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

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Stress hormones promote DNA damage in human oral keratinocytes

Araçatuba - SP
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Stress hormones promote DNA damage In human oral keratinocytes

Tese apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho'' - UNESP, para obtenção do título de 'Doutor em Odontologia. Área de Concentração: Estomatologia.

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1. Estresse psicológico 2. Norepinefrina 3. Glucocorticoide 4. Dano no DNA 5. Apoptose 6. Queratinócitos 7. Neoplasias bucais 8. Neoplasias de cabeça e pescoço I. T.

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“Tente uma, duas, três vezes e se possível tente a quarta, a quinta e quantas vezes for necessário. Só não desista nas primeiras tentativas, a persistência é amiga da conquista. Se você quer chegar onde a maioria não chega, faça o que a maioria não faz.”

– Bill Gates

“Daqui a 20 anos você estará mais decepcionado pelas coisas que você não fez, do que pelas que fez. Então, jogue fora suas amarras, navegue para longe do porto seguro, pegue os ventos em suas velas. Explore, sonhe, descubra.”

– Mark Twain

Resumo

Valente VB. Hormônios do estresse promovem dano no DNA de queratinócitos humanos de boca [tese]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2020.

RESUMO

O estresse crônico aumenta os níveis sistêmicos dos hormônios do estresse norepinefrina e cortisol. Assim como o carcinógeno específico do tabaco NNK (4-(metilnitrosamina)-1-(3-piridil)-1-butanona), estes hormônios podem induzir danos expressivos no DNA, o que contribui para o desenvolvimento do câncer. No entanto, é desconhecido se os hormônios do estresse possuem efeitos genotóxicos em queratinócitos de boca. Este estudo investigou os efeitos dos hormônios do estresse sobre o dano no DNA de uma linhagem celular de queratinócitos humanos de boca (NOK-SI). Células NOK-SI estimuladas com norepinefrina ou cortisol apresentaram maior dano no DNA que as células não tratadas. O dano induzido pela norepinefrina foi revertido pelo pré-tratamento das células com um beta-bloqueador. Células tratadas com NNK combinado à norepinefrina apresentaram níveis reduzidos das caspases 3 e 7. O cortisol também reduziu a atividade das enzimas pro-apoptóticas em relação às células não estimuladas. O dano no DNA promovido pelo NNK e cortisol e pela combinação de ambos levou ao acúmulo de γH2AX intracelular. Os efeitos causados pelo NNK e cortisol foram bloqueados com propranolol e com o antagonista do receptor de glicorcorticoide RU486, respectivamente. As quebras no DNA induzidas pela norepinefrina, na presença ou ausência de NNK, resultaram em maiores níveis celulares de 8OHdG. Este efeito também foi induzido via receptores beta-adrenérgicos. Os hormônios do estresse induzem danos no DNA de queratinócitos de boca e poderiam contribuir para a carcinogênese bucal.

Palavras-chave: Estresse Psicológico. Norepinefrina. Glucocorticoide. Dano no DNA. Apoptose. Queratinócitos. Neoplasias Bucais. Câncer de Boca. Neoplasias de Cabeça e Pescoço. Câncer de Cabeça e Pescoço. Câncer. Biomarcadores. Tumorigênese. Carcinogênese.

Abstract

Valente VB. Stress hormones promote DNA damage in human oral keratinocytes [thesis]. Araçatuba: UNESP - São Paulo State University; 2020.

ABSTRACT

Chronic stress increases the systemic levels of stress hormones norepinephrine and cortisol. As well tobacco-specific carcinogen NNK (4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone), they can induce expressive DNA damage contributing to the cancer development. However, it is unknown whether stress hormones have genotoxic effects in oral keratinocytes. This study investigated the effects of stress hormones on DNA damage in a human oral keratinocyte cell line (NOK-SI). NOK-SI cells stimulated with norepinephrine or cortisol showed higher DNA damage than untreated cells. Norepinephrine-induced DNA damage was reversed by pre-treatment with beta-adrenergic blocker propranolol. Cells treated with NNK combined to norepinephrine displayed reduced levels of caspases 3 and 7. Cortisol also reduced the activity of pro-apoptotic enzymes. DNA damage promoted by NNK or cortisol and carcinogen combined to the hormone led to intracellular γH2AX accumulation. The effects caused by NNK and cortisol were abolished by propranolol and glucocorticoid receptor antagonist RU486, respectively. DNA breaks induced by norepinephrine in the presence or absence of NNK resulted in higher 8OHdG cellular levels. This effect was also induced through beta-adrenergic receptors. Stress hormones induce DNA damage of oral keratinocytes and could contribute to oral carcinogenesis.

Keywords: Psychological Stress. Norepinephrine. Glucocorticoid. DNA Damage. Apoptosis. Keratinocytes. Oral Neoplasms. Oral Cancer. Head and Neck Neoplasms. Head and Neck Cancer. Cancer, Biomarkers. Tumorigenesis. Carcinogenesis.

Lista de figuras

LISTA DE FIGURAS

statistically significant difference ($p<0.0001$). NNK = 4 (N-metil-N-nitrosamine)-1-(3-piridil)-butano-1-one). NE = norepinephrine. PROP = propranolol.

Figure. 5. Cortisol increased the γH2AX nuclear expression in oral epithelial cells. (A) Control. (B) Cortisol. (C) NNK and cortisol. (D) Cortisol and RU486. (E) NNK, cortisol and RU486. (F) Cortisol increased the γH2AX nuclear expression levels in the presence or absence of carcinogen NNK. Glucocorticoid receptor blocker RU486 inhibited the increased DNA damage caused by the hormone. The results are expressed as the mean ± standard error of the mean (SEM). Upper- and lower cases equal letters indicate a statistically significant difference ($p<0.0001$). NNK = 4 (N-metil-N-nitrosamine)-1-(3-piridil)-butano-1-one). CORT = cortisol.

Figure 6. Effects of stress hormones on oxidative DNA damage in oral epithelial cells. (A) Norepinephrine up-regulated the 8OHdG levels in the presence or absence of carcinogen NNK. Beta-blocker propranolol inhibited the increased oxidative DNA damage caused by the hormone combined to NNK. (B) Cortisol did not change the 8OHdG levels secreted by the NOK-SI cells. The results are expressed as the mean ± standard error of the mean (SEM). Upper- and lower cases equal letters indicate a statistically significant difference ($p<0.05$). NNK = 4 (N-metil-N-nitrosamine)-1-(3-piridil)-butano-1-one). NE = norepinefrina. PROP = propranolol. CORT = cortisol.

Supplementary Fig. 1. Comet assay (A-E, 24h-exposure; F-J, 72h-exposure). (A,F) Control. (B,G) NNK. (C,H) Norepinephrine. (D,I) NNK and norepinephrine. (E) Norepinephrine induced DNA damage in the oral epithelial cells. (J) NNK in the presence or absence of norepinephrine promoted an expressive DNA damage in the NOK-SI cells. The results are expressed as the mean ± standard error of the mean (SEM). Upper- and lower cases equal letters indicate a statistically significant difference ($p<0.05$). NNK = 4 (N-metil-N-nitrosamine)-1-(3-piridil)-butano-1-one). NE = norepinephrine.

Lista de abreviaturas

LISTA DE ABREVIATURAS

3T3 - 3-day transfer inoculum 3×10^5 cells

8OHdG - 8-hydroxy-2'-deoxyguanosine

AKT - Protein kinase B

ANOVA - Analysis of variance

AP1 - Activator protein 1

ATF - Activating transcription factor

BCL-2 - B-cell lymphoma 2

BSA - Bovine serum albumin

cAMP - Cyclic 3′-5′ adenosine monophosphate

CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CORT - Cortisol

CREB - cAMP response element binding protein

DMEM - Dulbecco modified eagle medium

DNA - Deoxyribonucleic acid

ELISA - Enzyme-linked immunosorbent assay

EPAC - Exchange protein activated by adenylyl cyclase

ERK - Extracellular signal-regulated kinase

ETS - Erythroblast transformation specific

FAPESP - São Paulo Research Foundation

FBS - Fetal bovine serum

HCl - Hydrogen chloride

MAP - Mitogen-activated protein

MDM2 - Mouse double minute 2 homolog

MEK - Mitogen-activated protein kinase

MPK1 - Mitogen-activated protein kinase 1

Na_2EDTA - Ethylenediaminetetraacetic acid disodium

NaCl - Sodium chloride

NE - Norepinephrine

NNK - 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone)

NOK-SI - Normal oral keratinocyte-spontaneously immortalized

PBS - Phosphate-buffered saline

PI3K - Phosphoinositide 3-kinase

PKA - Protein kinase A

PROP - Propranolol

PVA - Polyvinyl alcohol

Raf - Rapidly accelerated fibrosarcoma

ROS - Reactive oxygen species

SEM - Standard error of the mean

SGK1 - Serum and glucocorticoid-regulated kinase 1

STAT3 - Signal transducer and activator of transcription 3

TUNEL - Terminal deoxynucleotidyl transferase dUTP nick-end labelling

UNESP - São Paulo State University

γH2AX - H2A histone family member X

SUMÁRIO

Stress hormones promote DNA damage in human oral keratinocytes[1]

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Introduction

1 INTRODUCTION

Psychological stress up-regulates the circulating levels of stress hormones norepinephrine and cortisol[1]. Exposure to these hormones released from chronic stress response has been associated to an enhanced risk of developing diseases such as cancer[1,2]. In somatic cells, norepinephrine and cortisol may induce DNA damage through the activation of beta-adrenergic and glucocorticoid receptors, respectively[3,4]. Both mechanisms trigger similar effects to those produced by the tobacco smoke carcinogens[5,6]. A significant damage in the integrity of DNA may lead to genome mutations and affect oncogenic mechanisms predisposing to cell malignant transformation[2,5].

Chronic stress and stress hormones may cause a significant DNA damage accompanied by a higher production of phosphorylated histone H2AX (γH2AX)[7,8] and 8-hydroxy-2'-deoxyguanosine (8OHdG)[2,9]. Both molecules are considered mutagenic biomarkers of the DNA damage and significantly enhance the occurrence of tumorigenic mutations into the genome of somatic cells, which may become malignant[2,10]. Moreover, stress hormones can inhibit the apoptosis of somatic cells[11,12] allowing their replication with DNA damage. This inhibition is also considered another crucial mechanism related to the acquisition of malignant phenotype[2,3]. The activity of caspases is measured to assess cell apoptosis and may become downregulated after chronic exposure to the stress hormones and carcinogenic agents[13,14].

Oral cancer represents the sixth most common malignancy worldwide and its occurrence has been widely associated to the tobacco smoking[15]. In addition, chronic stress and stress hormones have also been investigated in oral cancer patients and preclinical models of the disease[16-20]. We recently demonstrated that biobehavioral factors are related to increased circulating norepinephrine levels in these patients.[16] In an orthotopic model, chronic stress up-regulated the plasma levels of catecholamines and glucocorticoids, which would contribute to increase tumor size and invasiveness[17]. In rats underwent chemical carcinogenesis, we showed that the stress hormones levels in the normal microenvironment predict the risk of developing oral cancer[18]. An expressive DNA damage caused by the chemical substances such

as nicotine and tobacco-specific nitrosamines may be considered one of the first molecular events for oral cancer occurrence[5]. However, it remains unknown whether stress hormones have genotoxic effects in oral epithelial cells.

In the current study, we tested the hypothesis that the exposure of an oral keratinocyte cell line (NOK-SI) to the stress hormones in the presence or absence of chemical carcinogen NNK (4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone), a tobacco-specific nitrosamine, would induce DNA damage. In addition, these cells were also evaluated for the production of molecules associated to DNA damage and apoptosis.

Results

2 RESULTS

2.1 Norepinephrine and cortisol induce DNA damage in oral epithelial cells

TUNEL assay was performed to evaluate whether carcinogen NNK and stress hormones would promote DNA damage in NOK-SI cells. After 72h of treatment, group stimulated with norepinephrine displayed higher percentage of positive-TUNEL cells than untreated group ($p<0.0001$; Fig. 1A). Norepinephrine-induced DNA damage was blocked by the pre-treatment with nonselective beta-blocker propranolol ($p<0.0001$; Fig. 1A). Cells stimulated with cortisol showed an increase of the DNA damage when compared to control cells ($p=0.0004$; Fig. 1B). However, this effect was not abolished by the glucocorticoid receptor antagonist RU486 ($p>0.05$; Fig. 1B). NNK treatment did not induced DNA damage when compared to the untreated group ($p>0.05$; Fig. 1A). Likewise, stimulation with carcinogen NNK and norepinephrine or cortisol did not promote DNA breaks when compared to untreated group ($p>0.05$; Fig. 1A,B).

In another experimental set, the genotoxic effects of carcinogen NNK and norepinephrine on DNA damage of NOK-SI cells were assessed using the comet assay. After 24h of exposure, it was noted an increase in the tail DNA percentage of cells treated with hormone ($p<0.0001$; Supplementary Fig. 1C,E). After 72h of exposure, cells stimulated with NNK displayed an increase in the DNA damage concerning the untreated cells ($p<0.0001$; Supplementary Fig. 1G,J). NNK and norepinephrine also enhanced the DNA damage of NOK-SI cells when compared to the untreated control group ($p<0.0001$; Supplementary Fig. 1I,J).

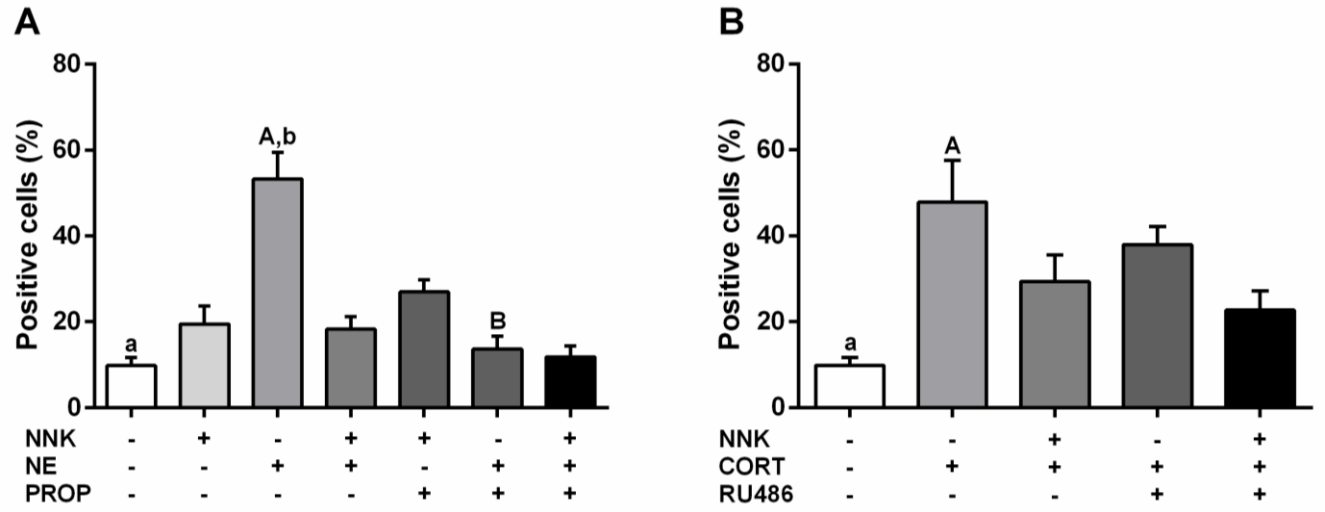
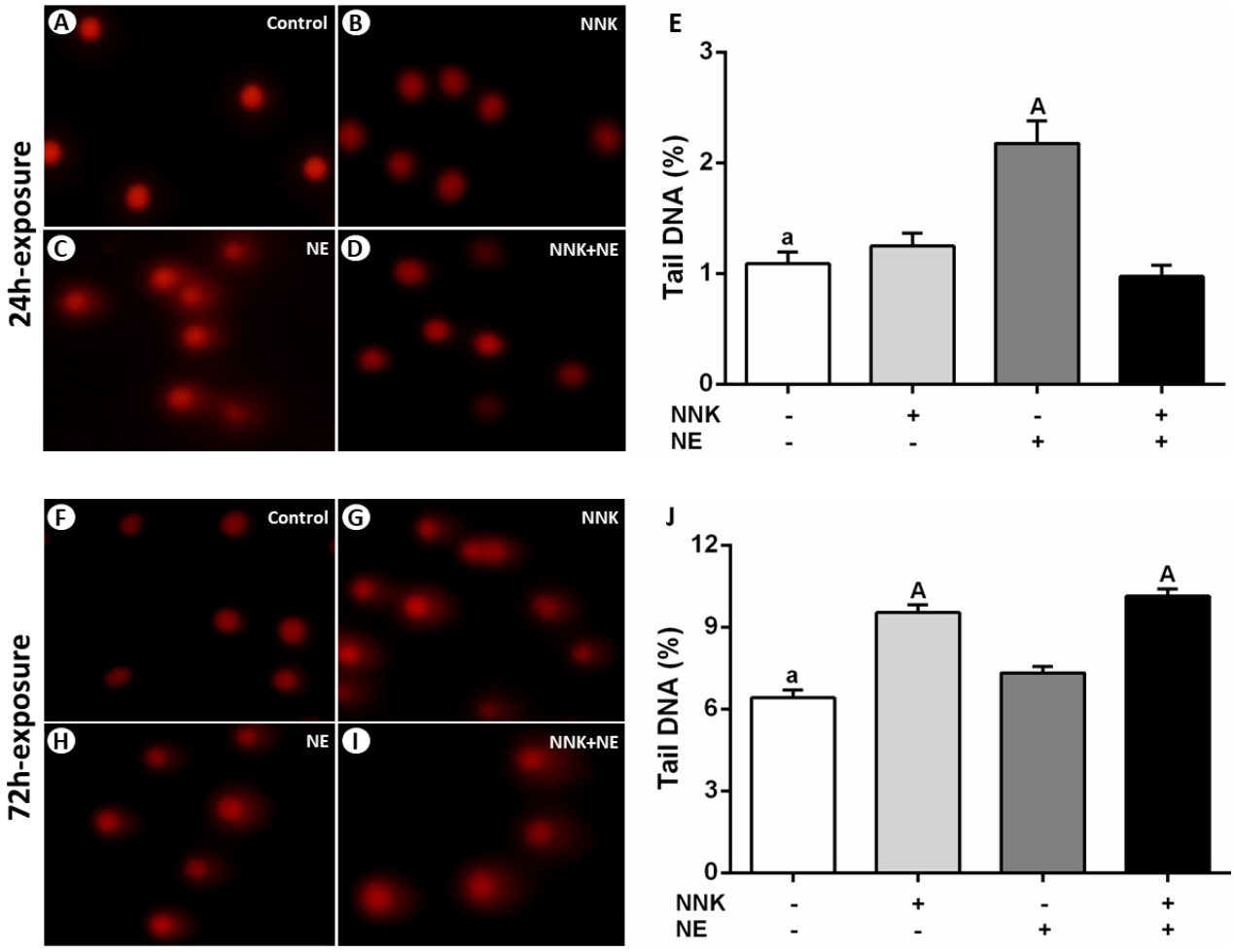


Figure. 1. Stress hormones promote DNA damage in oral keratinocytes. (A) Norepinephrine increased the DNA damage in oral epithelial cells and this effect was abolished by the non-selective beta-blocker propranolol. (B) Cortisol increased the DNA fragmentation of oral keratinocytes. The results are expressed as the mean ± standard error of the mean (SEM). Upper- and lower cases equal letters indicate a statistically significant difference ($p<0.05$). NNK = 4 (N-metil-N-nitrosamine)-1-(3-piridil)-butano-1-one). NE = norepinephrine. PROP = propranolol. CORT = cortisol.



Supplementary Fig. 1. Comet assay (A-E, 24h-exposure; F-J, 72h-exposure). (A,F) Control. (B,G) NNK. (C,H) Norepinephrine. (D,I) NNK and norepinephrine. (E) Norepinephrine induced DNA damage in the oral epithelial cells. (J) NNK in the presence or absence of norepinephrine promoted an expressive DNA damage in the NOK-SI cells. The results are expressed as the mean ± standard error of the mean (SEM). Upper- and lower cases equal letters indicate a statistically significant difference ($p<0.05$). NNK = 4(N-metil-N-nitrosamine)-1-(3-piridil)-butano-1-one). NE = norepinephrine.

2.2 Cortisol inhibits the activity of apoptotic enzymes in oral keratinocytes

The effects of NNK and stress hormones on the activity of caspases 3 and 7 were measured in NOK-SI cells. Carcinogen NNK or norepinephrine did not change the levels of caspases concerning the untreated group ($p>0.05$; Fig. 2B,C,H). Nevertheless, cells treated with NNK combined to the norepinephrine showed lower levels of the apoptotic enzymes ($p<0.0001$; Fig. 2D,H). Cells treated with cortisol displayed lower levels of caspases 3 and 7 than untreated cells ($p<0.0001$; Fig. 3B,F). However, this effect was not inhibited by the glucocorticoid receptor antagonist RU486 ($p>0.05$; Fig. 3D,L).

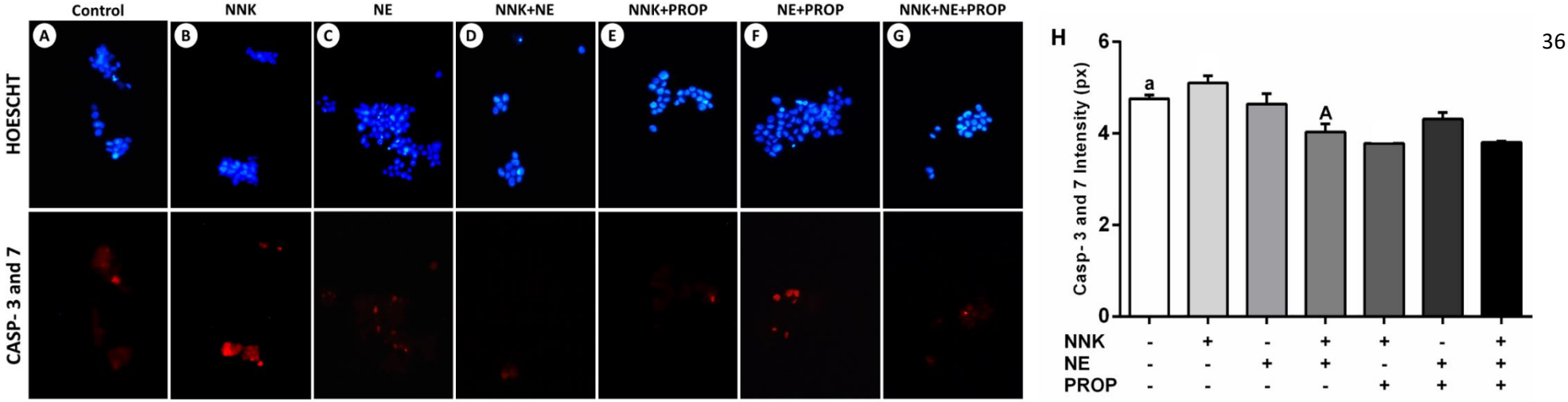


Figure 2. Effects of tobacco-specific nitrosamine NNK and norepinephrine on the activity of the caspases 3 and 7. (A) Control. (B) NNK. (C) Norepinephrine. (D) NNK and norepinephrine. (E) NNK and propranolol. (F) Norepinephrine and propranolol. (G) NNK, norepinephrine and propranolol. (H) Combined treatment of NNK with norepinephrine inhibited the activity of the pro-apoptotic enzymes. This effect was not abolished by the beta-adrenergic blocker propranolol. The results are expressed as the mean ± standard error of the mean (SEM). Upper- and lower cases equal letters indicate a statistically significant difference ($p<0.0001$). NNK = 4 (N-metil-N-nitrosamine)-1-(3-piridil)-butano-1-one). NE = norepinephrine. PROP = propranolol.

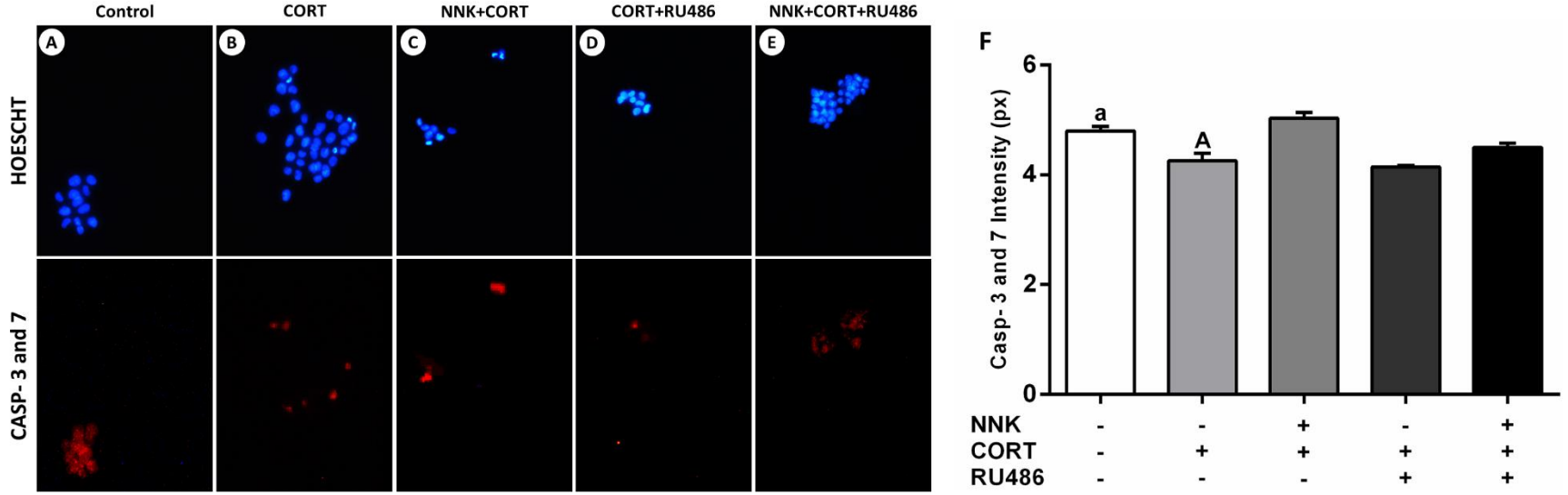


Figure 3. Cortisol inhibits the activity of caspases 3 and 7. (A) Control. (B) Cortisol. (C) NNK and cortisol. (D) Cortisol and RU486. (E) NNK, cortisol and RU486. (F) Cortisol inhibited the activity of pro-apoptotic enzymes. This effect was not abolished by the glucocorticoid receptor blocker RU486. The results are expressed as the mean ± standard error of the mean (SEM). Upper- and lower cases equal letters indicate a statistically significant difference ($p<0.0001$). NNK = 4 (N-metil-N-nitrosamine)-1-(3-piridil)-butano-1-one). CORT = cortisol.

2.3 DNA damage induced by tobacco-derived nitrosamine NNK and cortisol in oral keratinocytes is associated with intracellular γH2AX accumulation

Immunofluorescence assays were performed to assess the γH2AX cellular accumulation levels in NOK-SI cells stimulated with NNK and stress hormones. After 4h of stimulation, cells treated with NNK displayed an increase in the γH2AX accumulation when compared to untreated cells ($p<0.0001$; Fig. 4B,H). The NNK-induced γH2AX accumulation was inhibited by the pre-incubation with beta-adrenergic antagonist propranolol ($p<0.0001$; Fig. 4E,H). NOK-SI cells stimulated only with norepinephrine did not display significant changes in the γH2AX accumulation when compared to control cells ($p>0.05$; Fig. 4C,H). On the other hand, cells treated only with cortisol or hormone combined with NNK showed a higher γH2AX accumulation when compared to untreated cells ($p<0.0001$; Fig. 5B,C,F). Pre-incubation with glucocorticoid receptor antagonist RU486 inhibited these genotoxic effects ($p<0.0001$; Fig. 5D,E).

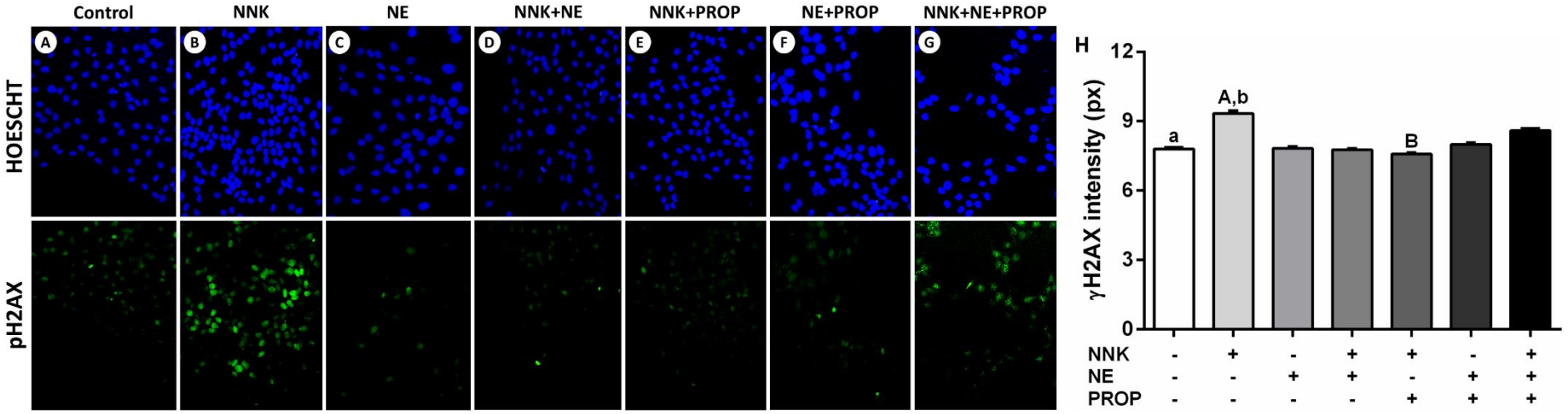


Figure 4. Exposure to the NNK and norepinephrine and γH2AX nuclear expression in oral keratinocytes. (A) Control. (B) NNK. (C) Norepinephrine. (D) NNK and norepinephrine. (E) NNK and propranolol. (F) Norepinephrine and propranolol. (G) NNK, norepinephrine and propranolol. (H) NNK increased the γH2AX nuclear expression levels. Beta-blocker propranolol inhibited the increased DNA damage caused by the carcinogen. The results are expressed as the mean ± standard error of the mean (SEM). Upper- and lower cases equal letters indicate a statistically significant difference ($p<0.0001$). NNK = 4 (N-metil-N-nitrosamine)-1-(3-piridil)-butano-1-one). NE = norepinephrine. PROP = propranolol.

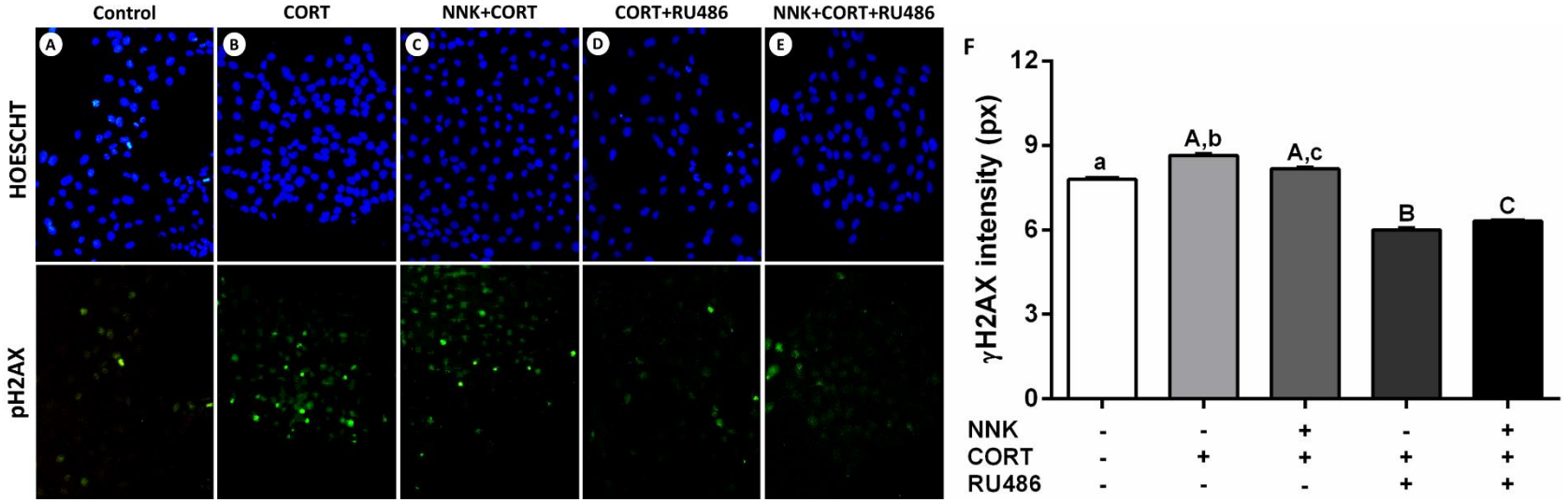


Figure. 5. Cortisol increased the γH2AX nuclear expression in oral epithelial cells. (A) Control. (B) Cortisol. (C) NNK and cortisol. (D) Cortisol and RU486. (E) NNK, cortisol and RU486. (F) Cortisol increased the γH2AX nuclear expression levels in the presence or absence of carcinogen NNK. Glucocorticoid receptor blocker RU486 inhibited the increased DNA damage caused by the hormone. The results are expressed as the mean ± standard error of the mean (SEM). Upper- and lower cases equal letters indicate a statistically significant difference ($p<0.0001$). NNK = 4 (N-metil-N-nitrosamine)-1-(3-piridil)-butano-1-one). CORT = cortisol.

2.4 Norepinephrine increases the 8OHdG levels in the presence or absence of carcinogen NNK in oral keratinocytes

To assess oxidative DNA damage levels induced by stress hormones in NOK-SI cells, the 8OHdG concentrations were measured in the cell culture medium. After 4h of treatment, cells stimulated only with norepinephrine or hormone associated to the carcinogen NNK secreted higher 8OHdG levels in the culture supernatant than untreated cells ($p<0.0001$; Fig. 6A). Treatment with the hormone alone resulted in an approximately four-fold increase in the 8OHdG levels. The effects of norepinephrine alone or combined with NNK on the 8OHdG levels were abolished partially and totally by the beta-adrenergic antagonist propranolol, respectively ($p<0.05$; $p<0.001$; Fig. 6A). NOK-SI cells treated with carcinogen NNK showed no significant changes in the 8OHdG levels when compared to untreated cells ($p>0.05$; Fig. 6A). On the other hand, cortisol did not change the 8OHdG levels secreted by the oral keratinocytes when compared to non-stimulated cells ($p>0.05$; Fig. 6B).

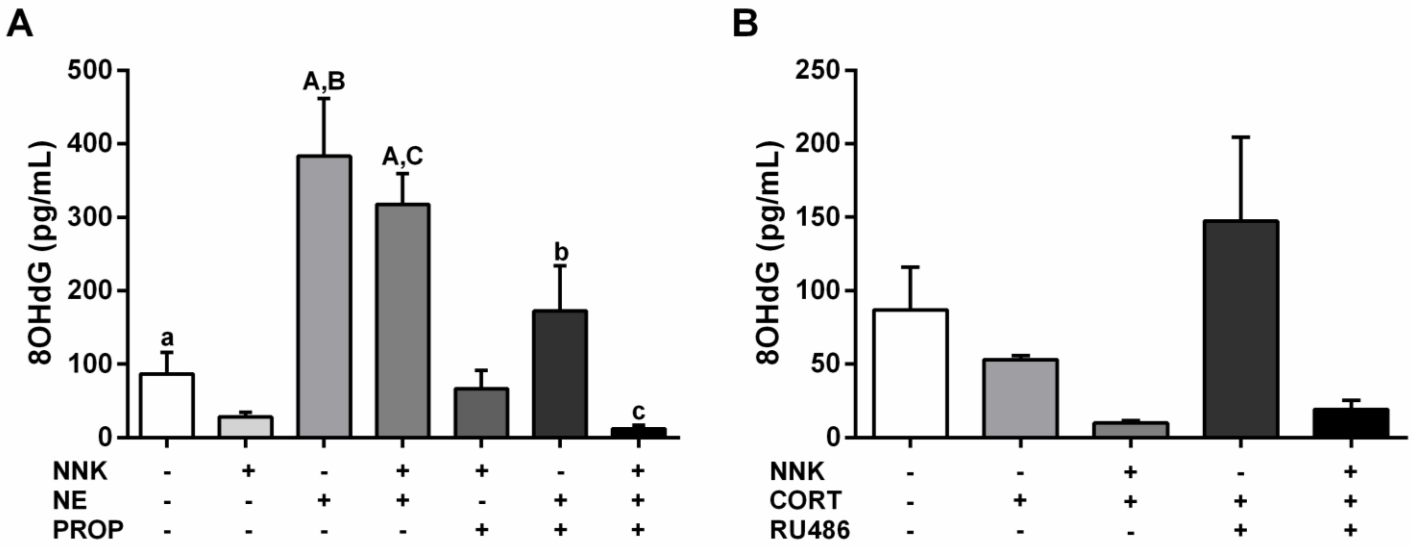


Figure 6. Effects of stress hormones on oxidative DNA damage in oral epithelial cells. (A) Norepinephrine up-regulated the 8OHdG levels in the presence or absence of carcinogen NNK. Beta-blocker propranolol inhibited the increased oxidative DNA damage caused by the hormone combined to NNK. (B) Cortisol did not change the 8OHdG levels secreted by the NOK-SI cells. The results are expressed as the mean ± standard error of the mean (SEM). Upper- and lower cases equal letters indicate a statistically significant difference ($p<0.05$). NNK = 4 (N-metil-N-nitrosamine)-1-(3-piridil)-butano-1-one). NE = norepinefrina. PROP = propranolol. CORT = cortisol.

Discussion

3 DISCUSSION

Investigations have showed that stress hormones may act on oral cancer promoting tumor cell proliferation and invasion[17-20]. Although our previous study has suggested that stress hormones may also predict oral carcinogenesis in a preclinical model[18], the mechanisms associated with stress-induced malignant transformation of oral epithelial cells were unknown. In the current study, we used the NOK-SI cell line to confirm the hypothesis that stress hormones and tobacco-specific nitrosamine NNK promote DNA damage and reduce the apoptotic activity of human oral keratinocytes. Our results demonstrated that norepinephrine and NNK acted on beta-adrenergic receptors while cortisol activated the glucocorticoid receptor to produce tumorigenic effects in the NOK-SI cells. In this context, we showed the genotoxic action of stress hormones in the presence or absence of NNK, which has not yet been elucidated in oral keratinocytes.

The role of stress hormones on the promotion of malignant phenotype in normal cells has been increasingly investigated in recent years[2-4,7,21-24]. In these cells, DNA damage is considered a crucial event to trigger the malignant transformation[2,5]. After several cell divisions, an accumulation of damaged DNA may result in irreversible genome mutations commonly found in tumor cells[2]. In the current study, norepinephrine induced an expressive DNA damage of human oral keratinocytes. Similarly, some evidences also show that adrenergic hormone has also genotoxic effects in normal cells, which may contribute to the tumorigenesis. Flint et al.[4] demonstrated that norepinephrine treatment promoted DNA damage in mouse fibroblasts and this effect was abolished by using beta-adrenergic blocker propranolol. Likewise, we induced DNA damage of oral epithelial cells after norepinephrine exposure and reversed this effect with the beta-adrenergic blocker use. Some signaling pathways by which norepinephrine could be promoting DNA damage have been suggested. In a study, norepinephrine treatment promoted DNA damage of normal and cancer cells via PI3K/AKT/MDM2/p53 signaling pathway[7]. Sun et al.[21] also demonstrated that exposure to norepinephrine can induce DNA damage of embryonic stem cells through the activation of cyclic 3′-5′ adenosine

monophosphate (cAMP) and protein kinase A (PKA), molecules that constitute the cAMP/PKA pathway. Similarly to our study, these investigations also used norepinephrine at 10 µM to produce an increased DNA damage, which may be considered a high concentration that simulate stress conditions.

Activation of the beta-adrenergic receptors by the norepinephrine can result in the upregulation of cAMP/PKA pathway, which increases mitochondrial oxidative phosphorylation and reactive oxygen species (ROS) production[2,25]. ROS may damage mitochondrial and nuclear DNA and produce a wide range of DNA lesions such as strand breaks, thymine glycol, base loss and base damage including 8OHdG[2]. In the current study, oxidative DNA damage induced by norepinephrine in the presence or absence of carcinogen NNK resulted in a higher secretion of 8OHdG cellular levels. This event was dependent of beta-adrenergic receptors. Another investigation found higher 8OHdG cellular levels in the DNA genomic of cardiac myoblasts after exposure to norepinephrine[26]. 8OHdG has been considered the most common mutagenic biomarker associated to oxidative DNA damage[2]. It is closely related to the development of several types of cancer in humans and has also been used to estimate the DNA damage after exposure to carcinogenic agents[27]. In oral cancer patients, elevated salivary 8OHdG levels have already been related to tumor progression and also seem to be involved in the pathogenesis of the disease[28,29]. The increased 8OHdG immunoexpression in non-tumor tissues contribute to the chemical oral carcinogenesis in preclinical models[30,31].

In the current study, norepinephrine combined to nitrosamine NNK inhibited the apoptosis of NOK-SI cells by decreasing the activity of caspases 3 and 7. This inhibition would permit an uncontrolled proliferation of keratinocytes with damaged DNA, whose tumorigenic mutations could lead to the oral cancer development. The reduced expression of caspase 3 in oral cancer tissues has been related to malignant transformation[32]. A recent investigation demonstrated that norepinephrine protected against apoptosis of mesenchymal stem cells by inhibiting the expression of caspase 3 via AKT/BCL-2[11]. It has also been suggested that norepinephrine regulates cell apoptosis through the activation of cAMP/PKA pathway via beta-adrenergic receptors[33]. There is substantial

evidence that norepinephrine released from circulating blood and local sympathetic nerve fibers bind to beta-adrenergic receptors on the cell membrane and induce the synthesis of cAMP, responsible for triggering oncogenic mechanisms[33]. cAMP activates PKA that phosphorylates several proteins including transcription factors of the ATF/CREB and GATA families[33,34]. Moreover, cAMP-induced up-regulation of Exchange Protein activated by Adenylyl Cyclase (EPAC) triggers the Ras/Raf/ MEK/ERK pathway regulating the transcription factors of the AP1 and ETS families[33,35]. In malignant cells, all these mechanisms mediated by cAMP influence the production of cytokines and growth factors, as well as cell morphology, motility and proliferation.[33] Beta-adrenergic signaling activated by the norepinephrine may also up-regulate the phosphorylation of the transcription factor STAT3, which modulates tumorigenic pathways[36].

In epithelial cells of the upper aerodigestive tract, DNA damage promoted by tobacco-specific carcinogenic substances has been considered a crucial event for malignant transformation[5]. In the current study, comet assay showed that tobacco-specific nitrosamine NNK induced DNA damage of the NOK-SI cells after 72h-treatment. However, TUNEL assay demonstrated that this period of exposure to nitrosamine did not promote significant fragmentation of the acid nucleic in these cells. These findings suggest that a long period of exposure to NNK would be needed to promote the induction of malignant phenotype in NOK-SI cells. In bronchial epithelial cells, Shen et al.[37], for example, induced a malignant phenotype after 192 hours of exposure to nitrosamine. In our study, TUNEL assay showed that NNK almost inhibited the genotoxic effect caused by the norepinephrine. Likewise, after 24h-exposure, comet assay revealed that tobacco nitrosamine almost inhibited the norepinephrine-induced DNA damage. These findings suggest that the NNK and norepinephrine competed for binding to the beta-adrenergic receptors. Beta-adrenergic signaling activated by the norepinephrine, which resulted in genotoxic effects, may have been inhibited when nitrosamine NNK binded to the receptors. Furthermore, the sensitivity of the beta-adrenergic receptors may be downregulated when they are exposed to several agonists, such as catecholamines and NNK[6]. In human cells, NNK is enzymatically converted into metabolites, which bind to the DNA molecule and

induce tumorigenic mutations[5,6]. These genotoxic effects can be mediated by nicotinic acetylcholine and beta-adrenergic receptors[6]. Although the tumorigenic action of nicotinic receptors induced by the NNK binding has been demonstrated in oral epithelial cells[38-41], the role of beta-adrenergic signaling activated by the nitrosamine still need to be investigated. In other types of normal epithelial cells[42,43], the tumorigenic action of beta-adrenergic receptors induced by NNK binding has already been evaluated. Beta-adrenergic receptors activated by the nitrosamine may mediate, for example, the levels of ERK1/2 MAP kinases in mammary cells[42]. In small airway cells, beta-adrenergic signaling activation by NNK can up-regulate ERK1/2 and ATF1/CREB phosphorylation via PKA[43].

Cortisol secreted into the circulation binds to the glucocorticoid receptor in the cytoplasm and translocate it to the nucleus where they modulate the transcription of several genes by associating to DNA response elements and/or to other transcription factors[44]. Cortisol may lead to phosphorylation of MDM2 that down-regulates the function of the tumor suppression protein p53, mediator responsible for regulating several mechanisms associated to the DNA repair[2,24]. The persistent accumulation of DNA damage enhances the risk of malignant transformation predisposing the tumor cell development[2]. These findings concerning the MDM2/p53 signaling pathway were demonstrated in mouse embryonic fibroblasts after treatment of the cells with corticosterone, a glucocorticoid similar to cortisol found in mice and rats[24]. Another investigation showed that cortisol at 1000 nM, considered a pharmacological concentration, promoted approximately a four to five-fold increase in DNA damage of 3T3 mouse fibroblasts after 10 minutes of exposure to the hormone, and this effect was reversed using antagonist RU486[3]. Our results showed that cortisol at 100 nM in the presence or absence of NNK promoted a higher γH2AX cellular accumulation and this event was completely reversed by RU486 treatment. In the current study, the concentration designed for cortisol simulated chronic stress conditions[19]. Recent studies have shown that cortisol exposure also promote an accumulation of γH2AX in breast cancer cells[8,45,46]. In these investigations, the analysis of γH2AX phosphorylation was used as an indicator of DNA damage induced by the cortisol, whose effects were blocked with

RU486 use[8,45,46]. These findings suggest that cortisol produces genotoxic effects mediated via glucocorticoid receptor. Currently, there is a lack of consensus regarding the cortisol concentration to induce these effects in normal and tumor cells. The quantification of γH2AX levels can also be used to determinate the DNA lesions resulting from exposure to environmental carcinogens such as nitrosamines[10,47]. In the current study, NNK promoted an increase in the γH2AX nuclear expression in oral keratinocytes and this genotoxic effect was abrogated by the beta-adrenergic blockade with propranolol. In oral carcinogenesis, tissue γH2AX immunoexpression increases significantly before malignant transformation being considered an indicator for oral cancer development[48].

The activation of glucocorticoid receptor may lead to transcription of genes associated to cell survival pathways and inhibition of apoptosis depending on cell type[12,44]. The present study shows that cortisol promotes anti-apoptotic effects in NOK-SI cells by reducing the activity of caspases 3 and 7. In addition to DNA damage, this anti-apoptotic effect induced by the glucocorticoid could contribute to a proliferative malignant behavior in these cells. In breast cancer cell lines, dexamethasone (a glucocorticoid) at 1000 nM, inhibited chemotherapy-induced apoptosis and promoted the gene expression of SGK1 and MKP1, whose proteins are associated to cell survival[12]. After glucocorticoid exposure, proapoptotic genes, caspases 3, 8 and 9 and pro-apoptotic BCL-2 family members were downregulated[12]. In another study, dexamethasone inhibited the activity of caspases in lung and cervical cancer cell lines preventing therapy-induced tumor reduction in orthotopic model[49]. Similarly to these findings, the current study demonstrates that cortisol in a stress concentration may affect cell death of normal oral keratinocytes. These cells might then proliferate continuously with damaged DNA becoming malignant.

This study showed that stress hormones may induce DNA damage of oral keratinocytes. The evidences suggest that norepinephrine can activate the beta-adrenergic signaling to increase oxidative DNA damage. ROS generation related to the DNA damage was successfully evaluated by measuring the cellular 8OHdG levels. Our findings also suggest that cortisol produces an

increased DNA damage accompanied by a higher nuclear γH2AX accumulation. This mechanism may be mediated by the glucocorticoid receptor. We treated the NOK-SI cells with norepinephrine or cortisol at high concentrations that simulate chronic stress conditions in humans. Furthermore, these cells were also treated with the tobacco-specific nitrosamine NNK at a circulating concentration commonly found in chronic smokers. In our experiments, we have succeed in evaluating the genotoxic role of each hormone in the presence or absence of NNK, which is not possible to verify clinically. Although this study has brought advances regarding the use of in vitro model to better understand the role of stress hormones on the initial steps of oral carcinogenesis process in humans, it has also some limitations that need to be considered. It was not possible to determine the minimal amount of damaged DNA responsible to induce biological responses. The comet assay was considered a supplementary experiment to investigate the DNA damage induced by the norepinephrine. Therefore, it was not performed after cortisol treatment. The cells were also not tested with different concentrations of cortisol once it seems to have dual effects on tumorigenic processes, as we previously reported[18,19].

To date, no study has evaluated the genotoxic effects of stress hormones associated to tobacco-specific nitrosamines on the promotion of genomic lesions in normal oral keratinocytes. Our findings show that high concentrations of norepinephrine and cortisol that simulate chronic stress cause expressive DNA damage of NOK-SI cells, predisposing them to malignant transformation. The occurrence of this event may be confirmed by the evaluation of the increased cellular levels of biomarkers associated to the DNA damage. The genotoxic effects caused by the stress hormones in epithelial cells could increase the risk of developing oral cancer.

Material and Methods

4 MATERIAL AND METHODS

4.1 Cells and culture conditions

Normal Oral Keratinocyte-Spontaneously Immortalized (NOK-SI) cell line was kindly provided by the Dr. Aline Satie Takamiya (São Paulo State University - UNESP, Araçatuba, São Paulo, Brazil). NOK-SI cells were cultured in Dulbecco Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA, EUA) containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate and 4.5 g/L glucose. All reagents used in this study were purchased from Sigma Aldrich (St. Louis, MO, USA). The culture medium was supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, 100 µg/mL penicillin and 0.1% gentamicin. NOK-SI cells were grown in 75-cm^2 culture flasks (Greiner Bio-One, Kremsmünster, Austria) at 37ºC in humidified atmosphere of 5% (v/v) CO_2. After reaching approximately 70% cell density, the cells were trypsinized, seeded in 6-well plates and then maintained under the same conditions. In these plates, NOK-SI cells were stimulated with NNK and/or stress hormones after reaching about 50% cell density.

4.2 Carcinogen and hormone treatment

The growth medium was replaced with NNK and/or norepinephrine at 10 µM or cortisol at 100 nM, according to previous studies[19,50]. These concentrations simulate chronic stress conditions in humans. The blocking of hormone receptors was performed by pre-incubating cells with beta-adrenergic receptor antagonist propranolol and glucocorticoid receptor antagonist RU486. Both antagonists were diluted in the culture medium to achieve the concentration of 10 µM. Cells were incubated in the presence of antagonists for 1h prior to the addition of NNK and/or norepinephrine or cortisol. NOK-SI cells were cultured without FBS overnight and then exposed to the NNK and/or hormone for 4h in culture medium without FBS. Moreover, cells were also cultured with 10% FBS and stimulated with carcinogen and/or hormone for 72h in a culture medium with 10% FBS. In this case, the stimuli were replaced every

24h along with the renewal of the culture medium. Preliminary experiments showed that the suppression of FBS for 72h, even in the presence of stress hormones, promoted morphological changes and high mortality rate in NOK-SI cells.

4.3 Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL assay)

The cells were washed in phosphate-buffered saline solution (PBS) with 1 mg/mL of polyvinyl alcohol (PBS/PVA) and fixed using a 4% paraformaldehyde solution for 1h at room temperature. Then, NOK-SI cells were permeabilized using 0.5% Triton X-100 for 30 min and washed with PBS/PVA. For the positive control, untreated cells were incubated with DNAse I (50 IU/mL) for 1h at room temperature while cells from the experimental groups were maintained in PBS/PVA. Cells were incubated in 30 µL of TUNEL reaction mixture (In-Situ Cell Death Detection Kit (Roche Diagnostics Corp., Indianapolis, IN, USA) for 1h at room temperature, according to the manufacturer's recommendations. For the negative control, untreated cells were incubated with label solution only. The cells were washed in PBS/PVA and stained with Hoechst 33342 (1 µg/mL) for 10 min at room temperature. Then, they were washed again in PBS/PVA and transferred to the glass slides using the Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Coverslips were placed. The cells were analyzed using an inverted epifluorescence microscope (Olympus America Inc, Center Valley, PA, USA). The percentage of TUNEL-positive cells showing fragmented DNA was then determined by a blinded researcher.

4.4 Single cell gel electrophoresis (comet assay)

Adherent cells were trypsinized from the plates, mixed in 0.5% low-melting agarose and placed in glass slides pre-coated with 1% agarose gel. The

slides were immersed in lysis solution (1M Tris, 1M NaCl, 1M Na_2EDTA, 1% Triton X-100) with proteinase K for 2h at 50ºC. Then, they underwent electrophoresis at 25 V for 30 min and neutralized with 0.4 M Tris-HCl for 5 min. The cells were then stained with ethidium bromide and photographed using an inverted microscope (Olympus America Inc, Center Valley, PA, USA) with an excitation wavelength of 495 nm and an emission wavelength of 520 nm. In each group, a total of 100 cells was analyzed. The percentage of DNA in the comet tail was determined using the CASP software (University of Wroclaw, Wroclaw, Poland) by a blinded researcher to the experimental groups.

4.5 Activity of caspases-3 and -7

The activity of caspases-3 and -7 was measured in the NOK-SI cells using the Image-iT LIVE Red Caspase-3 and -7 Detection Kit (Molecular Probes, Invitrogen, OR, USA) according to the manufacturer's instructions. The cells were incubated in 10-µL of a fluorescent inhibitor of caspases-3 and 7 for 1h at 37ºC in a humid chamber. Cells were washed with PBS buffer solution and fixed in 4% paraformaldehyde solution for 40 min at room temperature. The cells were then stained with Hoechst 33342 (1 µg/mL) and the slides mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Stained cells were photographed using an inverted microscope (Olympus America Inc, Center Valley, PA, USA). A blinded researcher to the experimental groups quantified the fluorescence signal intensities (pixels) of the stained cells in the recorded images running the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

4.6 Measurement of 8-hydroxy-2'-deoxyguanosine (8OHdG) content

The 8-hydroxy-2'-deoxyguanosine (8OHdG) levels were measured in the culture medium using a specific kit (DNA/RNA Oxidative Damage; Cayman

Chemical, Michigan, USA) by the method Enzyme-Linked Immunosorbent Assay (ELISA). The ELISA assay sensitivity was 30 pg/mL for 8OHdG. This assay was performed in triplicate according to the manufacturer's recommendations.

4.7 Immunoexpression of phospho-H2AX (γH2AX)

Cells were fixed with 4% paraformaldehyde solution in glass-bottom tissue culture plates and maintained in methanol for 20 min at 4ºC. Non-specific binding sites were then blocked by using 1% bovine serum albumin (BSA) for 2h at room temperature. Cells were incubated overnight at 4ºC with primary antibody for anti-phospho-H2AX (1:200; Cell Signaling Technology, Beverly, MA, USA). Then, the secondary antibody was incubated for 1h at room temperature. The cells were stained with Hoechst 33342 (1 µg/mL) and the slides mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). NOK-SI cells were photographed using an inverted microscope (Olympus America Inc, Center Valley, PA, USA) and a blinded researcher to the experimental groups quantified the fluorescence signal intensities (pixels) of the stained cells in the recorded images by using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

4.8 Statistical analysis

GraphPad Prism 8.21 (GraphPad Software Inc., San Diego, CA, USA) was used to perform statistical analysis. The one-way analysis of variance (ANOVA) with post-hoc Tukey test for multiple comparisons evaluated possible differences between the groups. The results were presented as Mean ± Standard Error of Mean (SEM). Differences were considered statistically significant when $p<0.05$.

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Author Contributions

V.B.V. Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Writing the original draft. D.M.C., G.M.K. and G.B.N. Data curation; Investigation; Methodology. K.C.T., E.R.B. and G.I.M. Visualization, Investigation and Resources. G.Z.M. and S.H.P.O. Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Review and editing. D.G.B. Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Review and editing.

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Anexos

Anexo A - Normas de publicação da Scientific Reports

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