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MELATONIN AS A PREVENTIVE TREATMENT OF TONGUE MUCOSA ALTERATIONS IN AN ANIMAL MODEL OF OROFACIAL RADIATION.

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ABSTRACT

Objective: To evaluate the effect of melatonin as a protective treatment for the tongue in irradiated rats. **Materials and Methods:** Male Sprague Dawley rats were subjected to a single session of 50 Gy radiation and treated with melatonin 30 minutes before and after the radiotherapy session. A clinical evaluation was carried out a week and a half, third- and sixth-week post-treatment; finally, a tongue biopsy was taken for a histopathological study in the third and sixth weeks after radiation. **Results:** Clinical evaluation shows a clear trend, that preventive administration of melatonin could facilitate the recovery of mucosal tissue after radiation. Additionally, cellular infiltrate was 40% fewer in the melatonin-treated group compared to the control, as well as the number of the congested vessel were fewer. **Conclusion:** These findings showed for the first time the preventive role of melatonin in the tongue mucosa reducing the changes associated with mucositis, inflammatory infiltrate, and congestive blood vessels.

Keywords: radiotherapy; radioprotection, melatonin; oral mucositis.

CLINICAL SIGNIFICANCE:

Melatonin could be a promising candidate pharmaceutical strategy to protect the cells of the lingual mucosa from the side effects of radiotherapy applied to the rat jaw.

INTRODUCTION.

Radiation therapy (RT) plays a key role in the treatment of patients with head and neck cancer (HNC)[1]. This cancer includes epithelial malignant tumors of the upper aerodigestive system including the oral cavity[2]. This pathology represents 3% of all malignant neoplasms, with an estimated 60,000 new cases each year and 12,000 annual deaths, in the United States, while in Latin America 7% of deaths are reported by this entity [3]. Approximately 80% of all HNC patients receive RT at least once during the course of their disease [4].

The main mechanism of action of RT is to restrict the reproductive potential of tumor cells inducing cell death through apoptosis, necrosis, mitotic catastrophe, senescence and autophagy [5]. Radiation therapy gives rise to adverse reactions that appear during or after treatment in the oral cavity, these damages can be acute that appear during or immediately after treatment, such as oral mucositis (OM) or chronic, which appear month or years after treatment as osteoradionecrosis (ORN) [6,7].

One of the most frequent early complications in the oral cavity after receiving radiotherapy is mucositis, which presents as erythema, atrophy, ulceration with or without pseudomembranes, bleeding, pain and burning, which appear 7-10 days after the initiation of treatment in relation to the radiation dose received; erythema appears from 20 Gy, and the most serious stages from 30 Gy. Therefore, OM is often a dose-limiting factor in cancer treatment, which can lead to delays or even interruption of the treatment protocol, negatively affecting the control of disease progression [4,8].

Melatonin (MLT) (N-acetyl-5-methoxytryptamine) is a hormone synthesized by the mammalian pineal body, Harder's gland, gastrointestinal tract, testes, and lymphocytes [9,10], having both immunomodulatory and anti-inflammatory and antitumor effects [11]. Melatonin and its metabolites are powerful antioxidants, as they are capable of reducing oxidative stress by various mechanisms, including direct free radical scavenging,

detoxification of highly reactive oxidants with the donation of an electron [11], improves mitochondrial homeostasis and gene regulation by increasing the expression of antioxidant enzymes, and suppressing pro-oxidant enzymes [11]. In this sense, the possibility of using melatonin as an antioxidant and radioprotective treatment has begun to be studied in various experimental models, in order to reduce the side effects produced by both chemo and radiotherapy, however, the effect of the melatonin on oral cavity specially on tongue mucositis are poor studied, it is for this reason that in this investigation an animal model was designed allowing to evaluate the radioprotective effect of melatonin on the tongue mucosa.

MATERIALS AND METHODS.

Animals and experimental design.

Twenty (20) male Sprague Dawley rats were used, between 300-350 grams, acquired in the animal facility of the Faculty of Veterinary Sciences, (ULA, Mérida), which were manipulated according to the bioethical guidelines of the international guide for handling of experimental animals, according to the Helsinki Treaty, and the project was approved by the evaluation comitte of Instituto de Investigaciones de la Facultad de Odontología (IIFO). The animals were kept individually in plastic boxes. The temperature of the nursery room was kept at 24-26 ºC and changes / hour of the room air were guaranteed. The humidity was regulated and the light / dark cycles lasted 12 hours, respectively. Water and food were supplied *ad libitum* and the experiments were carried out during the light period. The animals were randomly divided into 4 experimental groups with 5 animals per group (figure 1):

RT-PBS: animals that received radiotherapy and injected with pyrogen-free saline intraperitoneally (i.p.) 30 minutes before and 30 minutes after being subjected to radiation.

RT-MLT: animals that were subjected to the irradiation protocol, which received pretreatment with 50 mg / kg of melatonin via i.p. 30 minutes before and 30 minutes after the radiotherapy session. PBS: Animals that were not subjected to radiotherapy (unconditioned) and injected with pyrogen-free saline via i.p. 30 minutes before and after the radiation simulation. MLT: Animals that were not subjected to radiotherapy (unconditioned) and injected with 50 mg / kg of melatothine via i.p. 30 minutes before and after the radiation simulation.

Irradiation protocol.

The animals were anesthetized with 10% ketamine and 2% xylazine in a proportion of 50/5 mg / kg by i.p. for the radiation procedure, which consisted of a single 50 Gy dose [12], delivered at a rate of 1.6 Gy / min, for an approximate exposure time of 30 minutes per group. As irradiator, a Cobalt-60 bomb (INVAP brand, model TERADI-80) belonging to the Radiotherapy and Nuclear Medicine Service of the Autonomous Service Hospital Universitario de Maracaibo (SAHUM) was used. Animals were positioned at the edge of a radiation field of size 30x30 cm^2, exposing only the lower right jaw of each one and blocking the upper jaw while the rest of the body was excluded from the field. Animals belonging to the RT-MLT groups received 50 mg / kg of melatonin (Sigma; USA) [12], 30 minutes before the radiation session and then 30 minutes after [13,14], while those in the MLT group were injected with 50 mg / kg of melatonin via i.p. as a pharmacological control, 30 minutes before and after the radiation simulation.

Clinical evaluation.

The oral cavity of the animals was examined after radiation at three different times, at one and a half weeks, three weeks, and six weeks, where animals were anesthetized with (ketamine 50 mg / kg (Virbac®) and xylazine 5 mg / kg) i.p. and the buccal mucosa was

evaluated, considering the presence of mucositis on the tongue for which the animal was placed face up, a white light lamp, a spatula and a clamp were used; post-radiation clinical changes were evaluated following the scale of oral mucositis used by Jasper et al [15] in a rat model.

Histopathological analysis.

In the third week post-radiation, at the end of the clinical evaluation, a small biopsy of the tongue of the animals was taken for histopathological analysis, also at the sixth week post-radiation, where the animals were perfused after the clinical evaluation and tongue samples were taken, for both cases lateral cuts were made of the tongue on the right side, later they were fixed in 10% paraformaldehyde for 48 hours and embedded in paraffin, cuts were made between 2-5 µm of the tongue biopsy in frontal direction using a microtome. Finally, they were deparaffinized with xylene, stained with Hematoxylin and eosin (H & E) and observed under light microscopy at 40X magnification. The following histological variables were analyzed: cellular infiltrate and the number of congestive blood vessels present.

Statistical analysis.

The analysis was carried out using the GraphPad prism version 8 software. Qualitative variables were expressed as frequencies and percentages and compared using Fisher's exact test. Quantitative variables were compared using Friedman's nonparametric analysis of variance with Dunn's posttest. The results are expressed as the mean ± standard deviation. The level of significance was established at $p < 0.05$.

RESULTS

Melatonin facilitates the recovery of the oral mucosa in irradiated animals.

To evaluate the preventive role of melatonin pretreatment on the mucosa of irradiated animals, clinical evaluations were carried out where it was observed that all the animals that were irradiated presented changes in the tongue associated with mucositis that were reported on an ordinal scale from normal (grade zero) to scaly mucosa, with epithelial denudation and the presence of one or more ulcers, with complete exposure of the connective tissue (grade three). In the first week and a half of post radiation evaluation, 40% of the irradiated control animals (RT-PBS group) exhibited between grade 1 and 3 of mucositis while the other 60% had no tongue disorders. In the third week, the signs of mucositis decreased to only 40% in grade 1 and by the sixth week to 20%. The animals treated with melatonin (RT-MLT group) showed a similar behavior to the first week of evaluation, during which 60% of the animals presented between grade 2 and 3 of mucositis while the other 40% did not present any affection, however, by the third week, animals showed a clear decrease in the degree of mucositis where it was only observed in one animal, which corresponds to 20% of the group, being completely absent at the sixth week post radiation (Table 1), which suggests a clear trend, that the preventive administration of melatonin could facilitate the recovery of mucosal tissue after radiation. The non-irradiated groups (PBS and MLT) did not present mucositis on any of the evaluation days (Figure 2).

Melatonin pretreatment prevents the severity of cellular infiltration in tongue biopsies of irradiated animals.

To relate the clinical findings observed in the animals with the possible histological affections of the tongue, biopsies were performed at the third- and sixth-week post-radiation, and it was found that in the third week post-radiation, both groups of irradiated animals presented mild and moderate cellular infiltrate in 50%; characterized by polymorphonuclear cells. However, in tongue biopsies at 6 weeks post-radiation, the

animals that received pretreatment with melatonin presented mild cellular infiltrate in 40% and moderate in 60%, while the untreated group presented moderate cellular infiltrate in 80% and severe in 20%, which suggests that the pretreatment with melatonin could be preventing the severity of the cellular infiltrate in the tongue of the irradiated animals (figure 3).

Melatonin prevents damage of blood vessel. the number of congestive vessels was analyzed from tongue biopsies and it was possible to show that both irradiated groups presented abnormalities, observing a greater number of congested vessels in the RT-PBS group (2.8 ± 1.6) with a significant increase at 6 weeks (5.8 ± 2.6) with respect to PBS and MLT controls, $p = 0.0265$ for both cases. Similarly, RT-MLT group presented a lower number of congested blood vessels (2.2 ± 1.1 for the third week and 4.2 ± 1.3 for the sixth week) compared to the RT-PBS group, although this decrease was not significant $p > 0.999$. In the same way, the control groups did not show affections at the blood vessel level (Figure 4). These data suggest that radiation induce damage on the tongue blood vessel and the melatonin tend to prevent this alteration.

DISCUSSION.

One of the consequences or side effects of radiotherapy in HNC is the changes associated with mucositis, and for which there is no prophylaxis or definitive treatment available[16]. Mucositis is an inflammatory reaction that affects the entire gastrointestinal tract, it occurs as a consequence of a series of biological events that begin in the submucosa and progress towards the epithelium [8].

Melatonin is a low toxicity antioxidant and anti-inflammatory that has shown oncostatic effects, it is not only a simple antioxidant, it also has radioprotective and radiosensitive effects [17]. Melatonin has been shown to exert a modulating effect on irradiated tissue, due to its antioxidant, anti-tumor, anti-inflammatory and anti-apoptotic functions [18]. It also

exerts a protective effect on the oral mucosa of patients receiving radiation, by preventing the mucositis, through its antioxidant and anti-inflammatory properties[18].

It is known that radiation induced reactive oxygen species is known to activate the NFκβ pathway including cytokines and pro-inflammatory enzymes, thus stimulating the immune system, which induces mitochondrial dysfunction and apoptosis in irradiated tissue, these damaged mitochondria release ROS, which activate the NLRP3 inflammosome pathway, which activates caspase-1, which produces mature IL-1β. Together, NF-κβ and NLRP3-dependent inflammation and apoptosis cause oral mucositis [19]. A study by Ortiz et al [20] demonstrated that melatonin applied as a gel to the oral mucosa of irradiated rats protects mitochondria and inhibits the NF-κβ activation pathways and the NLRP3 inflammosome, reducing inflammation and apoptosis and, therefore, preventing the development of oral mucositis. Unlike this work, in this study the radiation protocol was directed towards the mandible, as a model of osteoradionecrosis; however, it was possible to observe the affections in the tongue, as in the Ortiz study, where the radiation was directly in the tongue, which promotes the side effects generated by radiation and the possible effect of melatonin as a protector against the appearance of these events.

However, little scientific literature has been published regarding the effect of melatonin on the tongue of irradiated rats, therefore this result reports for the first time that pretreatment with melatonin could be exerting a protective effect on the tongue mucosa of irradiated rats. The results of the present study showed that all the irradiated animals presented mucositis, however, in the irradiated animals treated with intraperitoneal melatonin they presented a decrease in the signs of mucositis, even when there was no statistically significant difference, a tendency to decrease in clinical signs, which suggests a possible participation of melatonin as a radioprotective agent, developing its properties as an anti-inflammatory agent, and as an antioxidant [11].

These facts could be due to the dose and treatment protocol used in the model, which is consistent with a study conducted with 39 patients diagnosed with HNC who received chemoradiation treatment, which were additionally treated daily with 20 mg of melatonin, where there were no significant differences between the group that received melatonin treatment and the placebo group, which was attributed by the authors to insufficient doses and treatment time [21].

In the same way, our results are consistent with those previously reported by Fernández et al[17] who observed that rats subjected to tongue radiation showed signs of mucosal damage, similar results were also observed when the radiation was applied directly to the intestine. However, animals irradiated and treated with melatonin gel showed a reduction in intestinal morphological changes. Similarly, our results also coincide with that evidenced by Najafi et al[22], who demonstrated that whole-body radiation in rats leads to the development of moderate mucositis in the duodenum and that receiving treatment with melatonin or melatonin plus metformin can reduce the severity of mucositis.

About the histological analysis, we evidenced cellular infiltrate in tongue biopsies of irradiated rats, in different degrees such as mild, moderate, and severe, however, the group of animals that received melatonin decreased the infiltrate of inflammatory cells, which corresponds to the decrease in clinical findings observed in this same group of animals, this could be attributed to the anti-inflammatory effect of this hormone. This fact coincides, as observed by previous work [22] where the group of animals that received radiation, presented a slight infiltration of inflammatory cells and the group that was treated with melatonin before and after radiation was able to decrease cell infiltration.

Regarding the damage to the blood vessels, in this research it was evidenced that irradiated animals presented alteration on blood vessels level, while the group of rats who received melatonin presented fewer congested blood vessels, although this decrease was not significant. These results are consistent with a study conducted in gamma-irradiated

rats, which caused significant damage to blood vessels in the duodenum, which when treated with melatonin significantly attenuated blood vessel congestion compared to untreated irradiated rats [22].

According to the results obtained in this study, it could be concluded that ionizing radiation alters the structure of the tongue, this damage is probably caused by indirect effects. Likewise, melatonin could be a promise candidate drug that protect the cells of the lingual mucosa from the side effects of radiotherapy applied to the rat jaw. Therefore, melatonin could represent an innovative adjuvant strategy in the treatment of head and neck cancer, combining its oncostatic and cytoprotective effects, even though future studies should be carried out with a greater number of animals that allow corroborating the possibility of using melatonin as adjunctive treatment in HNC.

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Table 1. Mucositis on the tongue of animals irradiated with and without MLT.

MUCOSITIS	**1 ½ w**			**3w**			**6 w**		
(n=5)	RT-PBS	RT-MLT	P	RT-PBS	RT-MLT	P	RT-PBS	RT-MLT	P
NORMAL	3 (60%)	2 (40%)	>0,9999	3 (60%)	4 (80%)	>0,9999	4 (80%)	5 (100%)	>0,9999
MUCOSITIS	2 (40%)	3 (60%)		2 (40%)	1 (20%)		1 (20%)	-	

w: evaluation time in weeks

P: p value from Fisher's exact test

RT-PBS: animals who received radiation protocol and treatment with vehicle (PBS)

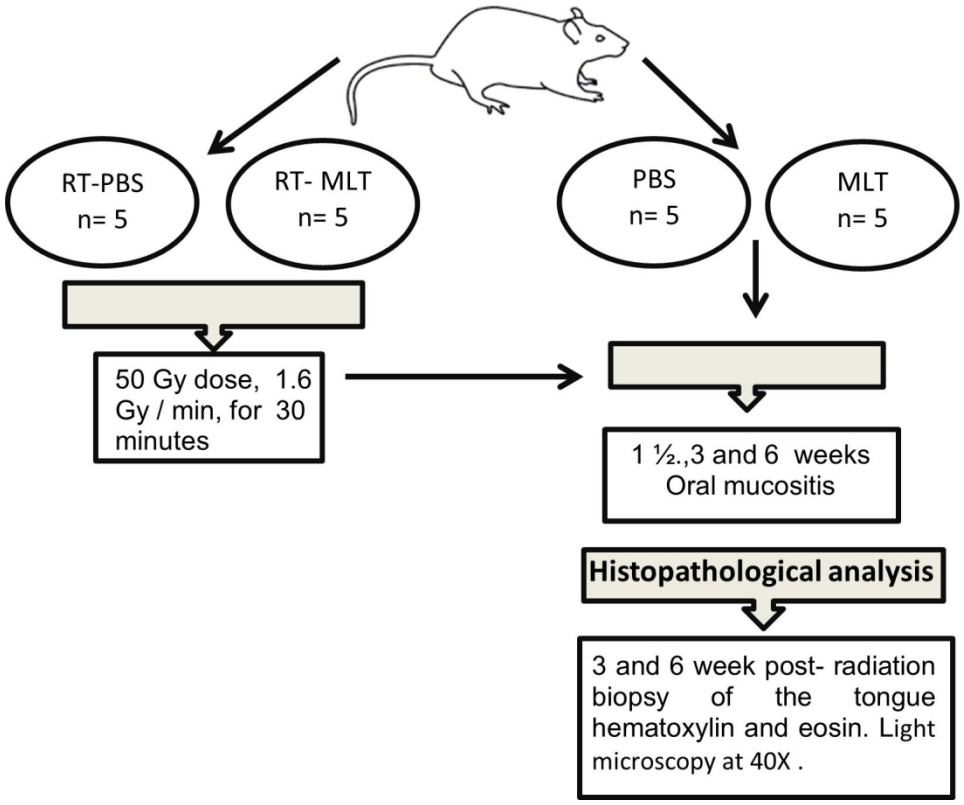
RT-MLT: animals who received radiation protocol and treatment with melatonin (MLT)

Figure 1. Experimental design.

Figure 2. Radiation-associated clinical changes in the tongue mucosa. Representative photographs of irradiated animals that received 50 mg / kg of melatonin 30 minutes before and after radiation (RT-MLT) and vehicle (RT-PBS) in the first and a half-week, third- and sixth-week post radiation are shown. In the same way, photographs of animals that were not irradiated, belonging to the PBS and MLT groups as controls (CTRL), are shown. White arrows indicate the presence of alterations in the mucosa of the tongue, such as a change in color, the presence of an ulcer or a pseudomembrane.

Figure 3. Pretreatment with melatonin prevents cellular infiltration in tongue tissue of irradiated Sprague Dawley rats. The upper panel show tongue tissue with absence of cellular infiltrate (CTRL) at 3- and 6-week post radiation (3W and 6W respectively). The middle and bottom panel shows presence of inflammatory cellular infiltrate such as polymorphonuclear and macrophages cells (arrows) in tongue tissue of animals belonging to the RT-PBS and RT-MLT groups, respectively. The columns 2 and 4 are a magnification of columns 1 and 3, respectively. Hematoxylin and eosin staining, at 10X and 40X ~~approximate~~ magnification in light microscope.

Figure 4. Graphic representation of the effect of radiation and melatonin treatment on the blood vessels of rat tongue biopsies. The number of congested blood vessels present in tongue biopsies at 3 and 6 weeks after radiation is shown in the RT-PBS and RT-MLT groups, as well as the absence of congested blood vessels in the PBS and MLT control groups. Results expressed as the mean ± standard deviation. Friedman test with Dunn's posttest, the * represents significant differences with a value of $p < 0.05$.



Experimental design

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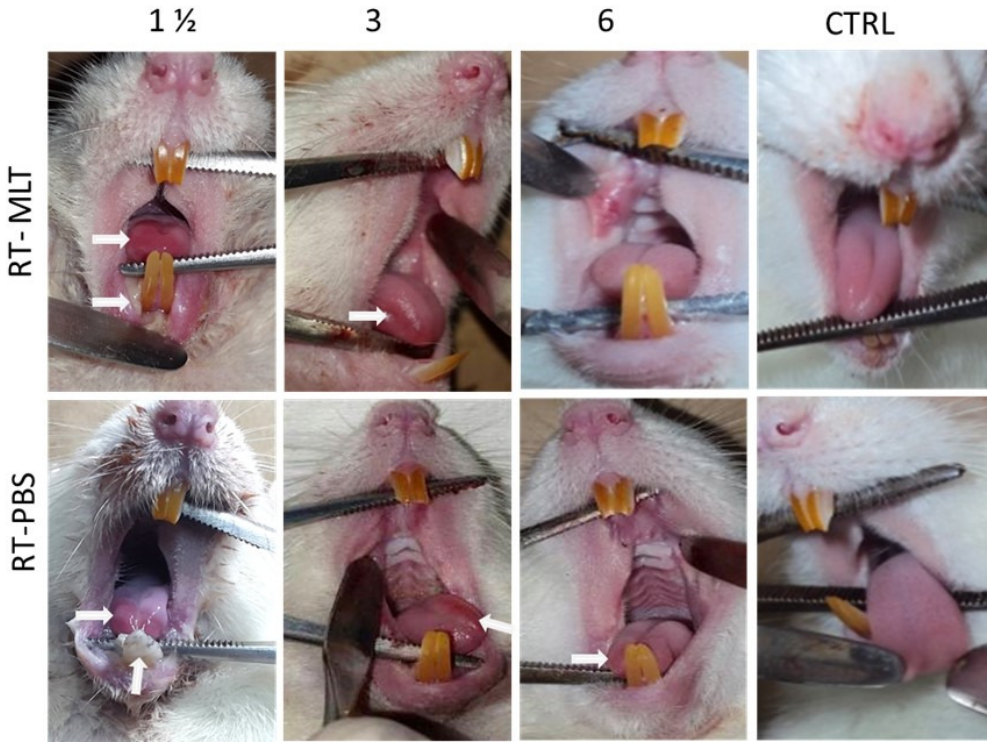

Radiation-associated clinical changes in the tongue mucosa. Representative photographs of irradiated animals that received 50 mg / kg of melatonin 30 minutes before and after radiation (RT-MLT) and vehicle (RT-PBS) in the first and a half-week, third- and sixth-week post radiation are shown. In the same way, photographs of animals that were not irradiated, belonging to the PBS and MLT groups as controls (CTRL), are shown. White arrows indicate the presence of alterations in the mucosa of the tongue, such as a change in color, the presence of an ulcer or a pseudomembrane.

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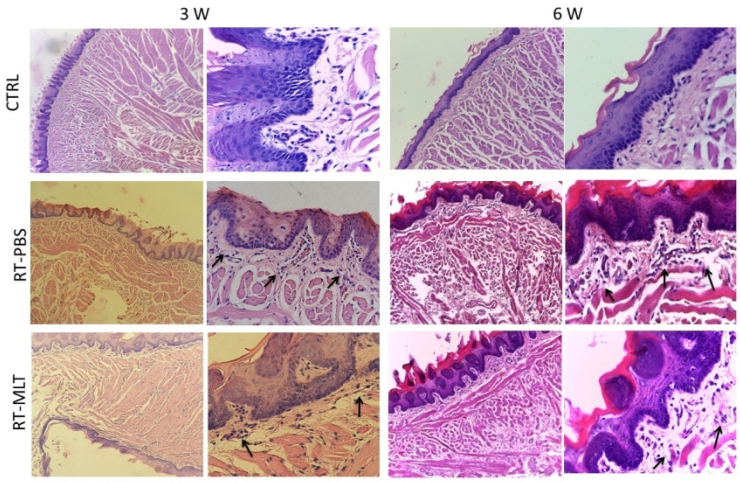

Pretreatment with melatonin prevents cellular infiltration in tongue tissue of irradiated Sprague Dawley rats. The upper panel show tongue tissue with absence of cellular infiltrate (CTRL) at 3- and 6-week post radiation (3W and 6W respectively). The middle and bottom panel shows presence of inflammatory cellular infiltrate (arrows) in tongue tissue of animals belonging to the RT-PBS and RT-MLT groups, respectively. The columns 2 and 4 are a magnification of columns 1 and 3, respectively. Hematoxylin and eosin staining, 40X approximate magnification in light microscope.

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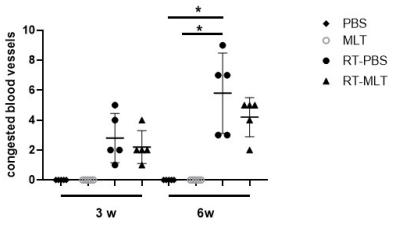

Graphic representation of the effect of radiation and melatonin treatment on the blood vessels of rat tongue biopsies. The number of congested blood vessels present in tongue biopsies at 3 and 6 weeks after radiation is shown in the RT-PBS and RT-MLT groups, as well as the absence of congested blood vessels in the PBS and MLT control groups. Results expressed as the mean ± standard deviation. Friedman test with Dunn's posttest, the * represents significant differences with a value of $p < 0.05$.

878x494mm (37 x 37 DPI)