Giseli Mitsuy Kayahara

Melatonin suppression increases the incidence and progression of chemically induced oral cancer in rats

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Orientador: Prof. Dr. Daniel Galera Bernabé.

Coorientadores: Prof. Dr. Marcelo Macedo Crivelini e Profa. Dra. Kellen Cristine Tjioe

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Dedicatória

DEDICATÓRIA

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À minha família, por sempre ser o meu amparo;

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Epígrafe

EPÍGRAFE

Conheça todas as teorias, domine todas as técnicas, mas ao tocar uma alma humana, seja apenas outra alma humana.

Carl Jung

Resumo

Kayahara GM. **SUPRESSÃO DE MELATONINA AUMENTA A INCIDÊNCIA E PROGRESSÃO DO CÂNCER DE BOCA INDUZIDO QUIMICAMENTE EM RATOS** [dissertação]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2019

RESUMO

Estudos sugerem que a supressão de melatonina e disfunção circadiana em trabalhadores noturnos podem estar relacionadas ao desenvolvimento e à progressão do câncer. Pesquisas têm mostrado também que a incidência tumoral pode ser aumentada pela pinealectomia. Entretanto, nenhum estudo avaliou a influência da cirurgia de pinealectomia sobre o desenvolvimento e a progressão do câncer de boca. No presente estudo, nós investigamos os efeitos da supressão de melatonina sobre a ocorrência e a progressão tumoral um modelo pré-clínico de câncer de boca induzido quimicamente. Nós demonstramos, pela primeira vez, que ratos pinealectomizados tiveram maior ocorrência de carcinoma espinocelular de boca, comparado aos animais controle. Ratos pinealectomizados também exibiram volume e espessura tumorais cerca de 3 e 2 vezes maior que animais sham, respectivamente. Além disso, pinealectomia induziu atrofia do epitélio não-tumoral adjacente às lesões bucais. Os ratos pinealectomizados apresentaram maior resposta inflamatória no front de invasão tumoral, caracterizada principalmente pelo aumento do número de eosinófilos e macrófagos associados ao tumor. Tumores de ratos submetidos à pinealectomia exibiram maior imunoexpressão de ERK1/2 e p53 no microambiente tumoral. Estes resultados revelam que a supressão de melatonina acelera o desenvolvimento e a progressão do câncer de boca associado ao

aumento de eosinófilos e macrófagos no front de invasão tumoral e maior ex[illegible]o de ERK1/2 e p53 no microambiente tumoral.

Palavras-chave: Melatonina; Câncer de boca; Câncer de cabeça e pescoço; Carcinoma espinocelular

Abstract

Kayahara GM. **MELATONIN SUPPRESSION INCREASES THE INCIDENCE AND PROGRESSION OF CHEMICALLY INDUCED ORAL CANCER IN RATS** [dissertation]. São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil; 2019.

ABSTRACT

Studies suggest that melatonin suppression and circadian dysfunction in shift workers can be related to cancer risk. Furthermore, investigations have shown that pinealectomy promotes higher tumor incidence in rats. However, no study evaluated the influence of pinealectomy surgery on oral cancer onset and progression. In the current study, we investigated the effects of melatonin suppression on tumor occurrence and progression in a preclinical model of oral cancer. We demonstrated for the first time that pinealectomized rats had higher oral squamous cell carcinoma occurrence than sham animals. Furthermore, pinealectomized animals displayed tumor volume and thickness about 3 times and twice higher than sham-operated rats, respectively. Moreover, pinealectomy induced atrophy of non-tumor epithelium adjacent to the oral lesions. Pinealectomized rats showed higher mean number of tumor-associated macrophages and eosinophils in the carcinoma invasion front. In addition, tumors from pinealectomized rats displayed increased immunoexpression of ERK1/2 and p53 in the tumor microenvironment. These results reveal that melatonin suppression promotes higher oral cancer occurrence and progression

associated with increasing of inflammatory cells and ERK1/2 and p53 expressions in the tumor microenvironment.

Keywords: Melatonin; Mouth neoplasms; Head and neck neoplasms; Carcinoma, Squamous Cell

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anxiety-like behavior from sham and PNT groups before and after carcinogenesis. Bars represent the mean ± SEM. p>0.05. (sham, n= 11; PNT, n= 12).

Figure 3. Epithelial thickness of non-tumor epithelium adjacent to the tongue lesions derived from 4NQO treatment. A-C) Student's test showed no statistical differences regarding to epithelial thickness, corneal thickness and total thickness of non-tumor epithelium immediately adjacent to the tongue lesions derived from 4NQO treatment in PNT and sham animals**. Epithelial thickness of non-tumor epithelium in the distant sites from the lesion. D)** There were no differences in non-tumor oral epithelial thickness between both groups. **E)** PNT rats displayed lower corneal thickness of non-tumor epithelium than sham group. **F)** Student's t-test revealed that PNT group had decreased total thickness of non-tumor epithelium compared to sham rats. **G and H)** Total epithelial thickness of non-tumor epithelium immediately adjacent to the lesion from sham and PNT group, respectively (H&E, original magnification x100). **I and J)** Total epithelial thickness of non-tumor epithelium distant to the lesion from sham and PNT rats, respectively (H&E, original magnification x100). Bars represent the mean ± SEM. *p<0.05 (sham-PNT, n=10; PNT, n=10).

Figure 4. Inflammatory response in the tumor invasion front. Student's t-test showed no differences in the average number of leukocytes **(A)**, neutrophils **(C)**, mast cells **(E)** and lymphocytes **(G)** in the OSCCs from sham and PNT animals. PNT rats displayed increased average number of tumor-associated eosinophils **(I)** and macrophages **(K)** compared to sham animals. Tumor size was not associated with the average number of neutrophils **(D)** and mast cells **(F)** in the invasion front in sham and PNT rats. Advanced tumor-bearing PNT rats had higher average number of leukocytes **(B)**, lymphocytes **(H)** and eosinophils **(J)**. Early or advanced tumor-bearing PNT rats exhibited increased average number of macrophages than Sham

rats **(L)**. *p<0.05. Bars represent the mean ± SEM. *p<0.05. **ES:** Early stage. **AS:** Advanced stage. (sham, n=5; PNT, n=11).

Figure 5. Expression of tumor progression-related genes and melatonin receptors and immunostaining of PKA, ERK1/2 and p53 in the OSCC microenvironment. A – F) Student's t-test showed no differences between sham and PNT OSCCs for mRNA expression of VEGF, NFkB, CDKN2A-p16, MMP2, MMP9 andMTNR1a. **G)** Student's t-test showed no statistic differences between both groups for PKA expression in OSCCs. **H)** PNT rats had increased tumor expression of ERK1/2 compared to sham animals. **I)** PNT animals displayed higher tumor expression of nuclear p53 than sham rats. Immunoexpression of PKA **(J and K)**, ERK1/2 **(L and M)** and p53 **(N and O)** in OSCC invasion front from sham and PNT rats, respectively (original magnification x400). Bars represent the mean ± SEM. *p<0.05. (sham-PNT, n= 7; PNT, n= 9).

Lista de Abreviaturas

Lista de abreviaturas

4-NQO – 4-nitroquinoline-1-oxide

Akt - Serine/threonine-specific protein kinase

ANOVA – Analysis of variance

Bcl-2 – B-cell lymphoma protein 2

CDKN2a-p16 – cyclin-dependent kinase Inhibitor 2A

cDNA – Complementary DNA

DMBA – 7,12-Dimethylbenz(a)anthracene

DNA – Deoxyribonucleic acid

ERK1/2 – Extracellular signal-regulated kinases 1 e 2

EZM – Elevated zero maze

FST – Forced swimming test

H&E – Hematoxylin and eosin

H_2O_2 – Hydrogen peroxide

HIF-1α – Hypoxia-inducible factor 1-alpha

HNC – Head and neck cancer

IL-17 – Interleukin 17

IL6 – Interleukin 6

LSD1 – Histone lysine-specific demethylase

MAPK – Mitogen-activated protein kinase

mM – millimolar

mm^3 – cubic millimeter

MMP-2 – Metalloproteinase 2

MMP-9 – Metalloproteinase 9

mRNA – Messenger ribonucleic acid

MT1 – Melatonin receptor 1

MT2- Melatonin recaeptor 2

MTRN1a – Melatonin Receptor 1A

MTRN1b – Melatonin Receptor 1B

NF-κB – Factor nuclear kappa B

OSCC – Oral squamous cell carcinoma

PBS – Phosphate buffer solution

PCNA – Proliferating cell nuclear antigen

PCR – Polymerase chain reaction

PCR – Polymerase chain reaction

PKA – Protein kinase A

PNT – Pinealectomy

RNA – Ribonucleic acid

ROCK-1 – Rho-associated protein kinase 1

ROS – Reactive oxygen species

RQ – Relative Quantity

RT-PCR – Real time polymerase chain reaction

SCN – Suprachiasmatic nucleus

SEM – Standard error media

SNS – Sympathetic nervous system

TNF-α – Tumor necrosis factor alpha

USA – United States of America

VEGF – Vascular endothelial growth factor

Vs – Versus

WHO – World Health Organization

µm – micrometer

Sumário

Sumário

Title: Melatonin suppression increases the incidence and progression of chemically induced oral cancer

Short title: Melatonin suppression accelerates cancer onset

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Formatted according to the rules of **Endocrine-Related Cancer**

Introduction

1. Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone released by the pineal gland in response to darkness (Li et al., 2017). Melatonin secretion is controlled by the suprachiasmatic nucleus (SCN), which relays photoperiodic information to the pineal via sympathetic nervous system (SNS) (Tan et al., 2015). However, melatonin is also synthetized in many sites such as gastrointestinal tract, skin, retina, and bone marrow (Acuña-Castroviejo et al., 2014). In addition to the circadian rhythm control, pineal hormone has antioxidant, anti-inflammatory and oncostatic activities (Karaaslan et al. 2015). Recently, studies have shown that melatonin may inhibit breast cancer progression (Mao et al., 2012; Jardim-Perassi et al., 2014). Melatonin may affect tumor growth by reducing cell proliferation and angiogenesis and inhibiting DNA damage, besides increasing the activity of tumor suppressor genes and apoptosis of tumor cells (Panzer and Viljoen, 1997). A study showed that lung metastasis of gastric cancer may be inhibited by melatonin through downregulation of MMP-2, MMP-9, and NF-κB expressions (Wang et al., 2019). Few investigations analyzed the effects of melatonin suppression on cancer

onset in pre-clinical models. Pinealectomized rats had higher incidence of DMBA-induced mammary cancer than sham-operated animals (Tamarkin et al., 1981; Shah et al., 1984). However, no studies have investigated the impact of pinealectomy on other types of cancer.

Melatonin may regulate cell proliferation and apoptosis in cancer through the p53 signaling pathway (Santoro et al. 2013). In breast and colon cancers cell lines, melatonin inhibited cell proliferation and prevented DNA damage of tumor cells through the p53 activation (Santoro et al., 2013). Furthermore, melatonin administration inhibited lymphoma development in $Trp53^{-/-}$ mice, compared to non-treated group (Huang et al., 2015). Melatonin also may influence tumor progression through the ERK1/2 modulation (Cagnol & Chambard 2010). On cancer, ERK1/2 may mediate several events including apoptosis, cell proliferation and metastasis (Olea-Flores et al., 2019). A recent study showed in an orthotopic model of oral cancer, that melatonin administration reduced the p-ERK levels in the tumor microenvironment (Liu et al., 2018). Moreover, melatonin increases the sensibility of esophageal cancer to fluorouracil chemotherapy through ERK signaling pathway inhibition (Lu et al., 2016).

Inflammation play a critical role in cancer onset and progression (Coussens & Werb, 2002). Liu et al. (2019) demonstrated that oncology patients with high systemic inflammation index had lower disease-free survival and decreased distant metastasis-free survival compared to patients with low inflammation levels. Systemic inflammation has also been associated with an increase in depth tumor invasion, greater risk of regional metastasis, and advanced clinical stage in patients with esophageal cancer (Zhang et al., 2019).

Melatonin is considered an important molecule with anti-inflammatory features (Najafi et al., 2017). The hormone suppresses the increase of TNF-α and IL6 proinflammatory cytokines in ovary cancer rats (Chuffa et al., 2015). Furthermore, melatonin administration promoted lower Infiltration of eosinophils, IL-17+ inflammatory cells and Foxp3+ cells in the tumor tissue of hamsters with chemically induced cholangiocarcinoma (Wongsena et al., 2018).

Currently, head and neck cancer (HNC) is the 3th most incident and the 7th leading cause of cancer death worldwide, with oral squamous cell carcinoma (OSCC) being its main subtype (Bray et al., 2018). Melatonin treatment may inhibit tumor progression and metastasis, as well as improve responses of head and neck oncology patients to chemotherapy treatment (Yeh et al., 2016; Lu et al., 2016; Shen et al., 2018). *In vitro* studies show that pineal hormone affects the motility of OSCC cell lines by inhibiting MMP-9 and VEGF transcription, which are molecules known to influence tumor progression (Goncalves et al., 2014; Yeh et al., 2016). Furthermore, melatonin significantly suppresses cell proliferation in dose- and time-dependent manner in an orthotopic model of oral cancer and *in vitro* by reducing histone lysine-specific demethylase (LSD1) expression (Yang et al., 2017). Other investigations have demonstrated that melatonin may be a good adjuvant therapy for cancer treatment (Li et al., 2017; Lissoni et al. 1999). Lissoni et al (1999) revealed that HNC patients concomitantly treated with melatonin and chemotherapy displayed higher 1-year survival rate and increased tumor remission rate than those who received chemotherapy alone. Despite evidences of the melatonin effects on the cancer progression, its role on the tumorigenesis is poorly known. In this research, we used a preclinical oral carcinogenesis model to test the hypothesis that the

melatonin suppression would promote higher chemically induced cancer occurrence and progression in rats.

Material and Methods

2. Material and methods

2.1 Animals and experimental design

In order to assess whether the suppression of melatonin affects the oral cancer onset and progression, twenty-three male Wistar rats were housed in groups of 4 animals per cage (25.9 × 47.6 × 20.9 cm, polypropylene) and kept under standardized conditions (22±2 °C; 12/12h light/dark cycle; lights on at 7:00 h). The animals were divided into 2 groups: **PNT:** 12 rats submitted to pinealectomy surgery; **Sham:** 11 rats submitted to sham-pinealectomy surgery. Fifteen days after pinealectomy or sham surgeries, all animals were undergoing to chemically induced oral carcinogenesis for 16 weeks. The rats had their depressive-like and anxiety-like behaviors tested through forced swimming test (FST) and elevated zero maze (EZM), respectively. At the end of the experimental period, the animals were euthanized for evaluation of oral cancer incidence and progression. All the experimental protocols were approved by the

Animal Ethics Committee of the São Paulo State University, School of Dentistry, Araçatuba, SP, Brazil (Protocol Number: 00522-2017).

2.2 Pinealectomy

Pinealectomy surgery was performed as described by Hoffman & Reiter (1965). The rats were anesthetized with intraperitoneal injection of Ketamine Chlorhydrate 10% (50mg/kg body weight) and Xilazine Chlorhydrate 2% (5mg/kg body weight) and subjected to pinealectomy (PNT) or to a sham-surgery. A sagittal incision was made on the scalp anteroposteriorly along the midline. Skin and muscles were turned away in order to expose the lambdoid (λ) suture. With a trephine (5 mm in diameter), a bone window was created on the confluence of the superior sagittal and transverse venous sinuses and the bone-disk was removed. Thereafter, the superficial pineal gland was withdrawal. Then, the bone-disk was replaced, and the skin was sutured with surgical suture thread (Shalon® 4-0). The animals of sham group were submitted to the same procedures, however, the pineal gland was not removed.

2.3 Behavioral phenotyping

Behavioral evaluation was accomplished in two moments during the experimental period: two weeks after the surgery and before starting carcinogenic induction and after carcinogen treatment. The evaluation of depressive- and anxiety-like behavioral phenotypes was performed by one blind experienced observer for groups and experimental conditions.

2.3.1 Forced swimming test (FST)

The FST was accomplished as described by Porsolt et. al (1977). Firstly, the rats were forced to swim for 15 minutes in a glass cylinder (13 cm in

diameter × 24 cm in height) filled with water (25ºC±2) (pre-test). On the next day, the rats were forced swim again at the same conditions of pre-test for 6 minutes. The performance of animals was recorded with a camera positioned 100 centimeters away from the cylinder. To evaluate the depressive-like behavior, immobility time of the rats was analyzed. The immobility time was considered as absence of escape-oriented behaviors.

2.3.2 Elevated zero maze (EZM)

EZM is an annular platform elevated 50 cm above the floor. The apparatus has a diameter of 105 cm, divided into two opposite open arms and two opposite closed arms. For the test, the rats were placed individually into one of close arms of the EZM. The behavior of animals was recorded for 10 minutes with a camera positioned above of apparatus. The anxiety-like behavior was analyzed by time spent in the open arms (Shepherd et al. 1994).

2.4 Oral carcinogenesis model

Two weeks after the pinealectomy or sham surgery, all rats were submitted to oral carcinogenesis. For tumor induction, the rats were treated with 50 ppm of 4-nitroquinoline-1-oxide (4NQO) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in drinking water for 16 weeks (Valente et al. 2018). Animals had free access to food (Purina®, Paulínia-SP, Brazil) and carcinogen solution which were replenished twice a week. After the experimental period, the rats were euthanized and the tongues with carcinogen-induced lesions were extracted for histopathological, immunohistochemistry and molecular analysis.

2.5 Histopathological analysis

To histopathological analysis, the tongues were longitudinally sectioned and fixed in 10% buffered formaldehyde solution (Merck, Darmstadt, Germany) during 48h. After, the tissues were alcohol dehydrated and paraffin embedded. Histological sections (3 μm of thickness) were obtained and stained with hematoxylin and eosin (H&E). The tongue lesions were classified in leukoplakia or OSCC well, moderately or poorly differentiated, according to the World Health Organization (WHO) classification (El-Naggar et al., 2017). Leukoplakia is a precursor lesion of OSCC and for its diagnosis the following microscopy were considered: epithelial atrophy, acanthosis with or without hyperkeratosis and epithelial dysplasia (van der Waal et al., 1997). The epithelial dysplasia was classified into mild, moderate or severe (El-Naggar et al., 2017). Microscopic examination was performed by an experienced oral pathologist who was blind to the experimental groups.

2.6 Evaluation of tumor thickness and volume

The three-dimensional measurements of the tumor were obtained with a digital caliper. The tumor volume was calculated in mm^3 using the formula: depth x width × length (Valente et al., 2018). To evaluate tumor thickness, OSCC slides were photographed on a microscope equipped with a digital camera (Zeiss Axio imager Z1 microscope, Carl Zeiss, Munchen-Hallbergmoos, Germany). The measurements in μm were performed from the deepest point of tumor invasion to the surface of the lesion using the ImageJ software (Valente et al., 2018).

2.7 Epithelial thickness measurement of non-tumor oral epithelium

To examine the effects of melatonin suppression on morphologic features of non-tumor oral epithelium, H&E slides were analyzed at x100 magnification. Non-tumor oral epithelium thickness adjacent to the tongue lesions was evaluated by measuring (in μm) the distance between basal cell layer and granular layer. Corneal thickness was assessed by measuring the distance between the lucid layer and corneal layer surface. Total epithelial thickness was measured between the basal cell layer and apical epithelial surface (epithelium + corneal layer) (Alvisi et al., 2018). On each slide, 4 fields were analyzed: two fields immediately adjacent to the tongue lesions and two distant fields from the lesion site. For statistical analysis, the mean thickness of epithelium and corneal layer were calculated between two fields immediately adjacent to the tongue lesions and between two distant fields from the lesion. All the measurements were performed by a blind researcher to experimental groups.

2.8 Inflammatory cells quantification in the tumor invasion front

To evaluate the influence of melatonin suppression on tumor inflammatory response in sham and PNT rats, the average number of neutrophils, eosinophils, macrophages, lymphocytes and mast cells were quantified in OSCC slides stained with H&E, at 1000x magnification (Leica DM2500, Leica Biosystems, Wetzlar, Germany). On each slide, five fields of tumor invasion front were assessed. The analysis was performed by a blind researcher to experimental groups.

2.9 Immunohistochemistry

To immunohistochemistry evaluation, histological sections of OSCC were deparaffinized and rehydration. Heat-induced epitope retrieval was performed by 10mM citrate buffer, pH 6.0, for 20 minutes at 55°C. Blockade of endogenous peroxidase activity was accomplished by 3% H_2O_2, for 20 minutes. After, the slides were washed with PBS solution (pH 7.2). Sections were then incubed with primary antibody anti-p53 (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PKA (dilution 1:3000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-ERK1/2 (dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Following, the sections were incubated with Histofine antibody polymer conjugated with horseradish peroxidase (Nichirei Biosciences, Tokyo, Japan) for 30 minutes. The slides were washed with PBS solution and a chromogenic substrate (3,3′,5,5′-tetramethylbenzidine) was incubated for 5 minutes. Reactions were stopped in deionized water. Counter-staining was performed by Harris's hematoxylin for 20 seconds followed dehydration and covered with coverslip for microscopic examination. Sections stained by immunohistochemistry were evaluated for the percentage of immunopositive cells in the tumor invasion front. A total of 1000 tumor cells were counted in 2-4 fields at 400x magnification. The results were expressed as the percentage of positive cells (%). All the analyzes were performed by a blind examiner to experimental conditions.

2.10 Expression of tumor progression-related genes and melatonin receptors

To assess the association between melatonin suppression and the expression of the tumor progression-related genes, real time-PCR was

performed to evaluate the mRNA expression levels for VEGF, NFkB, MMP-2, MMP-9 and CDKN2a-p16. The mRNA expression of the melatonin receptors MTNR1a and MTNR2a also were examined in the tumors from both experimental groups. Once collected, the tumor specimens were washed in saline solution, immersed in TRIzol (Invitrogen Life Technologies, USA) and stored at -80ºC. Total RNA was extracted for synthesis of complementary DNA (cDNA). RNA quantity and quality were evaluated by Nanodrop 2000 (Thermo Scientific, Wilmington, USA). cDNA was synthesized using the High Capacity RNA to cDNA kit (Invitrogen Life Technologies). TaqMan™ RT-PCR assay measured the mRNA levels by the StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The primers used were VEGF (Rn01511601_m1), NFkB (Rn01310378_g1), MMP–2 (Rn01538170_m1), MMP–9 (Rn00579162_m1), CDKN2a-p16 (Rn00580664_m1), MTNR1a (Rn01488022_m1) and MTNR1b (Rn01447987_m1). ß-actin (Rn00562253_m1) gene was used as endogenous control. mRNA Relative Quantity (RQ) for each target gene was calculated using comparative Ct method. The assays were performed in duplicate.

2.11 Statistical analysis

GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, CA, USA) software was used to perform the statistical analysis. Chi-square test was performed to assess the OSCC incidence in the both experimental groups. To determine differences between sham and PNT rats for tumor volume and thickness, epithelial thickness, number of inflammatory cells, mRNA and protein levels and behavioral measures, Student's t-test was accomplished. Analysis of variance (One-way ANOVA) with post-test Tukey–Kramer multiple comparison

analysis was performed to determine differences between mean of inflammatory cells of both groups, according to the tumor volume. All values are given as the mean ± SEM and the level of statistical significance was set at a p value less than 0.05 ($p<0.05$) for all statistical tests.

Results

3. Results

3.1 Melatonin suppression induces oral cancer occurrence and progression

All PNT and sham rats were submitted to chemically induced carcinogenesis to investigate the effect of melatonin suppression on oral cancer occurrence. After carcinogenic induction, PNT rats displayed higher OSCC occurrence than sham animals (p=0.0029) (Fig. 1I). According to WHO criteria, all PNT rats (100%) had well-differentiated OSCC. Differently, in the sham group 54.5% displayed leukoplakia, while only 45.4% developed well-differentiated OSCC. We also assessed the OSCC progression from rats of both groups. The results showed that melatonin suppression promoted higher tumor growth. PNT rats exhibited higher OSCC volume and thickness than sham rats. The tumor volume was about 3 times higher in PNT animals (73.76 ± 11.7 mm^3) compared to sham animals (27.0 ± 5.19 mm^3) (p=0.0314) (Fig.1J). Furthermore, tumor thickness was twice greater in PNT rats (1344 ± 196.1 μm) than sham rats (600.6 ±172.7 μm) (p=0.0341) (Fig.1K).

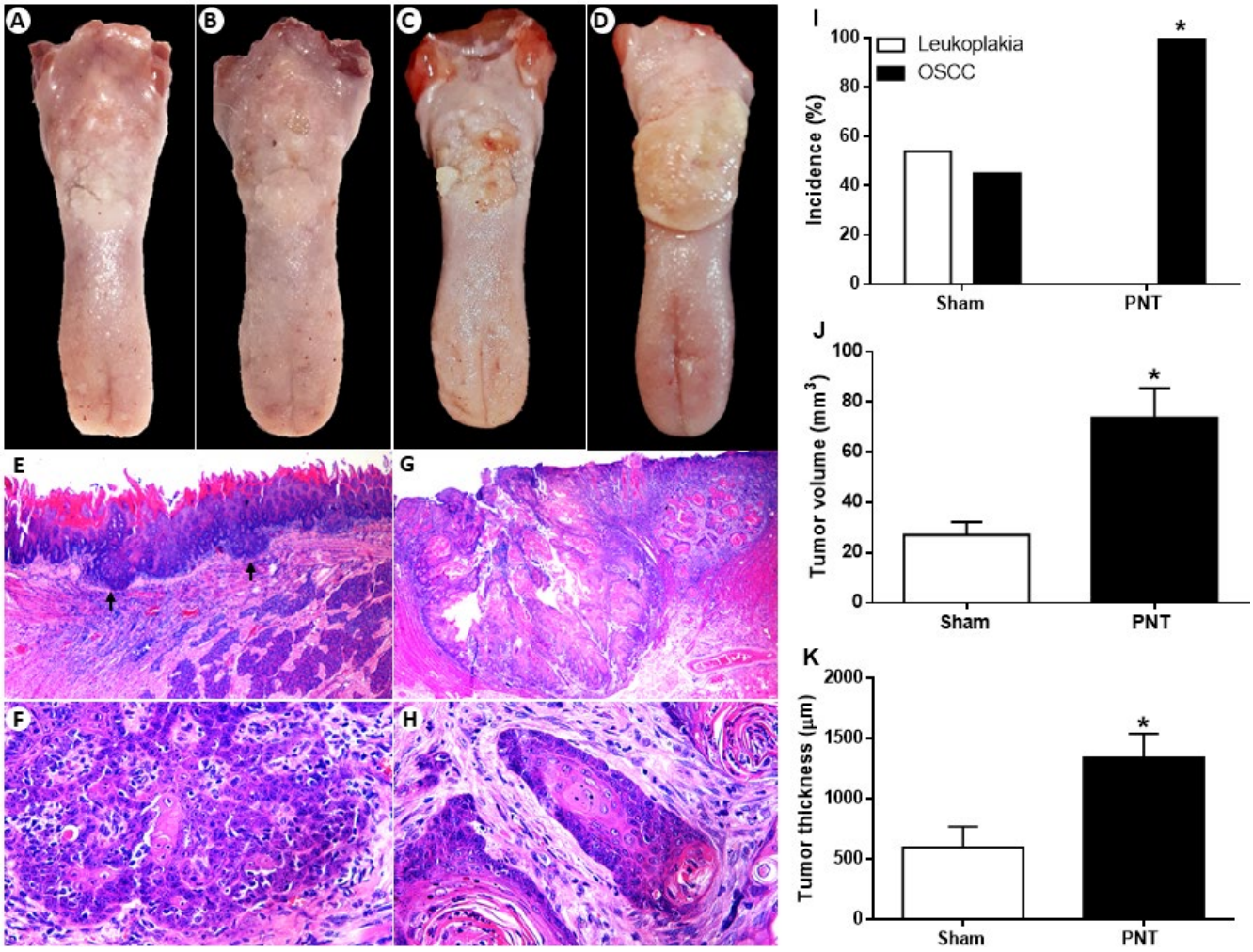

Figure 1. Clinical features of OSCCs derived from 4NQO treatment in sham (A and B) and PNT rats (C and D). A). Small irregular white plates. **B)** Discrete ulcerative lesion. **C)** Ulcerative lesion displaying white and reddish surface. **D)** Extensive ulcer with yellowish-white areas. **Histopathological features of tongue tumors from sham (E and F) and PNT rats (G and H) (H&E staining). E)** Well-differentiated OSCC (black arrows) (original magnification x250). **F)** Tumor cells showing hyperchromatism and dyskeratosis (original magnification x400). **G)** Extensive Well-differentiate OSCC (original magnification x250). **H)** Islands of well-differentiated tumor cells with nuclear pleomorphism and keratin pearls (original magnification x400). **Occurrence and progression of OSCC from sham and PNT animals. I)** Chi-square test revealed that PNT rats had a higher occurrence of OSCC than sham rats. **J)** Student's t-test showed that PNT group exhibited increased tumor volume compared to sham group. **K)** PNT rats exhibited higher tumor thickness than sham animals. (sham, n=11; PNT, n=12;) Bars represent the mean ± SEM. *p<0.05

3.2 Effects of pinealectomy on the depressive- and anxiety-like behaviors

To evaluate the behavioral alterations induced by pinealectomy, all rats were tested for depressive- and anxiety-like behaviors through FST and EZM, respectively. Although PNT rats displayed low-depressive (immobility time in FST, 104.3 ± 16.01s) and high-anxious behavior (time in the open arms in EZM test, 13.9 ± 4.105s) compared to sham animals (164.0 ± 28.08s and 21.0 ± 3.719s, respectively) tests did not reveal statistical difference between the groups ($p>0.05$) (Fig. 2A-B). After carcinogenesis, there were also no differences between PNT and sham groups in the depressive- (PNT, 134.8 ± 19.11s *vs* sham,145.0 ± 11.29s) and anxiety-like (PNT, 12.08 ± 5.28s *vs* sham, 17.00 ± 7.49s) behavioral phenotype ($p>0.05$) (Fig. 2A-B).

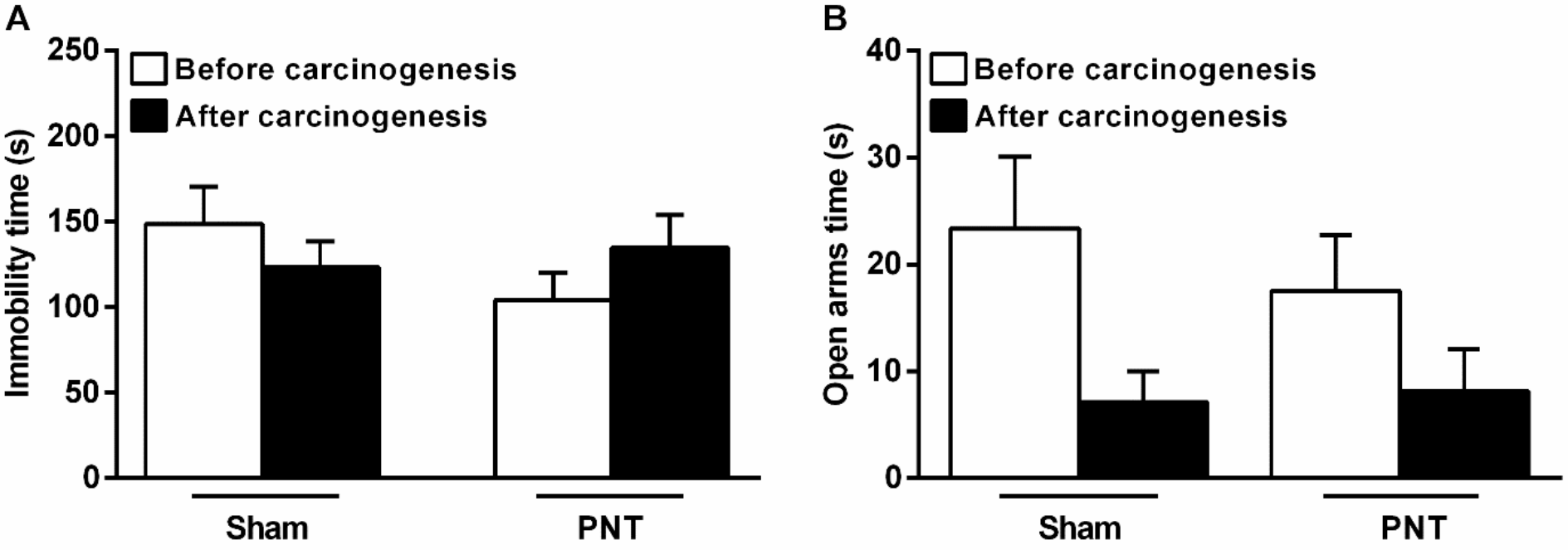

Figure 2. Anxiety- and depressive-like behavior from sham and PNT rats. A) There were no differences in the depressive-like behavior from sham and PNT rats before and after carcinogenesis. **B)** Student's t-test revealed no differences in the anxiety-like behavior from sham and PNT groups before and after carcinogenesis. Bars represent the mean ± SEM. $p>0.05$. (sham, n= 11; PNT, n= 12).

3.3 Melatonin suppression promotes atrophy of non-tumor oral epithelium

To assess the effects of pinealectomy on the morphologic features of non-tumor epithelium, thickness of oral epithelium adjacent to the tongue lesions was measured. After carcinogenic induction, sham and PNT rats showed no differences in the thickness of epithelium (sham: 98.10 ± 4.689 μm *vs* PNT: 82.25 ± 7.628 μm), keratin layer (sham: 49.22 ± 3.739 μm vs PNT: 49.23 ± 4.697 μm) and total thickness (sham: 110.5 ± 5.195 μm vs PNT: 101.3 ± 12.12 μm) immediately adjacent to the oral lesion ($p>0.05$) (Fig.3A-C). When we evaluated the non-tumor oral epithelium distant from the tongue lesions, there were no statistical differences in the epithelial thickness of sham (52.70 ± 3.596 μm) and PNT (45.81 ± 2.965 μm) groups ($p>0.05$) (Fig.3D). However, PNT animals displayed lower corneal thickness (18.19 ± 1.451 μm) than sham rats (25.98 ± 2.477 μm) ($p= 0.0143$) (Fig. 3E). The total thickness of non-tumor oral epithelium also was reduced in the PNT rats (64 ± 4.007 μm) compared to sham animals (78.67 ± 4.725 μm) ($p=0.0292$) (Fig. 3F).

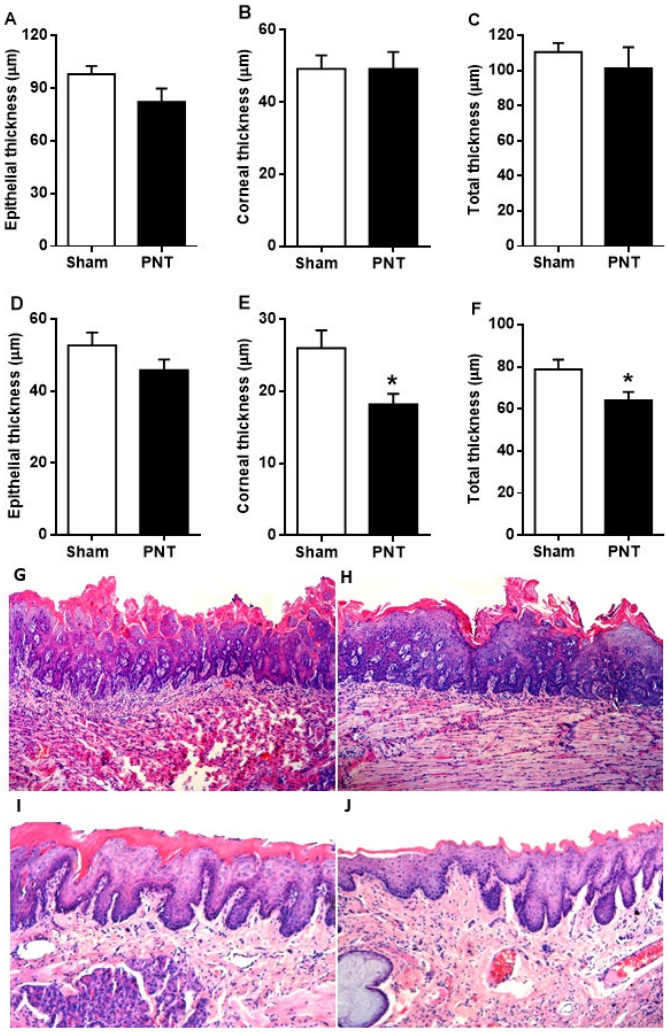

Figure 3. Epithelial thickness of non-tumor epithelium adjacent to the tongue lesions derived from 4NQO treatment. A-C) Student's test showed no statistical differences regarding to epithelial thickness, corneal thickness and total thickness of non-tumor epithelium immediately adjacent to the tongue lesions derived from 4NQO treatment in PNT and sham animals**. Epithelial thickness of non-tumor epithelium in the distant sites from the lesion. D)** There were no differences in non-tumor oral epithelial thickness between both groups. **E)** PNT rats displayed lower corneal thickness of non-tumor epithelium than sham group. **F)** Student's t-test revealed that PNT group had decreased total thickness of non-tumor epithelium compared to sham rats. **G and H)** Total epithelial thickness of non-tumor epithelium immediately adjacent to the lesion from sham and PNT group, respectively (H&E, original magnification x100). **I and J)** Total epithelial thickness of non-tumor epithelium distant to the lesion from sham and PNT rats, respectively (H&E, original magnification x100). Bars represent the mean ± SEM. *p<0.05 (sham-PNT, n=10; PNT, n=10).

3.4 Melatonin suppression promotes increase of inflammatory cells in the tumor invasion front

The effects of pinealectomy on inflammatory response were evaluated in the tumor invasion front of the OSCCs from both groups. When we assess the average number of leukocytes, there were no significant differences between sham (32.37 ± 4.104) and PNT rats (49.20 ± 6.368) ($p>0.05$) (Fig. 4A). We also evaluated individually the different cells involved in the inflammatory response. Student's t-test revealed that PNT rats displayed increased average number of eosinophils (PNT, 1.444 ± 0.2597 *vs* sham,0.2800 ± 0.1020) ($p=0.0074$)(Fig. 4I) and macrophages (PNT, 10.53 ± 0.6379 *vs* sham, 6.924 ± 0.5910) ($p=0.0014$) (Fig. 4K) compared to sham animals. However, there were no significant differences between both groups to the average number of neutrophils (PNT, 11.74 ± 2.931 *vs* sham, 3.695 ± 1.434) ($p>0.05$) (Fig.4C), mast cells (PNT, 7.545 ± 0.8918 *vs* sham, 8.438 ± 2.014) ($p>0.05$)(Fig.4E) and lymphocytes (PNT, 17.45 ± 1.604 *vs* sham, 14.14 ± 1.358) ($p>0.05$) (Fig.4G). In our results, PNT rats displayed OSCC volume approximately three times higher than sham group. However, some PNT animals had incipient lesions similar to non-pinealectomized rats. Thus, to also examine the association of tumor stage with the number of inflammatory cells in the invasion front, carcinomas from PNT rats were classified into early or advanced stages. This classification was performed on basis of a median-split of the tumor volume. Advanced tumor-bearing PNT rats displayed higher average number of leukocytes ($p=0.0308$) (Fig. 4B), lymphocytes ($p=0.0341$) (Fig. 4H) and eosinophils ($p=0.0277$) (Fig. 4J) than sham rats. Independently of tumor stage, mean number of

macrophages was increased in the invasive front from PNT rats compared to sham animals (p= 0.0056) (Fig. 4L).

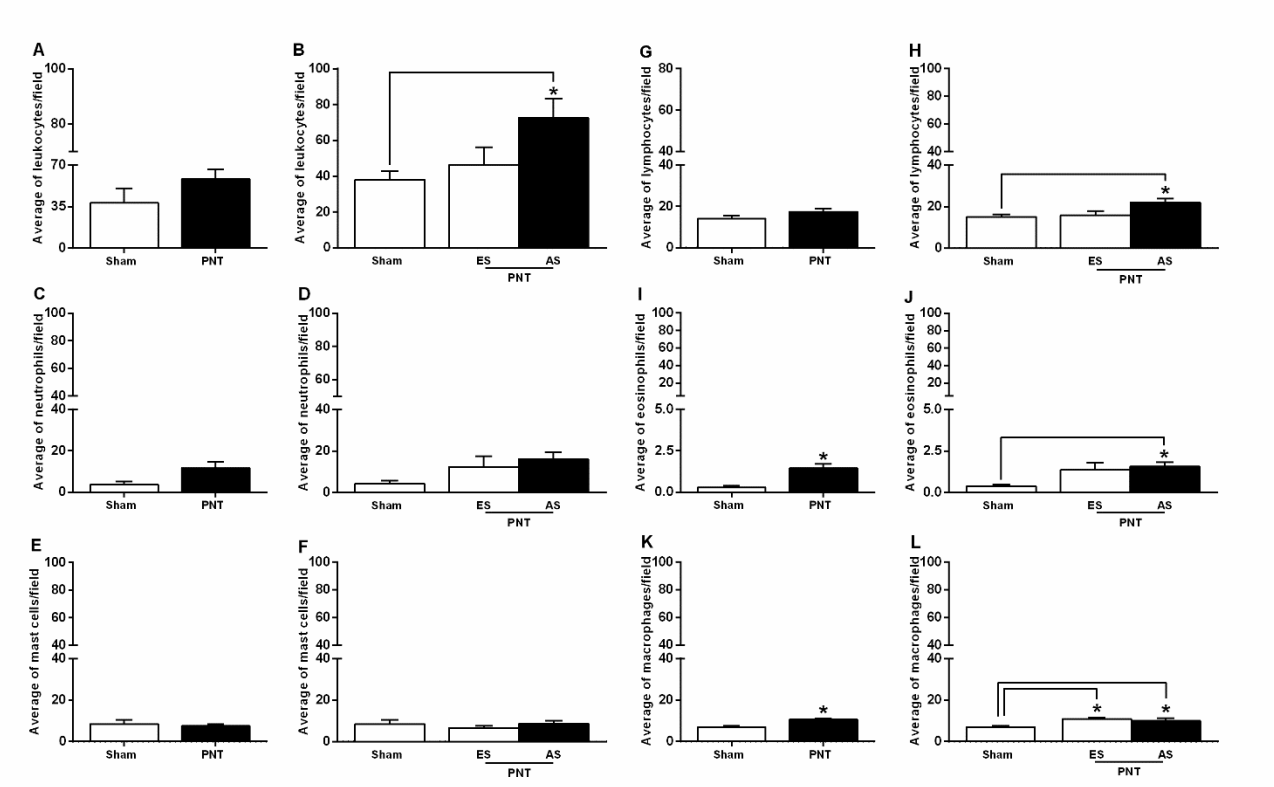

Figure 4. Inflammatory response in the tumor invasion front. Student's t-test showed no differences in the average number of leukocytes **(A)**, neutrophils **(C)**, mast cells **(E)** and lymphocytes **(G)** in the OSCCs from sham and PNT animals. PNT rats displayed increased average number of tumor-associated eosinophils **(I)** and macrophages **(K)** compared to sham animals. Tumor size was not associated with the average number of neutrophils **(D)** and mast cells **(F)** in the invasion front in sham and PNT rats. Advanced tumor-bearing PNT rats had higher average number of leukocytes **(B)**, lymphocytes **(H)** and eosinophils **(J)**. Early or advanced tumor-bearing PNT rats exhibited increased average number of macrophages than Sham rats **(L)**. *p<0.05. Bars represent the mean ± SEM. *p<0.05. **ES:** Early stage. **AS:** Advanced stage. (sham, n=5; PNT, n=11).

3.5 Tumors from PNT rats display higher p53 and ERK1/2 expression in the OSCC invasion front

Tumor progression-related genes and melatonin receptors expression were evaluated in the tumor microenvironment from sham and PNT rats. The mRNA levels of VEGF, NFkB, MMP2, MMP9, CDKN2a-p16 and MTNR1a were higher in OSCC specimens from PNT rats, however, these results did not reach statistical significance ($p>0.05$) (Fig. 5A – F). MTNR1b was expressed only in three samples, two from sham group and one from PNT animals (data not shown). The expression of carcinogenesis-related proteins PKA, ERK½ and p53 was assessed by immunohistochemistry. There were no differences to immunoexpression of PKA in the tumor invasion front between sham (27.80 ± 3.001%) and PNT rats (28.33 ± 3.709%) ($p>0.05$) (Fig. 5G). ERK1/2 immunoexpression was increased in tumor front from PNT animals (57,05 ± 4,645%), compared to sham rats (43,87 ± 3,177%) ($p=0.0451$) (Fig. 5H). Similarly, PNT rats exhibited higher p53 expression in the OSCC invasion front (44,35 ± 4,330%) than sham rats (32,99 ± 1,438%) ($p=0.0430$) (Fig. 5I).

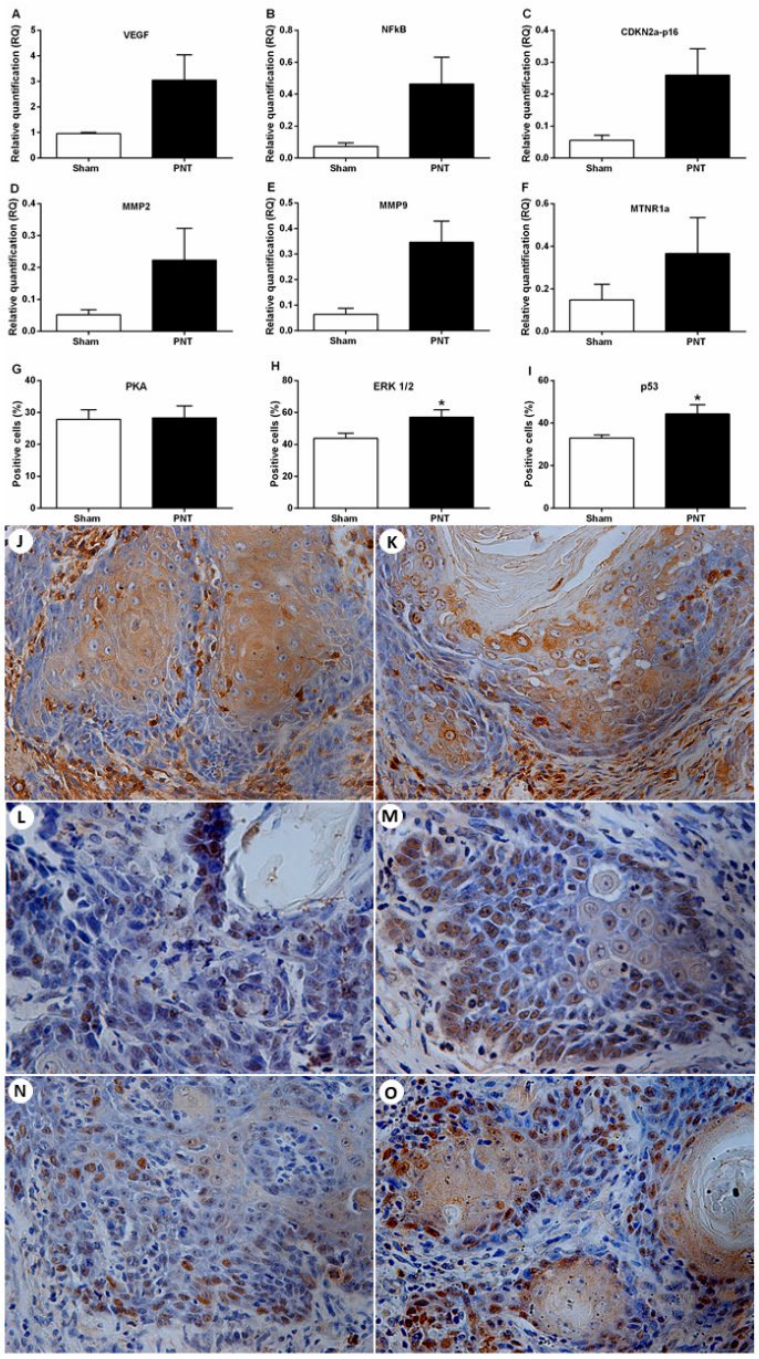

Figure 5. Expression of tumor progression-related genes and melatonin receptors and immunostaining of PKA, ERK ½ and p53 in the OSCC microenvironment. A – F) Student's t-test showed no differences between sham and PNT OSCCs for mRNA expression of VEGF, NFkB, CDKN2A-p16, MMP2, MMP9 andMTNR1a. **G)** Student's t-test showed no statistic differences between both groups for PKA expression in OSCCs. **H)** PNT rats had increased tumor expression of ERK1/2 compared to sham animals. **I)** PNT animals displayed higher tumor expression of nuclear p53 than sham rats. Immunoexpression of PKA **(J and K)**, ERK1/2 **(L and M)** and p53 **(N and O)** in OSCC invasion front from sham and PNT rats, respectively (original magnification x400). Bars represent the mean ± SEM. *p<0.05. (sham-PNT, n= 7; PNT, n= 9).

Discussion

4. Discussion

In order to evaluate the role of melatonin deficiency on oral cancer occurrence and progression, we used a pinealectomy model to induce the hormone systemic suppression in rats. Pinealectomy is a classical model of suppression of the melatonin serum concentrations in rodents (Lewy et al., 1980). In humans, suppression of melatonin release occurs, for example, in shift workers due artificial light at night exposition (Hunter & Figueiro, 2017; Touitou et al., 2017; Razavi et al., 2019). Our results provided the first evidences that melatonin suppression may influence oral cancer onset and progression. We demonstrated that rats submitted to pinealectomy displayed increased OSCC incidence compared to rats underwent sham-surgery. Moreover, PNT rats had OSCC volume and thickness about 3 times and twice higher than sham animals, respectively. Studies that have evaluated the influence of pinealectomy on cancer progression are scarce. Only El-Domeiri & Das Gupta (1976) showed that pinealectomy resulted in higher melanoma growth in hamsters (El-Domeiri & Das Gupta, 1976). Recently, a growing number of studies have investigated the effects of melatonin on cancer progression and treatment (Lissoni et al,1999; Amin et al., 2016). Some studies show that melatonin has oncostatic effects, due to its ability to inhibit angiogenesis, cell proliferation and metastatic process (Yeh et al., 2017; Gonçalves et al., 2014). In HNC, many investigations have shown different signaling pathways in which melatonin may inhibit the tumor progression. Liu et al. (2018), for example, demonstrated that pineal hormone inhibits the OSCC growth by inactivating ROS-dependet Akt pathway and reducing expression of cyclin D1, PCNA, and Bcl-2, resulting in lower cell proliferation and decreased

epithelial-mesenchymal transition. Melatonin also may inhibit OSCC metastasis (Goncalves et al. 2014; Yeh et al. 2016). For this, pineal hormone decreases expression of pro-angiogenic genes HIF-1α and VEGF and pro-metastatic gene ROCK-1 (Goncalves et al. 2014), as well as, promotes transcriptional suppression of the MMP-9 gene mediated by decreased histone acetylation (Yeh et al. 2016). Thus, we suggest that pinealectomy induces opposite effects to the melatonin treatment in tumor cells, promoting cell proliferation and increased OSCC progression. On the other hand, researches have showed that decreased levels of melatonin may be a risk factor for mammary cancer onset (Tamarkin et al., 1981; Shah et al., 1984; Devore et al., 2017). Nevertheless, to the best of our knowledge, this is the first study to demonstrate the influence of melatonin suppression on oral carcinogenesis.

In preclinical studies, melatonin suppression may induce behavioral and neurobiological alterations in rodents (Tchekalarova et al., 2016). In our investigation, we assessed the role of pinealectomy on behavior of rats submitted to chemical carcinogenesis. Here, we did not find significant differences in the anxiety- and depressive-like behavior between both groups, evaluated two weeks after surgery. Following oral carcinogenesis, there were also no differences between behavioral measures from sham and PNT rats. Regarding to anxiety-like behavior, there are inconsistent results among the different studies. Bustamante-García et al. (2014) demonstrated that pinealectomy increased anxiety-like behavior in rats. On the other hand, investigations of Tchekalarova et al. (2016) showed that pinealectomy decreased anxiety-like behavior one month after surgery, however, this significance was lost 3 months after pinealectomy. This variability between

studies may be related to the methodological conditions, tests applied and time-window between pinealectomy and behavioral test. In relation to depressive-like behavior, studies show that PNT rats display higher levels of depressive-like behavior compared to non-pinealectomized animals and this is reversed by administration of exogenous melatonin (Tchekalarova et al., 2016). No study evaluated the pinealectomy effects on behavior of rats submitted to carcinogenic induction. However, we suggest that exposure of both groups to the chemical carcinogen 4NQO could induce high anxiety-like behavior in all animals, overlapping effects of pinealectomy.

Pinealectomy can also promote morphometric and biochemical changes in the skin epithelial tissue, such as atrophy of the epithelium and decreased levels of antioxidant enzymes (Esrefoglua et al., 2005). Here, we hypothesized that the molecular events related to oral carcinogenesis resulting from pinealectomy could be accompanied by morphological changes in the oral epithelium. Our results showed that immediately adjacent to the tongue lesions, PNT rats displayed a lower epithelial thickness than sham animals, but these results did not reach statistical significance. The analysis in this area of oral epithelium could have been influenced by the intense cell proliferation that occur in this site. When we evaluated a distant region from lesion site, we observed an atrophy of corneal layer and total epithelial thickness from PNT rats compared to sham animals. Pineal hormone is a powerful free radical scavenger (Tan et al., 2002). Unlike, melatonin suppression promotes increase of reactive oxygen species (ROS)concentrations and reduces levels of antioxidant enzymes in the skin of rats (Esrefoglua et al., 2005). In non-transformed cells, high ROS tend to promote cell growth arrest and/or cell death

(Martindale & Holbrook, 2012). In this context, we suggest that the epithelial atrophy displayed by PNT rats, results from lower keratinocytes proliferation caused by a possible increase in ROS in the tongue microenvironment after pinealectomy. Decreased epithelial thickness is associated to higher susceptibility to harmful agents, allowing the penetration of carcinogenic substances through the mucosa (Wight & Ogden, 1998). In a *post-mortem* study, Valentine et al. (1985) showed that predisposing factors for oral cancer, such as increased alcohol and tobacco consumption, were associated with a reduction in tongue epithelial thickness. Therefore, a lower epithelial thickness in PNT rats would allow a higher infiltration of carcinogen 4NQO in tongue epithelium, leading to increased DNA damage and, consequently, favoring higher OSCC incidence.

Cancer development and progression may be influenced by inflammatory responses (Coussens and Werb, 2002). Immune cells can have dual effects on tumorigenesis, eliminating tumor cells or promoting cancer growth (Gajewski et al. 2013). Here, we demonstrated that melatonin suppression induced increased average number of eosinophils and macrophages in the tumor microenvironment. Moreover, only large tumors of PNT rats had increased mean number of lymphocytes in the tumor invasion front. Likewise, previous investigations with non-cancer preclinical model showed that pinealectomy promoted an increase in tissue number of eosinophils and macrophages (Oner et al., 2004; Dair et al., 2008). On the other hand, melatonin administration suppressed the number of eosinophils in cholangiocarcinoma samples (Wongsena et al., 2018). The presence of eosinophils in the mammary cancer microenvironment enhanced tumor growth

and lung metastasis (Panagopoulos et al., 2017). In human OSCC specimens, tumor-associated macrophages are positively associated to tumor size, nodal metastasis, invasive behavior, and invasive depth, while tumor-associated tissue eosinophilia was positively correlated with advanced disease stage, and tumor invasion depth (Kouketsu et al., 2019; De Paz et al., 2019). Thus, our findings suggest that melatonin suppression induced increase of inflammatory cells in the tumor microenvironment, which contributed to OSCC progression in PNT rats.

Cancer progression may be modulated by melatonin through receptor-dependent mechanisms (Jockers et al., 2016). In this study, mRNA levels for MTNR1a and MTNR1b were detected in the OSCC microenvironment. MTNR1a and MTNR1b are genes that encode MT1 and MT2 receptors respectively and studies have reported its expression in normal tongue microenvironment and OSCC cells (Ortiz et al., 2015; Nakamura et al., 2008). However, in oral cancer cells, melatonin receptors may suffer epigenetic silencing, resulting in its lower expression and increased tumor growth (Nakamura et al., 2008). Silencing of MTNR1a in OSCC specimens was correlated to tumor size and shorter overall survival (Nakamura et al., 2008). So, we suggest that the expression of MTNR1b in only some specimens of our samples may be due genetic alterations that would result in its gene silencing. Investigations have shown also that melatonin may inhibit the expression of tumor progression-related genes (Wang et al., 2018; Colombo et al., 2018). For example, expression of NFkB was reduced in breast cancer cells treated with melatonin (Colombo et al. 2018). Another study showed that melatonin may control cell invasion and metastasis in ovarian cancer patients by decrease in

MMP9 activity (Goncalves et al. 2014; Yeh et al. 2016). There were no studies that have evaluated the expression of cancer progression-related genes and proteins in pinealectomized rats. In our study, mRNA levels for VEGF, NFkB, CDKN2a-p16, MMP2, MMP9 genes were increased in the tumor microenvironment from PNT rats, but the results did not reach significance. This may be due to high cell heterogeneity found between OSCC samples. Higher tumors display a large quantity of tumor cells, whereas lower tumors also exhibit several stromal cells, such as inflammatory cells, fibroblasts and blood vessels.

Several signaling pathways related to cancer progression may be modulate by melatonin. In our research, melatonin suppression did not promote differences in PKA expression in OSSCs. Our results revealed that PNT rats had increased expression of ERK1/2 in the tumor invasion front. Modulation of MAPKs pathways may be involved in anticancer properties of this hormone (Cagnol & Chambard 2010. As a result of melatonin treatment, ERK1/2 may control proliferation, migration, differentiation and death of tumor cells (Cagnol, 2009). Recent studies showed that melatonin inhibited the ERK1/2 expression in esophageal carcinoma and oral cancer cells, decreasing tumor progression (Lu et al., 2016; Liu et al., 2018). In a human leiomyosarcoma xenografts model, high physiological levels of melatonin induced a dose-dependent suppression of ERK1/2 (Dauchy et al., 2009). In the current study, besides the increased ERK1/2 expression in tumors from PNT rats, we also demonstrated that pinealectomy induced higher nuclear expression of p53 in the invasive front. This event results from p53 gene mutations which allow its accumulation in the nucleus of tumor cells and, consequently, the survival of genetically unstable cells (Soussi 2000). Although so far there was no evidences linking

p53 nuclear accumulation and melatonin levels, hormonal treatment can increase the activity of p53 tumor suppressor signaling pathway, reducing the tumor growth (Amin et al., 2019). In short, melatonin may increase the activity of p53 pathways and decrease ERK1/2 phosphorylation. Although we did not administer melatonin to any group of animals, our results suggest that pinealectomy may promote chemically induced OSCC growth in rats through increase of ERK1/2 expression and nuclear accumulation of p53, and consequent loss of its function.

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Conclusion

5. Conclusion

Taken together, our results reveal for the first time that melatonin suppression may induce higher oral cancer occurrence and progression in a preclinical model. Our findings suggest that chemically induced OSCC development can be influenced by decreased epithelial thickness after pinealectomy. Furthermore, the accelerated tumor progression in pinealectomized rats could be mediated by increase of tumor-associated macrophages and eosinophils and oncogenic protein ERK1/2, besides of inactivating of p53.

Declaration of interest: None

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References

Referências

1. Acuña-Castroviejo D, Escames G, Venegas C, Díaz-Casado ME, Lima-Cabello E, López LC, Rosales-Corral S, Tan D, Reiter R 2014 Extrapineal melatonin: Sources, regulation, and potential functions. Cell. Mol. Life Sci. 71:2997–3025
2. Alvisi S, Baldassarre M, Gava G, Mancini I, Gagliardi M, Seracchioli R, Meriggiola MC 2018 Structure of Epithelial and Stromal Compartments of Vulvar and Vaginal Tissue From Women With Vulvo-Vaginal Atrophy Taking Ospemifene. J Sex Med.15(12):1776-1784
3. Amin AH, El-Missiry MA, Othman AI, Ali DA, Gouida MS, Ismail AH 2019 Ameliorative effects of melatonin against solid Ehrlich carcinoma progression in female mice. J Pineal Res. 8:e12585.
4. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A 2018 Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 6:394-424
5. Bustamante-García R, Lira-Rocha AS, Espejo-González O, Gómez-Martínez AE, Picazo O 2014 Anxiolytic-like effects of a new 1-N substituted analog of melatonin in pinealectomized rats. Prog Neuropsychopharmacol Biol Psychiatry. 51:133-9.
6. Cagnol S, Chambard JC 2010 ERK and cell death: mechanisms of ERK-induced cell death--apoptosis, autophagy and senescence. FEBS J. 1:2-21

7. Chuffa LG, Fioruci-Fontanelli BA, Mendes LO, Ferreira Seiva FR, Martinez M, Fávaro WJ, Domeniconi RF, Pinheiro PF, Delazari Dos Santos L, Martinez FE 2015 Melatonin attenuates the TLR4-mediated inflammatory response through MyD88- and TRIF-dependent signaling pathways in an in vivo model of ovarian cancer. BMC Cancer. 15:34
8. Colombo J, Jardim-Perassi BV, Ferreira JPS, Braga CZ, Sonehara NM, Júnior RP, Moschetta MG, Girol AP, Zuccari DAPC 2018 Melatonin Differentially Modulates NF-κB Expression in Breast and Liver Cancer Cells. Anticancer Agents Med Chem. 12:1688-1694.
9. Coussens LM, Werb Z 2002 Inflammation and cancer. Nature. 6917:860-7
10. Dair EL, Simoes RS, Simões MJ, Romeu LR, Oliveira-Filho RM, Haidar MA, Baracat EC, Soares JM Jr 2008 Effects of melatonin on the endometrial morphology and embryo implantation in rats. Fertil Steril. 5 Suppl:1299-305
11. Dauchy RT, Blask DE, Dauchy EM, Davidson LK, Tirrell PC, Greene MW, Tirrell RP, Hill CR, Sauer LA 2009 Antineoplastic effects of melatonin on a rare malignancy of mesenchymal origin: melatonin receptor-mediated inhibition of signal transduction, linoleic acid metabolism and growth in tissue-isolated human leiomyosarcoma xenografts. J Pineal Res. Aug;47(1):32-42
12. De Paz D, Chang KP, Kao HK, Lao WW, Huang YC, Chang YL, Huang Y 2019 Clinical Implications of Tumor-Associated Tissue Eosinophilia in Tongue Squamous Cell Carcinoma. Laryngoscope. 5:1123-1129
13. Devore EE, Warner ET, Eliassen AH, Brown SB, Beck AH, Hankinson SE, Schernhammer ES 2017 Urinary Melatonin in Relation to Postmenopausal

Breast Cancer Risk According to Melatonin 1 Receptor Status. Cancer Epidemiol Biomarkers Prev. 3:413-419

14. El-Domeiri AA, Das Gupta TK 1976 The influence of pineal ablation and administration of melatonin on growth and spread of hamster melanoma. J Surg Oncol. 3:197-205
15. El-Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ 2017 WHO classification of head and neck tumours (9th ed.), IARC, Lyon
16. Eşrefoglu M, Seyhan M, Gül M, Parlakpinar H, Batçioğlu K, Uyumlu B 2005 Potent therapeutic effect of melatonin on aging skin in pinealectomized rats. J Pineal Res. 3:231-7
17. Gajewski TF, Schreiber H, Fu YX 2013 Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol. 10:1014-22
18. Goncalves NN, Rodrigues RV, Jardim-Perassi BV, Moschetta MG, Lopes JR, Colombo J, Zuccari DA 2014 Molecular markers of angiogenesis and metastasis in lines of oral carcinoma after treatment with melatonin. Anticancer Agents Med Chem. 9:1302-11
19. Hoffman RA, Reiter RJ 1965 Rapid pinealectomy in hamsters and other small rodents. Anat Rec.1:19-21
20. Huang HS, Chu SC, Hsu CF, Chen PC, Ding DC, Chang MY, Chu TY 2015 Mutagenic, surviving and tumorigenic effects of follicular fluid in the context of p53 loss: initiation of fimbria carcinogenesis. Carcinogenesis. 11:1419-28
21. Hunter CM, Figueiro MG. Measuring Light at Night and Melatonin Levels in Shift Workers: A Review of the Literature. Biol Res Nurs. 2017; 4:365-374.
22. Jardim-Perassi BV, Arbab AS, Ferreira LC, Borin TF, Varma NR, Iskander AS, Shankar A, Ali MM, de Campos Zuccari DA 2014 Effect of melatonin on

tumor growth and angiogenesis in xenograft model of breast cancer. PLoS One. 9:e85311

23. Jockers R, Delagrange P, Dubocovich ML, Markus RP, Renault N, Tosini G, Cecon E, Zlotos DP 2016 Update on melatonin receptors: IUPHAR Review 20. Br J Pharmacol. 173(18):2702-25
24. Karaaslan C, Suzen S 2015 Antioxidant properties of melatonin and its potential action in diseases. Curr Top Med Chem. 9:894-903
25. Kouketsu A, Sato I, Oikawa M, Shimizu Y, Saito H, Tashiro K, Yamashita Y, Takahashi T, Kumamoto H 2019 Regulatory T cells and M2-polarized tumour-associated macrophages are associated with the oncogenesis and progression of oral squamous cell carcinoma. Int J Oral Maxillofac Surg. 19: 31084-7
26. Lewy AJ, Tetsuo M, Markey SP, Goodwin FK, Kopin IJ 1980 Pinealectomy abolishes plasma melatonin in the rat. J Clin Endocrinol Metab. 1:204-5.
27. Li Y, Li S, Zhou Y, Meng X, Zhang JJ, Xu DP, Li HB 2017 Melatonin for the prevention and treatment of cancer. Oncotarget. 2017; 24:39896-39921
28. Lissoni P, Barni S, Mandalà M, Ardizzoia A, Paolorossi F, Vaghi M, Longarini R, Malugani F, Tancini G 1999 Decreased toxicity and increased efficacy of cancer chemotherapy using the pineal hormone melatonin in metastatic solid tumour patients with poor clinical status. Eur J Cancer. 12:1688-92
29. Liu J, Shi Z, Bai Y, Liu L, Cheng K 2019 Prognostic significance of systemic immune-inflammation index in triple-negative breast cancer. Cancer Manag Res. 11:4471-4480.
30. Liu R, Wang HL, Deng MJ, Wen XJ, Mo YY, Chen FM, Zou CL, Duan WF, Li L, Nie X 2018 Melatonin Inhibits Reactive Oxygen Species-Driven

Proliferation, Epithelial-Mesenchymal Transition, and Vasculogenic Mimicry in Oral Cancer. Oxid Med Cell Longev. 2018:3510970.

31. Lu YX, Chen DL, Wang DS, Chen LZ, Mo HY, Sheng H, Bai L, Wu QN, Yu HE, Xie D, et al. 2016 Melatonin enhances sensitivity to fluorouracil in oesophageal squamous cell carcinoma through inhibition of Erk and Akt pathway. Cell Death Dis. 10:e2432.
32. Mao L, Dauchy RT, Blask DE, Slakey LM, Xiang S, Yuan L, Dauchy EM, Shan B, Brainard GC, Hanifin JP, et al. 2012 Circadian gating of epithelialto-mesenchymal transition in breast cancer cells via melatonin-regulation of GSK3beta. Mol Endocrinol. 26:1808–1820
33. Marshall KA, Reiter RJ, Poeggeler B, Aruoma OI, Halliwell B 1996 Evaluation of the antioxidant activity of melatonin in vitro. Free Radic Biol Med. 3:307-15
34. Martindale JL, Holbrook NJ 2002 Cellular response to oxidative stress: signaling for suicide and survival. J. Cell. Physiol.192: 1–15.
35. Najafi M, Shirazi A, Motevaseli E, Rezaeyan AH, Salajegheh A, Rezapoor S 2017 Melatonin as an anti-inflammatory agent in radiotherapy. Inflammopharmacology. 25:403–13.
36. Nakamura E, Kozaki K, Tsuda H, Suzuki E, Pimkhaokham A, Yamamoto G, Irie T, Tachikawa T, Amagasa T, Inazawa J, et al. 2008 Frequent silencing of a putative tumor suppressor gene melatonin receptor 1 A (MTNR1A) in oral squamous-cell carcinoma. Cancer Sci. 99(7):1390-400
37. Olea-Flores M, Zuñiga-Eulogio MD, Mendoza-Catalán MA, Rodríguez-Ruiz HA, Castañeda-Saucedo E, Ortuño-Pineda C, Padilla-Benavides T, Navarro-Tito N 2019 Extracellular-Signal Regulated Kinase: A Central Molecule Driving Epithelial-Mesenchymal Transition in Cancer. Int J Mol Sci. 20(12)

38. Oner H, Kus I, Oner J, Ogetürk M, Ozan E, Ayar A 2004 Possible effects of melatonin on thymus gland after pinealectomy in rats. Neuro Endocrinol Lett. 1-2:115-8.
39. Ortiz F, Acuña-Castroviejo D, Doerrier C, Dayoub JC, López LC, Venegas C, García JA, López A, Volt H, Luna-Sánchez M, et al. 2015 Melatonin blunts the mitochondrial/NLRP3 connection and protects against radiation-induced oral mucositis. J Pineal Res. Jan;58(1):34-49
40. Panagopoulos V, Leach DA, Zinonos I, Ponomarev V, Licari G, Liapis V, Ingman WV, Anderson P, DeNichilo MO, Evdokiou A 2017 Inflammatory peroxidases promote breast cancer progression in mice via regulation of the tumour microenvironment. Int J Oncol. 4:1191-1200
41. Panzer A, Viljoen M 1997 The validity of melatonin as an oncostatic agent. J Pineal Res. 4:184-202
42. Porsolt RD, Bertin A, Jalfre M 1977 Behavioral despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther. 2:327-36
43. Razavi P, Devore EE, Bajaj A, Lockley SW, Figueiro MG, Ricchiuti V, Gauderman WJ, Hankinson SE, Willett WC, Schernhammer E. Shift Work, Chronotype, and Melatonin Rhythm in Nurses. Cancer Epidemiol Biomarkers Prev. 2019
44. Santoro R, Mori F; Marani M; Grasso G; Cambria MA, Blandino G; Muti P; Strano S 2013 Blockage of melatonin receptors impairs p53-mediated prevention of DNA damage accumulation. Carcinogenesis. 34: 1051–1061
45. Shah PN, Mhatre MC, Kothari LS 1984 Effect of melatonin on mammary carcinogenesis in intact and pinealectomized rats in varying photoperiods. Cancer Res. 8:3403-7.

46. Shen YQ, Guerra-Librero A, Fernandez-Gil BI, Florido J, García-López S, Martinez-Ruiz L, Mendivil-Perez M, Soto-Mercado V, Acuña-Castroviejo D, Ortega-Arellano H, et al. 2018 Combination of melatonin and rapamycin for head and neck cancer therapy: Suppression of AKT/mTOR pathway activation, and activation of mitophagy and apoptosis via mitochondrial function regulation. J Pineal Res. 64(3)
47. Shepherd JK, Grewal SS, Fletcher A, Bill DJ, Dourish CT 1994 Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety. Psychopharmacology (Berl). 116(1):56-64
48. Soussi T 2000 p53 Antibodies in the sera of patients with various types of cancer: a review. Cancer Res. 7:1777-88
49. Tamarkin L, Cohen M, Roselle D, Reichert C, Lippman M, Chabner B 1981 Melatonin inhibition and pinealectomy enhancement of 7,12-dimethylbenz(a)anthracene-inducedvmammary tumors in the rat. Cancer Res. 1:4432-6
50. Tan DX, Manchester LC, Esteban-Zubero E, Zhou Z, Reiter RJ 2015 Melatonin as a Potent and Inducible Endogenous Antioxidant: Synthesis and Metabolism. Molecules. 10:18886-906
51. Tchekalarova J, Nenchovska Z, Atanasova D, Atanasova M, Kortenska L, Stefanova M, Alova L, Lazarov N 2016 Consequences of long-term treatment with agomelatine on depressive-like behavior and neurobiological abnormalities in pinealectomized rats. Behav Brain Res. 302:11-28
52. Touitou Y, Reinberg A, Touitou D. Association between light at night, melatonin secretion, sleep deprivation, and the internal clock: Health impacts and mechanisms of circadian disruption. Life Sci. 2017; 173:94-106

53. Valente VB, Verza FA, Lopes FYK, Ferreira JZ, Dos Santos PSP, Sundefeld MLMM, Biasoli ÉR, Miyahara GI, Soubhia AMP, de Andrade M, de Oliveira SHP, et al. 2018 Stress hormones concentrations in the normal microenvironment predict risk for chemically induced cancer in rats. Psychoneuroendocrinology. 89:229-238
54. Valentine JA, Scott J, West CR, St Hill CA 1985 A histological analysis of the early effects of alcohol and tobacco usage on human lingual epithelium. J Oral Pathol.14(8):654-65
55. van der Waal I, Schepman KP, van der Meij EH, Smeele LE 1997 Oral leukoplakia: a clinicopathological review. Oral Oncol. 5:291-301
56. Wang X, Wang B, Xie J, Hou D, Zhang H, Huang H 2018 Melatonin inhibits epithelial‐to‐mesenchymal transition in gastric cancer cells via attenuation of IL‐1β/NF‐κB/MMP2/MMP9 signaling. Int J Mol Med. 4:2221-2228
57. Wang X, Wang B, Zhan W, Kang L, Zhang S, Chen C, Hou D, You R, Huang H 2019 Melatonin inhibits lung metastasis of gastric cancer in vivo. Biomed Pharmacother. 117:109018
58. Wight AJ, Ogden GR 1998 Possible mechanisms by which alcohol may influence the development of oral cancer--a review. Oral Oncol. 1998; 6:441-7.
59. Wongsena W, Charoensuk L, Dangtakot R, Pinlaor P, Intuyod K, Pinlaor S 2018 Melatonin suppresses eosinophils and Th17 cells in hamsters treated with a combination of human liver fluke infection and a chemical carcinogen. Pharmacol Rep.1:98-105.
60. Yang CY, Lin CK, Tsao CH, Hsieh CC, Lin GJ, Ma KH, Shieh YS, Sytwu HK, Chen YW 2017 Melatonin exerts anti-oral cancer effect via suppressing

LSD1 in patient-derived tumor xenograft models. Oncotarget. 20:33756-33769.

61. Yeh CM, Lin CW, Yang JS, Yang WE, Su SC, Yang SF 2016 Melatonin inhibits TPA-induced oral cancer cell migration by suppressing matrix metalloproteinase-9 activation through the histone acetylation. Oncotarget. 7:21952–21967

62. Zhang Y, Xiao G, Wang R 2019 Clinical significance of systemic immune-inflammation index (SII) and C-reactive protein-to-albumin ratio (CAR) in patients with esophageal cancer: a meta-analysis. Cancer Manag Res. 11:4185-4200

Anexo A

Anexo A

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Análise dos efeitos da supressão de melatonina sobre a carcinogênese bucal quimicamente induzida em ratos: Estudo histopatológico, imunoistoquímico, molecular e comportamental"**, Processo FOA nº 00522-2017, sob responsabilidade de Daniel Galera Bernabé apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 17 de Outubro de 2017.

VALIDADE DESTE CERTIFICADO: 30 de Agosto de 2019.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 30 de Setembro de 2019.

CERTIFICATE

We certify that the study entitled **"Analysis of melatonin suppression on chemically induced oral carcinogenesis in rats: Histopathological, immunohistochemical, molecular and behavioral study."**, Protocol FOA nº 00522-2017, under the supervision of Daniel Galera Bernabe presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on October 17, 2017.

VALIDITY OF THIS CERTIFICATE: August 30, 2019.
DATE OF SUBMISSION OF THE FINAL REPORT: September 30, 2019.

Prof. Ass. Dr. Leonardo Perez Faverani
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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Anexo B

Anexo B

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