Universidade Estadual Paulista "Júlio de Mesquita Filho"
Faculdade de Odontologia de Araçatuba (FOA-UNESP)
Programa de Pós-Graduação em Odontologia
Área de Concentração: Estomatologia

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Níveis dos hormônios do estresse no microambiente: influência sobre a ocorrência do tumor e modulação durante a carcinogênese quimicamente induzida em ratos

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Orientador: Prof. Dr. Daniel Galera Bernabé

Co-orientadora: Profa. Dra. Sandra Helena Penha de Oliveira

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DEDICATÓRIA

DEDICATÓRIA

Dedico este estudo...

...à todos os pacientes que vivem ou que já viveram a experiência de adoecer com o câncer...

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AGRADEÇO...

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EPÍGRAFE

EPÍGRAFE

“Embora ninguém possa voltar atrás e fazer um novo começo, qualquer um pode começar agora e fazer um novo fim.”

Francisco Cândido Xavier

RESUMO

Valente VB. Níveis dos hormônios do estresse no microambiente: influência sobre a ocorrência do tumor e modulação durante a carcinogênese quimicamente induzida em ratos. [dissertação]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2016.

Resumo

Evidências mostram que os hormônios relacionados ao estresse podem influenciar a progressão do câncer, mas o papel destes mediadores sobre o processo de carcinogênese no microambiente tecidual, em condições naturais, é pouco compreendido. Neste estudo, nós utilizamos um modelo de carcinogênese bucal em ratos para testar a hipótese de que os níveis de hormônios relacionados ao estresse no microambiente tecidual em condições naturais (sem estresse) pré-indução carcinogênica influenciam a ocorrência e progressão do carcinoma espinocelular (CEC) de língua. Quarenta e oito ratos machos Wistar foram submetidos a uma biópsia de tecido lingual normal previamente à indução carcinogênica e os níveis teciduais de norepinefrina, corticosterona, ACTH e BDNF foram mensurados. Três semanas depois os animais foram tratados com o carcinógeno químico 4-nitroquinolina-1-óxido (4NQO) por 20 semanas e a ocorrência de CEC ou Leucoplasia (lesão precursora do CEC) na língua foi analisada microscopicamente. Níveis basais aumentados pré-carcinogênese de norepinefrina e BDNF e níveis reduzidos de corticosterona foram preditivos para ocorrência de CEC. Níveis basais elevados de norepinefrina foram associados à uma expressão reduzida de RNAm para CDKN2a-p16 nos CECs. Níveis teciduais de corticosterona e BDNF nas leucoplasias e corticosterona no CEC foram significativamente mais elevados em relação a mucosa normal pré-carcinogênese. Níveis elevados de norepinefrina no microambiente dos CECs foram associados a um maior volume e espessura do tumor. Da mesma forma, níveis elevados de norepinefrina, ACTH e BDNF no CEC foram associados a uma menor intensidade do infiltrado linfoplasmocitário subjacente ao tumor. Além disso, maior expressão de RNAm para IL-6 no CEC foi correlacionada à níveis elevados de corticosterona pós-carcinogênese. Este estudo mostra as primeiras evidências in vivo de que os níveis basais de hormônios do estresse no microambiente do tecido normal podem ser preditivos para a incidência do câncer quimicamente induzido. Além disso, a ação do carcinógeno pode modular os níveis hormonais no microambiente tecidual e estes podem estar associados à progressão do tumor. Em suma, estes dados sugerem que a susceptibilidade ao início e progressão do carcinoma quimicamente induzido pode ser diretamente influenciada por diferenças individuais endócrinas no microambiente pré-carcinogênese.

Palavras-chave: Neoplasias, carcinogênese, estresse fisiológico, norepinefrina, corticosterona, hormônio adrenocorticotrópico, fator neurotrófico derivado do encéfalo, neoplasias de cabeça e pescoço

ABSTRACT

Valente VB. Stress hormones levels in the microenvironment: influence on tumor occurrence and modulation during chemically induced carcinogenesis in mice. [dissertation]. Araçatuba: UNESP – São Paulo State University; 2016.

Abstract

Evidence show that stress-related hormones may influence cancer progression, but their role in tissue microenvironment on the carcinogenesis is poorly understood. In this study, we have used an oral carcinogenesis model in rats to test the hypothesis that stress-related hormones levels in the tissue microenvironment in natural conditions pre-carcinogenic induction could influence the oral squamous cell carcinoma (OSCC) occurrence and progression. Forty-eight male Wistar rats were underwent to a normal tongue tissue biopsy before carcinogenic induction and tissue levels of norepinephrine, corticosterone, ACTH and BDNF were measured. Three weeks later the animals were treated with chemical carcinogen 4-nitroquinoline-1-oxide (4NQO) for 20 weeks and the OSCC or oral leukoplakia (non-cancerous lesions) occurrence into tongue was evaluated. Increased concentrations of norepinephrine and BDNF, and reduced corticosterone levels in the pre-carcinogen microenvironment were predictive for OSCC occurrence. Increased pre-carcinogen norepinephrine concentrations were associated to a CDKN2a-p16 mRNA lower expression in OSCCs. Tissue levels of corticosterone and BDNF in oral leukoplakia and corticosterone in OSCC were significantly higher than pre-carcinogenesis normal mucosa. Increased norepinephrine concentrations in OSCC microenvironment were associated to a higher tumor volume and thickness. Likewise, increased levels of norepinephrine, ACTH and BDNF in OSCC were associated to a lesser intensity of the lymphoplasmocytic infiltrate underlying to tumor. Furthermore, IL-6 mRNA enhanced expression was correlated to increased corticosterone levels post-carcinogenesis. This study shows the first *in vivo* evidence that pre-carcinogen stress hormones levels in the microenvironment of the normal tissue may be predictive for chemically induced cancer occurrence. Moreover, the carcinogen action can modulate hormonal levels in the tissue microenvironment, which can be associated to tumor progression. In short, these data suggest that the susceptibility to onset and progression of chemically induced carcinomas may be directly influenced by endocrine individual differences in pre-carcinogenesis microenvironment.

Keywords: Neoplasias, carcinogenesis, physiological stress, norepinephrine, corticosterone, adrenocorticotropic hormone, brain-derived neurotrophic factor, head and neck neoplasias

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differentiated OSCC with intense inflammatory infiltrate underlying to neoplastic area. (G) Extensive ulcerative lesion reaching half of the tongue. (H) Highly infiltrative well-differentiated OSCC.

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Figure 5. Simple linear regression analysis among pre-carcinogen norepinephrine (A), corticosterone (B), ACTH (C) and BDNF (D) concentrations, and oral leukoplakia and OSCC occurrence. Pre-carcinogen norepinephrine and BDNF levels in the tongue microenvironment predicted oral cancer (*$p<0.05$). Student's test-t between stress-related hormones levels means pre-carcinogenic induction, which were used to produce respective bar graphs; *$p<0.05$.

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Figure 6. Student's test-*t* between pre- and post-carcinogen levels of norepinephrine (A), corticosterone (B), ACTH (C) and BDNF (D). Increased post-carcinogen corticosterone concentrations were found in the tumor and leukoplastic lesions (*$p<0.05$). Higher post-carcinogen BDNF levels were found only in the leukoplastic lesions compared to pre-carcinogen normal mucosa; *$p<0.05$.

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Figure 7. Correlation analysis showing a positive correlation between pre-carcinogen stress-related hormones levels (A, C, E, G, I and K; *$p<0.001$). A positive correlation was also observed in the post-carcinogen concentrations of

norepinephrine vs ACTH (D; *p<0.001), corticosterone vs ACTH (H; *p=0.035) and ACTH vs BDNF (L; *p<0.001).

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11ßHSD2 = 11β-hydroxysteroid dehydrogenase type II

4NQO = 4-nitroquinoline-1-oxide

ACTH = adrenocorticotropic hormone

AKT = protein kinase B

BDNF = brain-derived neurotrophic factor

C57BL6/J = C57 black 6/J

CA = California

cAMP = adenosina 3',5'-monofosfato cíclico

CDK-4 = cyclin-dependent kinase 4

CDK-6 = cyclin-dependent kinase 6

CDKN2A = cyclin-dependent kinase Inhibitor 2A

cDNA = complementary deoxyribonucleic acid

CNS = central nervous system

Co. = company

COX-2 = cytochrome c oxidase subunit 2

CRF = corticotropin-releasing factor

CV = coefficient of variation

DNA = deoxyribonucleic acid

E-cadherin = epithelial cadherin

ECM = extracellular matrix

eg. = for example

EGF = epidermal growth factor

ELISA = enzyme-linked immunosorbent assay

FGF = fibroblast growth factor

GA = Georgia

H&E = hematoxylin and eosin

HGF = hepatocyte growth factor

HPA = hypothalamic-pituitary-adrenal

IL-12 = interleukin 12

IL-6 = interleukin-6

Inc. = Incorporation

LASSO = least absolute shrinkage and selection operator

Ltd. = limited

MA = Massachusetts

MAPK = mitogen-activated protein kinase

MDMD 2 = murine double minute 2

MMP-2 = matrix metalloproteinase 2

MMP-9 = matrix metalloproteinase 9

MMPs = matrix metalloproteinases

MO = Missouri

mRNA = messenger ribonucleic acid

NC = North Carolina

NIH = National Institutes of Health

NK = natural killer

No = number

OSCC = oral squamous cell carcinoma

PBS = phosphate-buffered saline

PKA = protein kinase A

RNA = ribonucleic acid

RNase = ribonuclease

RQ = relative quantity

RT = room temperature

RT-PCR = reverse transcription polymerase chain reaction

SNS = sympathetic nervous system

SP = São Paulo

St. = Saint

Stat 3 = signal transducer and activator of transcription 3

Statistical Analysis System = SAS

TGF-α = transforming growth factor alpha

TGF-β = transforming growth factor beta

Th1 = type 1 helper cells

TrkB = tyrosine receptor kinase B

Twist = twist-related protein 1

TX = Texas

UK = United Kingdom

UNESP = Univ Estadual Paulista

USA = United States of America

UV = ultraviolet

VEGF = vascular endothelial growth factor

Wnt-1 = proto-oncogene protein Wnt-1

SUMÁRIO

INTRODUÇÃO

Introduction*

Studies have shown that hormones resulting from chronic stress may influence cancer progression (1-4). The two main pathways that have been investigated for mediating the emotional stress effects on cancer are the hypothalamic-pituitary-adrenal (HPA) axis (5) and sympathetic nervous system (SNS) (6). HPA axis activation occurs when neurons localized in the paraventricular nucleus secrete corticotropin-releasing factor (CRF); then, CRF stimulates the secretion of adrenocorticotropic hormone (ACTH), which induces the adrenal glands to synthesize cortisol (2,5). In parallel, SNS activation during chronic stress can induce a dysregulated production of catecholamines norepinephrine and epinephrine by the central nervous system (CNS) and adrenal gland (2,6).

Stress hormones act on tumor cells deregulating the production of cytokines, chemokines and growth factors, which are related with cancer progression (1-4). Studies have shown that norepinephrine and epinephrine enhance the expression of genes involved in tumor angiogenesis and proliferation such as vascular endothelial growth factor (VEGF) (7,8) and interleukin-6 (IL-6) (9-11), as well as deregulate the production of matrix metalloproteinases (MMPs) (12-14) types 2 and 9; proteins that increase tumor invasion and metastasis occurrence. Catecholamines derived from SNS hyperactivity suppress the cytotoxic T lymphocytes and natural killer (NK) cell

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*Normalização segundo o periódico "Carcinogenesis" (Anexo B)

responses (15), and activate the expression of genes by oncogenic virus (16). Pre-clinical studies have demonstrated that stress-related hormones can affect ovarian (7,8,10,12,13), breast (17), lung (18) and colon (19) cancer progression. Clinical investigations have showed that patients with advanced-stage ovarian (9), colorectal (20), and head and neck (21) cancer have significant increased cortisol levels compared to patients with early-stage disease or healthy controls. However, there are no clinical evidences that stress-related hormones levels can influence cancer occurrence. Few pre-clinical investigations have demonstrated that stress-related hormones also may act on the regular cells promoting cancer development (22,23). Long exposures in dose response experiments with norepinephrine and epinephrine induced significant increases in the tumorigenicity of mouse fibroblasts (22). Chronic stress promoted tumor development (lymphomas and sarcomas) in C57BL6/J male mice by the attenuation of p53 protein levels and transcriptional activity, which were mediated by increased levels of corticosterone in the serum (23).

Brain-derived neurotrophic factor (BDNF) is a neurotrophin distributed mainly in the CNS, where it act as trophic factor for dopaminergic and cholinergic neurons. Evidences have shown that BDNF expression in the CNS can be modulated by stress (24). BDNF and its receptor TrkB have an important role as autocrine modulators being expressed in several organs (24). Acute and chronic stress may increase plasma BDNF levels in pre-clinical models (25,26). Studies have demonstrated that BDNF expression is involved with tumor cell proliferation and invasion process (27,28). BDNF has been identified in head and neck (27),

liver (28), prostate (29) and bladder (30) cancers, suggesting a role of neurotrophins in cancer development and progression.

Tumor microenvironment is an aberrant tissue environment composed by tumor epithelial cells and specialized mesenchymal cells (inflammatory cells, myoepithelial and endothelial cells, myofibroblasts and fibroblasts) surrounding by an altered extracellular matrix (ECM), which plays an important role in cancer development and progression (31,32). Interactions between the mesenchymal and epithelium cells through the morphogenic factors (eg. Wnt-1, HGF and TGF-β) and growth factors (eg. FGF, EGF and TGF-α) secretion are altered in carcinomas and can influence the carcinogenesis (32). Although evidences show that stress and its neurohormones may influence cancer progression, there are no studies that have assessed the stress-related hormones levels in the tissue microenvironment before cancer and their influence on carcinogenesis and tumor progression. Chemical carcinogens exposure may affect important cellular metabolic functions as the production and release of different biological mediators (33,34). However, it is unclear how chemical carcinogens could act modulating stress hormones concentrations in the tumor microenvironment under carcinogenesis induction.

In the present study, we have used a pre-clinical oral carcinogenesis model to test the hypothesis that stress-related hormones concentrations in the tissue microenvironment before tumor induction could influence the cancer occurrence, clinicopathological variables and tumor progression-related genes expression. Furthermore, the local concentrations of these mediators were compared pre- and post-carcinogenesis induction and hormones levels in cancer

microenvironment were analyzed for association to tumor progression-related variables.

MATERIAL E MÉTODO

Materials and methods

1. Experimental animals and maintenance conditions

All experimental procedures involving animals were in agreement to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Welfare Committee at the Araçatuba Dental School (UNESP - Univ Estadual Paulista, Araçatuba, SP, Brazil - Process 2014-00816 – ANEXO A). Forty-eight male Wistar rats, 12 weeks old, weighing between 250 and 300 g, were used for the experiments. The animals were group-housed, 3 per cage (25.9 × 47.6 × 20.9 cm, polypropylene), and kept in a constant room temperature (RT) (25 ± 2 ºC), humidity (55 ± 3 ºC), under a 12-hour light-dark cycle, and received *ad libitum* access to standard food (Purina, Paulínia, SP, Brazil) and drinking water.

2. Experimental design

In order to assess whether the stress-related hormones concentrations in the tongue microenvironment could influence oral carcinogenesis chemically induced, the experiments were divided in three main steps. In phase 1, all animals were underwent to a lingual tissue collection for characterizing the stress-related hormones levels in the microenvironment (pre-carcinogen hormonal levels). In phase 2, the same animals who have passed through phase 1 were underwent to chemically induced oral carcinogenesis. In phase 3, after 20 weeks of oral carcinogenesis, all animals were euthanized for evaluation of oral cancer occurrence and stress-related hormones concentrations in the microenvironment (post-carcinogen hormonal levels) (Figure 1).

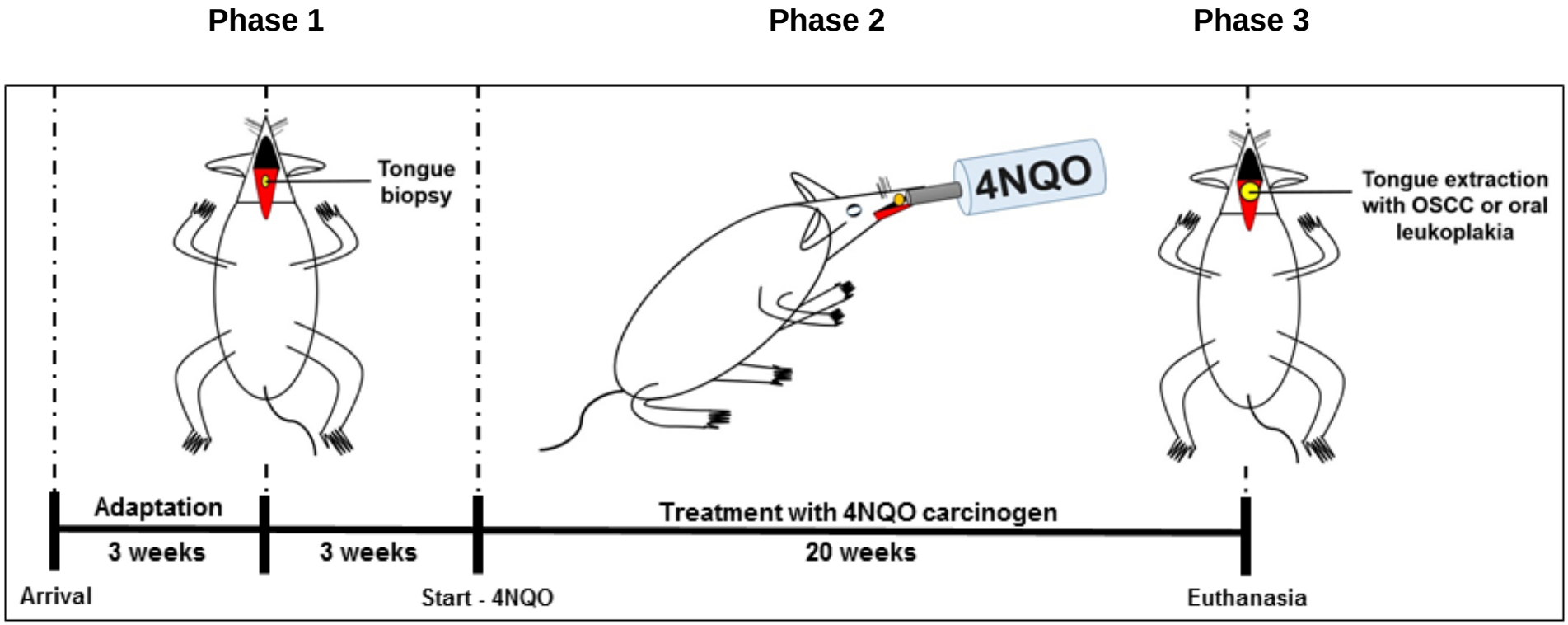

Figure 1. Illustrative representation of experimental design and study phases.

3. Individual characterization of the stress-related hormones levels in the tongue microenvironment pre-carcinogen induction

3.1. Anesthetic induction.

In the first phase of the study, to characterize individual differences in the stress-related hormones concentrations in the tongue microenvironment, all animals were submitted to a lingual biopsy under inhalatory anesthesia. The anesthetic induction was performed by induction-chamber (Figures 2A and 2B) containing isoflurane (Cristália, Itapira, SP, Brazil), with inspired fraction of 5%, diluted in 100% oxygen with 3L/min flow. An open non-rebreathing circuit (Anesthesia equipment Nikkei K – Takaoka, São Paulo, SP, Brazil) was used until the loss of muscular tonus (animal in the ventral decubitus position) (Figure 2B). In sequence, the rat was removed from induction-chamber and maintained under anesthesia with an adapted face mask, connected to the same induction system (Figure 2C). The anesthetic maintenance was performed with inspired fraction of 3% and with oxygen flow of 2L/min.

3.2. Tongue biopsy pre-carcinogenic induction.

With the animal anesthetized (Figure 2C), the tongue was tractioned using a suture thread (Figure 3A) and a biopsy was performed on the posterior region of tongue dorsum, with a 4 mm circular manual punch (Figure 3B) allowing the removal of a lingual mucosal fragment (Figures 3C, 3D and 3E). This region of the lingual mucosa was selected for the measurements of stress-related hormones, since it is the main location where carcinomas are developed in the chemically induced oral carcinogenesis model used in this study. After removing

the tongue fragment, the surgical wound was sutured with 4.0 silk thread (Ethicon Johnson & Johnson, São José dos Campos, SP, Brazil) (Figure 3F). The lingual mucosa sample was rinsed with physiological saline solution, and then stored at -80°C for hormones measurements. In the postoperative period, the animals were fed with softened rat chow, which was previously crunched in drinking water.

Figure 2. Anesthetic induction. A. Rat inside of Induction-chamber. B. Loss of muscular tonus (animal in the ventral decubitus position). C. The rat was removed from induction-chamber and maintained under anesthesia with an adapted face mask.

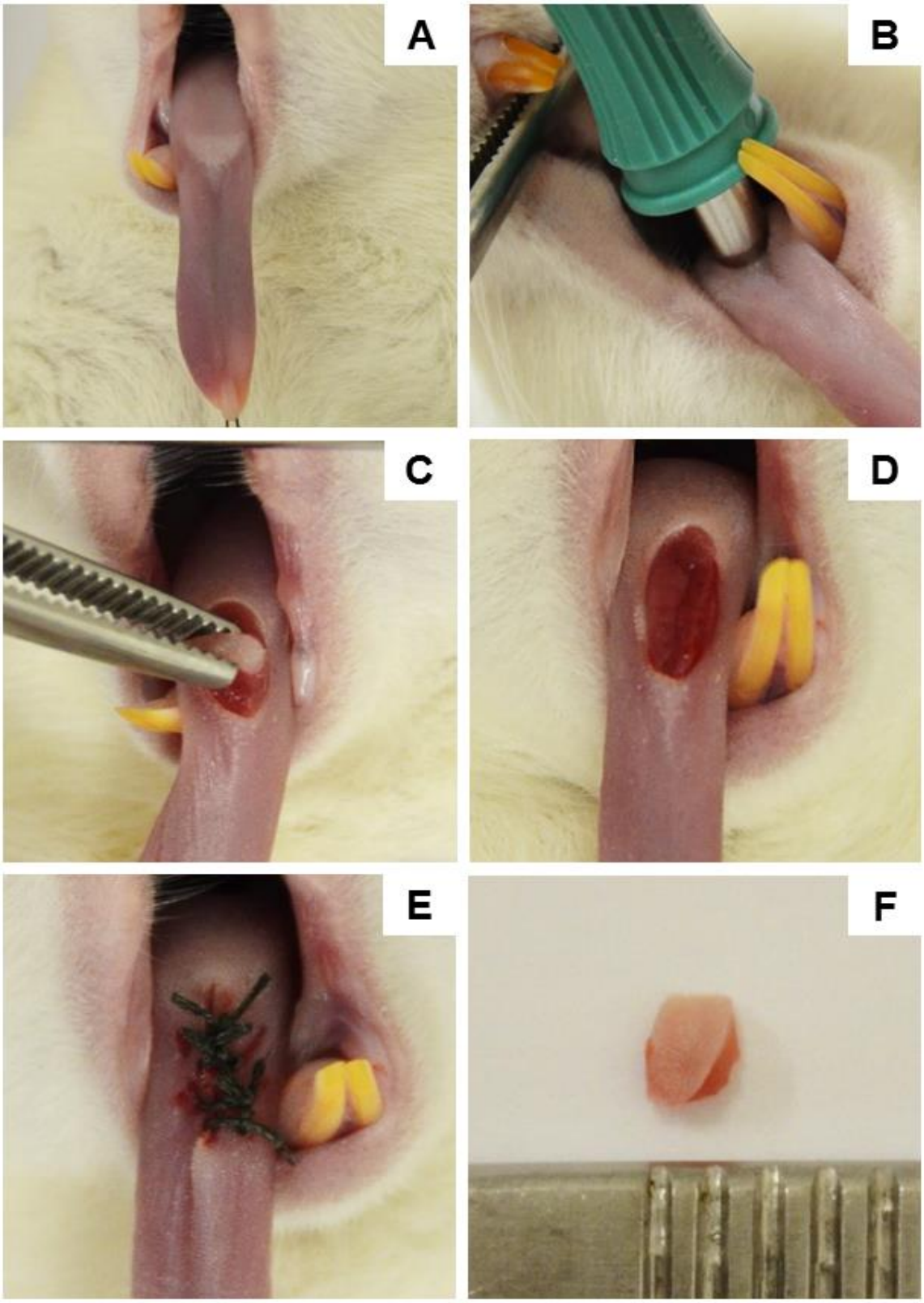

Figure 3. Tongue biopsy pre-carcinogenic induction. A. Normal tongue mucosa. B. Incision with a 4 mm circular manual punch. C. Removal of the fragment. D. Surgical wound. E. Sutures. F. Lingual mucosa fragment for laboratorial analyses.

3.3. Measurements of the stress-related hormones concentrations in the tongue microenvironment.

Sample preparation. For baseline stress-related hormones concentrations measurements in the tongue microenvironment, normal lingual mucosal samples were triturated in a PBS buffer containing a specific protease inhibitor cocktail (Calbiochem, San Diego, CA, USA). The tissue was rinsed with 1X PBS, and homogenized in 500 µL of protease inhibitor cocktail. Then, the homogenates were centrifuged for 5 minutes at 5000 xg. Lastly, the supernatant of each sample was removed, aliquoted and then stored at -80°C for stress-related hormones measurements.

Analysis of norepinephrine levels. Norepinephrine levels were quantified using a commercially available specific kit (Catalog. No: U0907Ge; Wuhan EIab Science Co. Ltd., China) through the enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's recommendations. The assay was performed in triplicate. The minimum detectable concentration of the assay (assay sensitivity) is less than 7.5 pg/mL, and intra-assay and inter-assay coefficient of variation (CV) are <4.3% and 7.5%, respectively.

Analysis of corticosterone, ACTH and BDNF levels. The corticosterone, ACTH and BDNF levels in normal lingual mucosa were measured using the Milliplex Multi-Analyte Profiling method, through Luminex xMAP™ technology. To perform these immunoassays, specific kits Milliplex™ (ACTH, corticosterone, Catalog. No: RSHMAG-69K; BDNF, Catalog. No: RPTMAG-86K; Millipore, Billerica, MA, USA) containing a 96-well plate each one were used. Firstly, 200 µL of assay buffer were added per well, and then the plates were shaken for 10

minutes. After decanting in RT, 25 µL of standard or control were added to appropriated wells, 25 µL of assay buffer were added to background and sample wells, and 25 µL of matrix were added to background, standards and control wells. After, 25 µL of samples were added to sample wells, and 25 µL of beads were added to each well. The plates were incubated overnight for 18 hours at 4ºC with shaking. Well contents were removed and they were washed 3X with 200 µL of wash buffer. Then, 50 µL of detection antibodies were added per well and the plates were incubated for 1 hour at RT. Afterward, 50 µL of streptavidin-phycoerythrin were added per well. The plates were incubated for more 30 minutes at RT, and then well contents were removed and they were washed 3X with 200 µL of wash buffer. Lastly, 100 µL of sheath fluid were added per well. All assays were run in triplicate. The assay sensitivities are 2914 pg/mL for corticosterone, 0.9 pg/mL for ACTH and 0.38 pg/mL for BDNF. The intra-assay and inter-assay CVs are <10% for corticosterone and ACTH, 1.7% and 12.2% for BDNF, respectively. The hormonal levels were quantified on the MAGPIX-XPONENT™ equipment (Millipore, Austin, TX, USA), and the data were analyzed using the Milliplex Analyst 5.1 software (Millipore, Billerica, MA, USA).

Normalization of hormonal levels. Total protein concentration in the tissue homogenates was measured using the method of Lowry et al. (35), and the data were used to normalize the stress-related hormones levels. The hormonal levels were expressed as pg/µg in 1 mL of tissue homogenate.

4. Oral carcinogenesis induction

Three weeks after the biopsy, all animals were anesthetized and checked before the start of oral carcinogenesis induction. All animals exhibited the dorsum

surface of the tongue healed, and thereby they were included in the next phase of study (chemically induced oral carcinogenesis). For tumor induction, animals were treated with 50 ppm of 4-nitroquinoline-1-oxide (4NQO) (Sigma-Aldrich, St. Louis, MO, USA) diluted in drinking water. All animals had free access to consume water with the carcinogen solution, which was replenished twice a week. In agreement with a previous study (36), animals were euthanized (by decapitation) after a 20 weeks period of carcinogenic induction. Then, in the third phase of the study, the tongues with induced-carcinogen lesions were extracted to perform histopathological analysis, measurements of the stress-related hormones levels in the microenvironment, and gene expression analysis by RT-PCR assay.

5. Histopathological analysis

For histopathological examination, the tongues were longitudinally sectioned and the tissues were fixed in 10% buffered formaldehyde for 48h, submitted to the routine laboratory procedures, embedded in paraffin blocks (sectioned at 4 μm), and stained with Hematoxylin and Eosin (H&E) (Merck, Darmstadt, Germany). The tongue lesions derived from the 4NQO treatment were classified in oral leukoplakia and oral squamous cell carcinoma (OSCC), wherein the leukoplakia is considered an early phase of OSCC development (37). For oral leukoplakia diagnosis were considered the microscopic features of atrophy of the epithelium, hyperplasia with or without hyperkeratosis, and epithelial dysplasia (38). According with World Health Organization (39) criteria, the epithelial dysplasia degree of the leukoplastic lesions was classified in mild, moderate or severe. In the OSCC specimens, histological grade of malignancy

was evaluated by two oral pathologists (by consensus) in accordance with Bryne's criteria (40), in which the histopathological features keratinization degree, nuclear pleomorphism, pattern of invasion and lymphoplasmocytic infiltrate are observed in the invasive front of tumors.

For measuring the tumor thickness, microscopic images of OSCC slides stained with H&E were captured on a specific microscope (DM4000B Leica, Wetzlar, Germany) equipped with a digital camera. The measurement was performed from the deepest point of tumor invasion to the surface of the ulcer or epithelium, which was obtained in microns using the Qwin Plus software (Leica Microsystem Imaging Solutions Ltd., Cambridge, UK).

6. Tumor volume and weight loss

The tumor volume was calculated using the anteroposterior, laterolateral and depth clinical invasion measurements, which were obtained in millimeters by a calibrated caliper. The final volume (in mm^3) was calculated by multiplying the three values found. Body weight of each animal was recorded weekly. Initial (before starting treatment with 4NQO) and final (before the animals' euthanasia) body measurements were used to calculated the weight loss during carcinogenic induction.

7. Stress-related hormones concentrations in the microenvironment post-carcinogen

To assess whether the chemically induced carcinogenesis promotes changes of the stress-related hormones levels in the tongue microenvironment, the norepinephrine, corticosterone, ACTH and BDNF post-carcinogen levels

were analyzed in the OSCC and oral leukoplakia tissues. For measuring these stress-related hormones, the same immunoassay techniques described above (ELISA and Milliplex Multi-Analyte Profiling) were used.

8. Expression of tumor progression-related genes by RT-PCR

In order to assess the associations among mRNA expression levels of five genes involved in the OSCC progression (7-14) and the pre- and post-carcinogen stress-related hormones levels in the microenvironment, the OSCC samples were analyzed by RT-PCR technique. Total RNA was extracted using the trizol reagent. Then, RNA samples were suspended in 20 µL of RNase free ultrapure water for subsequent synthesis of complementary DNA (cDNA). Thereafter, RNA quantity and quality were verified by spectrophotometry, and cDNA was synthesized with specific kit (High Capacity RNA to cDNA kit; Invitrogen Life Technologies) using 1 µg of total RNA according to the manufacturer's recommendations. mRNA levels were measured by TaqMan™ system in the StepOne Real-Time PCR™ equipment (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. The primers for IL-6 (Rn00569848_m1), MMP-2 (Rn01538170_m1), MMP-9 (Rn00579162_m1), VEGF (Rn01511601_m1) and CDKN2a-p16 (Rn00580664_m1) were analyzed. ß-actin (Rn00562253_m1) gene was used as endogenous control. The reactions were performed with a 20 µL final volume. Thermal cycling conditions followed all the manufacturer's recommendations. The expression levels of each gene in each sample were normalized to ß-actin mRNA levels. mRNA Relative Quantity (RQ) for each target gene was calculated using the comparative C_T method.

9. Statistical analysis

Statistical Analysis System 9.2 (SAS Institute Inc., Cary, NC, USA), GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, CA, USA), Epi Info 7 (Centers for Disease Control and Prevention, Atlanta, GA, USA) and RStudio, using R version 3.2.0 (Integrated Development for R. RStudio Inc., Boston, MA, USA) softwares were used to perform all statistical tests. Pre-carcinogen stress-related hormones data were submitted as independent variables to the simple linear regression and penalized regression analysis to predict OSCC occurrence. Then, the clinicopathological and gene expression data were entered into the penalized regression model as response variables using the R function 'glmnet'. The objective of the penalized regression is to reduce the number of covariates by taking into account their correlation, which avoid a large variability in the β's coefficient as well as reducing the number of predictors. The LASSO penalty was used in this analysis. Student's *t*-test was used to determine the differences between pre- and post-carcinogen hormones levels in the microenvironment. Furthermore, pre- and post-carcinogen stress hormones concentrations data were submitted to correlation analysis. Likewise, the same correlation test was used to evaluate associations among post-carcinogen hormones levels data and response variables. Level of statistical significance was set at a *p* value less than 0.05 for all statistical tests.

RESULTADOS

Results

1. Pre-carcinogen stress-related hormones concentrations in the tongue microenvironment are predictive for oral cancer occurrence.

After chemically induced oral carcinogenesis, 33 rats (68.75%) developed OSCC and 15 rats (31.25%) developed oral leukoplakias (Figure 4). All OSCCs were classified as well-differentiated tumors, according with the Bryne's criteria (40). Microscopic analysis showed that 80% of oral leukoplakias were diagnosed with moderate and severe epithelial dysplasia.

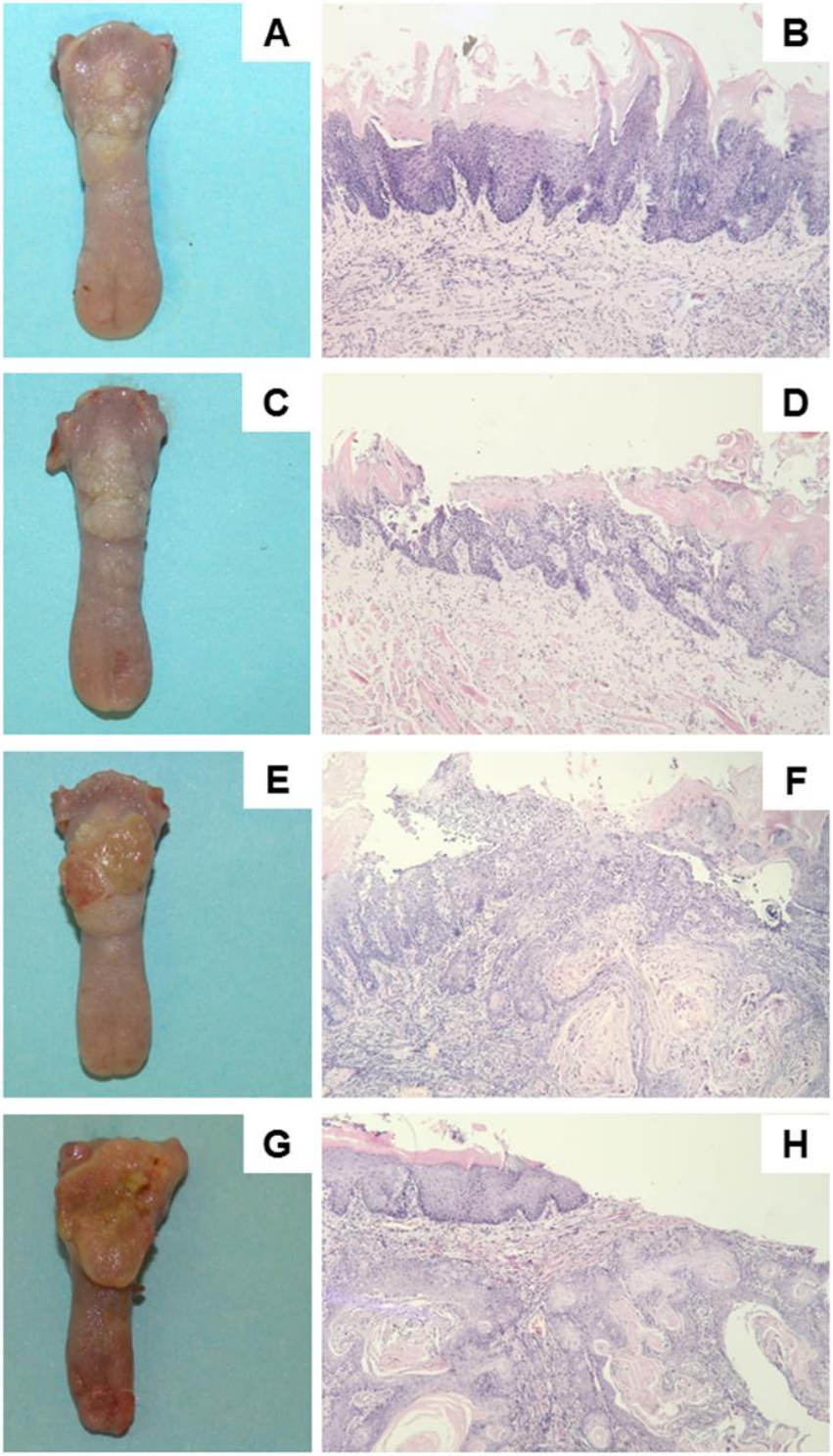

Figure 4. Clinical and histopathological features of oral leukoplakia and OSCC developed after chemically induced carcinogenesis by 4NQO (original magnification - 100x). (A) Small irregular yellowish-white plates. (B) Hyperplastic epithelium with mild dysplasia and hyperkeratosis. (C) Large heterogeneous white plate. (D) Moderate epithelial dysplasia with mild chronic inflammatory infiltrate. (E) Ulcerative lesion surrounded by few white areas. (F) Well-differentiated OSCC with intense inflammatory infiltrate underlying to neoplastic area. (G) Extensive ulcerative lesion reaching half of the tongue. (H) Highly infiltrative well-differentiated OSCC.

Simple linear regression analysis was performed to investigate whether the pre-carcinogen stress hormones levels in the microenvironment were predictive for OSCC occurrence. Increased pre-carcinogen concentrations of norepinephrine (OSCC, 0.1319 ± 0.0127 pg/μg; oral leukoplakia, 0.0834 ± 0.0085 pg/μg; p=0.023) and BDNF (OSCC, 0.2670 ± 0.0458 pg/μg; oral leukoplakia, 0.0818 ± 0.0212 pg/μg; p=0.021) in the tongue microenvironment were positively correlated with OSCC occurrence (Figures 5A and 5D). Nevertheless, pre-carcinogen concentrations of corticosterone (OSCC, 5.518 ± 0.7416 pg/μg; oral leukoplakia, 5.029 ± 0.6357 pg/μg; p=0.520) and ACTH (OSCC, 0.0053 ± 0.0009 pg/μg; oral leukoplakia, 0.0032 ± 0.0005 pg/μg; p=0.186) were not associated with oral cancer occurrence (Figures 5B and 5C). Likewise, penalized regression analysis was performed to identify the most relevant stress-related hormones to predict OSCC occurrence (Table I), and confirmed that higher pre-carcinogen norepinephrine concentrations were positively correlated with cancer occurrence (p=0.025). Increased pre-carcinogen BDNF concentrations were associated with a positive trend for oral cancer occurrence (p=0.068). Increased pre-carcinogen corticosterone concentrations were negatively correlated with OSCC occurrence (p=0.039).

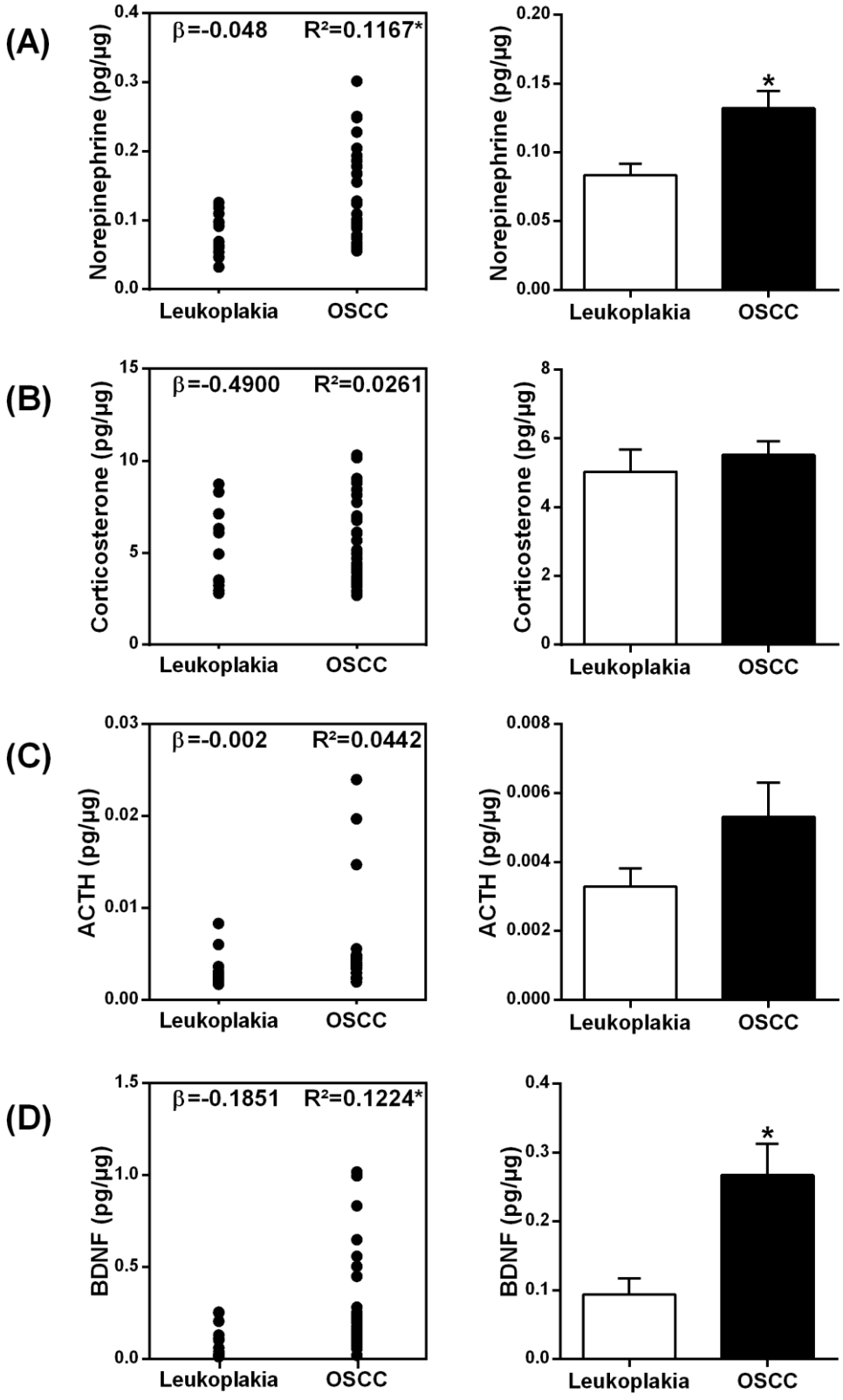

Figure 5. Simple linear regression analysis among pre-carcinogen norepinephrine (A), corticosterone (B), ACTH (C) and BDNF (D) concentrations, and oral leukoplakia and OSCC occurrence. Pre-carcinogen norepinephrine and BDNF levels in the tongue microenvironment predicted oral cancer (*p<0.05). Student's test-*t* between stress-related hormones levels means pre-carcinogenic induction, which were used to produce respective bar graphs; *p<0.05.

Table I. Pre-carcinogen stress-related hormones in the tongue microenvironment and OSCC occurrence risk.

	Estimate	z Value	p Value
Norepinephrine	35.40	2.420	0.025*
Corticosterone	-0.63	-2.056	0.039*
ACTH	195.62	0.692	0.489
BDNF	10.53	1.775	0.068

*Values considered statistically significant at $p<0.05$ (penalized regression analysis)

2. Pre-carcinogen stress-related hormones levels and their influences on the clinicopathological variables and tumor progression-related genes expression

Penalized regression analysis was conducted to elucidate the influence of pre-carcinogen stress-related hormones levels in the tongue microenvironment on the clinicopathological variables and mRNA levels for tumor progression-related genes (Table II) in OSCCs.

Clinicopathological variables. None of the pre-carcinogen hormones levels was correlated with clinicopathological variable such as tumor volume and thickness, and weight loss in rats who developed OSCC ($p>0.05$). Likewise, the OSCC histopathological features, such as keratinization degree, nuclear pleomorphism, pattern of invasion and lymphoplasmocytic infiltrate were not correlated with the pre-carcinogen concentrations of these stress hormones in the microenvironment ($p>0.05$). In the rats who developed oral leukoplakia, none of the hormones was correlated with epithelial dysplasia degree ($p>0.05$).

Gene expression features in OSCC. Increased concentrations of pre-carcinogen norepinephrine were predictive for reduced CDKN2a-p16 mRNA levels in the OSCC tissue ($p=0.0280$). However, the expressions of VEGF, MMP-2, MMP-9

and IL-6 were not correlated with the pre-carcinogen concentrations of stress hormones in the microenvironment ($p>0.05$).

Table II. Associations among pre-carcinogen stress-related hormones and clinicopathological variables and molecular features.

	Norepinephrine			Corticosterone			ACTH			BDNF		
Variables	**Estimate**	**t Value**	**p Value**	**Estimate**	**t Value**	**p Value**	**Estimate**	**t Value**	**p Value**	**Estimate**	**t Value**	**p Value**
Clinicopathological												
Tumor volume	1022.72	0.774	0.446	-21.35	-0.543	0.592	49587.91	1.415	0.169	-8.49	-0.017	0.986
Tumor thickness	7952.0	1.185	0.246	-180.47	-0.909	0.366	27559.27	1.559	0.131	-96.85	-0.039	0.969
Weight loss	-107.80	-0.647	0.521	-4.44	-0.997	0.325	1012.57	0.250	0.804	44.21	0.723	0.474
Keratinization degree	0.91	0.552	0.585	0.05	1.211	0.236	-42.77	-0.961	0.345	0.36	0.594	0.558
Nuclear pleomorphism	-8.90	-0.364	0.716	-1.16	-1.209	0.227	288.29	0.567	0.571	-10.08	-1.107	0.268
Pattern of invasion	-1.95	-0.487	0.630	-0.26	-1.890	0.069	105.30	1.186	0.245	0.82	0.802	0.429
Lymphoplasmocytic infiltrate	-16.47	-1.763	0.077	0.49	1.826	0.067	-348.29	-1.496	0.134	-0.42	-0.184	0.853
mRNA expression												
VEGF	-75.96	-1.450	0.168	2.05	1.218	0.242	-715.31	-0.390	0.702	-0.44	-0.030	0.976
MMP-2	-31.85	-1.706	0.109	0.73	1.231	0.237	-76.20	-0.117	0.909	2.02	0.385	0.705
MMP-9	-48.04	-0.305	0.765	2.43	0.481	0.638	-1567.41	-0.284	0.780	-7.24	-0.163	0.873
IL-6	-37.63	-0.385	0.706	-0.71	-0.221	0.828	-359.66	-0.102	0.920	3.44	0.145	0.887
CDKN2a-p16	-12.74	-2.420	0.028*	0.27	1.608	0.128	142.86	0.775	0.450	2.63	1.775	0.096

*Value considered statistically significant at $p<0.05$ (penalized regression analysis)

3. Differences between pre- and post-carcinogen stress-related hormones concentrations in the microenvironment

The modulation of stress-related hormones levels induced by 4NQO carcinogen treatment was evaluated in the microenvironment. For this, student's *t*-test was performed to identify differences between pre- and post-carcinogen stress-related hormones concentrations. Post-carcinogen corticosterone levels in OSCC (32.752 ± 7.558 pg/µg) and oral leukoplakia (19.259 ± 4.246 pg/µg) were higher than hormone levels found in the normal tongue microenvironment before carcinogenic induction (OSCC, 5.518 ± 0.401 pg/µg; oral leukoplakia, 5.028 ± 0.635 pg/µg) ($p<0.01$) (Figure 6B). Likewise, higher BDNF concentrations were found in the leukoplastic lesions post-carcinogen treatment (0.240 ± 0.037 pg/µg) compared to normal tissue pre-carcinogen treatment (0.093 ± 0.023 pg/µg) ($p=0.008$). Nevertheless, no significant differences were found between pre-carcinogen BDNF levels in the normal tissue (0.266 ± 0.045 pg/µg) and OSCC tissue (0.238 ± 0.019 pg/µg) ($p=0.653$) (Figure 6D). No differences were found between pre- and post-carcinogen norepinephrine (Figure 6A) and ACTH (Figure 6C) concentrations for OSCC and leukoplastic lesions ($p>0.05$). There were no differences regarding stress-related hormones concentrations between OSCC and oral leukoplakia ($p>0.05$) (Figure 6). When all hormonal data from oral leukoplakia and OSCC were analyzed together, pre-carcinogen ACTH levels in the tongue microenvironment were higher than post-carcinogen hormone levels ($p=0.014$) (data were not shown) indicating that differently from observed with corticosterone, ACTH levels in microenvironment decrease after carcinogen treatment.

Pre-carcinogen stress-related hormones levels were positively correlated to each other (p<0.001; Figures 7A, 7C, 7E, 7G, 7I and 7K). However, when the correlations among the stress hormones concentrations were analyzed in post-carcinogen period the only positive associations that were preserved were ACTH with each one of other three hormones studied (p<0.05; Figure 7D, 7H and 7L). Thus, these results indicate a misbalance in stress-related hormones secretion into microenvironment during carcinogenesis induction.

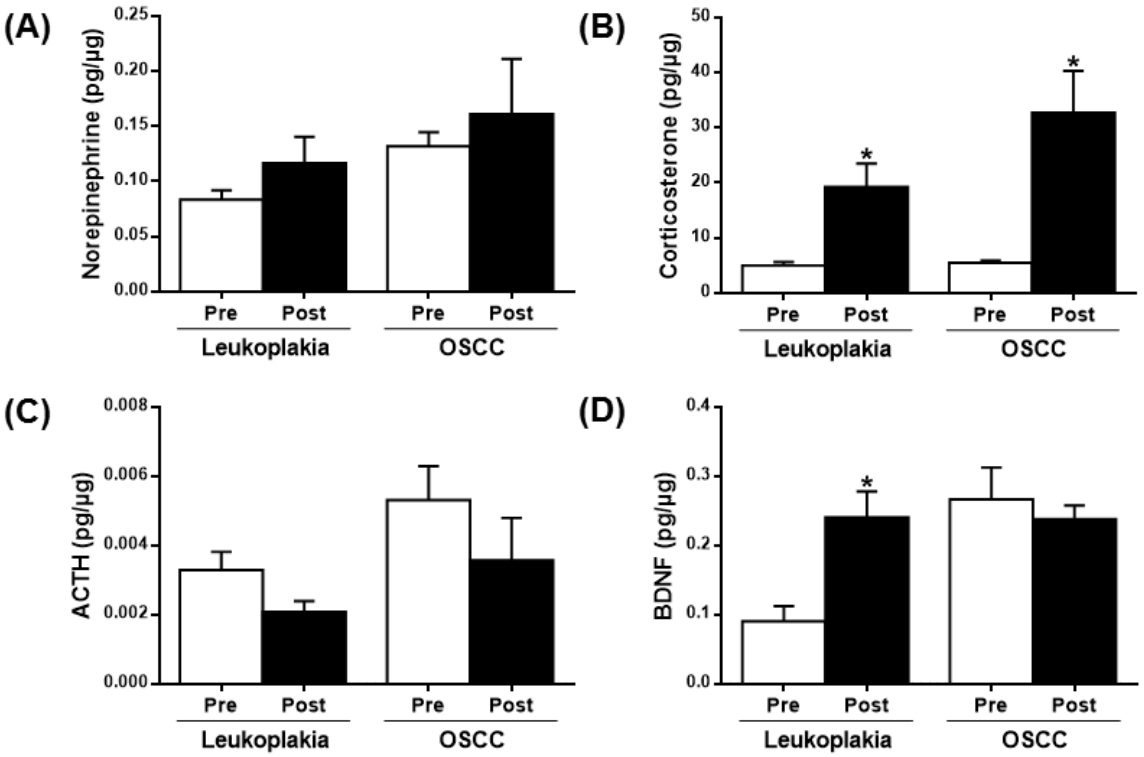

Figure 6. Student's test-*t* between pre- and post-carcinogen levels of norepinephrine (A), corticosterone (B), ACTH (C) and BDNF (D). Increased post-carcinogen corticosterone concentrations were found in the tumor and leukoplastic lesions (*p<0.05). Higher post-carcinogen BDNF levels were found only in the leukoplastic lesions compared to pre-carcinogen normal mucosa; *p<0.05.

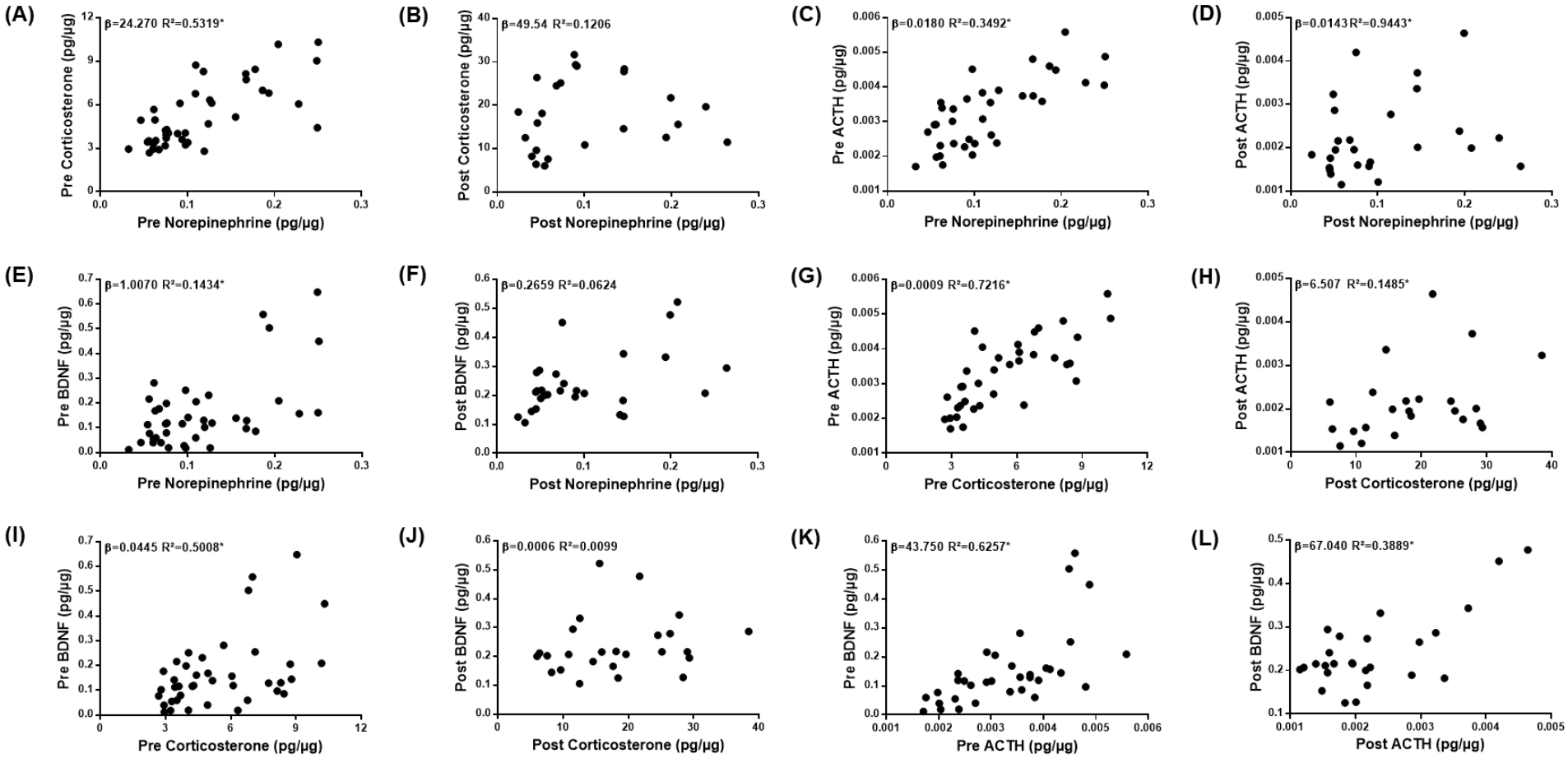

Figure 7. Correlation analysis showing a positive correlation between pre-carcinogen stress-related hormones levels (A, C, E, G, I and K; *p<0.001). A positive correlation was also observed in the post-carcinogen concentrations of norepinephrine vs ACTH (D; *p<0.001), corticosterone vs ACTH (H; *p=0.035) and ACTH vs BDNF (L; *p<0.001).

4. Associations among post-carcinogen stress-related hormones levels in the OSCC microenvironment and clinicopathological and molecular variables

When clinicopathological variables from OSCCs were correlated with post-carcinogen stress-related hormones, higher norepinephrine concentrations were associated to increased tumor volume ($p<0.001$). Likewise, an increase in the tumor thickness was correlated with higher norepinephrine levels in OSCC microenvironment ($p=0.003$). Weight loss of the animals with OSCC during carcinogenesis period was not associated to the post-carcinogen stress-related hormones concentrations ($p>0.05$). In addition, the OSCCs microscopic features keratinization degree, nuclear pleomorphism and pattern of invasion were not associated with the hormones levels in the tumor microenvironment ($p>0.05$). Higher levels of norepinephrine ($p=0.015$), ACTH ($p=0.015$) and BDNF ($p=0.019$) were associated with a lesser intensity of the lymphoplasmocytic infiltrate underlying to tumor. In the rats who developed oral leukoplakia, post-carcinogen stress-related hormones levels were not associated to epithelial dysplasia degree ($p>0.05$). The associations among post-carcinogen stress-related hormones levels and clinicopathological features are shown in Table III. The VEGF, MMP-2, MMP-9 and CDKN2a-p16 expressions in OSCC microenvironment were not associated to stress hormones concentrations ($p>0.05$). However, IL-6 gene overexpression was associated with increased levels of corticosterone in the tumor tissue ($p<0.001$).

Table III. Associations among post-carcinogen stress-related hormones and clinicopathological variables and molecular features.

	Norepinephrine			Corticosterone			ACTH			BDNF		
Variables	**R**	**R^2**	**p Value**	**R**	**R^2**	**p Value**	**R**	**R^2**	**p Value**	**R**	**R^2**	**p Value**
Clinicopathological												
Tumor volume	0.852	0.7259	<0.001*	-0.060	0.0036	0.793	-0.064	0.0040	0.780	-0.096	0.0092	0.686
Tumor thickness	0.726	0.5270	0.003*	-0.039	0.0015	0.866	-0.063	0.0039	0.784	-0.080	0.0064	0.736
Weight loss	-0.073	0.0053	0.742	-0.294	0.0864	0.114	0.064	0.0040	0.534	0.092	0.0084	0.434
Keratinization degree	-0.056	0,0031	0.807	0.144	0,0207	0.544	0.049	0.0024	0.834	0.048	0.0023	0.842
Nuclear pleomorphism	-0.060	0.0036	0.793	0.195	0.0380	0.408	-0.115	0.0132	0.627	-0.110	0.0121	0.651
Pattern of invasion	-0.092	0.0084	0.690	-0.030	0.0009	0.900	-0.117	0.0136	0.621	-0.121	0.0146	0.621
Lymphoplasmocytic infiltrate	-0.437	0.1909	0.015*	-0.313	0.0979	0.178	-0.531	0.2819	0.015*	-0.529	0.2798	0.019*
mRNA expression												
VEGF	-0.220	0,0484	0.336	0.063	0.0039	0.785	-0.042	0.0017	0.855	-0.052	0.0027	0.824
MMP-2	-0.201	0.0404	0.381	0.270	0.0729	0.236	0.020	0.0004	0.930	0.022	0.0004	0.923
MMP-9	-0.063	0.0039	0.783	0.199	0.0396	0.386	-0.014	<0.0001	0.951	0.058	0.0033	0.808
IL-6	-0.033	0.0010	0.887	0.817	0.6674	<0.001*	-0.001	<0.0001	0.994	-0.037	0.0013	0.877
CDKN2a-p16	-0.094	0.0088	0.682	0.009	<0.0001	0.968	0.105	0.0110	0.647	0.090	0.0081	0.703

*Values considered statistically significant at $p<0.05$ (correlation analysis)

DISCUSSÃO

Discussion

A long time it has been speculated that stress-related hormones could influence the cancer progression and onset. Although studies increasingly solidifies understanding that stress can relevantly affect tumor progression (1-4), its influence on the cancer occurrence is poorly understood. In this context, what still more obscure is the role of stress-related hormones as co-factors in conjunction with carcinogenic substances on the cancer induction. Although recent findings on stress hormones action in tissue microenvironment be increasingly understood for malignant phenotype induction (22,23,41), until the moment there were no investigations that have evaluated if baseline (pre-carcinogen) levels of the stress-related hormones in the microenvironment would have predictive value for cancer development and progression. In the present study, we have used a classic model of chemically induced carcinogenesis (36) to evaluate the influence of stress-related hormones on the oral malignancies occurrence. Our results demonstrated the first *in vivo* evidence that pre-carcinogen concentrations of norepinephrine and BDFN and reduced pre-carcinogen corticosterone levels were predictive for oral cancer occurrence, although the BDNF results have not reach statistical significance in penalized regression analysis.

In our findings, animals who had increased norepinephrine concentrations in the pre-carcinogen tongue microenvironment have had about 35 times higher chance of developing tongue carcinomas. Investigations have showed that norepinephrine can induce significant alterations in molecular pathways of the

carcinogenesis process (14,41-44). A study involving a chronic stress model showed that norepinephrine and epinephrine act via both Gs-protein kinase A (PKA) and β-arrestin-mediated signaling pathways inducing DNA damage and suppressing p53 concentrations through the AKT-mediated activation of MDM2 (41), which may increase considerably the cellular tumorigenicity. Activated-norepinephrine receptors (beta-adrenergic signaling) can trigger other oncogenic intracellular processes, such as the increase of cyclic AMP (cAMP) levels (14), and RAS signaling pathway (42) and transcriptional factor Stat 3 hyperactivation (43). According to Fitzgerald (44), higher norepinephrine levels could stimulate its extracellular receptors increasing the chances of developing cancer. Likewise, it has also been shown that chronic exposure to norepinephrine causes an increase in DNA-damage in 3T3 mouse fibroblasts (22). Interestingly, pre-clinical studies using chemically induced carcinogenesis models have showed that drug administration or low protein diet consumption in stomach (45,46), colon (47,48) and liver (49) were directly associated with an increase of carcinogenesis in these tissues and these events were associated to increased norepinephrine levels in tumor microenvironment. The *in vivo* results of this study indicate that not stress-induced basal increased norepinephrine concentrations in the microenvironment may be an important factor to act in conjunction with carcinogenic substances in the activation of oncogenic signaling pathways.

Higher pre-carcinogen corticosterone concentrations found in the pre-carcinogen tongue microenvironment were negatively correlated with OSCC occurrence in penalized regression analysis. In a way, this data was unexpected. In our initial hypothesis, data regarding corticosterone levels influence on

carcinogenesis should be positively correlated to norepinephrine findings with higher hormonal levels predicting tumor occurrence. Pyter & Prendergast (51) found that plasma corticosterone levels before carcinogen treatment predicted mammary cancer onset. In the present study, we have not evaluated the pre-carcinogen systemic hormonal concentrations in order to compare them to tissue microenvironment levels and evaluate impact on OSCC occurrence. Anyway, some evidences can support the corticosterone-related effects on carcinogenesis observed in our experiment. In female rats, per example, dietary administration of corticosterone has influenced a dose-dependent reduction in the incidence, multiplicity and size of mammary carcinomas (51). Under hepatocarcinogenesis induction, corticosterone injections inhibited precancerous cells growth and increased specific survival in another pre-clinical study (52). In a mouse model, topical dexamethasone treatment promoted an inhibition of skin carcinogenesis (53). Furthermore, glucocorticoid-mediated COX-2 inhibition promoted 11β-hydroxysteroid dehydrogenase type II (11ßHSD2) inhibition in human lung cancer cells; *in vivo*, 11ßHSD2 inhibition decreased lung tumor growth and invasion, and was associated with higher tissue glucocorticoids levels (54). Further investigations are necessary to confirm the different modulation in the same pre-carcinogen microenvironment promoted by the norepinephrine and corticosterone on carcinogenesis like found in this study. Pre-carcinogen ACTH concentrations in tongue microenvironment, differently of norepinephrine and corticosterone, were not predictive for oral cancer occurrence.

Increased pre-carcinogen BDNF levels in the tongue microenvironment were positively correlated with OSCC development in the simple linear regression

analysis and showed a positive trend to significance in the penalized regression analysis. BDFN levels are increased in response to stress (25,26) and may activate the TrkB receptors of PC12 cells in the rodents' adrenal medulla, which can release norepinephrine and epinephrine into the bloodstream (25). BDNF is overexpressed in more than a half of human head and neck squamous cell carcinomas and can induce cellular migration and tumor invasion through of TrkB activation, which could be a critical component for tumor progression (27). Likewise, oncogenic signaling pathways are activated by the BDNF, such as the upregulation of the phosphoinositide-3 kinase AKT-cascade (55) and MAPK p42/44 phosphorylation (56), resulting in cAMP activation (55,56). In addition, TrkB overexpression results in an altered expression of E-cadherin (with downregulation, in epithelium) and Twist (with upregulation, in mesenchyme) in the microenvironment (27). Interestingly, Twist transcriptional activity in stroma through AKT activation induces severe alterations in the epithelial cells (57). Although the predictive influence of pre-carcinogen BDNF concentrations in the tongue microenvironment for OSCC occurrence has not reached statistical significance, the present study data might suggest a specific role of this biomarker in carcinogenesis.

Although norepinephrine has been predicted for oral cancer occurrence, none of the pre-carcinogen hormones in the tongue microenvironment has influenced the post-carcinogen clinicopathological variables, such as tumor volume and thickness or keratinization degree and nuclear pleomorphism. Nevertheless, higher pre-carcinogen norepinephrine levels were predictive for CDKN2a-p16 mRNA lower expression in the tumor microenvironment. CDKN2a-

p16 is an important tumor-suppressor gene and its alteration is a frequent event in the tongue OSCC development in humans (58). This gene encodes the p16 protein, which has the ability to inhibit the activity of the CDK-4 and CDK-6 (cyclin D complex) that are necessary for phosphorylation of retinoblastoma protein and the G1-S phase transition of cell cycle progression (58,59). In the human tumors, CDKN2a-p16 gene inactivation during carcinogenesis process is caused by the homozygous deletions, inactivating mutations and promoter hypermethylation and is associated with loss of p16 protein expression (59). By the first time, stress hormone norepinephrine has been linked to CDKN2a-p16 lower expression in the tumor microenvironment, which could has a role in oncogenic signaling pathways activation. On the other hand, Schraml et al. (60) demonstrated that p16 is transcriptionally activated in murine hematopoietic progenitor cells after treatment with alpha- (oxymetazoline) and beta-adrenergic (terbutaline sulfate) agonists.

For better understanding of stress-related hormones levels modulation in the microenvironment derived from chemically induced oral carcinogenesis, pre-carcinogen hormones concentrations were compared to post-carcinogen levels. Interestingly, the results demonstrated that treatment with 4NQO carcinogen increased the corticosterone concentrations in the oral leukoplakia and OSCC microenvironment compared to normal mucosa before carcinogen treatment. BDNF concentrations were also higher in the oral leukoplakia microenvironment than pre-carcinogen normal mucosa. A possible explanation for these features is that chronic exposure to the chemical carcinogen can lead to a dysregulated production and release of these stress-related hormones by the epithelial and mesenchymal cells in the tissue microenvironment during tumor induction. The

substantial increase of the corticosterone levels in oral leukoplakia and OSCC microenvironments were probably related to an increased cellular endocrine reactivity due to stimulus-stressor of the carcinogen. An investigation showed that persistent activation of HPA axis in melanoma cells is caused by UV radiation resulting in the production of glucocorticoids (34). Likewise, HPA axis could be hyperactivated in oral cancer cells in response to 4NQO carcinogen. When the ACTH post-carcinogen levels in oral leukoplakia and OSCC tissues were evaluated in conjunction, the levels of this hormone were reduced after carcinogen treatment, an opposite result to that observed with corticosterone. Thus, higher systemic and local post-carcinogen corticosterone concentrations could be inhibiting the ACTH production by the tumor microenvironment cells (negative feedback effect). In another study, Al-Wadei et al. (33) using pancreatic cancer cell lines and immortalized human pancreatic duct epithelial cell line demonstrated that these cell lines produced and released norepinephrine and epinephrine upon exposure to nicotine (a chemical carcinogen), and this mechanism was mediated by the nicotinic acetylcholine receptors. In this study, despite the carcinogenic induction has increased the norepinephrine levels, this result has not reach statistical significance.

Higher post-carcinogen norepinephrine levels in OSCC microenvironment were correlated with greater tumor volume and thickness, suggesting its involvement in oral cancer progression *in vivo*. A pre-clinical study in nude mice showed a positive correlation among chronic stress and increased plasma concentrations of catecholamines and glucocorticoid, greater tumor size, and more invasive growth of human oral carcinoma cells (61). Sood et al. (12) showed

that stress levels of norepinephrine and epinephrine increased the invasiveness of ovarian cancer cells lines. Likewise, under chronic stress, increased catecholamines concentrations induced tumor growth of ovarian carcinoma and angiogenesis in an orthotopic mouse model through the activation of cAMP-PKA by the β2 adrenergic receptor, with an overexpression of VEGF, MMP-2 and MMP-9 (8). Similarly, norepinephrine treatment increased invasiveness, VEGF, MMP-2 and MMP-9 levels in culture supernatants of nasopharyngeal carcinoma (14) and OSCC cells proliferation (11) in a dose-dependent response, and these effects were blocked by propranolol (a beta-blocker). Higher corticosterone concentrations in the tumor microenvironment were associated to enhanced IL-6 mRNA levels. Bernabé et al. (11) showed that cortisol stress levels tended to increase IL-6 expression in a human OSCC cell line. Another investigation showed that norepinephrine increased expression of IL-6 mRNA and protein levels in human ovarian carcinomas cells through Src tyrosine kinase-dependent mechanism (10).

Studies have shown that increased concentrations of the stress-related hormones induce lymphocytes apoptosis and impair the macrophages functions (1,62). Catecholamines and glucocorticoids inhibit IL-12 production by the antigen-presenting cells (monocytes, macrophages and dendritic cells), which promotes type 1 helper cells (Th1) response and cellular immunity (1,62). Therefore, the inhibition mechanism or selective suppression of the Th1 lymphocyte's functions impairs the cellular immune responses against some tumors (1). In the present study, increased post-carcinogen concentrations of norepinephrine, ACTH and BDNF were associated with a lesser intensity of the

lymphoplasmocytic infiltrate underlying to tumor, thereby suggesting a probable immunosuppressive effect of these stress-related hormones in the OSCC microenvironment, which could facilitate tumor advancement. ACTH hormone has been considered an immuno-regulator, which plays a central role in important immune responses, such as chemotaxis and phagocytosis (63), whereas BDNF can decrease the levels of NK cells (64). Although oral leukoplakia is classified as a potentially malignant disorder or even an intermediate process of OSCC development (37), pre- and post-carcinogen stress-related hormones levels were not associated to epithelial dysplasia degree. The small size of oral leukoplakia sample may have influenced in obtaining these negative results.

The present study is original in its purpose and methodology. This is the first time that a classic chemically induced carcinogenesis pre-clinical model has been used to test the hypothesis by which individual variability of basal hormonal levels in the tongue microenvironment could act in conjunction with carcinogen predisposing cancer development. In humans, the conduction of cohort prospective studies that allow to evaluate the influence of local and systemic stress-related hormones on cancer onset is not simple. Although it is an excellent resource for studying the influence of stress hormones on the carcinogenesis process, the collection of biological samples before cancer development from human normal tissues requires a large population sample and its follow up until the cancer occurrence. In this study, we had success in reproducing this methodological perspective in animals, in order to study the presence of stress-related hormones in the normal tissue microenvironment, which would be induced to cancer some time later. This methodology provides perspectives of its

use for the investigation of physiological and genetic individual alterations in the microenvironment and their effects on oral carcinogenesis.

Although pre-carcinogen stress-related hormones levels in the tongue microenvironment have been predicted for OSCC occurrence and progression, behavior tests were not performed for analyzing the rats' stress levels. Likewise, the systemic hormonal levels were not evaluated, which would be interesting for crossing them with the tongue microenvironment concentrations. Moreover, we have explored the stress hormones levels in the microenvironment, but not their receptor-depend action. The hormonal biological effects are directly dependent on the biochemistry affinity between the hormones and receptors. Thus, future experiments that block or potentiate the action of beta-adrenergic, glucocorticoid and TrkB receptors may expand the understanding of their agonists' role on the chemically induced carcinogenesis process.

The first evidences that basal pre-carcinogen stress-related hormones levels in the microenvironment in natural conditions may predict chemically induced cancer occurrence were shown in the present investigation. Increased norepinephrine levels (related inversely to corticosterone levels) in the tongue microenvironment before oral carcinogenesis induction were predictive for tumor occurrence. Likewise, BDNF proved to be a promising biomarker for oral cancer occurrence. Furthermore, carcinogen may act as a potent exogenous stressor increasing stress hormones levels in the microenvironment, which can affect cancer progression. Although they have been observed in a pre-clinical model, the results of the present investigation strongly suggest that endocrine individual differences in the tissue microenvironment before carcinogen exposure can be

related to a higher susceptibility to tumor development in chemically induced carcinogenesis.

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†Normalização segundo o periódico "Carcinogenesis" (Anexo B)

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ANEXO A

ANEXO A - Certificado da Comissão de Ética no Uso de Animais (CEUA).

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 **"Variabilidade individual da atividade do sistema nervoso simpático e análise de sua influência sobre a carcinogênese bucal induzida por 4-NQO"**, Processo FOA nº 2014-00816, 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 02 de Setembro de 2014.

VALIDADE DESTE CERTIFICADO: 31 de Julho de 2016.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 30 de Agosto de 2016.

CERTIFICATE

We certify that the study entitled **"Individual variability of sympathetic nervous system activity and analysis of its influence on the 4-nitroquinoline 1-oxide-induced oral carcinogenesis"**, Protocol FOA nº 2014-00816, under the supervision of Daniel Galera Bernabé presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on September 02, 2014.

VALIDITY OF THIS CERTIFICATE: July 31, 2016.
DATE OF SUBMISSION OF THE FINAL REPORT: August 30, 2016.

Profa. Dra. Maria Gisela Laranjeira
Coordenadora da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
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Fone (18) 3636-3234 Email CEUA: ceua@foa.unesp.br

ANEXO B

ANEXO F - Normas para publicação no periódico "Carcinogenesis".

Preparation of manuscripts

Manuscripts should be in their final form when they are submitted so that proofs will require only correction of typographical errors. Manuscripts must be submitted according to the manuscript template, available online here. A mini-style checklist is also available online here.

Sections of the manuscript

Regular full-length papers should be subdivided into the following sequence of sections: Title page, Abstract, Introduction, Materials and methods, Results, Discussion, Acknowledgements, References, Tables and Legends to Figures. In the journal the Materials and methods, Acknowledgements and References will be printed in smaller type to accommodate more text.

Authors should follow the below guidelines for manuscript length:

5000 words (excluding references and figure legends)

6 or fewer tables and/or figures (6 in total)

250 word limit for the abstract

50 references

In addition to the abstract, manuscripts should also a 40-word summary of the article's main point. This may be used to highlight the article in the contents list online and in e-mail alerts. Such a summary is not compulsory, but is likely to encourage readers browsing the table of contents to read your article.

Supplementary Material Only directly relevant material should be included in the full text of manuscripts. Supporting materials, which are not essential in the full text, but would nevertheless benefit the reader, can be published as online-only supplementary data. Supplementary data should be submitted for review, in a separate file from the manuscript. Authors should ensure that supplementary data is referred to in the main manuscript at an appropriate point in the text.

Language editing

Particularly if English is not your first language, before submitting your manuscript you may wish to have it edited for language. This is not a mandatory step, but may help to ensure that the academic content of your paper is fully understood by journal editors and/or reviewers. Language editing does not guarantee that your manuscript will be accepted for publication. If you would like information about one such service please click here. There are other specialist language editing companies that offer similar services and you can also use any of these. Authors are liable for all costs associated with such services.

General format

As referees may choose to print out your paper for reviewing purposes, all sections of the manuscript must be double-spaced with margins of 25mm (one

inch) left at the sides, top and bottom of each page. Number each page top right (Title Page is 1). Please avoid footnotes, use instead, and as sparingly as possible, parenthesis within brackets.

Abstract

The second page of every manuscript must contain only the Abstract, which should be a single paragraph not exceeding 250 words. Published papers will only have the first 250 words of their abstracts incorporated into Medline, text in excess of this limit will be lost. The Abstract should be comprehensible to readers *before* they have read the paper, and abbreviations and reference citations should be avoided.

Acknowledgements

The acknowledgements should be included at the end of the text. Personal acknowledgements should precede those of institutions or agencies.

References

Authors are responsible for the accuracy of the References. Published articles and those in press (state the journal which has accepted them) may be included. Arrange references in numerical order with the numbers in the text in brackets and on the line (not as superscripts). In the reference list please note that for more than one author the first author name should be mentioned and the initials follow the name; then et al. The date should precede the title; the title should be given in full; the name of the journal should be abbreviated according

to the World List of Scientific Periodicals and underlined to indicate italics. References should therefore be listed as follows:

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