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Faculdade de Odontologia de Araçatuba – UNESP

JÉSSICA ARAÚJO FIGUEIRA

Early life stress by maternal separation increases tumor onset and progression in a chemically induced oral cancer model

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Araçatuba – SP

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Figueira JA. Estresse precoce de vida por separação maternal aumenta a incidência e progressão de tumor num modelo de câncer bucal quimicamente induzido em ratos [dissertação]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2018.

Resumo

A ocorrência de eventos estressores nas fases iniciais de vida (estresse precoce de vida – EPV) pode afetar negativamente funções fisiológicas e psicológicas na fase adulta. Apesar de investigações pré-clínicas terem mostrado que o estresse crônico pode afetar a progressão do câncer, não há estudos que investigaram os efeitos do EPV na progressão do câncer bucal. No presente estudo, utilizamos um modelo animal de carcinogênese bucal induzida pelo carcinógeno 4-Nitroquinolona-1-Óxido (4NQO) para avaliar o impacto do EPV induzido por separação materna (SM) sobre a incidência e progressão do carcinoma espinocelular (CEC) de boca. As ninhadas submetidas ao protocolo de SM foram separadas de suas mães durante 3 horas por dia, do dia pós-natal 1 ao 21. Após os animais atingirem a idade adulta (90 dias), os grupos SM e controle foram tratados com 4NQO durante 120 dias. Análise histopatológica foi realizada para avaliar a incidência de CEC de boca e grau de malignidade do tumor entre os animais estressados e não estressados. Também foram avaliados o volume e espessura tumoral e o peso do baço e das glândulas adrenais. Os níveis plasmáticos de norepinefrina foram analisados por ELISA e a expressão de RNAm para os genes relacionados à progressão do CEC de boca (IL-6, TNF-alpha, VEGF, p53 e CDKN2A) foram analisados por PCR em tempo real. A SM no período pós-natal aumentou em aproximadamente 60% a ocorrência de CEC de boca na idade adulta. Os ratos submetidos à SM desenvolveram tumores mais espessos ($p=0.02$) e de maior volume ($p=0.03$) comparado ao grupo controle. Análise microscópica das características de malignidade dos CECs de boca mostraram pior padrão de invasão ($p=0.004$) e maior invasão perineural ($p=0.04$) nos tumores dos ratos estressados. O EPV no período neonatal diminuiu significativamente o peso do baço ($p=0.003$) e das glândulas adrenais ($p=0.03$) nos animais com câncer. Porém, não foi observada diferença nos níveis plasmáticos de norepinefrina. Os CECs dos ratos submetidos ao EPV apresentaram maior expressão de IL-6 ($p=0.04$) e menor expressão de p53 ($p=0.02$) em relação aos tumores dos ratos não estressados. Nossos achados fornecem a primeira evidência de que o EPV pode aumentar a incidência e progressão do câncer de boca quimicamente induzido, e sugerem que estes efeitos podem estar associados à uma desregulação da expressão de IL-6 e p53.

Palavras-chave: Estresse psicológico; câncer; câncer de boca; carcinoma espinocelular; carcinogênese.

Figueira JA. Early life stress by maternal separation increases tumor onset and progression in a chemically induced oral cancer model [dissertation]. São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil; 2018.

Abstract

Early life stress (ELS) may negatively affect the behavior and physiological functions in adulthood. Despite pre-clinical investigation have shown that chronic stress may affect cancer progression, there are no studies which have investigated ELS effects on oral cancer progression. In the present study, we used an oral carcinogenesis animal model induced by carcinogen 4-Nitroquinoline-1-oxide (4NQO) to assess the impact of ELS induced by maternal separation (MS) on the oral squamous cell carcinoma (OSCC) occurrence and progression. The litters underwent MS protocol were separated from their dam for 3 hours daily, during postnatal day 1-21. After animals reach adulthood (90 days), MS and control groups were treated with 4NQO during 120 days. Histopathological analysis was performed to evaluate the OSCC incidence and malignant degree between stressed and non-stressed rats. The volume and tumor thickness and the spleen and adrenal glands weight were also evaluated. Plasma norepinephrine levels were analyzed by ELISA and mRNA expression for OSCC progression-related genes (IL-6, TNF- α , VEGF, p53 and CDKN2A) were analyzed by real-time PCR. MS in neonatal period increased in almost 60% chemically induced OSCC occurrence in adulthood. Rats exposed to MS developed thicker ($p=0.02$) and larger tumors ($p=0.03$) compared to non-stressed rats. OSCCs from ELS rats showed worst pattern of invasion ($p=0.004$) and more perineural invasion ($p=0.04$) ELS also significantly reduced spleen ($p=0.003$) and adrenal glands ($p=0.03$) weight in cancer rats. ELS induced overexpression of IL-6 ($p=0.04$) and attenuated p53 expression ($p=0.04$) in OSCCs. Our results provides the first evidences that ELS may increase oral cancer onset and progression, and suggest that this effect can be associated with altered expression of IL-6 and p53.

Key-words: Psychological stress; cancer; mouth cancer; squamous cell carcinoma; carcinogenesis.

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Lista de Abreviações

SNS = Sympathetic nervous system

HPA = Hypothalamic-pituitary-adrenal

VEGF = Vascular endothelial growth factor

TNF-alpha = Tumor necrosis factor-alpha

IL-6 = Interleukin-6

MMPs = Matrix metalloproteinases

ELS = Early life stress

MS = Maternal separation

NK= Natural killer

HNC = Head and neck cancer

OSCC = oral squamous cell carcinoma

4NQO = 4-nitroquinolone-1-oxide

OL = Oral leukoplakia

WHO = World health organization

RT-PCR = Reverse transcription-polimerase chain reaction

ELISA = Enzyme-linked immunosorbent assay

RNA = Ribonucleic acid

miRNA = Micro ribonucleic acid

mRNA = Messenger ribonucleic acid

DNA = Deoxyribonucleic acid

cDNA = Complementary deoxyribonucleic acid

CT = Threshold cycle

SEM = Standard error of the mean

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Early life stress by maternal separation increases tumor onset and progression in a chemically induced oral cancer model

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1. Introduction*

Psychological stress has been associated to cancer initiation, growth and metastasis [Soung et al., 2015; Shin et al., 2016; Cole et al., 2015; Antoni et al., 2006]. Most of *in-vitro* and pre-clinical studies have shown that stress and its neurohormones can accelerate cancer cell proliferation [Soung et al., 2015; Shin et al., 2016; Cole et al., 2015; Antoni et al., 2006; Al-Azri et al., 2014; Capoccia et al., 2015; Madden et al., 2013; Shi et al., 2015; Smith et al., 2017; Xie et al., 2015], while their effects on tumor onset are still poorly understood. Stressful events induce increased secretion of hormones which have been implicated in cancer progression (eg. catecholamines and cortisol) through activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal axis (HPA) [Armaiz-Pena et al., 2008; Shin et al., 2016].

Stress can suppress immune functions against cancer (presentation of tumor antigens, proliferation of T lymphocytes, and humoral and cell-mediated immune response) [Dhabhar et al., 1997; Elenkov et al., 2002; Glaser et al., 2001], and promotes resistance to apoptosis through the activation of anti-apoptotic genes [Herr et al., 2003; Pasquier et al., 2013]. Stress hormones (eg. epinephrine and norepinephrine) may also induce cancer progression by modulating the expression of tumor angiogenesis-related genes (eg. vascular endothelial growth factor – VEGF) and cell proliferation (tumor necrosis factor-alpha – TNF-alpha and interleukin-6 – IL-6), as well as overexpression of proteins involved in tumor invasion and metastasis (matrix metalloproteinases – MMPs,

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types 2 and 9) [Lutgendorf et al., 2003; Lutgendorf et al., 2008a; Yang et al., 2008; Sood et al., 2006; Yang et al., 2009].

There are several pre-clinical models to investigate the effects of stress on different biological conditions. For example, chronic stress can be induced by physical restraint [Rasmussen et al., 1957], agglomeration [Edwards et al., 1977], presence of predator (cat-rat) [Hamilton et al., 1964], shock [Borysenko et al., 1980], confrontation with intruders [Olivier et al., 1992] and social isolation [Krishnan et al., 2011]. When stress events occur during early life (eg. postnatal period and childhood), they can affect behavior and physiological functions, such as growth, metabolism, reproduction, besides inflammatory and immune responses in adulthood [Nishi et al., 2013; Silberman et al., 2016]. Early life stress (ELS) may induce permanent effects on organism [Nish et al., 2013; Silberman et al., 2016] and increases the risk for developing diseases like neuropsychiatric disorders, type 2 diabetes and cardiovascular disease [Kendler et al., 2002; Eriksson et al., 2014].

Maternal Separation (MS) is the main model among rodents for investigating the ELS impact on neuroendocrine stress-induced responses and psychiatric disorders (eg. depressive and anxiety like behaviors) [Andersen et al., 2015; Millstein and Holmes, 2007; Romeo et al., 2003; Schmidt et al., 2011; Vetulani et al., 2013]. Evidences show that MS during the postnatal period increases the activity of neurohormonal stress pathways (SNS and HPA axis) [Holmes et al., 2005; Lippmann et al., 2007], altering the levels of catecholamines and corticosterone [Gogberashvili et al., 2007; Esquivel et al., 2009; Sterley et al., 2013; Aisa et al., 2007; Marais et al., 2008; Daniels et al., 2009]. Past studies explored the effects of MS-induced ELS on cancer onset and progression. La

Barba et al. in 1969, 1971 and 1972 carried out initial investigations on the effects of MS on cancer progression through inoculation of Ehrlich ascites carcinoma cells in Balb/c mice [La Barba et al., 1969; 1971; 1972]. However, they did not find significant effects of MS on the survival and mortality rates [La Barba et al., 1969; 1971; 1972]. More recently, one study showed that MS followed by chronic restraint stress reduced the natural killer (NK) cells cytotoxicity and increased tumor metastases in a breast cancer pre-clinical model [Nakamura et al., 2011]. Using a chemically induced mammary cancer model in mice, Boyd et al. demonstrated that psychosocial stress during the neonatal period accelerated chemically induced mammary tumorigenesis in adulthood [Boyd et al., 2010].

Head and neck cancer (HNC) comprises the seventh most common cancer worldwide, being oral squamous cell carcinomas (OSCCs) the most frequent subtype of HNC [Day et al., 2005; Rettig & D'Souza, 2015]. OSCC commonly affects elderly men and its main risk factors are chronic tobacco and alcohol consumption [Day et al., 2015; Rettig & D'Souza, 2015]. Despite the known influence of chronic stress on tumor progression in some cancer pre-clinical models [Thaker et al., 2006; Lamkin et al., 2012; Kim-Fuchs et al., 2014; Zhao et al., 2015], few studies have analyzed the effects of stress and its neurohormones on OSCCs progression. Xie et al. using an orthotropic model in mice showed that chronic stress by restraint accelerated tumor growth and increased the invasiveness of OSCC cells [Xie et al., 2015]. However, Rivera et al. did not observed significant effects of restraint stress on tumor incidence in chemically induced OSCC mice model. [Riviera et al., 2001]. Recently, we have shown that pre-carcinogen stress hormones levels in the normal microenvironment may be predictive for chemically induced cancer in rats

[Valente et al. 2017], which would hypothesize that early stressors could affect oral carcinogenesis. Although ELS is a risk factor for neurobiological and psychiatric disorders and recent evidences show its impact on tumor immunity and progression, no studies which have investigated the effects of ELS on oral cancer progression. In this study, we have used a chemically induced oral carcinogenesis model to investigate the impact of MS-induced stress on OSCC onset and progression.

2. Materials and Methods

2.1 Animals and experimental conditions

All experiments involving animals were performed according to the guidelines of principles for the use of animals in the laboratory and the study was approved by the Animal Ethics Committee from the Araçatuba School of Dentistry – UNESP. For the experiment, 93 male pups (*Rattus norvegicus albinus*, Wistar) were used. To generate the animal population, male Wistar rats were kept individually in boxes with two females for mating. All animals were kept in climatized environment (25 ± 2 °C), 12h light/dark cycle with food (Purina®, Paulínia-SP, Brazil) and water available *ad libitum*.

2.2 Experimental design

The study was conducted with two experimental groups: 1) MS group – 46 male Wistar pups submitted to ELS by maternal separation; 2) Control group – 47 male Wistar pups not submitted to ELS by maternal separation. Both groups were maintained under the same conditions described above. After 21 days of ELS by MS in the postnatal period, litters were weaned and separated by gender on the 22nd day post-birth. Only the male rats of each group were used for the later phases of the experiment. When rats from both groups reached adulthood (90 days of age), they were induced to oral carcinogenesis during 120 days.

2.3 Maternal separation

After birth (P0), litters were randomly divided into two groups (MS group and control group). Litters receiving maternal separation were separated from the dam for 3h/day (08h00 – 11h00), during 21 days (P1 - P21). Dams were removed from the cage and placed in another cage. Pups were then moved to a clean

cage in another room with a thermal blanket at 32°C (to prevent hypothermia), so that the separation involved a loss of olfactory, tactile and auditory contact with the dam. After the 3-hour separation period, pups returned to their dam's cage. Animals from control group were not disturbed during this period, except to be moved to clean cages. Pups were weaned on P22 and housed with littermates in three or four per cage until they were 90 days old, when then they were submitted to oral carcinogenesis.

2.4 Oral carcinogenesis model

For oral carcinogenesis induction, animals from both groups were treated with 50 ppm of 4-nitroquinolone-1-oxide (4NQO) (Sigma-Aldrich, St. Louis, USA), diluted in drinking water, starting at P90. All animals only had access to water containing 4NQO. Animals were euthanized by decapitation after 120 days of carcinogenic induction, and tongues from all animals were extracted for histopathological analysis. 4NQO treatment in drinking water is the main oral carcinogenesis model used in rodents due its close similarity to histological and molecular changes observed in human oral cancer [Knoija et al., 2006]. Oral tumors can affect any region in the oral cavity, but they occur predominantly in posterior area of the tongue.

2.5 Histopathological analysis

After extraction, tongues were longitudinally sectioned for histopathological analysis. Tissues were fixed in 10% buffered formaldehyde (Merck, Darmstadt, Germany) for 48 hours, embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin (H&E, Merck). Tongue lesions derived from carcinogenic treatment were classified in oral leukoplakia (OL) and OSCC. OL is considered

an early phase of OSCC development [Warnakulasuriya et al., 2007]. The World Health Organization (WHO) criteria were used to classify the epithelial dysplasia degree of OL in mild, moderate or severe [Warnakulasuriya et al., 2007]. For invasive OSCCs, histological grade of malignancy was evaluated according to Bryne's criteria, in which histopathological features like keratinization degree, nuclear pleomorphism, pattern of invasion and host response (lymphoplasmocytic infiltrate) were analyzed in the invasive front of tumors [Bryne et al., 1989]. Invasive OSCCs were also analyzed according to a classification usually used to investigate tumor aggressiveness in humans [Brandwein-Gensler et al., 2005]. In this classification, histologic risk of local recurrence (low, intermediate and high-risk) and overall survival probability (good, intermediate and poor) are assessed according to the pattern of invasion, lymphocytic response and perineural invasion on tumor margin status [Brandwein-Gensler et al., 2005]. Histopathological analysis was performed by an experienced oral pathologist, who was blind to the experimental groups.

2.6 Thickness and tumor volume

Tumor thickness measurement was microscopically obtained from HE-stained OSCC sections (DM 400 B, Leica, Wetzlar, Germany). Measurement was taken from the deepest point of tumor invasion to the surface of the ulcer or epithelium and was obtained (in mm) using the Qwin Plus software (Leica Microsystem Imaging Solutions Ltd., Cambridge, UK). Tumor volume was calculated using clinical measurements (anteroposterior and laterolateral measured by a calibrated caliper) and microscopic depth invasion. Final volume, in cubic millimeters (mm³), was calculated by multiplying the three measurements.

2.7 Body weight variation, spleen and adrenal gland weight

Initial body weight (before starting 4NQO treatment) and final body weight (before the rat's euthanasia) were used to calculate weight variation during carcinogenic induction. Spleen and adrenal glands were weighed and values were adjusted for the body weight of each animal.

2.8 Measurement of plasma norepinephrine levels

Trunk blood samples were collected from decapitated unanesthetized rats in EDTA tubes and centrifuged at 1500g, 4°C, for 10 minutes. Plasma samples were separated and stored at -80°C after collection until assayed. Plasma norepinephrine concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kits (Elabscience, Houston, TX, USA), according to the manufacturer's recommendations.

2.9 Analysis of tumor progression-related genes expression by RT-PCR

Total RNA was extracted using mirVANA miRNA Isolation kit (Life Technologies), according to the manufacturer's recommendations. RNA quantity was verified by spectrophotometry (Nanodrop, Roche) and complementary DNA (cDNA) was synthesized with a 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 PCRTM equipment (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. Primers for IL-6 (Rn00569848_m1), p53 (Rn 00755717_m1), VEGF (Rn01511601_m1), TNF-alpha (Rn00562055_m1) and CDKN2A (Rn 00580669_m1) were used for analysis. β-actin (Rn00562253_m1) gene was used as endogenous control. Thermal cycling conditions followed all the manufacturer's recommendations.

Expression levels for each gene in each sample were normalized to β -actin mRNA levels. Results were analyzed using the comparative threshold cycle (CT) method, and were presented on percentage of increase of mRNA expression compared to control.

2.10 Statistical analysis

GraphPad prism 6.01 (GraphPad Software Inc. Dan Diego, CA, USA) was used to perform all statistic tests. In order to evaluated whether ELS by MS affected OSCC incidence and microscopic malignancy degree, Chi-squared test was used. Student's test-t was used to determine whether there were differences between the experimental groups regarding clinicopathological variables, plasma norepinephrine levels and mRNA expression for tumor progression related genes. Results are shown as mean \pm standard error of the mean (SEM). Significance level was set at 5% ($p < 0.05$).

3. Results

In the MS and control groups the mortality rate during 4NQO treatment was of 15.2% and 12.7%, respectively. Rats from both groups who died before reaching experimental period of oral carcinogenesis were excluded from the study. The remaining 80 animals (39 from MS group and 41 from control group) were used to investigate the effects of ELS on oral carcinogenesis.

3.1 Early life stress by maternal separation increases chemically induced OSCC incidence

Rats exposed to maternal separation displayed higher incidence of OSCC compared to controls. While in the control group only 15 rats (37%) had OSCC and 26 (63%) had OL, in the MS group OSCC was diagnosed in 23 rats (59%) and 16 rats had OL (41%) (Figs. 1 and 2). ELS increased OSCC incidence in almost 60% ($p=0.0450$) (Table 1). According to WHO classification, most of OSCCs from both groups were graded as well-differentiated (MS, 84.6% vs control, 75%) and all OLs had severe dysplasia. Tumors from the MS and control groups did not differ in relation to malignancy degree according WHO criteria ($p>0.05$) (data not shown).

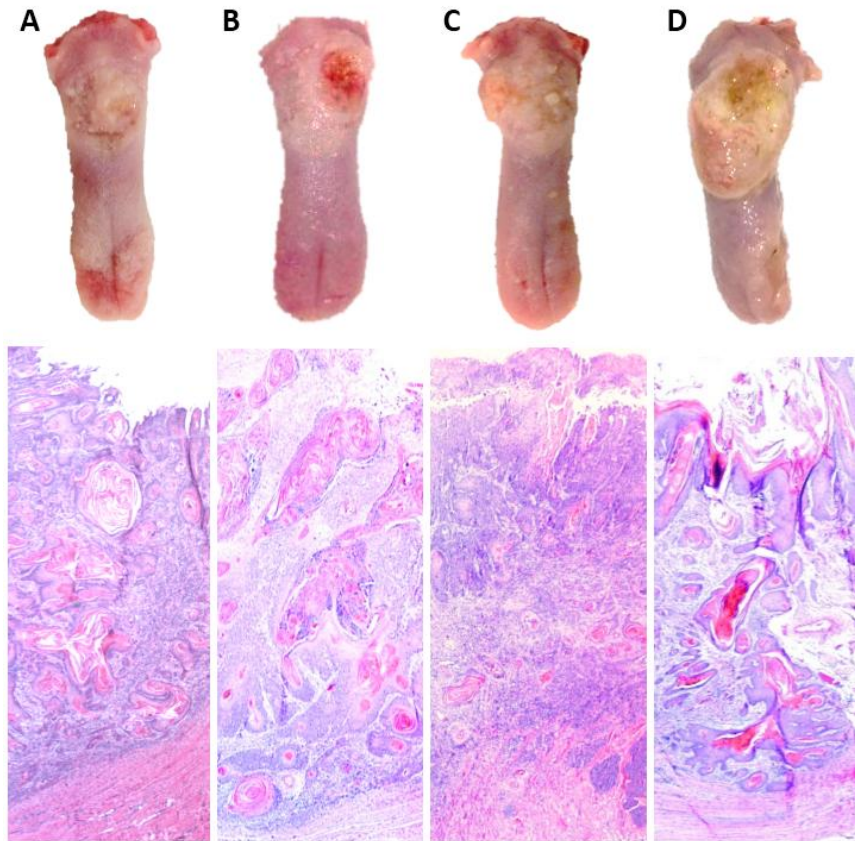


Figure 1. Clinical and histopathological features of chemically induced OSCC in control rats (A and B) and MS rats (C and D). (A) White plate with erosive and ulcerative areas. (B) Extensive deep ulcer. (C) Heterogeneous white plate and vegetative ulcer. (D) Large ulcerated lesion. Microscopic examination revealed OSCCs with well-differentiated cells arranged in islands of varying size, keratin pearls and chronic inflammatory infiltrate in the tumor stroma (H&E staining, original magnification x100).

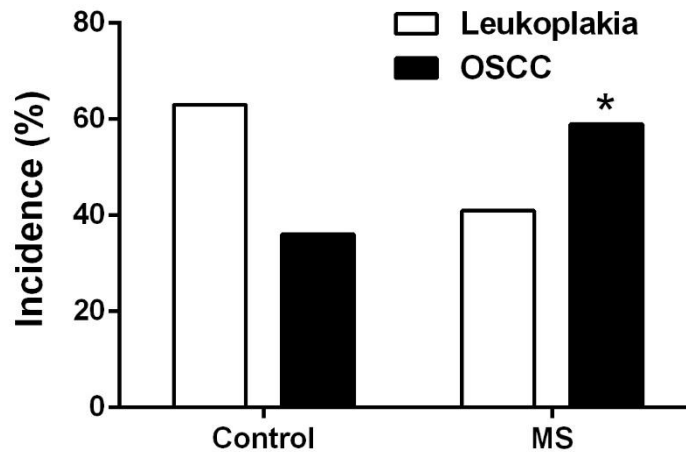


Figure 2. Incidence of OSCC and OL. Rats exposed to maternal separation displayed higher OSCC incidence compared with controls (MS, 59% vs control, 37%) (* $p=0.0450$). Bar graphs represent the percentage of OSCC and OL occurrence for both groups. * $p<0.05$.

Table 1. OSCC and OL incidence in MS and control groups.

Variables	Control	MS	p Value
OSCC	15 (37%)	23 (59%)	*0.0450
Leukoplakia	26 (63%)	16 (41%)	

Chi-square analysis.

3.2 Early life stress induces reduction of spleen weight in oral cancer rats

There was no difference in body weight variation between both groups (Fig. 3A).

Neonatal MS impairs spleen weight in rats who developed OSCC ($0,001828 \pm 8,392e-005g$) compared to control ($0,002438 \pm 0,0002004g$) ($p=0.0032$) (Fig. 3B).

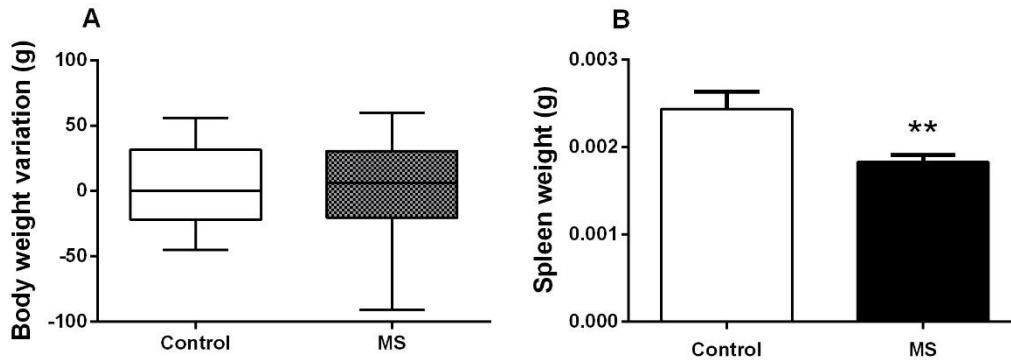


Figure 3. Body weight variation and spleen weight. (A) There were no differences in body weight variation between both groups. (B) Oral cancer rats who had been exposed to MS (n=23) showed lower spleen weight than non-stressed control animals with OSCC (n=15). Student's test-*t*; bar graphs represent mean with labeled error bars (\pm SEM). ** $p < 0.05$.

3.3 Adrenal gland weight and plasma norepinephrine levels

Neonatal MS impairs adrenal gland weight in rats with oral cancer ($5,977e-005 \pm 2,335e-006$ g) compared to non-stressed oral cancer rats ($6,964e-005 \pm 4,152e-006$ g) ($p=0.0318$) (Fig. 4A). Plasma norepinephrine levels from oral cancer rats were measured by ELISA assay. There were no significant differences in plasma norepinephrine levels between maternal separated oral cancer rats ($1560 \pm 72,36$ pg/mL) and control rats ($1602 \pm 161,3$ pg/mL) ($p=0.7901$) (Fig. 4B).

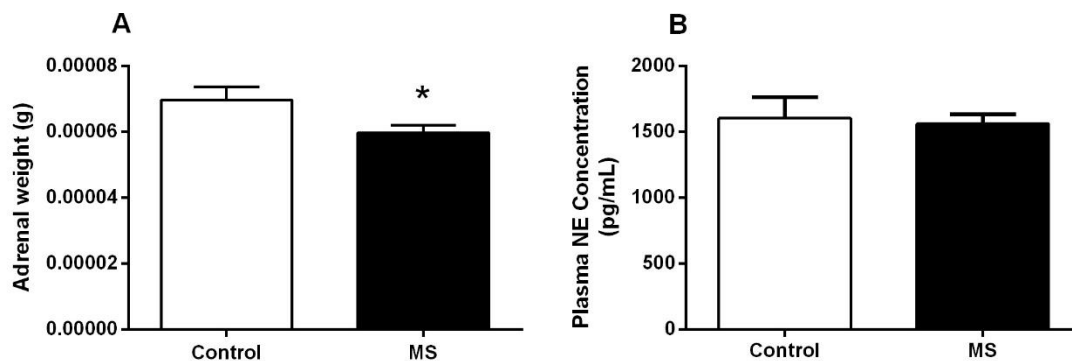


Figure 4. Adrenal gland weight and plasma norepinephrine levels. (A) Oral cancer rats exposed to MS (n=23) showed lower adrenal gland weight than non-stressed oral cancer rats (n=15). **(B)** Plasma from MS oral cancer rats (n=23) and non-stressed oral cancer rats (n=15) were tested for norepinephrine levels by ELISA. There was no significant difference in the plasma norepinephrine levels between both groups. Student's test-*t*; bar graphs represent mean with labeled error bars (\pm SEM). * $p < 0.05$.

3.4 Rats submitted to maternal separation displayed thicker and larger tumors

OSCCs from stressed rats by MS were thicker (2.858 ± 0.2788 mm) compared to control rats (1.877 ± 0.3228 mm) ($p = 0.0236$) (Fig. 5A). Rats from MS group also developed thrice-fold larger tumors (223.6 ± 46.11 mm³) than the control group (75.55 ± 14.45 mm³) ($p = 0.0359$) (Fig. 5B).

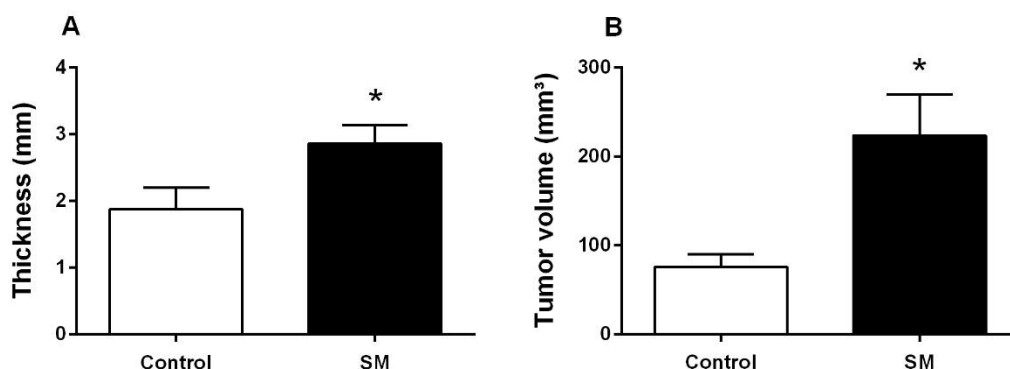


Figure 5. Tumor thickness and volume. (A) Student's test-*t* revealed that animals submitted to maternal separation had thicker tumors compared to control animals. **(B)** MS group also displayed large tumors than control group. Bar graphs represent mean \pm SEM. * $p < 0.05$.

3.5 Effects of maternal separation on the microscopic features of malignancy in OSCCs

3.5.1 Bryne's Multifactorial Grading System

According to Bryne's criteria, the majority invasive OSCCs from the MS group had moderated keratinization degree (54%) and host response (46%). Similarly, control group had the same proportion of high and moderated keratinization degree (50% high and 50% moderated) and half of OSCCs displayed high host response (50%). Most of invasive OSCCs from both groups showed little nuclear pleomorphism (MS, 69% vs control, 62.5%), patterns of invasion in small groups of tumor cells (MS, 54% vs control, 50%) and low aggressiveness (MS, 69% vs control, 87.5%). There were no statistical differences between groups for any microscopic variables according Bryne's criteria ($p>0.05$). Although the control group showed a higher percentage of low grade of aggressive tumors than MS group according to Bryne's final score, there were not statistical differences between the groups ($p=0.606$) (Table 2).

Table 2. OSCC microscopic features according to Bryne's Multifactorial Grading System

Variables	Control	MS	p Value
Keratinization degree			
High	4 (50%)	3 (23%)	0.2303
Moderate	4 (50%)	7 (54%)	
Minimal	0 (0%)	3 (23%)	
None	0 (0%)	0 (0%)	
Nuclear pleomorphism			
Little	5 (62.5%)	9 (69%)	0.9224
Moderate	2 (25%)	3 (23%)	
Intense	1 (12.5%)	1 (8%)	
Extreme	0 (0%)	0 (0%)	
Pattern of invasion			
Well-delineated	1 (12.5%)	1 (7.5%)	0.8454
Infiltrating	3 (37.5%)	4 (31%)	
Small groups	4 (50%)	7 (54%)	
Marked	0 (0%)	1 (7.5%)	
Host response			
High	4 (50%)	4 (31%)	0.2573
Moderate	3 (37.5%)	6 (46%)	
Slight	0 (0%)	3 (23%)	
None	1 (12.5%)	0 (0%)	
Agressiveness			
Low	7 (87.5%)	9 (69%)	0.6065
Intermediate	1 (12.5%)	4 (31%)	
High	0 (0%)	0 (0%)	

Chi-square analysis.

3.5.2 Brandwein-Gensler's histologic risk assessment

Accordance to Brandwein-Gensler's criteria, all invasive OSCCs from MS group showed a pattern of invasion with invasive islands with less than 15 tumor cells or single tumor cells invading, while only half of invasive OSCCs from control group showed this same pattern ($p=0.0046$). Most of invasive OSCCs from the rats exposed to MS showed perineural invasion in nerves smaller than 1mm (46%) compared to control rats of which vast majority of them (87.5%) did not display perineural invasion ($p=0.0460$). In regards to the risk of local recurrence and overall survival probability, MS rats had high risk of local recurrence (MS, 46% vs control, 25%) and poor survival probability (MS, 46% vs control, 25%) compared to control rats, however these results did not reach statistical significance ($p=0.3212$) (Table 3).

Table 3. OSCC microscopic features according to the Brandwein-Gensler's histologic risk assessment.

Variables	Control	MS	p Value
Pattern of invasion			
Broad, "fingers", large islands or invasive island > 15 cells	4 (50%)	0 (0%)	
Invasive islands < 15 cells, strands or single-cells	4 (50%)	13 (100%)	*0.0046
Widely dispersed	0 (0%)	0 (0%)	
Lymphocytic Response			
Dense continuous band	5 (62.5%)	3 (23%)	
Large patches	2 (25%)	7 (54%)	0.1947
Little or none	1 (12.5%)	3 (23%)	
Perineural invasion			
None	7 (87.5%)	7 (54%)	
< 1 mm	0 (0%)	6 (46%)	*0.0460
> 1 mm	1 (12.5%)	0 (0%)	
Risk of local recurrence			
Low	1 (12.5%)	0 (0%)	
Intermediate	5 (62.5%)	7 (54%)	0.3212
High	2 (25%)	6 (46%)	
Overall survival probability			
Good	1 (12.5%)	0 (0%)	
Intermediate	5 (62.5%)	7 (54%)	0.3212
Poor	2 (25%)	6 (46%)	

Chi-square analysis.

3.6 Maternal separation increased IL-6 and reduced p53 gene expression in OSCCs.

RT-PCR assay was performed to evaluate whether ELS by MS induced changes in the expression of OSCC progression-related genes (IL-6, TNF-alpha, VEGF, p53 and CDKN2A). OSCCs from rats exposed to MS had almost six times more expression of IL-6 mRNA ($565 \pm 233\%$) compared to tumors from the control animals ($p=0,0454$) (Fig. 6A). TNF-alpha ($223,2 \pm 91.58\%$) and VEGF mRNA ($227.4 \pm 126.2\%$) expressions were also higher in OSCCs from MS rats, but these results did not reach statistical significance ($p>0.05$) (Figure 6B and C). When the expression of tumor suppressor genes CDKN2A and p53 were analyzed, results showed that MS significantly reduced p53 mRNA expression by almost 45% ($-44.68 \pm 5.81\%$) in OSCCs compared to tumors from non-stressed rats ($p=0.0210$), but not significantly affected CDKN2A mRNA expression ($379 \pm 214.1\%$) ($p>0.05$) (Fig. 6D).

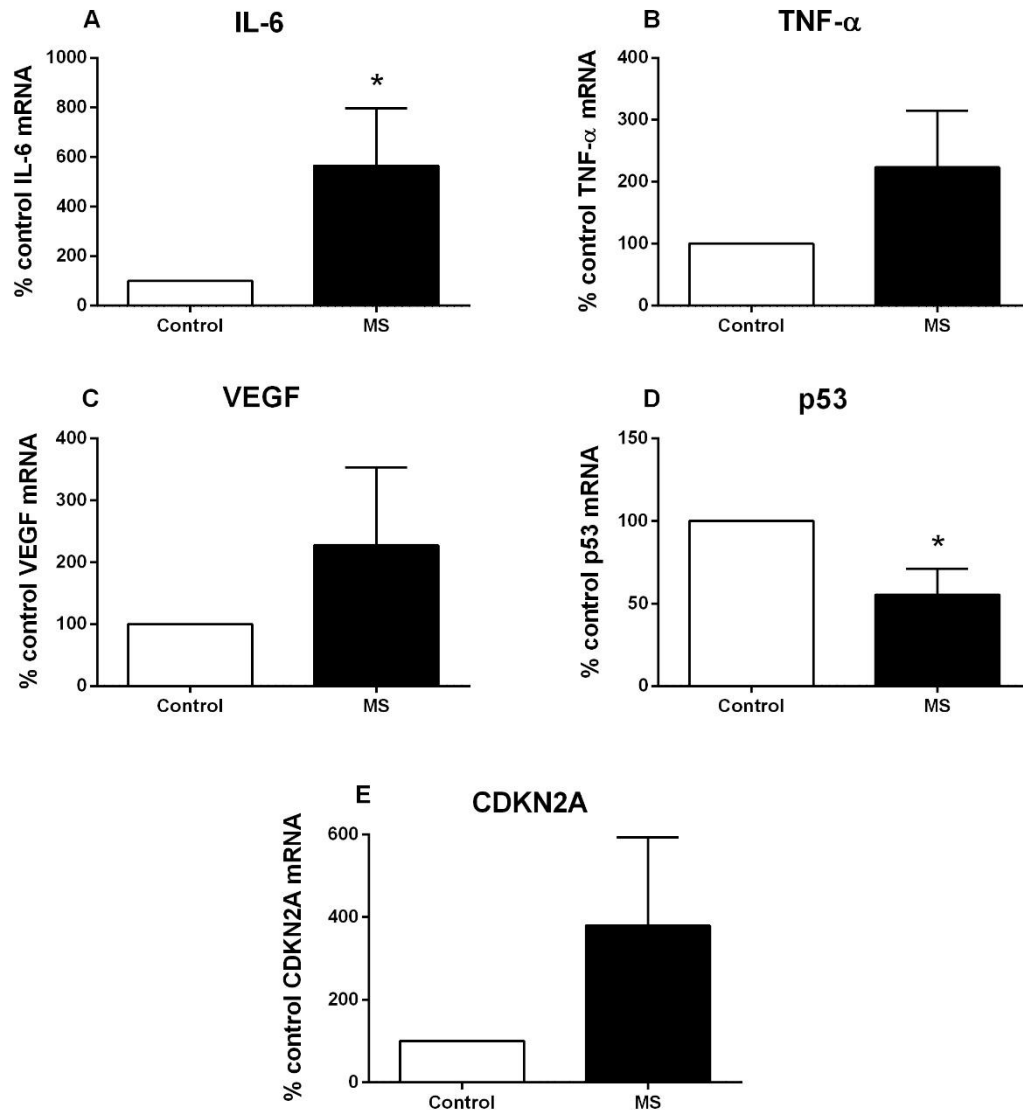


Figure 6. MS promoted upregulation of IL-6 mRNA and downregulation of p53 mRNA in the OSCCs. Rt-PCR assay was performed to evaluate the MS effects on the expression of OSCC progression-related genes IL-6 (A), TNF-alpha (B), VEGF (C), p53 (D) e CDKN2A (E) (MS, n=10 vs control, n=10). Student's test-*t*; bar graphs represent mean of increase percentage of mRNA expression compared to control (\pm SEM). **p*<0.05.

4. Discussion

In the present study, we have shown for the first time that ELS by MS during the neonatal period increased chemically induced OSCC occurrence in adulthood. Rats submitted to MS displayed almost 60% higher OSCCs occurrence when compared to non-stressed rats. Results found by Nakamura et al. [2011], in a breast cancer pre-clinical model indicated that MS can impaired NK cytotoxicity on cancer, hence on tumor immunity, increasing tumor metastasis. Recently, Boyd et al. [2010] observed that psychosocial stress during the neonatal period increased mammary tumorigenesis in adulthood. Although only a few studies have investigated the impact of ELS on cancer, several pre-clinical studies have shown that chronic stress may directly impacts different types of cancer by increasing tumor incidence and progression [Feng et al., 2012; Saul et al., 2005; Thaker et al., 2006; Lamkin et al., 2012; Kim-Fuchs et al., 2014; Zhao et al., 2015]. However, only two studies evaluated the implication of chronic restraint stress on OSCCs development [Rivera et al., 2011; Xie et al., 2015]. Rivera et al. [2011] showed that chronic restraint stress did not increase OSCCs incidence in a chemically induced oral carcinogenesis model. On the other hand, Xie et al., [2015] using an OSCC orthotropic model in mice, found that chronic restraint stress induced tumor growth and angiogenesis.

In addition to increasing OSCC occurrence, ELS by MS also promoted significant tumor growth. We observed that maternally separated rats had tumors 1.5-fold thicker and thrice-fold larger compared to non-stressed rats. Some studies have demonstrated that stress-related sympathetic neurotransmitters, such as norepinephrine and epinephrine, are powerful modulators of growth in several types of cancer [Tilan et al., 2010; Kim-Fuchs et al., 2014; Zhao et al.,

2015; Xie et al., 2015]. Stress-related tumor growth through β -adrenergic signaling might occur following the release of angiogenic factors from tumor cells and other cell types from tumor microenvironment [Tilan et al., 2010], as well as proteins involved in tumor invasion (eg. MMPs) [Lutgendorf et al., 2008a]. In our previous study, we demonstrated that norepinephrine, in a concentration that simulates stress conditions, increased OSCC cell proliferation [Bernabé et al., 2011]. Activation of the HPA axis by chronic stress have also been associated to tumor growth [Xie et al., 2015]. Studies with ovarian and oral cancer cells demonstrated that a cortisol concentration that simulates stress condition increased VEGF and IL-6 secretion, respectively. [Lutgendorf et al., 2003; Bernabé et al., 2011]. To our knowledge, this is the first time it has been shown that ELS can influence tumor growth in a pre-clinical chemically induced oral cancer model.

Although in our study rats submitted to ELS showed higher percentage of tumors with advanced stage of aggressiveness than non-stressed rats according to Bryne's criteria, this finding did not reach statistical significance. In a chemically induced OSCC rat model, Rivera et al. [2011] did not find differences on tumor severity between stressed and non-stressed animals using Bryne's analysis. Boyd et al. [2010] analyzed the invasive potential of mammary tumors and observed that mice submitted to MS had a greater number of invasive mammary carcinomas, which could be related to higher ER α expression (a nuclear receptor activated by estrogen) in tumors from stressed mice. In our study, microscopic analysis according to Brandwein-Gensler's criteria revealed a striking worst pattern of invasion in tumors developed by maternally separated rats. Moreover, OSSCs from rats stressed during the neonatal period showed higher occurrence

of perineural invasion. These results indicate a higher tumor severity on early life stressed rat when compared to non-stressed rats, given the microscopic findings as worst pattern of invasion and perineural invasion have been previously associated to cancer cell dissemination and metastasis [Liebig et al., 2009; Li et al., 2013].

Our results showed no significant differences between the groups in body weight variation during oral carcinogenesis induction period. In the few previous studies, which have investigated the ELS impact on cancer in pre-clinical models, there are no consistent results regarding ELS-induced body weight changes. [Nakamura et al., 2011; La Barba et al., 1971; La Barba et al., 1969]. This may be explained by the variations in MS methodology, such as frequency, duration and life period of separation among the different studies. Moreover, the cancer induction model and time of carcinogenesis or weighing period also may influence body weight. The protocol used in our study (3 hours of MS on post-natal days 1-21), did not result in significant body weight changes after MS followed by oral carcinogenesis. In the present study, MS induced a reduction on spleen weight in rats submitted to chemically induced oral carcinogenesis. Roque et al. [2014] observed that ELS by MS did not induce changes in spleen weight in adulthood, while Xiao et al. [2014] showed that MS reduced spleen mass in animals without cancer. Since the spleen is a lymphatic organ responsible for lymphocyte production, it is possible that ELS impairs spleen immune function. In fact, Roque et al. [2014] showed that MS reduced the percentage of CD8C T cells in the spleen.

Evidences show that ELS may induce long-lasting changes in behavioural and neuroendocrine parameters [Slotten et al., 2006]. Our results revealed that

ELS rats had reduced adrenal gland weight compared to non-stressed rats. Similar results were also observed in a previous investigation with non-cancer rats submitted to MS in neonatal period [Slotten et al., 2006]. These findings seem to differ from those found in chronically stressed animals in adulthood, who usually developed adrenal hypertrophy after stress induction [Ulrich-Lai et al., 2006]. We did not find increased systemic norepinephrine response in cancer rats submitted to MS, which was unexpected based in previous studies with non-cancer rats exposed to ELS and chronic stress [Gogberashvili et al., 2007; Esquivel et al., 2009; Uresin et al., 2004; Xie et al., 2015]. A pre-clinical study showed that maternally separated rats with metastasis exposed to a second stressor stimulus (restraint) had a lower corticosterone response compared to animals only exposed to MS [Nakamura et al., 2011]. We suggest that MS produces a stress pre-sensitization during the neonatal period resulting in an impaired SNS and HPA axes responses in adulthood. Therefore, when submitted to carcinogenesis during adulthood (which can also be a stressor factor), MS rats could have a less effective response on adrenal glands hypertrophy and blood catecholamine secretion compared to non-stressed animals. Future studies are needed to address whether norepinephrine concentrations in peripheral tumor tissue from cancer rats previously exposed to ELS are similar or not those observed to plasma.

IL-6 is a pro-inflammatory cytokine, secreted by immune and cancer cells, which mediates chronic inflammation and play a significant role in cancer onset and progression [Kumari et al., 2016; Gasche et al., 2011]. In OSCC, IL-6 may regulate cell proliferation and differentiation, and patient survival. [Wang et al., 2002; Shinriki et al., 2009 and 2011; Chen et al., 2012]. Furthermore, high levels

of circulating IL-6 have been associated with poor prognosis and shorter survival in OSCC patients [Guo et al., 2012]. An *in vivo* study demonstrated that chronic stress may lead to increased circulating IL-6 levels in non-cancer patients [Kiecolt-Glaser et al., 2003]. Stress-related hormones may also upregulate IL-6 expression in cancer cells [Nilsson et al., 2007; Yang et al., 2009; Bernabé et al., 2011]. In a previous *in-vitro* study, we showed that increased norepinephrine concentration, which would simulate stress conditions, enhanced IL-6 mRNA expression and protein production in OSCC cell lines [Bernabé et al., 2011]. Furthermore, stress hormone-induced OSCC cell proliferation was mediated through IL-6. Recently, Kim et al. [2017] observed that rats stressed by MS displayed higher plasma IL-6 levels compared to non-stressed rats. To our knowledge, the present study shows for the first time that ELS by MS increases IL-6 expression in cancer microenvironment. Brighenti et al. [2014], found a new pathway connecting IL-6 to cancer, showing that IL-6 downregulated expression of p53 in normal and tumor cells. Interesting, along with the increase of IL-6 expression in OSCC from MS rats, we also found a reduction of 45% in p53 expression in the same tissue compared to tumors from non-stressed rats. Tumor suppressor gene p53 plays a key role in genomic stability and cancer prevention [Liu et al., 2015]. Frequently, p53 is mutated in tumors and its activity is highly compromised in all cancer types [Meek, 2015]. Feng et al. [2012] demonstrated that attenuation of p53 function is mediated by elevated stress-related hormones levels, and that decreased function of p53 had a relevant role in chronic stress-induced tumorigenesis in mice. On the other hand, a pre-clinical study observed that p53 expression in non-cancer mammary glands from adult mice was not differentially affected by neonatal stress [Boyd et al., 2010].

In our hypothesis, effects generated by ELS leads to an increase of IL-6 and initiate reactions that decrease the expression of p53. Based on previous studies, IL-6 overexpression regulates positively the expression of proteins related to cell cycle, apoptosis and cell transformation (eg. c-myc, MDM2), which are associated to p53 pathway and tumorigenesis of different types of cancer [Brighenti et al. 2014; Deisenroth et al., 2010; Zhang et al., 2009; Shi et al., 2008 and 2011]. Therefore, IL-6 overexpression mediating a decrease of p53 could contribute to tumor development.

5. Conclusion

This study provides the first evidence that ELS increase tumor onset and progression in a chemically induced oral cancer model. Furthermore, our findings suggest that IL-6 overexpression and decrease of p53 expression could be a relevant part of the underlying mechanism behind the effects of ELS on oral carcinogenesis.

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Anexo A

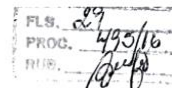
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CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA



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 da influência do estresse precoce de vida sobre a carcinogênese bucal: estudo histopatológico, molecular e comportamental em animais"**, Processo FOA nº 00495-2016, 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 2016.

VALIDADE DESTE CERTIFICADO: 02 de Outubro de 2018.

DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 02 de Novembro de 2018.

CERTIFICATE

We certify that the study entitled **"Influence of early life stress on oral carcinogenesis: histopathology, molecular and behavioral studies in animals"**, Protocol FOA nº 00495-2016, 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, 2016.

VALIDITY OF THIS CERTIFICATE: October 02, 2018.

DATE OF SUBMISSION OF THE FINAL REPORT: November 02, 2018.

[Handwritten Signature]
Profa. Ass. Dra. Maria Gisela Lafanjeira
Coordenadora da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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Anexo B

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