoptosis, the cycle progresses to phase four—the ulceration phase. During this stage, damage to the epithelial barrier allows bacterial translocation and wound colonization. Additionally, the loss of stem cells in the intestinal crypts impairs epithelial cell renewal, leading to ulcer formation. Bacteria penetrate the submucosa, further exacerbating inflammation and activating pro-apoptotic genes (Sougiannis *et al.*, 2021; Akbarali *et al.*, 2022). Finally, the healing phase begins. This phase is characterized by the restoration of normal epithelial proliferation and barrier function, marking the resolution of mucositis (Sougiannis *et al.*, 2021; Akbarali *et al.*, 2022).

3. Introduction to the agents/drugs utilized in animal research

Diethylnitrosamine (DEN) is a pro-carcinogen commonly used to develop hepatocellular carcinoma (HCC) in model mice (Zhang *et al.*, 2020). HCC is a primary liver tumor that usually develops

in a background of chronic liver disease (Khaled *et al.*, 2022). DEN is administered to young mice and converts to an active carcinogen in the liver that causes DNA and oxidative damage (Zhang *et al.*, 2020).

Doxorubicin (DOX) is a chemotherapeutic agent used in this study. It is considered one of the most commonly used anticancer drugs in its class (anthracycline derivatives) due to its effectiveness in combating a wide range of cancers (Peter *et al.*, 2022). However, its strong therapeutic properties are limited by its hydrophilic nature, short half-life, low bioavailability, and large volume of distribution, all of which necessitate high drug doses for effective treatment, leading to adverse side effects (Peter *et al.*, 2022).

Melatonin (N-acetyl-5-methoxytryptamine, MEL) is a tryptophan (TRP)-derived substituted indoleamine that is synthesized from serotonin in mainly in the pineal gland and GI tract (Claustrat & Leston, 2015; Liu *et al.*, 2022). This hormone is produced by two types of cells: pinealocytes and enterochromaffin cells (Minich *et al.*, 2022).

Pinealocytes are located in the pineal gland within the brain, while enterochromaffin cells are distributed throughout the GI tract (Minich *et al.*, 2022). The GI tract holds melatonin amounts that are at least 400 times higher than those found in the pineal gland (Tordjman *et al.*, 2017). Unlike pinealocytes, which are regulated by the light-dark cycle, enterochromaffin cells are influenced by food intake and digestion, especially TRP (Tordjman *et al.*, 2017; Minich *et al.*, 2022). This essential dietary amino acid plays a critical role in oxidative balance, inflammatory responses, physiological stress regulation and GI health (Davidson *et al.*, 2022). Protein rich food (chicken, fish and eggs) and plant-based sources such as beans, nuts and oatmeal are rich dietary sources of TRP (Davidson *et al.*, 2022).

In the pineal gland, melatonin is secreted during the night as its release is synchronized with the dark period (Claustrat & Leston, 2015). In the gut, melatonin is released in a paracrine and autocrine manner, enhancing gastric mucosal activity, circulation, and GI motility (Minich *et al.*, 2022). Serotonin, a key neurotransmitter, is synthesized from TRP via the 5-hydroxytryptophan (5-HTP) pathway. In the intestine, serotonin is then converted to N-acetylserotonin by the enzyme serotonin-N-acetyltransferase (SNAT). Finally, N-acetylserotonin is methylated by hydroxyindole-O-methyltransferase (HIOMT), also known as ASMT, to produce melatonin (Davidson *et al.*, 2022), see Figure 3.

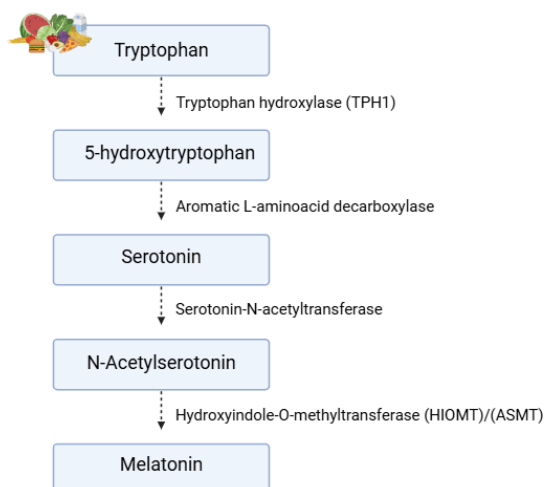


Figure 3: Pathway melatonin synthesis in the intestines. Created in <https://BioRender.com>

Gut melatonin levels remain unchanged after pinealectomy, indicating its independence from pineal production (Chen *et al.*, 2011). Notably, melatonin levels in the GI tract are 10 to 100 times higher than those found in the bloodstream (Tordjman *et al.*, 2017). Melatonin levels in the gut change with age (Chen *et al.*, 2011). In rats, levels peak at birth and decline by day 21, especially in the jejunum, ileum, and colon (Chen *et al.*, 2011). Later in life, melatonin increases again. In humans, melatonin levels in the blood peak at night, reaching 80–120 pg/mL, while daytime levels are much lower, typically between 10 and 20 pg/mL (Kolli *et al.*, 2024). On average, about 30 µg of melatonin is produced by the body each day. Around 80% of this production occurs at night (Kolli *et al.*, 2024).

From a pharmacokinetic perspective, melatonin is quickly metabolized, with a half-life of approximately 45 minutes (Harpsøe *et al.*, 2015). The liver clears more than 90% of the circulating melatonin (Claustrat & Leston, 2015). Melatonin is lipophilic, allowing it to easily cross cell membranes. Once released into the bloodstream, it can reach various body fluids, tissues, and cellular compartments, including saliva, urine, cerebrospinal fluid, preovulatory follicles, semen, amniotic fluid, and milk (Claustrat & Leston, 2015).

It is well-established that melatonin mediates signals associated with darkness (Claustrat & Leston, 2015). By activating the melatonin receptors 1 and 2 (MT1 and MT2), melatonin can influence sleep-wake cycles, regulate biological rhythms, and modulate cellular and neurophysiological functions. However, melatonin has been associated with several other functions that may aid in reducing chemotherapeutics-induced intestinal injury.

Melatonin is considered one of the most effective antioxidants. Its metabolites can neutralize up to ten ROS, whereas most antioxidants can only counteract a few (Minich *et al.*, 2022). Studies in rats revealed that melatonin was significantly more effective than vitamins C and E in protecting tissues against damage caused by oxidative stress (Meng *et al.*, 2017). Additionally, melatonin exhibits anti-

excitatory properties, and stimulates the regeneration of epithelial cells (Minich *et al.*, 2022).

Furthermore, melatonin plays a role in energy expenditure, body mass regulation, and bone density enhancement (Tordjman *et al.*, 2017). Ulcer prevention and immune regulation are also key functions of melatonin (Tordjman *et al.*, 2017). Previous studies have found that melatonin has been widely used to treat various diseases due to its antioxidant, anti-inflammation, antidiabetic, and anti-obesity properties. Indeed, melatonin has been demonstrated to alleviate many liver diseases, such as heavy metal-induced liver injury, liver inflammation, and liver fibrosis (Liu *et al.*, 2022).

These crucial roles help explain why melatonin may be a potential preventing agent for patients with intestinal damage, especially in the small intestine where its antioxidant, anti-inflammatory, and immune-modulating properties could help protect and repair the epithelial lining. This study aims to investigate the potential protective effect of melatonin following DOX chemotherapy. This bachelor's thesis is part of a broader research project and focuses specifically on the middle section of the small intestine, the jejunum.

MATERIALS AND METHODS

1. Animals

This study was approved by the local animal ethics committee at Uppsala University (approval number 5.8.18-22137/2023), and all procedures were conducted in accordance with ethical standards. In line with the principles of the 3Rs—Replacement, Reduction, and Refinement—this study was designed to minimize animal use while maximizing scientific value by using multiple tissue samples.

Six weeks old male Sv129-mice were obtained from Janvier and housed in the animal facility at the Uppsala University Biomedical Centre (Sweden). These mice were used as an HCC-model and were induced by intraperitoneal injection of DEN (35 mg/kg body weight) every two weeks. All mice have been weighed before starting any procedure. The control group received equivalent volumes of saline

to match the handling and stress conditions of the experimental groups. After week 25, the treatments began for a period of five weeks. DOX was given by intraperitoneal injection with doses of 5 mg/kg body weight, two times a week. Melatonin was administered to one group at an approximate dose of 50 mg/kg body weight via drinking *water ad libitum*. A separate group received a combination treatment of DOX and melatonin following the same concentrations and procedures (Figure 4). Throughout the entire treatment phase, mice were continuously monitored to assess their overall well-being. However, due to the development of severe ulcers and a marked decline in health status, all animals were humanely euthanized before the scheduled end. On three consecutive days, the mice were anesthetized with 4-5% isoflurane followed by cervical dislocation. The last DOX dose was given seven days before the animals were euthanized. To maximize the value of each animal, plasma, liver, heart and intestinal tissues were collected for various studies within the research group. The intestine was divided into four segments: duodenum, jejunum, ileum and colon. This project focuses specifically on the jejunum. The technique used for the jejunum involved creating 'Swiss rolls'. It involves rolling the intestine into a spiral shape before fixation and sectioning. This technique is an effective approach for large-scale preparation of intestinal tissue for both histological and morphological analysis (Bialkowska *et al.*, 2016).

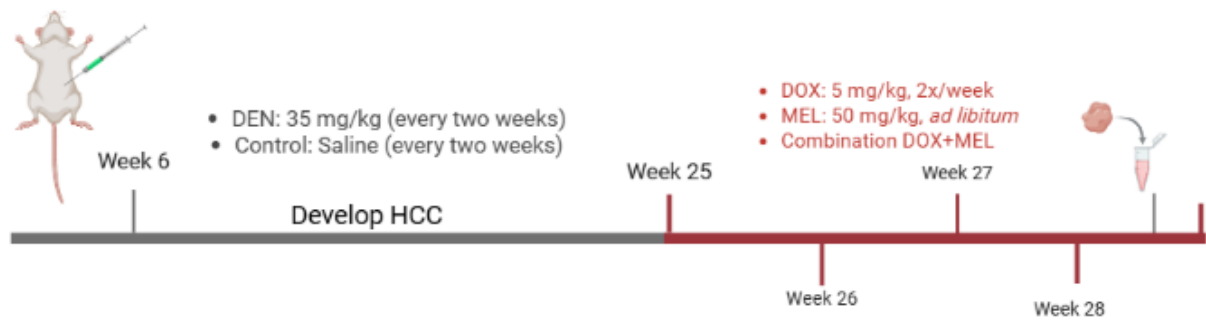


Figure 4: Timeline of the animal study from the first injection of DEN (saline for the control group). Treatment period was originally planned for five weeks, but mice were sacrifice already after week 28 due to ulceration and mouse health decline. Created in <https://BioRender.com>.

2. Study design

Five different groups of SV129 mice were used in this study, each consisting of 10 mice housed in two cages. This results in a total of 10 subgroups, each containing five mice, labeled from A to E (Table 1).

Table 1: Study design of SV129-mice. Five different treated groups of 10 mice with group 1 as control. Group 2 only received DEN. Group 3 got DEN and DOX. Group 4 received DEN and melatonin. The last group got a combination of DEN, DOX and melatonin.

Groups (n=10)	Subgroups (n=5, labeled A-E)	Properties
1	1+2	CONTROL
2	3+4	HCC (negative control)
3	5+6	HCC+DOX
4	11+12	HCC+MEL
5	13+14	HCC+DOX+MEL

3. Staining procedures and analysis

All samples were fixed in 10% formalin for 24h, and subsequently transferred to 70% ethanol and embedded in paraffin. Paraffin-embedded tissues were sectioned into 5 μ m slices using a microtome (ThermoScientific Microm HM355S) to obtain single-cell layers. For each mouse sample, four slides were prepared, each containing an average of three tissue sections. One series of samples was

processed for hematoxylin and eosin (H&E) staining, a second series for Sirius Red staining, and a third series for Alcian Blue staining. A fourth series was retained as a backup for potential staining failures or technical issues.

For deparaffinization and rehydration, samples were passed through two xylene baths, followed by a graded ethanol series (absolute, 95%, 80%, 75%, 70%), and finally two deionized water baths. Each step consisted of two baths: the first lasting 1 minute and the second 5 minutes. An exception was made for the water baths, both of which lasted 5 minutes. After all stainings, the samples were dehydrated through a graded ethanol series (95%, 100%), with each concentration applied twice: once for 1 minute, followed by 5 minutes. Finally, the tissues were cleared with two washes of xylene, each lasting 1 minute and 5 minutes, respectively. Mounting of the samples was done by using Pertex as a synthetic resin-based mounting medium. The samples were analyzed under a bright field microscope (Zeiss AXIO Scope.A1 Trinocular Pathology Microscope w/ 2.5x Objective) with a 10x lens. The images were acquired using ZEN-core-3.4 (Blue Edition) software integrated with the microscope system and image analysis was performed using Fiji.

With the H&E staining, nuclei are stained purple while the rest of the cytoplasm is colored red/pink. After deparaffinization and rehydration, samples were transferred into Mayer hematoxylin (hematoxylin 5g, potassium alum 100g, distilled water 1L, 95% ethanol 50ml, mercuric oxide 2.5g and glacial acetic acid 40ml), which gives the purple

colour to the nuclei, followed by a washing step of 10 minutes under lukewarm running water. Continuing with the eosin staining (Thermo Scientific and with a concentration of 0.5w/v) for 1 minute. Last steps are the dehydration, clearing and mounting. Five pictures were made of every sample (every mouse). From every picture, five different villi and crypts were chosen to take measurements.

A second staining procedure, Alcian blue staining, was performed to differentiate goblet cells within the tissue. For this staining deparaffinization and rehydration is needed. The samples were first incubated in 3% acetic acid for 3 minutes, with no rinsing step done afterward. Subsequently, Alcian blue 8GX (Thermo: J60122.09) was prepared at a concentration of 1g in 100mL of 3% acetic acid and applied to the samples, which were incubated for 30 minutes. This was followed by a brief 1-minute rinse. Counterstaining with Mayer's hematoxylin and subsequent dehydration, clearing and mounting were then carried out. Goblet cells were quantified by manually counting all cells exhibiting red staining, indicative of positive labeling, within the villi. Quantification was performed using colour deconvolution and thresholding of staining intensity. To maximize the detection of goblet cells, a threshold range of 0–180 was applied to the blue channel during image analysis. All captured images (five per sample) were evaluated, and five representative villi per image were selected as regions of interest for goblet cell quantification. An average was taken of the counted goblet cells per sample.

Sirius red staining was used to assess collagen content within the tissue. Examining the arrangement (or disorganization) of a collagen-rich matrix can be helpful for understanding or evaluating pathophysiological conditions. Sirius red staining was initiated with deparaffinization and rehydration. An incubation of 1 hour was used for the pico-sirius red (0.5g of Sirius RED F3B dissolved in 500ml of saturated aqueous picric acid) followed by a wash step in two changes of acidified water (5ml acetic acid glacial to 1L water). Consistent with the two prior staining protocols, the process concluded with dehydration, clearing and

mounting. Under bright-field microscopy, collagen appears red against a pale yellow background. For quantification, color deconvolution was applied (masson trichrome), followed by threshold adjustment on the red channel (range: 0–70) to isolate collagen-specific signals.

Counterstaining was carried out as follows. The samples were first stained with Sirius Red for one hour. Counterstaining was then performed by immersing the samples in hematoxylin for 3 minutes, followed by a 10-minute rinse under warm running water. Finally, the samples were dehydrated, cleared, and mounted.

RESULTS

To investigate the effect of different treatments in the jejunal segment, the villus length across the five groups was measured (Table 2). A one-way ANOVA test was performed to assess statistical differences between groups (Figure 5). No significant difference was observed between the control group and the mice with HCC. Administration of DOX (HCC+DOX) or MEL (HCC+MEL) did not significantly alter villus length compared to the HCC affected mice. However, combined treatment with both DOX and MEL (HCC+DOX+MEL) significantly increased villi length. Specifically, villi length in the HCC+DOX+MEL group was significantly longer than in the HCC group ($p = 0.049$) and HCC+MEL group ($p = 0.0023$).

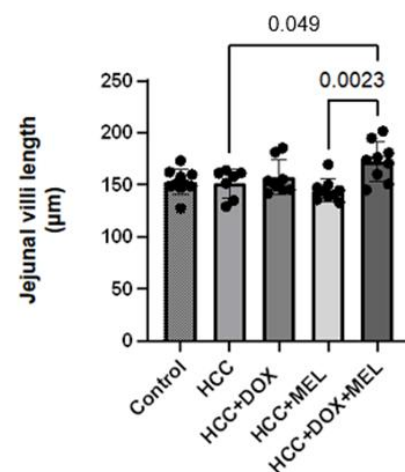
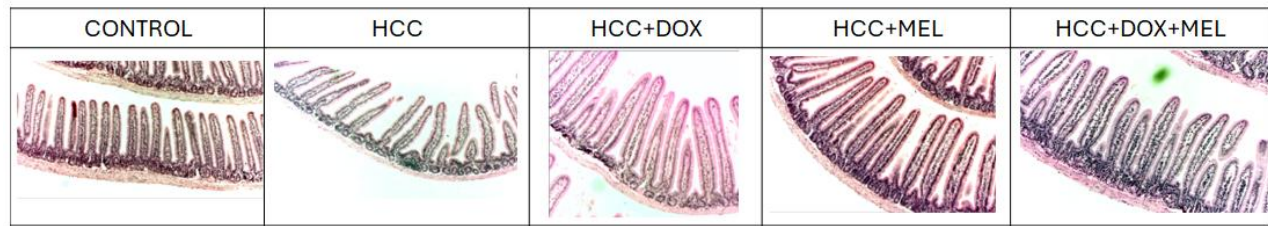


Figure 5: Effect of DOX and MEL treatment on villi length in the jejunum of HCC-mice. Bar graph showing the mean $\pm$ SEM villi length across five groups: Control, HCC, HCC+DOX, HCC+MEL, and HCC+DOX+MEL. Statistical significance was assessed using one-way ANOVA. Significant differences between groups are indicated: $p = 0.049$ and $p = 0.0023$.

Table 2: Effect of DOX and MEL treatment on villi length and crypt depth in the jejunum of HCC-mice. Representative pictures of the five groups: Control, HCC, HCC+DOX, HCC+MEL and HCC+DOX+MEL; stained with H&E to measure the villi length and crypt depth.



Crypt depth was also examined to assess the effects of melatonin on HCC-induced mice. The data were analyzed using a one-way ANOVA (Figure 6). No significant difference was observed between the control group and the HCC group. Administration of DOX, did not significantly affect crypt depth relative to the HCC group. Giving melatonin (HCC+MEL) alone did not give any significance in comparison with the HCC group. However, the combination of DOX and MEL significantly increased crypt depth relative to the HCC group ($p = 0.0067$), the HCC+DOX group ($p = 0.030$) and HCC+MEL group ($p = 0.024$).

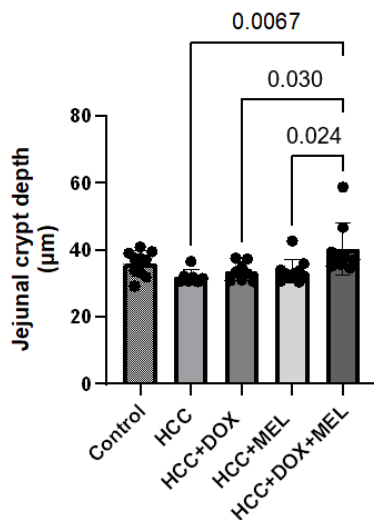


Figure 6: Effect of DOX and MEL treatment on crypt depth in the jejunum of HCC-mice. Bar graph showing the mean $\pm$ SEM crypt depth across five groups: Control, HCC, HCC+DOX, HCC+MEL, and HCC+DOX+MEL. Statistical significance was determined using one-way ANOVA. Significant differences between groups are indicated: $p = 0.0067$, $p = 0.030$ and $p = 0.024$.

To evaluate the effect of melatonin on mucosal protection, the number of goblet cells was quantified in both villi and crypts (Table 3). Statistical analysis was performed using one-way ANOVA test (Figure 7). DEN treatment alone did not significantly alter the number of goblet cells

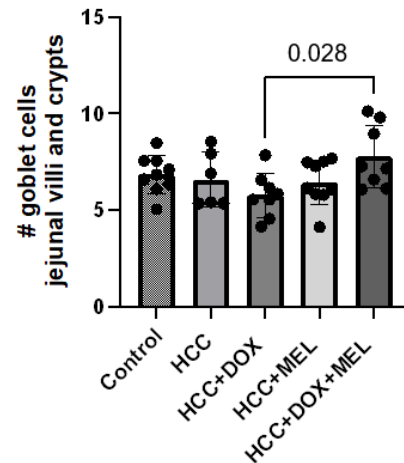


Figure 7: Effect of DOX and MEL treatment on the number of goblet cells in villi and crypts of the jejunum of HCC-mice. Bar graph showing the mean $\pm$ SEM number of goblet cells across five groups: Control, HCC, HCC+DOX, HCC+MEL, and HCC+DOX+MEL. Statistical analysis was performed using one-way ANOVA. A significant increase in goblet cell number was observed in the HCC+DOX+MEL group compared to HCC+DOX ($p = 0.028$).

compared to control animals. Similarly, treatment with DOX alone (HCC+DOX) or melatonin alone (HCC+MEL) did not lead to statistically significant changes in goblet cell numbers. However, combined treatment with DOX and melatonin (DEN+DOX+MEL) resulted in a significant increase in goblet cell counts compared to the DEN+MEL group ($p = 0.028$).

The intensity of the Sirius red staining varied between samples and the amount of tissue captured in each image was inconsistent. Additionally, the results suggested that the overall appearance of the sample was leaning towards a pink colour, making it unclear which areas truly represented positive Sirius red staining (Table 4). Because of this uncertainty and variability, an alternative method was evaluated. Counterstaining the Sirius red with hematoxylin was done to

Table 4: Representative images of different methods tried for quantification of collagen. Left, Sirius red. In the middle, the counterstaining as a combination of Sirius Red and hematoxylin. The image on the right shows the same tissue under polarized light, highlighting collagen fibers.



improve the contrast between the collagen and surrounding tissue. The tissue appeared purple after counterstaining, while collagen remained distinctly red. To enhance the distinction further, polarized light microscopy was used. This highlighted collagen against a dark background and provided additional confirmation of its localization. No additional collagen quantification was performed due to these difficulties.

DISCUSSION

This study investigated the potential protective effects of melatonin against chemotherapy-induced intestinal damage and toxicity. Mice with DEN-induced HCC were treated with the chemotherapeutic agent DOX in order to evaluate the effects on jejunal toxicity. Melatonin was added to the treatment regime, either alone or in combination with DOX to investigate its potential to reduce GI toxicity. Jejunal tissues were collected for morphological analysis and subjected to various histological stainings to assess differences in villus length, crypt depth, goblet cell numbers and collagen deposition.

The observed absence of differences between the control group (saline treated) and the HCC group across all measured parameters suggests that the chemical agent DEN does not exert intestinal toxicity. This results supports high validity of the animal model, as it shows that DEN does not affect intestinal morphology or toxicity.

For the jejunal villi length, a disparity between groups was expected. DOX treatment has previously been shown to reduce the villi length (Cano-Cebrián *et al.*, 2022). The lack of a more

distinct difference may be due to the extended period between the last DOX treatment and the end of the experiment. In the present study this was between seven and nine days. According to Dekaney *et al.*, 2009, chemotherapy-induced apoptosis, intestinal damage and repair occur in two distinct phases: an initial damage phase (0h to 72h post-injection) marked by loss of stem/progenitor cells, villus blunting, and crypt loss. The repair phase (72h to 168h) is characterized by increased proliferation, stem cell recovery, and restoration of villus and crypt structure, along with a rise in number of goblet, Paneth, and enteroendocrine cell populations. An alternative way to eliminate the differences in treatment duration between mouse groups would be to shift the entire process by three days for each group. Melatonin alone does not affect villus length. However, in combination with chemotherapy, it appears to exert a protective effect. This suggests that melatonin’s benefits become apparent under cellular stress induced by DOX. Interestingly, the expected damage from DOX is not reflected in a significant difference between the HCC and HCC+DOX groups. This unexpected result may be explained by the timing of sample collection, highlighting the importance of time as a critical factor in assessing treatment effects.

For the crypts, our data indicates that melatonin in combination with DOX promotes crypt regeneration (hypertrophy or hyperplasia) or crypt homeostasis in DEN-induced intestinal injury. Melatonin alone did not induce significant changes, suggesting its effect only present during conditions of DOX-induced stress. We expected a more pronounced effect between the groups. It is most

likely that the timing of tissue collection played a significant role in influencing the observed outcomes. After approximately seven days, the jejunal crypts appear to return to normal. This may explain the limited differences observed between the groups (Dahlgren *et al.*, 2021; Dekaney *et al.*, 2009). As previously mentioned, this issue could be addressed by shifting the entire experimental timeline by three days between groups.

An increase in goblet cell numbers is an interesting observation found in the group of animals receiving the combined treatment of DOX and melatonin. Still, the difference is modest, ranging approximately from six to nine cells. Our findings align with those reported by Dekaney *et al.*, 2009, who similarly observed minimal changes in goblet cell numbers throughout most of their time-course study. Notably, Dekaney *et al.*, 2009 reported a significant increase in goblet cells toward the end of the repair phase, a period marked by active intestinal regeneration and elevated cell proliferation. Their results suggest that, unlike other epithelial cell types, goblet cells are largely preserved during chemotherapy. Moreover, their secretory products play a crucial role in mucosal protection and repair, and their absence has been linked to more severe intestinal injury.

CONCLUSION

The aim of this study was to evaluate the potential protective effect of melatonin on the intestinal tract of mice following chemotherapy, using histological analysis with various staining techniques. Specifically, we assessed villi length, crypt depth, goblet cell counts, and collagen deposition in the jejunum. The results in the present study indicate that melatonin exerts an effect when cells are stressed by chemotherapy. The lack of pronounced differences may be due to suboptimal timing of mouse sacrifice. Due to experimental limitations, the data did not align with our expectations or with findings reported in the literature. Nevertheless, this study successfully optimized the Swiss roll technique for intestinal sectioning, enabling detailed morphological analysis of multiple intestinal regions. Future

research should focus on investigating the rate and timing of intestinal regeneration.

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