

Flávia Alves Verza

*Social isolation stress increases the incidence and growth
of chemically induced oral cancer in rats*



Araçatuba- SP

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chemically induced oral cancer in rats*

Dissertação apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista “Júlio de Mesquita Filho”, para obtenção do título de Mestre em Odontologia - Área de Concentração em Estomatologia.

Orientador: Professor Assistente Doutor Daniel Galera Bernabé.

Co- orientadora: Professora Adjunta Sandra Helena Penha de Oliveira.

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Dedicatória

Dedicatória

À minha família

Espero merecer todo o amor que tem por mim.

“Quando as raízes são profundas, não há razão para temer o vento.”

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Επίγραφε

Epígrafe

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Acredito nos que trabalham comigo.

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Farei o meu melhor a cada dia.

Somos o resultado das nossas crenças e sonhos.

Epígrafe



É exatamente disso que a vida é feita, de momentos.

Momentos que temos que passar, sendo bons ou ruins, para o nosso próprio aprendizado.

Nunca esquecendo do mais importante: **Nada nessa vida é por acaso.**

Absolutamente nada.

Por isso, temos que nos preocupar em fazer a nossa parte, da melhor forma possível.

A vida nem sempre segue a nossa vontade, mas ela é perfeita naquilo que tem que ser.

Chico Xavier

Resumo

Verza FA. Estresse por isolamento social aumenta a ocorrência e a progressão do câncer de boca induzido quimicamente em ratos. [dissertação]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2017.

Resumo

Estudos têm mostrado que o estresse crônico pode influenciar a progressão do câncer, porém sua influência sobre o início da doença é pouco compreendida. Dentre os eventos estressores, o estresse por isolamento social (EIS) afeta grande número de indivíduos, podendo induzir alterações neuro-hormonais e desordens emocionais. Embora estudos mostrem que o EIS pode aumentar a progressão de alguns tipos de neoplasias malignas, sua influência sobre o início e progressão do câncer de cabeça e pescoço é desconhecida. O objetivo deste estudo foi avaliar os efeitos do EIS sobre a incidência e progressão do carcinoma espinocelular (CEC) de boca induzido por 4-nitroquinolina 1-óxido (4NQO), bem como seus efeitos sobre o comportamento depressivo e a expressão de genes envolvidos na progressão do tumor. Sessenta ratos Wistar machos foram divididos em dois grupos de 30 animais: grupo isolado (ratos isolados socialmente desde os 21 dias de idade) e grupo controle (ratos mantidos em grupo). Ao atingirem 90 dias de idade, os animais de ambos os grupos foram submetidos à avaliação do comportamento depressivo pelos testes de suspensão pela cauda (TSC) e natação forçada (TNF). Logo em seguida, os animais foram submetidos à indução carcinogênica por meio da ingestão de 4NQO diluído na água de beber. Após 20 semanas, os animais foram avaliados quanto ao comportamento depressivo e posteriormente eutanasiados para análise histopatológica das lesões induzidas em língua. PCR em tempo real foi realizada para avaliar a expressão de RNAm para os genes TNF- α , IL-6, VEGF, MMP-2 e MMP-9 nos tumores de ambos os grupos. Os resultados mostraram que EIS aumentou a ocorrência de CEC de boca em 20.4% em relação ao grupo de ratos não estressados ($p=0.002$). Os tumores dos animais isolados apresentaram volume tumoral cerca de duas vezes maior ($p=0.0366$) em relação aos tumores dos animais do grupo controle. Os ratos estressados perderam mais peso corporal ($p=0.006$) e apresentaram menor peso do baço ($p=0.0121$) em relação aos ratos não estressados. EIS induziu aumento da expressão de RNAm para os genes TNF- α , IL-6, VEGF, MMP-2 e MMP-9, porém os resultados não atingiram significância estatística ($p>0.05$). O EIS induzido precocemente não induziu alterações significativas no comportamento depressivo mensurado pelo TSC e TNF antes e após a indução carcinogênica ($p>0.05$). Este estudo fornece as primeiras evidências de que o estresse crônico por isolamento social pode influenciar a incidência e progressão do CEC de boca induzido quimicamente em animais.

Palavras-Chave: Neoplasias; Neoplasias de cabeça e pescoço; Neoplasias bucais; Estresse psicológico; Isolamento social; Carcinogênese; Depressão.

Abstract

Verza FA. Social isolation stress increases onset and growth of chemically induced oral cancer in rats. [dissertação]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2017.

Abstract

Clinical and preclinical studies have shown that chronic stress may influence cancer progression, but its influence on the onset of the disease is poorly understood. Among the stressful events, social isolation stress (SIS) affects a large number of individuals, and may induce neurohormonal dysregulation and emotional disorders. Although investigations show that SIS may increase the progression of some types of malignancies, its influence on the onset and progression of head and neck cancer is unknown. In this study, we have evaluated the SIS effects on the occurrence and progression of induced oral squamous cell carcinoma (OSCC), as well as its effects on depression-like behavior and expression of genes involved in tumor progression. Sixty male Wistar rats were divided into two groups of 30 animals: isolated group (rats submitted to SIS) and grouped (rats non-stressed). In the SIS group, the animals remained individually isolated after completing 21 days of life, while in the control group the rats were kept in group. At 90 days of age, both groups were tested for depression-like behavior by the tail suspension (TST) and forced swimming (FST) tests. Afterwards, the animals were submitted to oral carcinogenesis induction with 4-nitroquinoline 1-oxide (4NQO) carcinogen diluted in drinking water. After 20 weeks, the animals were again tested for depressive behavior and euthanized for histopathological analysis in the tongue specimens. Real-time PCR was performed to evaluate mRNA expression for TNF-alpha, IL-6, VEGF, MMP-2 and MMP-9 genes in tumors from the both groups. The results showed that SIS increased OSCC occurrence by 20.4% in relation to control group ($p=0.002$). Isolated rats displayed tumor volume two-fold higher than grouped rats ($p=0.0366$). Stressed rats lost more body weight ($p=0.006$) and showed lower spleen weight ($p = 0.0121$) compared to non-stressed rats. SIS induced increase of mRNA expression for TNF-alpha, IL-6, VEGF, MMP-2 and MMP-9 genes, but these results did not reach statistical significance ($p>0.05$). SIS did not induce significant changes in depression-like behavior measured by TST and FST before and post-carcinogenesis induction ($p>0.05$). This study provides the first evidence that chronic stress by social isolation may influence the chemically induced OSCC occurrence and progression in animals.

Keywords: Neoplasms; Head and neck neoplasms; Oral neoplasms; Psychological stress; Social isolation; Carcinogenesis; Depression.

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Fig. 1. Clinical features of chemically induced OSCC (grouped rats, A and B vs isolated rats, C and D). (A) White plate with erosive areas. (B) Extensive white plate with a small ulcer. (C) Heterogeneous white plate. (D) Large ulcerated lesion. **Histopathological features (H&E staining, original magnification x100).** Histopathological examination revealed OSCCs with well-differentiated cells arranged in islands of varying size, keratin pearls and chronic inflammatory infiltrate in the tumor stroma.

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Fig. 2. Incidence of OSCC and oral leukoplakia. Chi-square analysis showed that socially isolated animals had a higher incidence of OSCC than group-housed animals ($p < 0.05$). Nevertheless, there was no significant difference between the groups regarding oral leukoplakia incidence ($p > 0.05$). Bar graphs represent the percentage of OSCC and oral leukoplakia for both groups.

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Fig. 6. Gene expression of TNF-alpha, IL-6, MMP-2, MMP-9 and VEGF in the OSCCs. **(A)** TNF-alpha, **(B)** IL-6, **(C)** MMP-2, **(D)** MMP-9 and **(E)** VEGF. There were no significant differences regarding the expression of tumor progression-related genes between groups ($p>0.05$). Student's test-*t*; bar graphs represent mean with labeled error bars ($\pm$ SEM).

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Lista de Abreviaturas

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4NQO – 4-nitroquinoline-1-oxide

ACTH – adrenocorticotropin

ADRs - adrenergic receptors

AKT – preoteín kinase A

ANS - autonomic nervous system

BDNF – brain-derived neurotrophic factor

cAMP – adenosine 3',5'-monofosfato cíclico

cDNA - complementary DNA

CEC – carcinoma espinocelular

CNS – central nervous system

CRH - corticotropin releasing hormone

CT - comparative threshold

CV – coefficient of variation

DNA – deoxyribonucleic acid

E - epinephrine

ECM – extracellular matrix

eg. – exemplo

EIS – estresse por isolamento social

FST – forced swimming test

H&E – hematoxylin and eosin

HPA – hypothalamic-pituitary-adrenal

IL-6 – interleukin-6

Ltd. - Limitada

MAPK – mitogen-activated protein kinase

MMP-2 – matrix metalloproteinase 2

MMP-9 – matrix metalloproteinase 9

mRNA – messenger ribonucleic acid

NE – norepinephrine

NK – natural killer

OSCC – oral squamous cell carcinoma

PKA – preotein kinase A

PND – Postnatal day

RNA – ribonucleic acid

RNase – ribonuclease

RQ - relative quantity

RT – room temperature

rt-PCR – reverse transcription polymerase chain reaction

SEM - standard error of the mean

SIS – social isolation stress

SNS – sympathetic nervous system

SP – São Paulo

TST – tail suspension test

UNESP – University Estadual Paulista

USA – United States of America

VEGF – vascular endothelial growth factor

vs - versus

WHO - World Health Organization

Sumário

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Social isolation stress increases the incidence and growth of chemically induced oral cancer in rats

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Verza FA. Social isolation stress increases onset and growth of chemically induced oral cancer in rats. [dissertação]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2017.

Abstract

Clinical and preclinical studies have shown that chronic stress may influence cancer progression, however its influence on the onset of the disease is poorly understood. Amongst the stressful events, social isolation stress (SIS) affects a large number of individuals, and may induce neurohormonal dysregulation and emotional disorders. Although investigations show that SIS may increase the progression of some types of malignancies, its influence on the onset and progression of head and neck cancer is unknown. In this study we have evaluated the SIS effects on the occurrence and progression of induced oral squamous cell carcinoma (OSCC), as well as its effects on depression-like behavior and expression of genes involved in tumor progression. Sixty male Wistar rats were divided into two groups of 30 animals: isolated group (rats submitted to SIS) and grouped (rats non-stressed). In the SIS group, the animals remained individually isolated after completing 21 days of life, while in the control group the rats were kept in group. At 90 days of age, both groups were tested for depression-like behavior by the tail suspension (TST) and forced swimming (FST) tests. Afterwards, the animals were submitted to oral carcinogenic induction with carcinogen 4-nitroquinoline 1-oxide (4NQO) diluted in drinking water. After 20 weeks, the animals were again tested for depressive behavior and euthanized for histopathological analysis in the tongue specimens. Real-time PCR was performed to evaluate mRNA expression for TNF-alpha, IL-6, VEGF, MMP-2 and MMP-9 genes in tumors from the both groups. The results showed that SIS increased OSCC occurrence by 20.4% in relation to control group ($p = 0.002$). Isolated rats displayed tumor volume two-fold higher than grouped rats ($p = 0.0366$). Stressed rats lost more weight ($p = 0.006$) and showed lower spleen volume ($p = 0.0121$) compared to non-stressed rats. SIS induced increase of mRNA expression for TNF-alpha, IL-6, VEGF, MMP-2 and MMP-9 genes, but these results did not reach statistical significance ($p > 0.05$). SIS did not induce significant changes in depression-like behavior measured by TST and FST before and after oral carcinogenesis induction ($p > 0.05$). This study provides the first evidence that chronic stress by social isolation may influence the chemically induced OSCC occurrence and progression in animals.

Keywords: Neoplasms; Head and neck neoplasms; Oral neoplasms; Psychological stress; Social isolation; Carcinogenesis; Depression.

Introduction

1. Introduction*

In the last decade, there has been a growing number of studies investigating the association between emotional disorders and cancer progression (Al-Azri et al., 2014; Capoccia et al., 2015; Madden et al., 2013; Rana et al., 2015; Shi et al., 2015; Smith et al. 2017; Xie et al., 2015). The responses to stress begin in the brain with the activation of the Central Nervous System (CNS) and secretion of several stress-related mediators via hypothalamic-pituitary-adrenocortical (HPA) axis and Sympathetic Nervous System (SNS) (Armaiz-Pena et al., 2008). The activation of the HPA axis due to stress occurs when neurons of the paraventricular nucleus of the hypothalamus secrete corticotropin releasing hormone (CRH) (Shin et al., 2016). This molecule is released into the bloodstream and reaches the anterior pituitary gland, which responds by releasing hormones such as adrenocorticotropin (ACTH) (Shin et al., 2016). The hormone ACTH is transported through the peripheral circulation to the adrenal glands, which synthesize and release cortisol into the tissue (Katz et al., 2003; Rana et al., 2015).

Chronic stress can induce a deregulated production of catecholamines such as norepinephrine (NE) and epinephrine (E) by the CNS and adrenal gland (Gunnar et al., 2007). These stress-related mediators may affect cancer progression by modulating cellular functions through adrenergic receptors (ADRs), which are found in many types of gastric (Shin et al., 2007), ovarian (Thaker et al., 2006), breast (Madden et al., 2013; Qin et al., 2015), and oral (Bernabé et al., 2011; Xie et al., 2015) cancers, and stromal cells in the tumor microenvironment (Cole et al., 2008; Eng et al., 2014). Activated adrenergic signaling may modify the activity of cancer cells influencing tumor angiogenesis, DNA repair system and inhibition of apoptosis (Antoni et al., 2006). For example, stress-related catecholamines NE and E increase the expression of genes involved in tumor angiogenesis and cell proliferation, such as vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6), as well as deregulate the production of matrix metalloproteinases (MMPs) types 2

* Normalização segundo a revista *Brain, Behavior, and Immunity* (Anexo B).

and 9; proteins that increase the potential for tumor invasion and metastasis (Lutgendorf et al., 2003; Lutgendorf et al., 2008a; Lutgendorf et al., 2008b; Yang et al., 2008; Sood et al., 2006; Yang et al., 2009).

Several stimuli in animals and humans may induce acute and chronic stress (Cacioppo et al., 2003; Dayas et al., 2001; Evans et al., 2012; Fone et al. 2008; Hermes et al; 2009; Lutgendorf et al., 2003; Sood et al., 2006). In general, these stressful events promote physical and psychological responses to the threat of an adverse condition. For example, stress responses may be triggered by physical stimulus (eg. pain, foot shock, immobilization), stressors that challenge cardiovascular homeostasis (eg. hemorrhage, exercise, heat exposure) and social stressors such as break interactions among individuals (eg. marital separation, death of partner, unemployment and social isolation) (Dayas et al., 2001). Investigations in the last decades have explored the effects of social isolation stress (SIS) in healthy people and individuals with illness (Al-Azri et al., 2014; Friedler et al., 2015; Holt-Lunstad et al., 2010; Karelina et al., 2011; Kroenke et al., 2003; Rana et al., 2015; Shi et al., 2015; Smith et al., 2017). Emotional stress due to social isolation does not affect only people who live alone. The breakup of relationships or even the feeling of loneliness can affect any individual, including those who reside with other people. Chronic stress by social isolation has direct impact on human health through Autonomous Nervous System (ANS) dysregulation, immune system and inflammatory markers (Campos et al., 2013; Rivera et al., 2011). Studies have shown that social isolation may increase mortality rates in humans (Al-Azri et al., 2014; House et al., 1988). In a meta-analysis investigation, researchers analyzed 148 surveys including more than 300 participants and found that people with strong "social relationships" survival rate were 50% higher than in isolated individuals (Holt-Lunstad et al., 2010).

Studies have shown that SIS can influence cancer progression (Capoccia et al., 2015; Hermes et al., 2009; Liu et al., 2005; Madden et al., 2013; Wu et al., 2000). For example, investigations revealed that SIS induced higher tumor growth in preclinical models of breast (Capoccia et al., 2015; Madden et al., 2013) and liver cancer (Liu et al., 2005). Increased cancer progression related to social isolation also has been seen in humans (Sprehn et al., 2009). SIS may affect tumor growth by altering the expression of genes associated with disease progression and affecting immune responses against the tumor cells (Liu et al., 2005; Williams et al., 2009). These effects of social isolation on

cancer progression are usually accompanied by alterations in the SNS with increased catecholamines levels found in the blood or tumor tissue (Capoccia et al., 2015; Liu et al., 2005). In addition to being able to influence the cancer progression, SIS may be predictive for emotional disorders such as depression (Reiche et al., 2004; Cacioppo et al., 2015; Wang et al., 2017). Although cancer patients display a higher occurrence of depressive symptoms after disease diagnosis (Raison & Miller, 2003; Shi et al., 2015; Smith et al., 2017) and evidences show that depressive phenotype may affect cancer progression (Penninx et al., 1998; Pyter et al., 2013), the relationships among SIS, depression and tumor occurrence in preclinical carcinogenesis models are poorly known

Oral cancer is the most common type of head and neck cancer and occurs more frequently in elderly men with a peak incidence in the fifth and sixth decades (Ferlay et al., 2010; Rettig & D'Souza, 2015). Squamous cell carcinomas (SCCs) constitute the majority of oral cancers, and the main risk factors for oral SCCs (OSCCs) are long-term tobacco and alcohol consumption (Zavras et al., 2001; Rettig & D'Souza, 2015). Few studies have analyzed the impact of chronic stress and its neurohormones on OSCC progression (Bernabé et al., 2011; Rana et al., 2015; Xie et al., 2015). Our previous study demonstrated that stress levels of norepinephrine increased cell proliferation and IL-6 secretion by the OSCC cell lines *in vitro* (Bernabé et al., 2011). This event occurs through beta-adrenergic receptors activation. Recently, Xie et al. (2015) using an OSCC orthotopic model in rats showed that restraint stress accelerated tumor growth. However, Rivera et al. (2011) showed that chronic restraint stress did not influence the incidence and severity of chemically induced OSCC by 4-nitroquinoline-1-oxide (4NQO) carcinogen. Until now, there are no studies, which have analyzed the effects of SIS on oral cancer occurrence and progression.

We hypothesized that social isolation could act as a cofactor of carcinogenic substances affecting oral carcinogenesis and increasing OSCC progression. In the present study, we have used an oral carcinogenesis preclinical model to investigate the impact of SIS on the occurrence of chemically induced OSCC in rats, as well as its role on depression-like behavior and tumor progression-related genes.

Materials and Methods

2. Materials and Methods

2.1 Animals and experimental conditions

All experiments involving animals were developed according to the guideline of principles for the use of animals in the laboratory and the study was approved by the Animal Ethics Committee from the Araçatuba Dental School - UNESP. Sixty Wistar male rats were kept under natural temperature conditions ($25 \pm 2^\circ \text{C}$), 12-h light and dark periods and *ad libitum* access to water and commercial diet (Purina[®], Paulínia-SP, Brazil).

2.2 Experimental design

The study was conducted with two experimental groups: 1) Isolated group: 28 male Wistar rats submitted to stress by social isolation (individually conditioned); 2) Grouped group (control): 28 male Wistar rats kept in groups of four animals per box. Both groups were maintained under the same conditions described above. When they reached adulthood (90 days of age), all the animals were submitted to tests for evaluating depression-like behavior levels. After behavioral tests, the animals from both groups were induced to oral carcinogenesis during 20 weeks. After completing 10 weeks of carcinogenesis, the animals were again submitted to behavioral tests. At the end of experimental period (before euthanasia) the animals were again underwent to depression behavioral tests.

2.3 Social isolation stress model

For induction of chronic stress by social isolation, the animals from isolated group were separated from the mother on the 21st postnatal day (PND), a fundamental period for the development of social behavior (Hermes et al., 2009; Williams et al., 2009; Wu et al., 2000). The animals were then kept isolated (one animal per box) in polypropylene boxes measuring 30 x 19 x 13 cm lined with shavings (Capoccia, et al. 2015; Liu, et al. 2005; Madden, et al. 2013; Williams, et al. 2009). Social isolation was maintained until the end of experimental period. For the control group, the animals were kept in group (4 animals) in polypropylene boxes measuring 41 x 34 x 16 cm lined with shavings. Both groups were maintained under the same external conditions previously mentioned. When they reached 90 days of life, the animals were evaluated for depressive-like behavior and later submitted to oral carcinogenesis.

2.4 Depressive-like behavior tests

Animals from both groups were tested for evaluation of depressive behavior prior to carcinogen treatment using the tail suspension and forced swimming tests. Both tests are based on the same principle: the animals to be exposed to an unavoidable situation. The animal's immobility times during the tests were used as depressed behavior measures.

Tail suspension test (TST). The TST was performed using a TST apparatus. The testing apparatus consisted of a 32 × 33 × 33 cm box with an open front side to allow visual observation during the test. The TST was initiated by securing rat by the tail with an adhesive tape positioned about 2.5 cm from the tail tip. Rats were suspended individually for 6 min and the duration of immobility behavior was determined. The time that they stay agitated or immobile was timed. At the beginning of the test, the

animals try to escape from suspension by vigorous movements and, after a few minutes, they tend to stay immobile. The immobility time was used to measure depression-like behavior (Porsolt, et al. 1977).

Forced swimming test (FST). The FST was performed 7 days after TST. FST was performed according to the protocol described by Porsolt, et al. (1977). The test was divided into two sessions: in the first session (pre-test), the rats were forced to swim for 15 minutes in a narrow cylinder (13 cm in diameter × 24 cm in height) containing water ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$). Pre-test was performed to adapt the animals for the test situation, thus providing higher results stability by raising the immobile behavior level during the second session. The second session (test itself) was performed 24 hours after the pre-test. In the second session, the animals were subjected to forced swimming test for 5 minutes under the same pre-test conditions and the agitation and immobility levels were measured with a specific chronometer according to the behavioral repetition time. Rats were considered immobile if they floated or made only those movements necessary to keep their heads above the surface of the water.

Both depressive-like behavior trials were videotaped for later analysis (AMCap DirectShow Video and Still Capture. Version 9.21). The videotaped test sessions were scored by two independent observers, who were blind to the experimental conditions. The two methods described previously were used to assess immobility time. Two days after FST, carcinogen treatment was started for oral cancer induction.

2.5 Oral carcinogenesis model

For oral cancer induction, the animals from groups were treated with 50 ppm of 4-nitroquinoline-1-oxide (4NQO) (Sigma-Aldrich, St. Louis, USA), diluted in drinking

water. All animals had free access to consume the water. The animals were euthanized by decapitation after 20 weeks (5 months) of carcinogenic induction (Katz, et al. 1985; Ribeiro, et al. 2004). Then, the tongue with the lesion was extracted for histopathological analysis and molecular assays.

2.6 Histopathological analysis

The tongues were longitudinally sectioned for histopathological analyzes. The tissues were fixed in 10% buffered formaldehyde (Merck, Darmstadt, Germany) for 48 hours, submitted to the routine laboratory procedures, embedded in paraffin blocks and stained with hematoxylin and eosin (H&E, Merck). The tongue lesions derived from carcinogen treatment were classified in oral leukoplakia and oral squamous cell carcinoma (OSCC). Oral leukoplakia is considered an early phase of OSCC development (Warnakulasuriya et al., 2007). For oral leukoplakia diagnosis, the following microscopic characteristics were considered: atrophy of the epithelium, hyperplasia with or without hyperkeratosis and epithelial dysplasia (van der Waal et al., 1997). According to World Health Organization (WHO Barnes et al., 2005) criteria, the epithelial dysplasia degree of the leukoplastic lesions was classified in mild, moderate or severe. Kujan's criteria were also used to evaluate the risk of malignant transformation of the oral leukoplakia specimens (Kujan, et al. 2006). In the OSCCs, histological grade of malignancy was evaluated by two experienced oral pathologists according with Bryne's criteria (Bryne, 1998), in which the histopathological features keratinization degree, nuclear pleomorphism, pattern of invasion and host response (lymphoplasmocytic infiltrate) were observed in the invasive front of tumors.

2.7 Tumor volume

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 mm³) was calculated by multiplying the three values found.

2.8 Body weight variation and spleen weight

Body weight of each animal was recorded weekly. Initial (before starting treatment with 4NQO) and final (before the animal's euthanasia) body measurements were used to calculate the weight variation during carcinogenic induction. Spleen weight was obtained right after the animal's euthanasia.

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

In order to assess whether SIS affects the mRNA expression levels of genes involved in the OSCC progression, real time PCR assay was performed. Total RNA was extracted using the TRIzol reagent. 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 a specific kit (High Capacity RNA to cDNA kit; Invitrogen Life Technologies) using 2 µg of total RNA according to the manufacturer's recommendations. mRNA levels were measured by TaqManTM system in the StepOne Real-Time PCRTM equipment (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. The primers for TNF-alpha (Rn00562055_m1),

IL-6 (Rn00569848_m1), MMP-2 (Rn01538170_m1), MMP-9 (Rn00579162_m1) and VEGF (Rn01511601_m1) were analyzed. β -actin (Rn00562253_m1) gene was used as endogenous control. The reactions were performed with a 20 μ L final volume. Thermal cycling conditions followed all the manufacturer's recommendations. The expression levels of each gene in each sample were normalized to β -actin mRNA levels. The results were analyzed using the comparative threshold cycle (CT) method.

2.10 Statistical analysis

GraphPad prism 6.01 (GraphPad Software Inc., San Diego, CA, USA), was used to perform all statistical tests. In order to evaluate whether SIS influences the OSCC incidence and malignancy degree, Chi-square analysis was used. Student's test-*t* was used to determine if there were differences between two groups regarding clinicopathological variables, depressive-like behavior and mRNA expression for tumor progression related genes. Results were presented in mean $\pm$ standard error of the mean (SEM). The critical level were set at 5% ($p < 0.05$).

Results

3. Results

3.1 Social isolation stress increases chemically induced OSCC incidence

First, we have investigated the effect of SIS on chemically induced OSCC incidence in male Wistar rats. In the group of socially isolated animals, 26 rats (92.8%) developed OSCC and only 2 rats (7.2%) had oral leukoplakia after 20 weeks of chemically induced carcinogenesis (Figs. 1 and 2). In the grouped animals, 21 rats (72.4%) developed OSCC while 8 animals (27.6%) had oral leukoplakia. According to WHO classification (Barnes, et al. 2005), all OSCCs of both groups were graduated as well-differentiated tumors. In the socially isolated group, there was an increase of 20.4% in OSCC incidence compared to non-stressed control group ($p=0.002$) (Fig. 2).

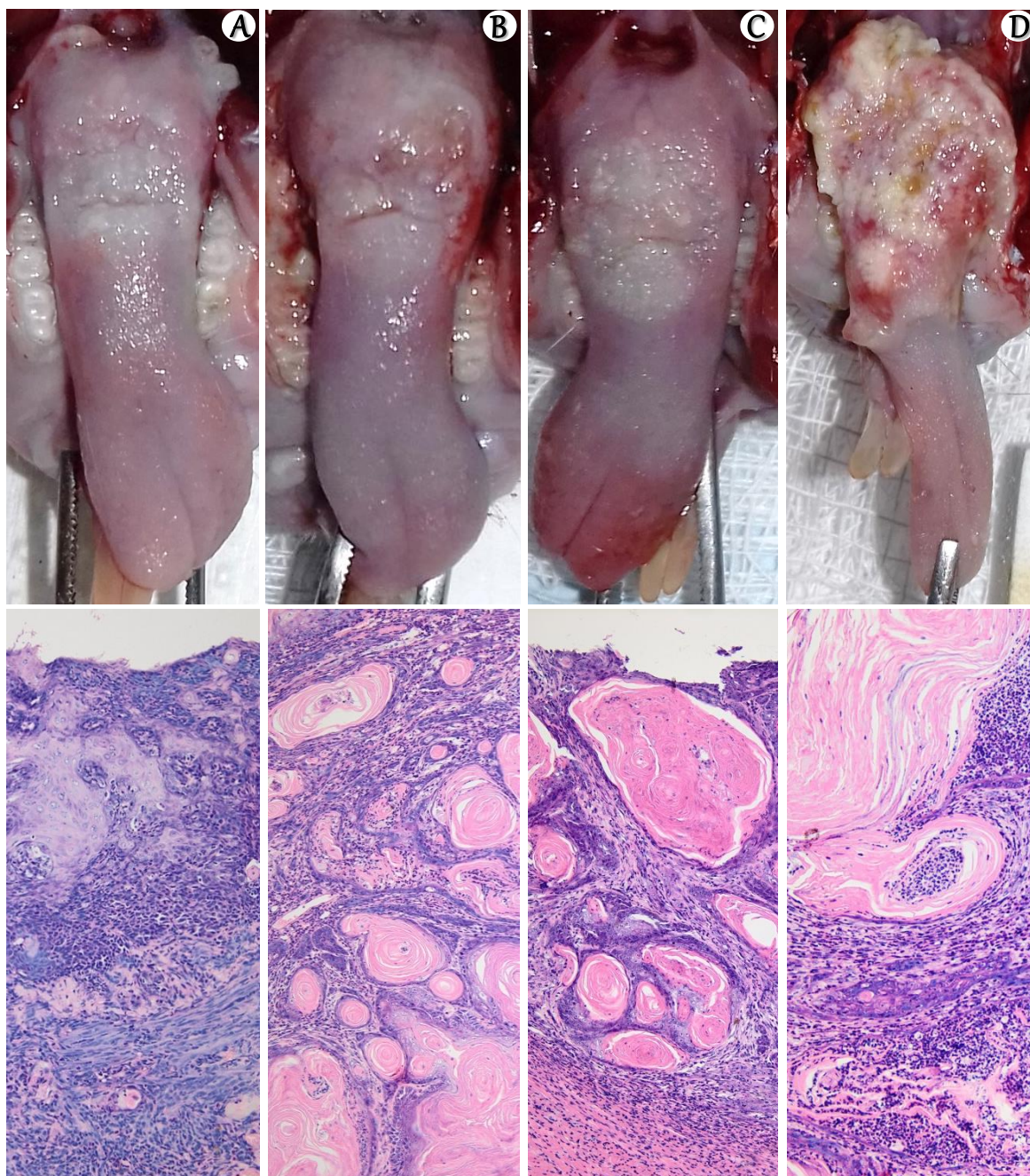


Fig. 1. Clinical features of chemically induced OSCC (grouped rats, A and B vs isolated rats, C and D). (A) White plate with erosive areas. (B) Extensive white plate with a small ulcer. (C) Heterogeneous white plate. (D) Large ulcerated lesion. Histopathological features (H&E staining, original magnification x100). Histopathological examination revealed OSCCs with well-differentiated cells arranged in islands of varying size, keratin pearls and chronic inflammatory infiltrate in the tumor stroma.

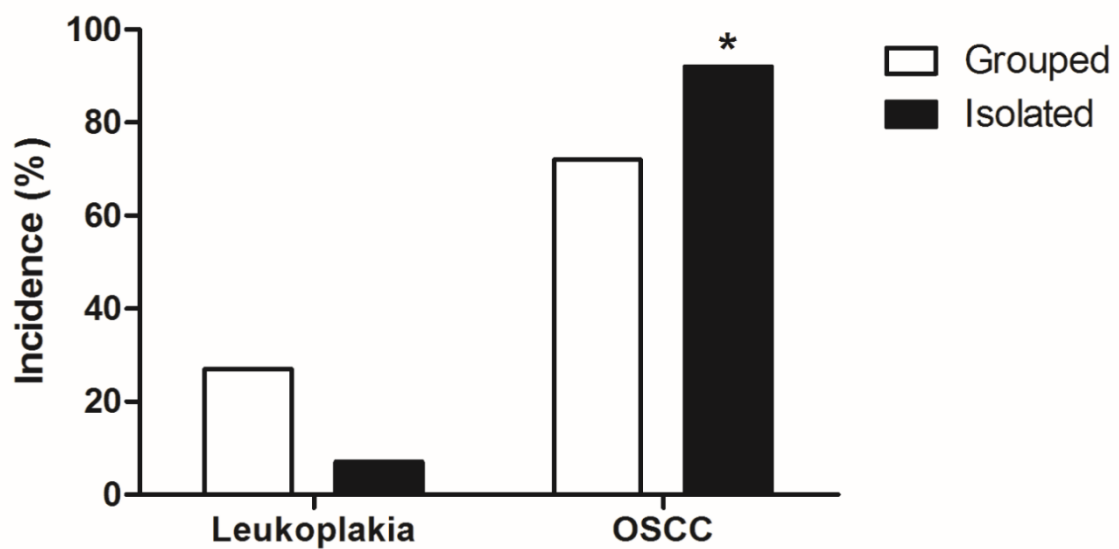


Fig. 2. Incidence of OSCC and oral leukoplakia. Chi-square analysis showed that socially isolated animals had a higher incidence of OSCC than group-housed animals ($p < 0.05$). Nevertheless, there was no significant difference between the groups regarding oral leukoplakia incidence ($p > 0.05$). Bar graphs represent the percentage of OSCC and oral leukoplakia for both groups.

3.2 Socially isolated rats display larger tumors than group-housed rats

Our results showed that rats subjected to SIS developed larger OSCC (646.8 ± 130.5 mm³) compared to group-housed rats (290.3 ± 62.87 mm³) ($p=0.0366$) (Fig. 3A). Stressed animals by social isolation exhibited OSCCs approximately two-fold larger than control group (Fig. 3A).

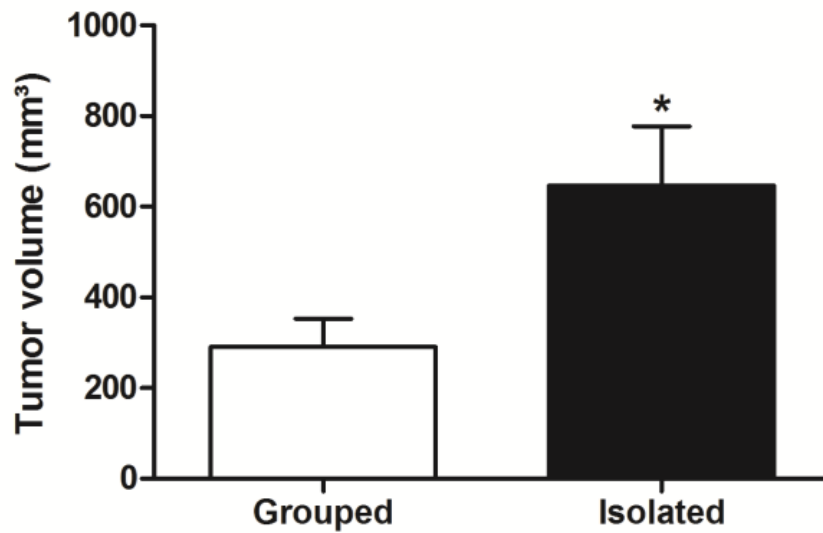


Fig. 3. Tumor volume. Student's test-*t* revealed that socially isolated rats had increased tumor volume compared to group-housed rats ($p=0.0366^*$). Bar graphs represent mean with labeled error bars ($\pm$ SEM).

3.3 Social isolation stress induces body weight loss and reduction of spleen weight in rats subject to oral carcinogenesis

Socially isolated rats lost weight while grouped animals gained body weight during the 20 weeks of carcinogenic induction (isolated, -29.46 ± 14.77 g vs grouped, 54.24 ± 17.33 g) ($p=0.006$) (Fig. 4A). At the end of carcinogen treatment, spleen weight was significantly reduced in the isolated rats (0.001508 ± 0.0001054 g) compared to control animals (0.001969 ± 0.0001242 g) ($p=0.0121$) (Fig. 4B).

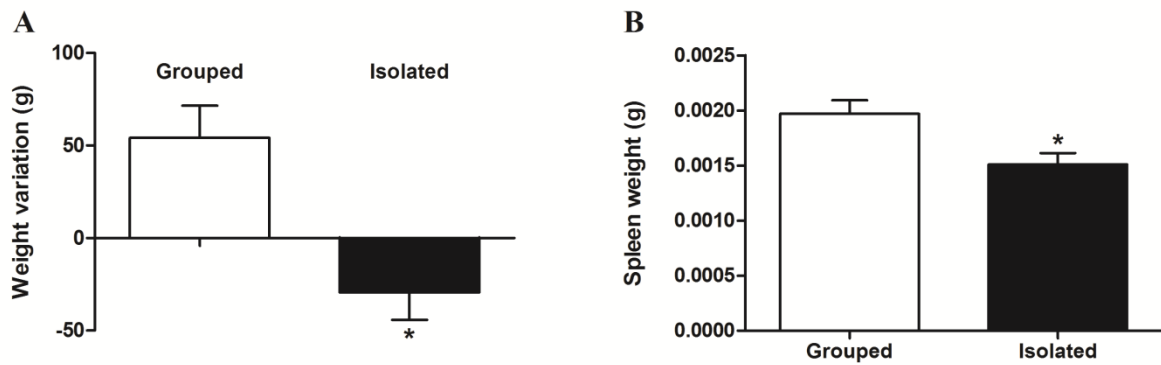


Fig. 4. Body weight variation and spleen weight. (A) Socially isolated rats showed weight loss while group-housed rats exhibited body weight gain during carcinogen treatment ($p=0.006$). (B) After carcinogenic induction, isolated rats showed lower spleen weight than grouped animals ($p=0.0121$). Student's test-*t*; bar graphs represent mean with labeled error bars ($\pm$ SEM).

3.4 Effects of social isolation stress on the microscopic features of malignancy in OSCCs

In both groups, the majority of OSCCs showed a high degree of keratinization (isolated, 54.6% vs grouped, 55%), little nuclear pleomorphism (isolated, 50% vs grouped, 65%), well-delineated infiltrating borders (isolated, 41% vs grouped, 60%) and inflammatory cell infiltration marked (isolated, 63.6% vs grouped, 55%) ($p>0.05$) (Table 1). According to the Bryne's criteria (Bryne, 1998), most OSCCs were classified as little aggressive tumors (isolated, 76.2% vs grouped, 66.6%) and therefore, there were no significant differences in the degree of tumor malignancy between isolated and grouped animals ($p=0.4945$) (Table 1). The majority of oral leukoplakia tissues was classified as high-risk for malignancy (isolated, 100% vs grouped, 83.3%) and no differences were found between groups ($p=0.6592$) (Table 2).

Table 1. OSCC microscopic features according to the Bryne's criteria.

Variables	Grouped	Isolated	p Value
Keratinization degree			
High	11 (55%)	12 (54.6%)	0.4494
Moderate	05 (25%)	08 (36.4%)	
Minimal	02 (10%)	00 (0%)	
None	02 (10%)	02 (9%)	
Nuclear pleomorphism			
Little	13 (65%)	11 (50%)	0.1174
Moderate	03 (15%)	09 (41%)	
Abundant	04 (20%)	01 (4.5%)	
Extreme	00 (0%)	01 (4.5%)	
Pattern of invasion			
Well-delineated	12 (60%)	09 (41%)	0.4594
Infiltrating	07 (35%)	09 (41%)	
In small groups	01 (5%)	03 (13.5%)	
Marked	00 (0%)	01 (4.5%)	
Host response			
Marked	11 (55%)	14 (63.6%)	0.4945
Moderate	09 (45%)	08 (36.4%)	
Slight	00 (0%)	00 (0%)	
None	00 (0%)	00 (0%)	
Aggressiveness			
Little	14 (66.6%)	16 (76.2%)	0.4945
Very	07 (33.4%)	05 (23.8%)	

Chi-square analysis.

Table 2. Malignancy risk of the leukoplakia tissues according to the Binary Grading System (Kujan's criteria).

Group	High-risk	Low-risk	p Value
Grouped	5 (83.3%)	1 (16.7%)	0.6592
Isolated	1 (100%)	0 (0%)	

Chi-square analysis.

3.5 Depressive-like behavior in rats subjected to social isolation stress

To assess whether SIS was able to increase depressive like-behavior in the rats who would be to subject to chemical oral carcinogenesis, the animals were tested for depressive-like behaviors before they had been treated with 4NQO carcinogen. SIS did not influence the depressive-like behavior ($p>0.05$) (Fig. 5). Although socially isolated rats have showed a higher depressive behavior than group-housed rats before cancer induction, the tail suspension (isolated, 177.7 ± 12.30 s vs grouped, 154.9 ± 12.13 s) ($p=0.1914$) (Fig. 5A) and forced swim (isolated, 174.6 ± 17.43 s vs grouped, 169.5 ± 16.87 s) ($p=0.8345$) (Fig. 5D) tests did not reveal significant differences. In addition, to evaluate whether carcinogenic induction promoted changes in depressive behavior, the animals were also submitted to the behavioral tests 10 and 20 weeks after carcinogen treatment. In any period, the 4NQO treatment did not promote significant changes in depressive-like behavior in both groups ($p>0.05$) (Figs. 5B, C, E and F).

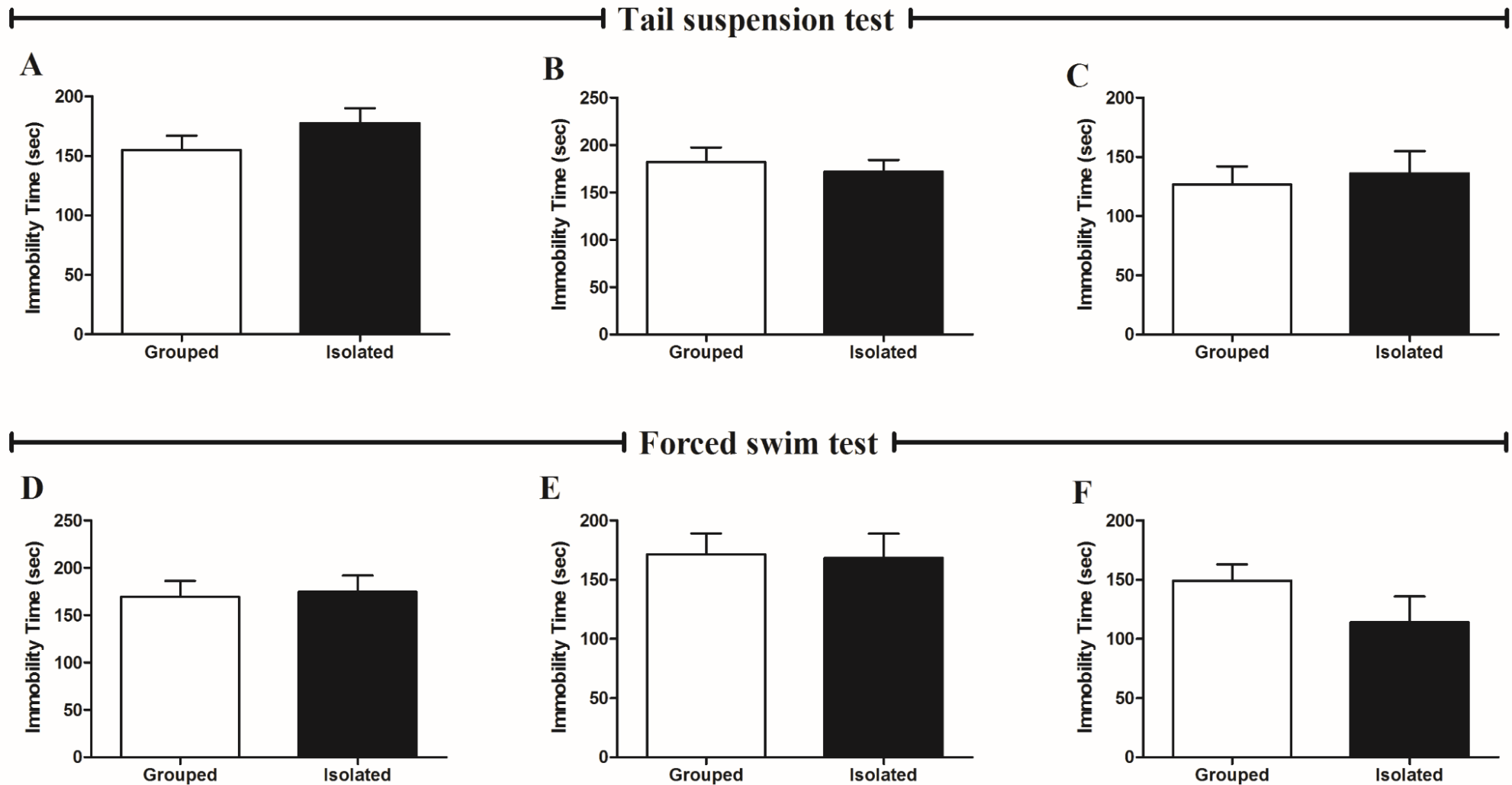


Fig. 5. Analysis of depressive behavior. (A and D) One-week pre-carcinogenic induction. (B and E) Ten weeks of carcinogenic induction. (C and F) Twenty weeks of carcinogenic induction. In all moments, there were no significant differences regarding the depressive-like behavior between groups ($p>0.05$). Student's test- t ; bar graphs represent mean with labeled error bars ($\pm$ SEM).

3.6 Expression of tumor progression-related genes in OSCCs from isolated and grouped rats

In OSCCs, rt-PCR assay was performed to evaluate whether chronic stress caused by social isolation induced changes in the expression of tumor progression-related genes (IL-6, TNF-alpha, MMP-2, MMP-9 and VEGF). Although gene expression has been considerably increased in the tumors from socially isolated rats, mRNA expression for proinflammatory cytokines TNF-alpha ($192.805 \pm 70.247\%$) ($p=0.8256$) (Fig. 6A) and IL-6 ($280 \pm 198.298\%$) ($p=0.4873$) (Fig. 6B), as well as for MMP-2 ($424.154 \pm 256.656\%$) ($p=0.5996$) (Fig. 6C), MMP-9 ($375.656 \pm 165.473\%$) ($p=0.7908$) (Fig. 6D) and VEGF ($938.031 \pm 599.473\%$) ($p=0.8502$) (Fig. 6E) were not significant when compared to control group.

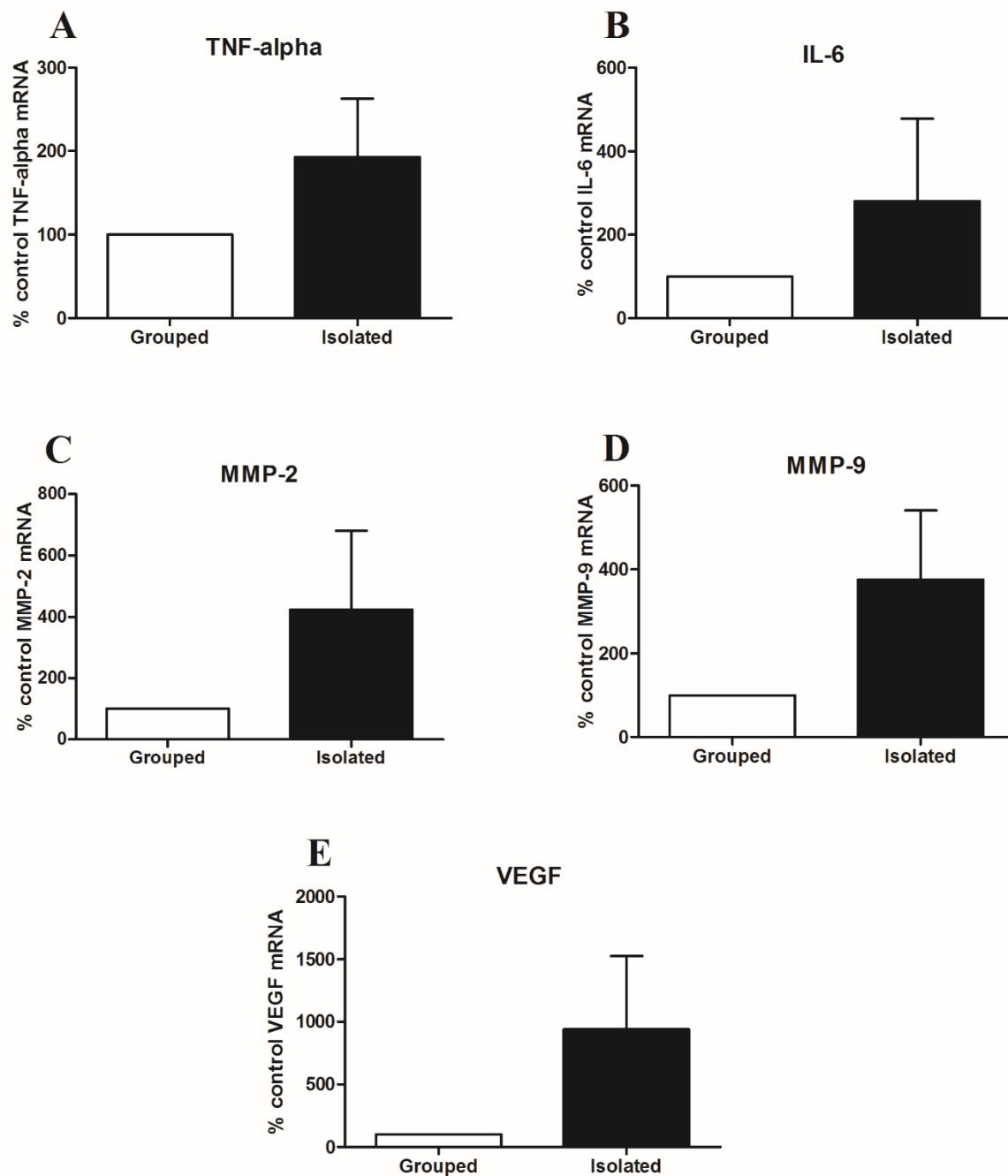


Fig. 6 Gene expression of TNF-alpha, IL-6, MMP-2, MMP-9 and VEGF in the OSCCs. (A) TNF-alpha, (B) IL-6, (C) MMP-2, (D) MMP-9 and (E) VEGF. There were no significant differences regarding the expression of tumor progression-related genes between groups ($p > 0.05$). Student's test-*t*; bar graphs represent mean with labeled error bars ($\pm$ SEM).

Discussion

4. Discussion

Evidences from clinical studies have shown that social factors can influence physical and mental health (Friedler et al., 2015). In this context, social isolation has been considered a potent psychological stressor characterized by the lack of social contacts, such as spouse, family and friends (Cacioppo et al., 2003; Friedler et al., 2015). When chronic, SIS may deregulate the HPA axis and autonomic nervous system (ANS) resulting in neuroendocrine changes, which can increase the incidence, morbidity and mortality of a great number of diseases seen in socially isolated individuals (Friedler et al., 2015). In humans, per example, an association between social isolation and more rapid cancer progression has been described (Kroenke et al., 2006; Sprehn et al., 2009). Likewise, social isolation stress has also been associated to tumor progression in pre-clinical models of ovarian (Lutgendorf et al., 2005), liver (Liu et al., 2005) and breast cancer (Capoccia et al., 2015; Madden et al., 2013; Williams et al., 2009). However, by our knowledge, there were no evidences that SIS could influence OSCC incidence and progression in rats submitted to chemically induced carcinogenesis.

Our results demonstrated that socially isolated rats had higher OSCC occurrence than group-housed animals. There was an increase of 20.4% in OSCC incidence in the socially isolated group. Few studies have evaluated the influence of chronic stress on oral cancer incidence and progression in vivo (Rivera et al., 2011; Xie et al., 2015). In rats, Rivera et al. (2011) showed that restraint stress did not influence the incidence and severity of chemically induced OSCC by carcinogen 4NQO. Nevertheless, Xie et al. (2015) demonstrated that restraint stress induced OSCC growth in a nude mice orthotopic model. In the present study, it is worth mentioning that SIS was initiated in

the post-weaning period, differently from restraint stress, which is usually carried out only in adulthood (Rivera et al., 2011; Xie et al., 2015). Interestingly, some biobehavioral negative effects derived from SIS may occur since post-weaning period to adulthood (Arakawa et al., 2003; Ferdman et al., 2007; Leng et al., 2004). Rat pups that do not develop in a social environment, per example, tend to be more anxious adults (Arakawa et al., 2003; Ferdman et al., 2007). Likewise, pro-tumorigenic mechanisms activated by SIS could affect the oral carcinogenesis, and these effects would be more significant when stress starts in the early stages of life.

In the present study, in addition to increased oral cancer incidence, SIS also promoted a significant OSCC clinical progression. Our results revealed that socially isolated rats had OSCCs approximately two-fold larger than group-housed rats. Similarly, in another pre-clinical study, singly-housed female mice developed chemically induced breast carcinomas with greater tumor volume and growth compared to grouped mice (Madden et al., 2013). In male Balb/c mice, Wu et al. (2000) showed that SIS resulted in an increased tumor growth of specific colon cancer cells after intrahepatic implantation. Furthermore, Williams et al. (2009) demonstrated that socially isolated female mice had greater mammary gland tumors than grouped animals. In addition to preclinical investigations, clinical studies have shown that privation of social support and marital status may affect cancer prognosis (Aizer et al., 2013). Two recent studies, one with head and neck cancer patients (Inverso et al., 2015) and another conducted only OSCC patients (Shi et al., 2017), showed that married patients had a lower occurrence of regional metastasis and better survival than widowed or divorced patients. Although recent findings have shown that SIS increases directly spleen weight in association with reduced splenic norepinephrine concentrations in an animal breast cancer model (Madden et al., 2013). Our results also showed that isolated animals had a

decrease in spleen weight after induced oral carcinogenesis. Madden et al. (2013) found that SIS increased spleen weight in association with reduced splenic norepinephrine concentrations in a breast cancer preclinical model. However, in this study spleen weight evaluation was made only three days after mice have been isolated. For other hand, chronic stress including SIS decreased spleen weigh and splenocytes activity in animals with or without cancer (Popović et al., 1999; Wu et al., 2000). It is well known that specific splenic macrophage populations are sensitive to neurohormonal changes derived from SIS and are important regulators of immune response during tumor progression (Cacioppo et al., 2015). Further studies are needed to evaluate the impact of SIS on the macrophage populations in the spleen and microenvironment of OSCC chemically induced. In the present study, isolated rats lost body weight compared to non-stressed rats. This finding may have been derived from the synergistic effect of SIS with the carcinogen on gastrointestinal system affecting rat's nutritional status, as well as associated to cancer-induced cachexia. However, some previous studies in preclinical cancer models have shown that individually housed animals often do not lose body weight after carcinogenic induction (Hermes et al., 2009; Weiss et al., 2004; Wu et al., 2000).

In animal models, chronic stress can still promote cancer progression associated to increased tissue expression of tumor progression-related genes (Thaker et al., 2006). In the present research, to evaluate whether SIS increased gene expression of pro-inflammatory cytokines TNF-alpha and IL-6 (cell proliferation and angiogenesis) (Bernabé et al., 2011; Yang et al., 2009), MMP-2, MMP-9 (cell migration) (Lutgendorf et al., 2008) and VEGF (angiogenesis) (Lutgendorf et al., 2003; Thaker et al., 2006; Yang et al., 2009) we have analyzed the expression of mRNA for each target gene by rt-PCR in the OSCCs samples. In our findings, mRNA expression for all tumor

progression-related genes was considerably higher in the OSCCs specimens from the isolated group, but the results were not significant. These negative results may be due to high data variability found in the gene expression analyses. Clinical and experimental investigations have demonstrated a well-established relationship between social factors and increased expression of tumor progression-related genes or proteins (Lutgendorf et al., 2002; Thaker et al., 2006; Wu et al., 2000; Yang et al., 2009). In patients with ovarian carcinoma, per example, higher levels of social support have been correlated with lower VEGF levels in the serum (Lutgendorf et al., 2002). In male mice, SIS can increase tumor angiogenesis, and this event may be associated with the up-regulation of angiogenesis-related factors, such as TNF-alpha and VEGF (Wu et al., 2000).

Chronic social isolation induces a variety of psychological symptoms in humans including depression (Cacioppo et al., 2015; Fone et al., 2008; Karelina et al., 2011). Likewise, SIS leads to several biobehavioral changes in the isolated animals, which usually develop a more depressive behavior than group-housed animals (Reiche et al., 2004; Wang et al., 2017). In our study, SIS did not influence the rat's depressive behavior measured by immobility time in TST and FST. Previous studies with non-cancer preclinical models have showed a more depressive-like behavior for socially isolated animals (Evans et al., 2012; Sun et al., 2013; Ma et al., 2016). However, in these studies, SIS was induced in adolescence or adulthood, after they had lived in group for an earlier period of their lives, differently from our study, in which the animals were isolated from post-weaning. In another study, Pisu et al. (2011) demonstrated that 4 weeks of SIS started in the post-weaning period induced shorter immobility time in the FST (less depressive-like behavior) compared to grouped rats. Our results also revealed that oral cancer occurrence did not induce depressive-like behavior neither in isolated group nor in control group. Lamkin et al. (2011) examined

the effect of tumor on depressive-like behavior in a mice ovarian cancer model. Depression-like behavior was analyzed through two different methods (sucrose intake and TST). Tumor induced a significant reduction in sucrose intake relative to control levels. However, like our findings with oral cancer, there was no significant effect of ovarian tumor on TST immobility. These data may indicate that although studies show that depressive behavior may be predictive for progression of some types of cancer (Penninx et al., 1998; Pyter et al., 2013), the tumor's effects on depressive behavior in preclinical models may be dependent on the sensitivity and type of method used to assess the status behavioral. Despite this, our data suggest that influence of SIS on OSCC onset and progression in rat oral cancer model is not dependent on depressive phenotype.

Conclusion

5. Conclusion

This study demonstrate for the first time that social isolation stress may increase OSCC occurrence and progression in a rat oral carcinogenesis model. Socially isolated rats display higher OSCC incidence and larger tumors than group-housed rats. Together, our results demonstrate that emotional disorders such as SIS can influence the onset and progression of OSCC. Future studies are needed to investigate the neurohormonal, inflammatory and immune mechanisms involved in social isolation-induced oral carcinogenesis and tumor progression.

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* Normalização segundo a revista *Brain, Behavior, and Immunity* (Anexo B).

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Anexo A

6. Anexo A



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"



CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

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

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado "**Análise da influência do estresse crônico por isolamento social sobre a carcinogênese bucal**", Processo FOA nº 2015-00687, 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 05 de Agosto de 2015.

VALIDADE DESTE CERTIFICADO: 10 de Março de 2016.

DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 10 de Abril de 2016.

CERTIFICATE

We certify that the study entitled "**Analysis of the influence of social isolation stress on oral carcinogenesis**", Protocol FOA nº 2015-00687, 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 August 05, 2015.

VALIDITY OF THIS CERTIFICATE: March 10, 2016.

DATE OF SUBMISSION OF THE FINAL REPORT: April 10, 2016.


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

Anexo B

7. Anexo B



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