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LONG-TERM EXPOSURE TO STRESS HORMONES INDUCES MALIGNANT PHENOTYPE IN ORAL KERATINOCYTES

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Trabalho de conclusão de curso apresentado à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos quesitos para obtenção do título de bacharel em Odontologia.

Orientador: Prof. Dr. Daniel Galera Bernabé

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SUMÁRIO

LONG-TERM EXPOSURE TO STRESS HORMONES INDUCES MALIGNANT PHENOTYPE IN ORAL KERATINOCYTES

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ABSTRACT

Psychological stress can promote cancer progression by increasing norepinephrine (NE) and epinephrine (EPI) local and systemic levels. However, little is known about the role of stress on the carcinogenesis process. This study explored the effects of long-term exposure to the stress-related catecholamines NE and EPI associated or not with the tobacco carcinogen NNK (4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone) on the malignant phenotype of human oral keratinocytes (NOK-SI). Occurrence of cellular atypia, cell migration, proliferation and expression of β-adrenergic (β-AR) receptors were analyzed after chronic treatment with NE, EPI and NNK. Morphological analysis showed that NE and EPI associated or not with NNK induced nuclear membrane irregularity, cellular atypia (cells with two or more atypias), and total mitosis in NOK-SI cells, as well as the carcinogen alone. NOK-SI oral keratinocytes with NE and EPI-induced atypia had higher proliferation rates. Increased cell migration was observed in NOK-SI cells chronically exposed to NE and EPI associated with NNK. Long-term treatment with stress hormones and tobacco carcinogen induced lower expression of β1-adrenergic (β1-AR) and β1-adrenergic (β2-AR) receptors, except treatment with NE which induced higher expression of the receptor β2-AR. This study shows for the first time that long-term exposure to the stress hormones NE and EPI associated or not with the tobacco carcinogen NNK induce malignancy-related phenotypic changes in NOK-SI human oral keratinocytes. The results suggest that stress-related catecholamines may have an impact on the oral carcinogenesis.

Keywords: stress, cancer, oral cancer, squamous cell carcinoma, keratinocytes, NNK, nitrosamines, catecholamines, norepinephrine, epinephrine, β-adrenergic receptors.

1. INTRODUCTION

Modern life is accompanied by stressors and while acute stress is beneficial to the organism, chronic stress has a negative effect on health. Chronic stress causes dysregulation in the hypothalamic-pituitary-adrenal (HPA) axis, resulting in an increase in cortisol production.[1] This dysregulation in cortisol production is accompanied by elevated levels of catecholamines, norepinephrine and epinephrine, as a result of activation of the sympathetic nervous system (SNS).[1] Psychological stress has been shown to enhance the progression of pancreatic cancer xenografts and increased serum and xenograft levels of norepinephrine, epinephrine, and cortisol.[2] Although the association of stress with cancer progression has been widely studied, few studies have evaluated the effects of stress on carcinogenesis. In normal oral human keratinocytes, norepinephrine and cortisol induced DNA damage at concentrations that mimic psychological stress in humans.[3]

According to the World Health Organization[4] cancer is the second leading cause of death in the world. Since in 2020 it was estimated more than 300 thousand new cases of oral cancer and almost 200 thousand deaths in the world.[5] In relation to oral cancer the main risk factor is the chronic use of tobacco and alcohol. Tobacco contains a wide variety of chemicals, including nicotine and carcinogens known as nicotine-derived nitrosamines.[6] Nitrosamine known as NNK has a promoting effect on mutations that can lead to uncontrolled cell proliferation, migration and invasion, making cells more resistant to cell cycle arrest and apoptosis.[7,8] Furthermore, like catecholamines, NNK is also a β-AR receptor agonist.[6] Studies show that β-ARs receptors play an important role in oral cancer.[9-15]

Long[16] and short[17] exposures to stress hormones induces expressive DNA damage in mouse fibroblasts, resulting in malignant transformation of these cells. Chronic stress also induced malignant transformation in bronchial epithelial cells.[18] Likewise, stimulation with NNK promotes malignant transformation in normal epithelial cells.[19,20] To our knowledge, there are no investigation focusing on the effects of stress hormones in conjunction with the tobacco carcinogen on normal oral epithelial cells. Nuclear changes such as abnormal nuclear size, nuclear contour irregularities, hyperchromasia and aberrant chromatin distribution are used to evaluate cellular malignancy.[21] In this study, we analyzed the occurrence of nuclear membrane irregularity, changes in nuclear size, increased mitoses and presence of atypical.

We proposed that the association of the tobacco carcinogen NNK with the stress-related catecholamines norepinephrine and epinephrine would potentiate the effect of the carcinogen. Here, we evaluate the effects of long-term exposure of norepinephrine and epinephrine associated or not with NNK on the malignant phenotype of normal oral human keratinocytes.

2. MATERIALS AND METHODS

Cells and culture conditions

The NOK-SI immortalized human oral keratinocyte cell line was provided by Dra. Aline Satie Takamiya (School of Dentistry of Araçatuba, São Paulo State University – UNESP, Araçatuba, São Paulo, Brazil). Cells were maintained and propagated in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen,

Carlsbad, CA, USA), 100 µg/mL penicillin, 100 µg/mL streptomycin, and 0.1% gentamicin, at 37 °C, in 5% CO2 humidified atmosphere.

Carcinogen and hormone treatment

Drugs used in this study were: NNK (Catalog number: 64091-91-4, Sigma, St. Louis, MO, EUA), norepinephrine (Catalog number: 108341-18-0, Merck, Darmstadt, Germany) e epinephrine (Catalog number: 329-63-5, Cayman Chemical, Ann Arbor, MI, EUA). The concentrations of catecholamines NE and EPI used in the experiments were defined based on a previous study.[10] Basal circulating levels of NE and EPI range from 10 pM to 1nM.[22] Stress conditions can increase these levels up to 10 µM in the tumor microenvironment.[22,23] NNK concentration was defined based on dose-response assays (data not shown). The stimuli were renovated every 48h along with the renewal of the culture medium for 30 days. Thus, the study was composed of 6 experimental groups: 1. Control group: unstimulated cells; 2. NNK group: cells treated only with the chemical carcinogen NNK at 50 µM; 3. NE group: cells treated only with the hormone norepinephrine at 10 µM; 4. EPI group: cells stimulated only with the hormone epinephrine at 10 µM; 5. NNK + NE group: cells stimulated with 50 µM NNK in association with 10 µM norepinephrine; and 6. NNK + EPI group: cells stimulated with NNK at 50 µM in association with epinephrine at 10 µM.

Morphological features of malignancy

To assess whether the effects of stress-related hormones in association or not with the chemical carcinogen NNK induce morphological changes related to the malignant phenotype, 7 x 10^5 NOK-SI cells from each experimental group were placed on common glass slides and after 24 hours were fixed with methanol

absolute for 20 minutes and stained with Hematoxylin and Eosin (H&E). Then, images were captured by the XC50 camera (Olympus, Shinjuku, TYO, JPN) coupled to a BX53 microscope (Olympus, Shinjuku, TYO, JPN). In all, 12 fields from each experimental group were evaluated by two evaluators blind to the experimental groups for the occurrence of nuclear size alteration, nuclear membrane irregularity, cellular atypia (cells with two or more atypias), atypical mitosis and total mitosis.[21,24,25]

BrdU cell proliferation assay

The BrdU assay was done using the BrdU Cell Proliferation Kit (Roche Diagnostics, Mannheim, BW, GER). The cells of each experimental group treated for 30 days with hormones and/or NNK were seeded in 96-well plates (1.8×10^4 cells/well) and cultured in 200 µL of DMEM culture medium with 10% SFB until they reached 80% confluence. The cell proliferative potential was then evaluated by the BrdU incorporation technique using the kit according to the manufacturer's recommendations. Colorimetric quantification was determined by spectrophotometry adjusted to a wavelength of 370 nM. The results were presented in optical density (OD).

Wound healing migration assay

The cells from the different experimental groups exposed to stress hormones and/or NNK for 30 days were seeded in 6-well plates (1×10^6 cells/well) and cultured in 2 ml of DMEM culture medium with 10% SFB until reach 80% confluence. A monolayer scratch was performed using a 200 µL pipette tip, creating a cell-free region in the center of each well. The cells were washed three times with phosphate-buffered saline (PBS), replaced with new DMEM containing

10% FBS and incubated at 37°C and 5% CO2 in a humidified incubator. The images were captured 24, 48 and 72 hours after the wounding using UC50 (Olympus, Shinjuku, TYO, JPN) camera attached to the CKX41 (Olympus, Shinjuku, TYO, JPN) microscope. The percentage of cell migration was characterized by the percentage decrease of the cell free area with the aid of the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

β1-AR and β2-AR expression in NOK-SI cells

Treated cells were seeded at a concentration of $7x10^5$ cells/well in 6-well plates containing coverslips. After adhering, cells were washed with PBS, fixed with 4% paraformaldehyde at room temperature (RT) and permeabilized with 100% methanol for 20 minutes at -20ºC. Subsequently, the cells were blocked for 1 hour with 3% bovine serum albumin (BSA) at room temperature and then incubated with primary antibody diluted in 1% BSA at 4ºC, in a humid chamber, overnight. The primary antibodies used in this study were β1-AR (Catalog number: ab3442, 1:100; Abcam, Cambridge, MA, USA) and β2-AR (Catalog number: ab182136, 1:100; Abcam, Cambridge, MA, USA). Next, the coverslips were washed with PBS and incubated with specific secondary antibody: anti-rabbit (Catalog number: A32731, 1:1000; Alexa Fluor 488, ThermoFisher Scientific, Carlsbad, CA, USA). After 1h incubation protected from light, the cells were washed with PBS and the coverslips were mounted with medium containing 4′, DAPI for nucleic acid staining. The signals expressed were detected and imaged with a BX53 microscope (Olympus, Shinjuku, TYO, JPN) associated with a XC50 camera (Olympus, Shinjuku, TYO, JPN). The fluorescence was quantified with software CellProfiler 4.0.7 (Broad Institute, Cambridge, MASS, USA).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). To assess differences among the experimental groups, One-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons was used. Results are presented as mean ± standard error of the mean (SEM). The critical level was set at 5% ($p<0.05$) to admit differences in values as statistically significant.

3. RESULTS

NNK and stress hormones induce morphological changes in oral keratinocytes

To evaluate the effects of NNK and stress-related hormones on the morphological changes of oral keratinocytes, NOK-SI cells were stimulated for 30 days with NE and EPI in the presence or absence of the NNK. Morphological analysis showed an increase in the occurrence of nuclear membrane irregularity in NOK-SI cells stimulated with the carcinogen NNK ($p<0.0001$; Figs. 1B and H), hormone NE $p<0.0001$;Figs. 1C and H), hormone EPI ($p= 0.0015$; Figs. 1D and H) and NNK associated with EPI ($p=0.0091$; Figs. 1F and H). By associating NNK with NE, the effect promoted by the carcinogen and the hormone alone was inhibited ($p=0.0200$; $p=0.0004$; Figs 1E and H). NE alone induced a higher percentage of atypical cells ($p=0.0136$; Figs. 1C and I). The occurrence of mitosis was higher in cells treated with NNK associated with EPI ($p=0.0192$; Figs. 1F and K). Although not reaching significance, cells treated with NNK or NE displayed higher occurrence of changes in nucleus size ($p>0.05$; Fig. 1G). Similarly, treatment with

EPI alone and with NNK associated with EPI induced a higher occurrence of atypical mitosis, but this result did not reach significance (p>0.05; Fig. 1J).

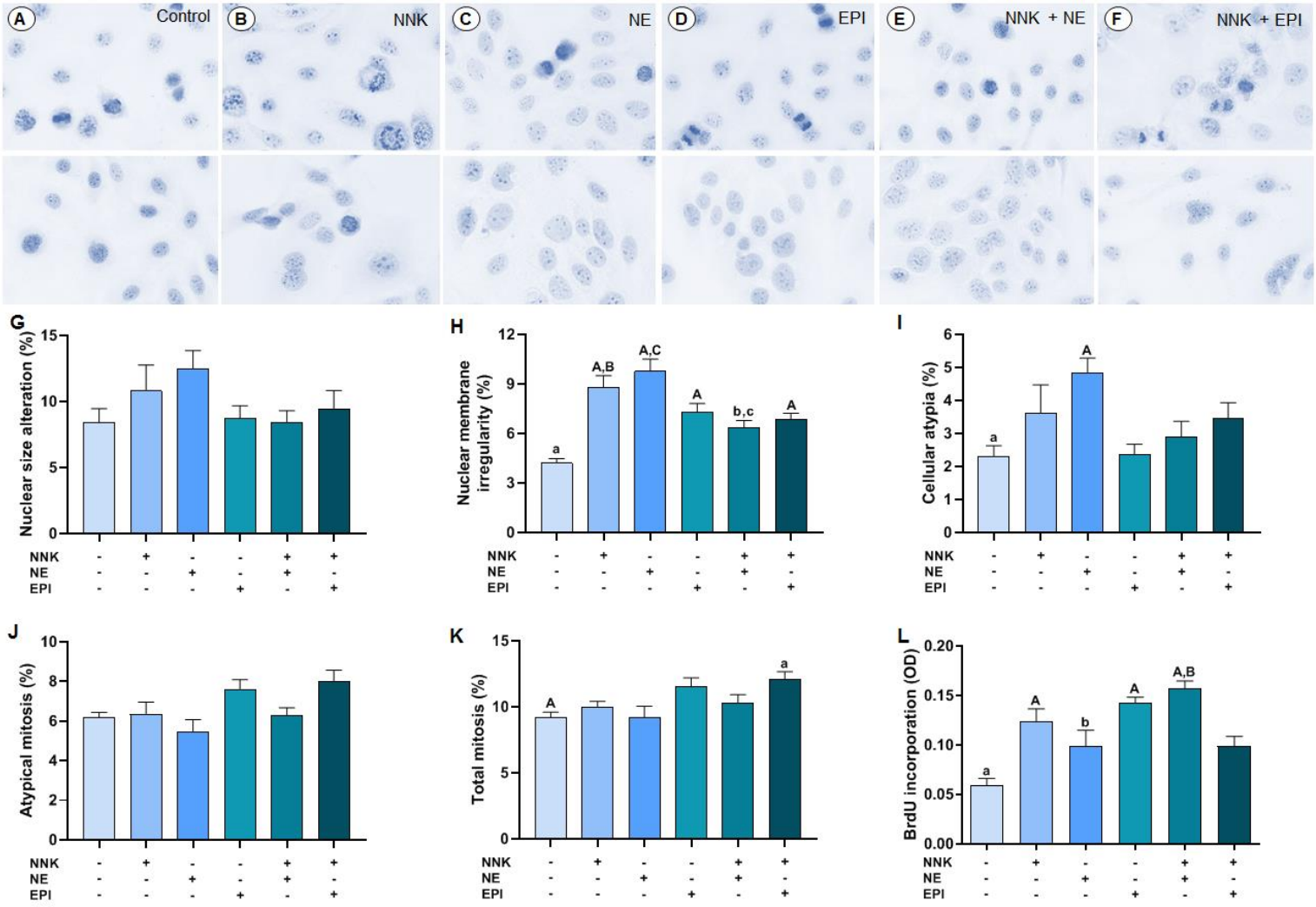

Figure 1. Effects of stress hormones in the presence and/or absence of the carcinogen NNK on the morphology and proliferation of NOK-SI cells. Keratinocytes NOK-SI derived from the oral mucosa were stimulated with NNK **(B)**, NE **(C)**, EPI **(D)**, NNK with NE **(E)** and NNK with EPI **(F)**. **(A)** Unstimulated cells were used as a control. **(G)** There was no significant difference between the experimental groups regarding the nuclear size alteration. **(H)** Cells treated with NNK, NE, EPI and NNK associated with EPI showed a higher rate of nuclear membrane irregularity compared to cells without treatment. **(I)** NE increased cellular atypia. **(J)** There was no significant difference between the experimental groups regarding the atypical mitosis. **(K)** Treatment with NNK associated with EPI increased the percentage of mitoses. **(L)** Cells treated with NNK, EPI and NNK associated with NE showed a higher rate of cell proliferation compared to cells without treatment. One-way ANOVA analysis was used to assess differences between experimental groups. The results are expressed as the mean ± SEM. Upper and lower cases equal letters indicate a statistically significant difference (p<0.05). H&E, original 400X magnification.

NNK and stress-related hormones induce increased cell proliferation of oral keratinocytes

To assess whether the morphological changes induced by NNK in oral keratinocytes NOK-SI are accompanied by changes in their proliferative potential after 30 days of sustained chronic exposure to stress-related hormones associated or not with NNK, we used the BrdU incorporation assay to assess cell proliferation rates. The results showed that the isolated treatment with NNK ($p= 0.0042$; Fig. 1L) and EPI ($p= 0.0008$; Fig. 1L) induced an increase in cell proliferation compared to unstimulated cells. Treatment only with NE also induced an increase in cell proliferation, but the result was not significant ($p>0.05$; Fig. 1L). However, when NE was associated with the carcinogen NNK there was a significant increase in the proliferative potential of NOK-SI cells ($p=0.0002$; Fig. 1L). In contrast, the association of EPI with NNK reduced the proliferative effect induced by EPI, but this inhibitory effect was not significant. ($p>0.05$; Fig. 1L).

Norepinephrine and epinephrine induce increased migration of oral epithelial cells

In order to verify the effects of sustained chronic exposure to NNK and stress-related hormones on cell migration, NOK-SI cells were evaluated for wound closure after 24, 48, and 72h. The results showed that after 24h ($p=0.0179$; Fig. 2G) and 72h ($p=0.0087$; Fig. 2I) EPI induced increase migration of NOK-SI cells. This EPI-induced increase in NOK-SI cell migration was inhibited when cells were stimulated by the hormone in conjunction with the carcinogen NNK ($p<0.0001$; Fig. 2H). After 24h, NNK in association with NE increased the migratory potential of oral epithelial cells ($p=0.0004$; Fig. 2G). The results also showed that NNK alone inhibited the migration of NOK-SI cells, although a significant inhibitory

effect was observed only after the 48h (p=0.0146; Fig. 2H). This effect was reversed when the carcinogen was associated with the NE hormone (p=0.0004; Fig. 2H).

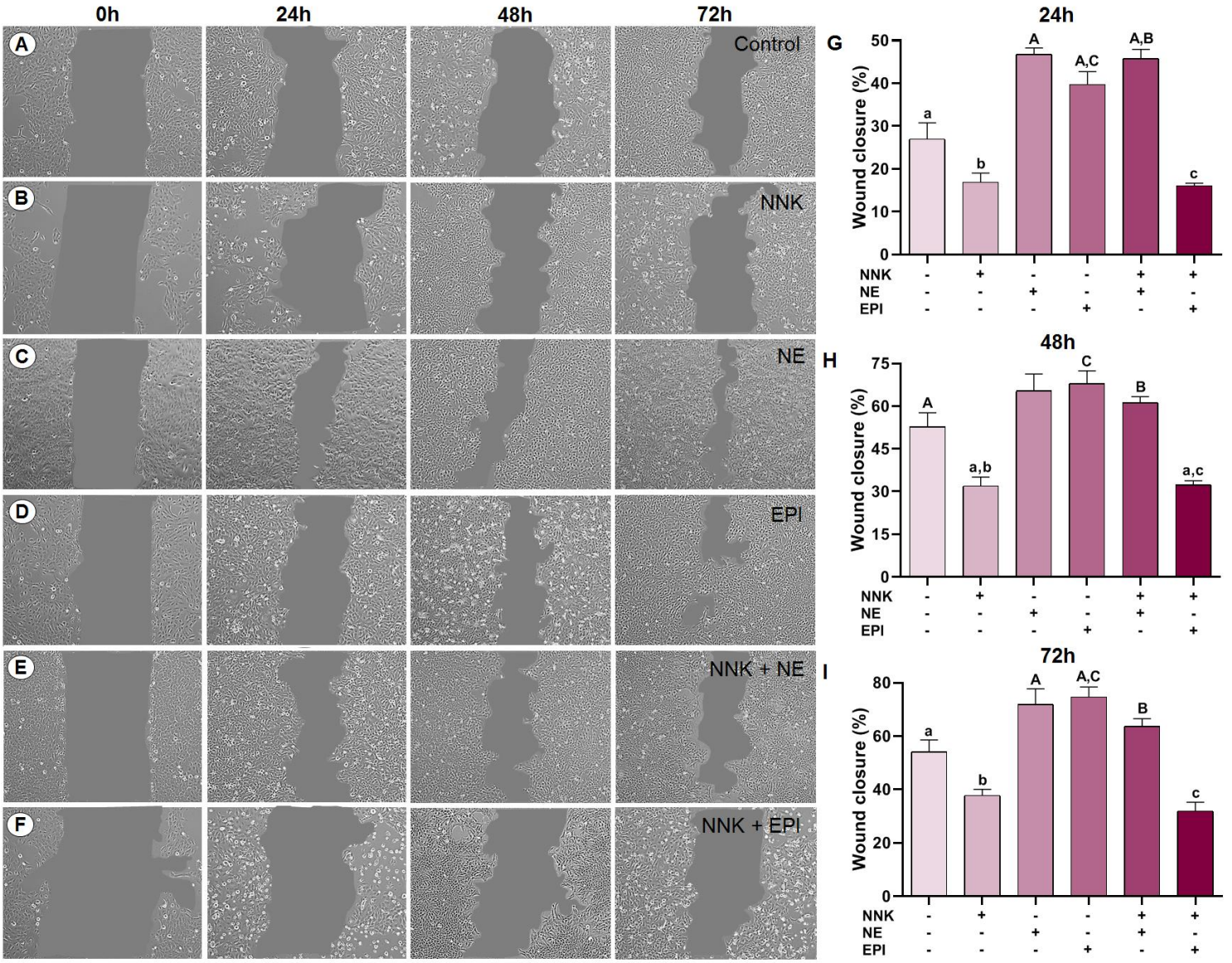

Figure 2. Stress hormones induce higher migration of NOK-SI cells. (A) Untreated cells. **(B)** NNK. **(C)** NE. **(D)** EPI. **(E)** NNK with NE. **(F)** NNK with EPI. **(G)** Cells treated with NE, EPI and NNK with NE showed higher migration after 24h when compared to untreated cells. **(H)** After 48h, cells treated with NNK and NNK with EPI had lower migration when compared to cells without treatment. **(I)** Cells from NE 10 μM and EPI 10 μM groups showed higher migration than cells from the control group. One-way ANOVA analysis was used to assess differences between experimental groups. The results are expressed as the mean ± SEM. Upper- and lower cases equal letters indicate a statistically significant difference ($p<0.05$). Original 100X magnification.

Sustained chronic exposure to NNK and stress-related hormones reduces the expression of β-adrenergic receptors in NOK-SI cells

Immunofluorescence assays were performed to evaluate the expression of β1-AR and β2-AR receptors in NOK-SI cells stimulated with NNK and stress-related hormones. The analysis of β1-AR receptor expression revealed higher expression of this receptor in the unstimulated cells compared to all treated cells ($p<0.0001$; Fig. 3A). When associating NNK with NE, the cells showed lower expression compared to NNK and NE alone ($p<0.0001$; Fig. 3A, C, D and F). The treatment with NNK associated with EPI induced lower expression of the β1-AR receptor when compared with NNK alone ($p<0.0001$; Fig. 3A, C and G). However, cells treated with NNK associated with EPI had increased expression of the β1-AR receptor when compared to cells treated with EPI alone ($p<0.0001$; Fig. 3A, E and G). treated cells with NE alone had higher β2-AR receptor expression compared to untreated cells ($p<0.0001$; Fig. 3H, I and K). However, by associating the hormone with NNK, the carcinogen inhibited the expression induced by the isolated NE ($p<0.0001$; Fig. 3H, K and M). When NOK-SI cells were chronically treated with the association of NNK with EPI, they display lower expression of the β2-AR receptor compared to cells treated with the hormone alone ($p<0.0001$; Fig. 3H, L and N).

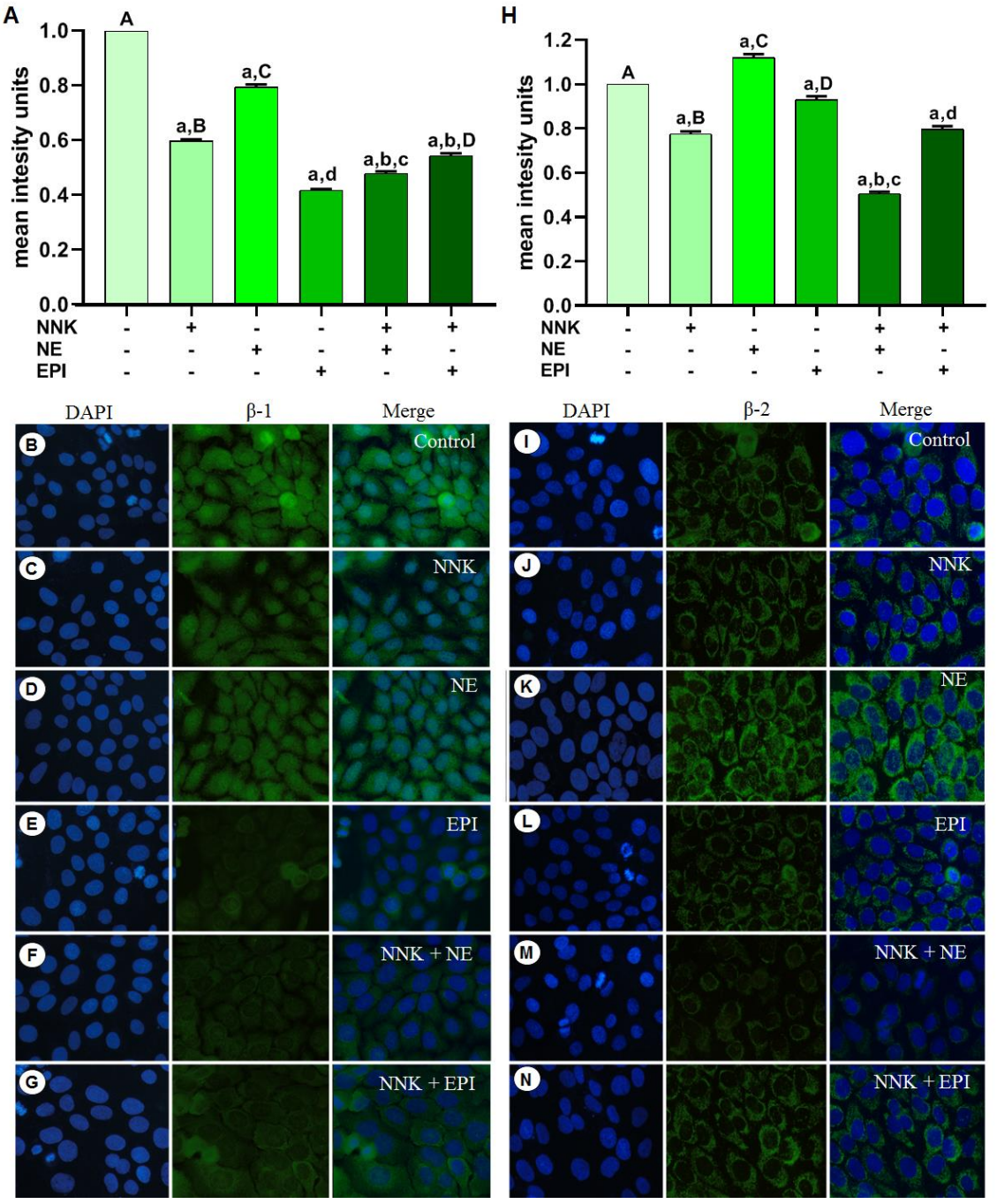

Figure 3. Expression of β1 and β2 adrenergic receptors after exposure to NNK and stress hormones. (A) Cells without treatment showed higher expression of the receptor, the association of NNK with NE inhibited the expression induced by the isolated hormone. **(H)** Cells treated only with NE showed higher expression of the receptor, but when the hormone was associated with NNK, the carcinogen inhibited the expression induced by the hormone, the association of NNK with NE inhibited the expression induced by the isolated hormone. **(B and I)** Untreated cells. **(C and J)** NNK. **(D and K)** NE. **(E and L)** EPI. **(F and M)** NNK with NE. **(G and N)** NNK with EPI. One-way ANOVA analysis was used to assess differences between experimental groups. The results are expressed as the mean ± SEM. Upper- and lower cases equal letters indicate a statistically significant difference ($p<0.05$). Original 400X magnification.

4. DISCUSSION

This study demonstrates for the first time that the stress-related catecholamines norepinephrine and epinephrine induce a malignant phenotype in normal oral keratinocytes, associate to morphological alterations, cell proliferation and migration. The distinction between benign and malignant cells is based on the morphological characteristics of the nucleus.[21] Nuclear membrane irregularity, changes in nuclear size, increased mitoses and presence of atypical mitoses are histopathological features of malignancy found in the process of malignant transformation.[21,24,25] Therefore, these characteristics were evaluated in our study to analyze the malignant phenotype in oral keratinocytes after long-term exposure to stress hormones and/or carcinogen. The results showed that nuclear membrane irregularity was the most significant alteration. Stress hormones, NE and EPI, alone induced a higher percentage of cells with nuclear membrane irregularity. Similar to our study, but in tumor cells, Zhang et al.,[26] showed that NE treatment induces morphological changes in colorectal and lung adenocarcinoma cells. EPI also induced morphological changes in astrocytes from the rat cortex by promoting changes in the membrane surface of these cells.[27]

As expected, our results showed that the tobacco carcinogen alone also induced a higher percentage of cells with nuclear membrane irregularity. Schwartz et al.[28] observed that cells from exfoliative cytology of smokers display high morphological changes when compared with cells from non-smokers. Furthermore, an in vitro study demonstrated that immortalized normal oral keratinocytes showed morphological changes after treatment with NNK.[29] In the current study, when associating the hormone EPI and the carcinogen NNK

increased percentage of cells with nuclear membrane irregularity and percentage of mitoses. Surprisingly, in our study, by associating NE with NNK, the effect of the hormone and the carcinogen on the nuclear membrane irregularity was inhibited. When we evaluated cells with two or more types of atypias, long-term treatment with NE induced a high number of atypias in the same cell. According to our results, both stress hormones and tobacco carcinogen are able to promote morphological changes associated with the process of malignant transformation of oral epithelial cells.

Cell cycle imbalance can result in deregulation of proliferation, favoring the process of carcinogenesis and/or tumor progression. In our study, stimulation with EPI alone increased the proliferation of oral keratinocytes. Zhang et al.,[30] also demonstrated that exposure to NE and EPI can increase proliferation of gastric cancer cells. However, in our study, the NE-induced proliferation did not reach significance. Despite this, when associating the hormone with the carcinogen, there was an increase in the proliferation of oral keratinocytes. Chronic exposure to NNK alone also induced increased cell proliferation. However, a study with bronchial epithelial cells showed a decrease in proliferation when stimulated cells with 50 μM of NNK. However, unlike our study, the treatment time was only 48 hours.[31] In contrast, a study with breast cancer cells showed increased expectations when chronically stimulating cells with a lower concentration of NNK.[19] One of the fundamental characteristics of cancer is its ability to sustain chronic proliferation.[32] Uncontrolled proliferation favors tumor growth, and when a malignant phenotype is achieved, the rate of proliferation exceeds the rate of apoptosis.[33] Our results show that stress hormones and

tobacco carcinogen induce a dysregulation of proliferation in NOK-SI oral keratinocytes.

Cell migration is a prerequisite for cancer invasion and metastasis. Our results showed that that treatment with stress hormones NE and EPI alone increases cell migration in oral keratinocytes. As in our study, Jeong et al.,[34] showed high migration of hepatocellular carcinoma and breast cancer cells after stimulation with 10 μM NE and EPI. By associating NE with NNK, the effect of the hormone on cell migration was maintained. Surprisingly, NNK alone inhibited cell migration. Conversely, Zhuang et al.,[35] demonstrated high migration of esophageal epithelial cells after treatment with NNK at lower concentrations than those used by us. We expected that the hormones NE and EPI, when associated with NNK, would potentiate the effect of the carcinogen on cellular atypia, proliferation and migration. However, when associating EPI with NNK, the carcinogen inhibited the effect promoted by the hormone alone. The same occurred with nuclear membrane irregularity when associating NE with NNK, as previously described. So, we hypothesized that there could be an desensitization of β-adrenergic (β-AR) receptors caused by long-term exposure with β-AR agonists, since both NNK and hormones act on these receptors. Therefore, we performed immunofluorescence assays to assess the effects of chronic exposure to stress hormones and tobacco carcinogen on the expression of β1-adrenergic (β1-AR) and β2-adrenergic (β2-AR) receptors.

Recent evidence has shown strong expression of β-AR receptors in oral tumors,[9-12,14] as well as blocking β-AR receptors can reduce tumor progression.[13] In our study, surprisingly, only NE induced an increase in the expression of the β2-AR receptor in NOK-SI keratinocytes. On the other hand, long-term exposure

to EPI and NNK reduced the expression of β1-AR and β2-AR receptors in NOK-SI cells. Higher expression of β2-AR has been reported compared to other β-AR receptors in oral cancer cell lines and in oral squamous cell carcinoma tissues, suggesting that β2-AR is the main β-AR in response to NE in oral squamous cell carcinoma.[14] In this same study, oral squamous cell carcinoma cells treated with NE had increased levels of β2-AR, while the expression of other adrenergic receptors was not altered.[14] However, in the cited study, the stimulation with NE was acute and in our study the cells were exposed to NE chronically. In line with our results, Kim et al.,[36] reported decreased β2-AR receptor expression in lung cells after long-term exposure to β-AR agonist. Furthermore, Yamaguchi et al.,[37] suggest that immobilization stress and chronic stimuli with β-AR agonist promote desensitization of the β1-AR receptor in rats.

5. CONCLUSION

Our results suggest that the stress hormones NE and EPI induce malignancy-related phenotypic changes in oral keratinocytes after sustained chronic exposure. Both NE and EPI induced morphological characteristics compatible with atypia found in the process of malignant transformation of oral epithelial cells. Furthermore, these morphological changes were accompanied by high migration and cell proliferation. Together, the results suggest that stress hormones may have an inducing role in oral carcinogenesis.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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