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Chemopreventive Activity of Systemically Administered Curcumin on Oral Cancer in the 4-Nitroquinoline 1-Oxide Model

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ABSTRACT

Curcumin has therapeutic potential in preventing several types of cancer, including colon, liver, prostate, and breast. The goal of this study was to evaluate the chemopreventive activity of systemically administered curcumin on oral carcinogenesis induced by 4-nitroquinolone-1-oxide (4-NQO). A total of 50 male albino rats, *Rattus norvegicus*, (Holtzman), were divided into five groups (n = 10 per group). Four of these groups were exposed to 50 ppm 4-NQO in their drinking water ad libitum for 8 or 12 weeks, two groups were treated with curcumin by oral gavage at 30 or 100 mg/kg per day, and one group was treated with corn oil (vehicle) only. The negative control group was euthanized at baseline. Tongues of all animals were removed after euthanasia and used in the subsequent analysis because the tongue is the primary site of carcinogenesis in this model. Descriptive histological analysis and immunohistochemistry for PCNA, Bcl-2, SOCS1 e-3, and STAT3 were performed to assess the oncogenic process. The gene expression of Vimentin, E-cadherin, N-cadherin, or TWIST1 was assessed using RT-qPCR as a representative of epithelial-mesenchymal transition (EMT) events. The administration of curcumin at 100 mg/kg during the 12 weeks markedly decreased the expression of PCNA, Bcl-2, SOCS1 e -3, and STAT3. Curcumin also minimized the cellular atypia under microscopic analysis and diminished the expression of the genes associated with EMT. These findings demonstrate that the systemic administration of curcumin has chemopreventive activity during oral carcinogenesis induced by 4-NQO. J. Cell. Biochem. 116: 787–796, 2015.

KEY WORDS: 4-NITROQUINOLINE-1-OXIDE; RAT; TONGUE; CURCUMIN; CHEMOPREVENTION

Oral cancer is the largest group cancer of the head and neck [Scully and Kirby, 2014] and third most frequent cancer, with a 5-year survival rate of less than 50% [Warnakulasuriya, 2009]. To better understand the process of carcinogenesis in their tissue and molecular aspects, models are routinely used in studies highlighting the 4-NQO, which consists of a chemical carcinogen within the group of polycyclic aromatic hydrocarbons (HPA), being widely used in the production of experimental carcinogenesis in animals because of its application to induce the formation of sequential stages of the carcinogenic process similar to that occurring in humans, which are made possible by the ability of 4-NQO to exert potent intracellular oxidative stress by generating 4-hidroxiaminoquinolona-N-oxide (4HAQO), a metabolic product which binds to DNA [Kanojia and Vaidya et al., 2006].

There is a high demand for identifying and developing chemical or herbal drugs that may increase the efficacy of the therapeutic approaches that have been established for some cancers and for reducing the deleterious side-effects of these therapies. One

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phytotherapy that is widely studied in cancer is curcumin, a polyphenolic compound derived from the rhizome of the plant Curcuma long.

Curcumin has various biological activities and excellent tolerance when administered systemically [Guimarães et al., 2011; Guimarães et al., 2012]. Some studies have reported the safety of using curcumin and its many antitumor activities in relation to many cancers, such as human head and neck squamous cell carcinoma, lung adenocarcinoma, and cell lines of oral squamous cell cancer cells [Aggarwal et al., 2004; Sharma et al., 2006; Chen et al., 2008].

Some activities of curcumin are reported, for example, inhibition cell proliferation, stimulates apoptosis in oral squamous cell cancer cell lines [Aggarwal et al., 2004; Sharma et al., 2006], potentiates apoptotic effects induced by chemotherapeutic agents [Kamat et al., 2007] and reduces the expression of STAT3 [Weissenberger et al., 2010]. However fewer studies have suggested that curcumin may play a role in tumorigenesis. The goal of this study was to evaluate the chemopreventive activity of systemically administered curcumin in the 4-nitroquinoline 1-oxide (4-NQO) model of oral cancer because it is a promising adjuvant in the treatment of several types of cancers.

MATERIAL AND METHODS

ANIMALS

All experimental protocols were approved by the Ethics Committee at the School of Dentistry at Araraquara-UNESP (Proc. CEUA No. 14/2012). A total of 50 male albino rats, *Rattus norvegicus*, (Holtzman), approximately 3–4 months old, were obtained from the Central Facility of Experimental Animals, UNESP Campus at Araraquara. The animals were randomly distributed into five groups ($n = 10$) and maintained at a temperature of 21°C ± 1, humidity of 65–75% and a controlled light cycle (12 h light-12 h dark), with feed and water available ad libitum. Four groups were treated with a solution of 4-NQO at 50 ppm (Sigma, USA) dissolved in the drinking water. The protocol used for the treatment with 4-NQO was based on the initiation-promotion protocol as described elsewhere [Ribeiro and Salvadori, 2007]. One group, the negative control group, was not subjected to any treatment and was sacrificed on the first day of the experiment [Vered et al., 2007; Noguti et al., 2012]. (Fig. 1)

TREATMENT WITH CURCUMIN

Two groups were exposed to 4-NQO and then treated with curcumin (Sigma/SLBD0850 V Life Science) by oral gavage, one group was

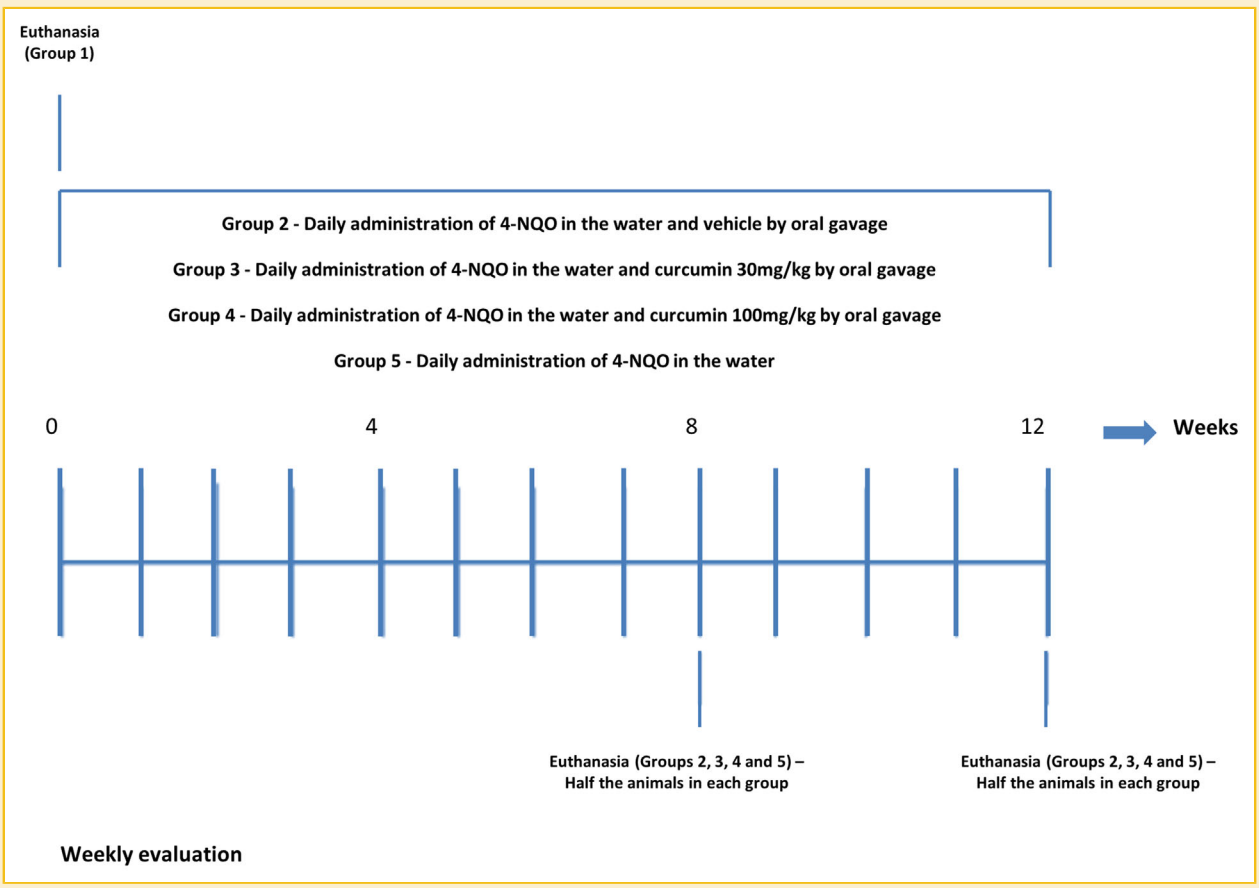

Figure 1. Distribution of groups of rats treated or not treated with 4-NQO. Of the groups submitted to 4-NQO, two groups were treated with 30 or 100 mg/kg body weight of curcumin and one group with vehicle (corn oil), daily by oral gavage, during the whole experiment. The rats were euthanized after 8 or 12 weeks.

administred 30 mg/kg and the group received 100 mg/kg of body weight, once per day during the 8 and 12 weeks. Curcumin was diluted in corn oil as a vehicle and the suspensions were prepared twice a week. A third group exposed to 4NQO was treated with corn oil (vehicle) only, using the same volume that was used for the larger dose of curcumin, during the same periods. (Fig. 1)

SAMPLE COLLECTION

After completing the experimental periods, all rats were euthanized using general anesthesia (ketamine, 80 mg/kg and xylazine, 20 mg/kg), and their tongues were removed. Tongues were treated in two different ways: (1) snap frozen in liquid nitrogen and kept in the −80°C until RT-qPCR analysis; or (2) fixed in 10% buffered formalin (pH 7.2) for approximately 48 hours and washed under running water for 24 h. Formalin-fixed specimens were embedded in paraffin to obtain semi-serial longitudinal sections that were 5 μm thick (antero-posterior). A total of 40 slices from each tongue were obtained per animal in each group, stained with hematoxylin and eosin (H&E), or used in the immunohistochemical analysis.

HISTOPATHOLOGICAL ANALYSIS (H&E)

Histopathological analysis was performed using a light microscope (Carl Zeiss, Germany). A trained and calibrated examiner who was unaware of the experimental groups to which each section belonged conducted the analysis. The grading of dysplasia and the detection of dysplastic cells followed the criteria established in the literature, focusing on tissue organization and cytological changes [Smith and Pindiborg, 1969]. The architectural changes in dysplastic epithelium were evaluated as follows: interpapillary bulbous or teardrop, loss of polarity of basal cells, irregular epithelial stratification, keratinization of individual cells or groups of cells of the spinous layer and loss of typical cell cohesion crests. The degree of oral dysplasia was recorded as described in the literature [Van Der Waal et al., 1997]: mild dysplasia: changes limited to the basal and parabasal layers; moderate dysplasia: changes from the basal layer to the middle portion of the spinous layer; and severe dysplasia: changes from the basal layer to an above average level of the epithelium. Histopathological analysis was performed by two independent observers, and a consensus was reached. The descriptive analysis indicated that most of the tissue changes associated with our model of 4NQO-induced oral cancer were located in the posterior region of the tongue, the area chosen for immunohistochemical and gene expression analysis.

IMMUNOHISTOCHEMISTRY

Histological sections from the posterior region of the tongue (five animals per group) were used to assess the expression of proliferating cell nuclear antigen (PCNA), apoptosis markers (Bcl -2), suppressor of cytokine signaling (SOCS3 and SOCS1) and signal transducer and activator of transcription (STAT3). The procedure for immunohistochemistry was standardized for each antibody as follows: PCNA (1:100 - Invitrogen 133900), Bcl-2 (1:200 - Abcam 7973), SOCS1 (1:200–9021 Santa Cruz Biotechnology), SOCS3 (1:200 - Abcam 16030), and STAT3 (1:200 - Santa Cruz Biotechnology). The target proteins were detected using a DAB-streptavidin (LSAB-2, Dako Cytomation) system. The sections were counter stained with hematoxylin for 15 s, and coverslips were mounted in Permount. Images were captured using a light microscope (Diastar-Cambridge Instruments) at 200× magnification. Semi-quantitative analysis was carried out by a trained and calibrated examiner who was unaware of the experimental groups, using the H-score method. To determine the H-score, both the intensity (0–3) of positive staining and the percentage (0–100) of positive cells were considered. For example, 10% of the cells with an intensity of 3, 30% of the cells with an intensity of 2, 20% of the cells with an intensity of 1, and 40% unstained cells result in an H-score of 110 $[(10 \times 3) + (30 \times 2) + (20 \times 1) + (40 \times 0) = 110]$. The H-score was determined in a minimum of three slides/specimen and four animals/experimental group. The data were combined for each target protein, for each experimental group, and in each experimental period.

RT–REAL TIME PCR

Samples from five animals from each experimental group were used, but only for the 12-week experimental period. Total RNA was extracted from tissue fragments dissected from the posterior region of freshly thawed tongues using a mini RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA purity was assessed by optical density (260/280 nm ratio >1.9; Nanodrop® 2000c, ThermoScientific, Canada). Samples were then treated with DNase (deoxyribonuclease I Amp Grade®, Invitrogen®, CA, USA), as directed by the manufacturer, and 700 ng of total RNA from each sample was used for synthesis of cDNA using the High-Capacity cDNA kit Reverse Transcription® (Applied Biosystems®, USA). The gene expression analysis was performed using primers and probes that were previously designed for the genes of interest and for an endogenous control gene that was not affected by the experimental procedure (GAPDH). The detection of target cDNA amplification via the release of the fluorophore linked to the TaqMan probe was performed using TaqMan chemistry (TaqMan Master Mix Fast, ®, Applied Biosystems®, USA) for each gene: TWIST1 (Twist homolog 1), Rn 00585470_s1; Vim (Vimentin), Rn00579738_m1; Cdh1 (E-cadherin 1), Rn00580109_m1; Cdh2 (N-cadherin 2), Rn00580099; GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and NM008084. The pre-optimized, standardized conditions of the qPCR reactions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Each sample was assayed in duplicate, and the data were analyzed using the thermocycler's software (StepOne Plus, Applied Biosystems, USA) with the relative quantification method (ΔΔCt), in which the normalized relative expression of target genes in the negative control animals was compared with the expression in the experimental groups.

DATA ANALYSIS

Immunohistochemistry and gene expression data were analyzed by statistical analysis using the GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA) software. ANOVA (one-way) test was used, followed by Tukey's test for multiple comparisons between groups. All tests were applied with a confidence level of 95% ($P < 0.05$) in this setting.

RESULTS

BODY MASS

Treatment with curcumin in the presence or absence of 4-NQO did not induce remarkable differences in body weight during the experimental periods evaluated in this setting. The weight gain for all animals after 8 or 12 weeks was regular, indicating that the experimental procedure did not interfere with basal metabolism of the animals.

CURCUMIN EFFECTIVELY PREVENTS DYSPLASIA AND ATYPIA OF TONGUE EPITHELIUM ASSOCIATED WITH 4NQO-INDUCED CARCINOGENESIS

Microscopically, tongues from the negative control group presented keratinized stratified squamous epithelium, with the presence of taste buds. The epithelium of the anterior portion of the tongue was more flat, with slightly prominent interdigitation and covered by a thin layer of keratin. The middle portion showed slight thickening of the epithelium and keratin layer. In the posterior region, particularly in the region of the filiform papillae, the epithelium protruded prominently with irregular shape that was covered by a thick layer of keratin (Figs. 2A and 3A). After 8 weeks of treatment with 4-NQO or 4-NQO + vehicle, all rats showed severe epithelial dysplasia, primarily in the posterior region of the tongue. Typically, the severity of dysplasia is reflected by the presence of atypical cells in the epithelial layer (Figures 2B and 2C). These changes were attenuated when animals were treated with 30 mg/Kg and 100 mg/kg of curcumin (Fig. 2D,E). After 12 weeks of treatment with 4-NQO or 4-NQO + vehicle, no remarkable differences were noticed; atypical cells were not more prominent compared with the 8-week group (Fig. 3B,C). In one case, there was a cleft epithelium with disruption of the granular layer (Fig. 3B). After treatment with 30 mg/kg of curcumin, we observed a decrease in atypical cells in different layers of the epithelium, and treatment with 100 mg/Kg of curcumin markedly diminished the number of atypical cells (Fig. 3D,E).

CURCUMIN DECREASES THE EXPRESSION OF SELECTED ORAL CANCER BIOMARKERS IN 4NQO-INDUCED EARLY CARCINOGENESIS

PCNA from the negative control group showed an H-score of 273. Immunopositive cells were restricted to the basal cell layer of the epithelium. After 8 weeks of treatment with 4-NQO or 4-NQO + vehicle, the expression of PCNA increased by 147% (H-score = 402) and 138% (H-score = 378), respectively. After 12 weeks, this increase was 111% (H-score = 305) and 96% (H-score = 264), respectively, compared with the negative control group ($P < 0,05$). These results indicate that exposure to 4-NQO increased proliferation in the tongue epithelium and the corn oil vehicle had no significant effect. Treatment with curcumin for 8 weeks at either 30 or 100 mg/kg did not significantly alter the values of the H-score for PCNA compared with the groups treated with 4-NQO or 4-NQO + vehicle ($P > 0.05$). However, after 12 weeks of treatment with 100 mg/kg of curcumin, the intensity of PCNA labeling (H-score 229) was significantly ($P < 0.05$) decreased compared with the group treated with 30 mg/kg curcumin (H-score = 387); this intensity was not significantly different from the negative control group.

As expected, the expression of anti-apoptotic Bcl-2 was very weak in the negative control group, and the Bcl-2-immunopositive

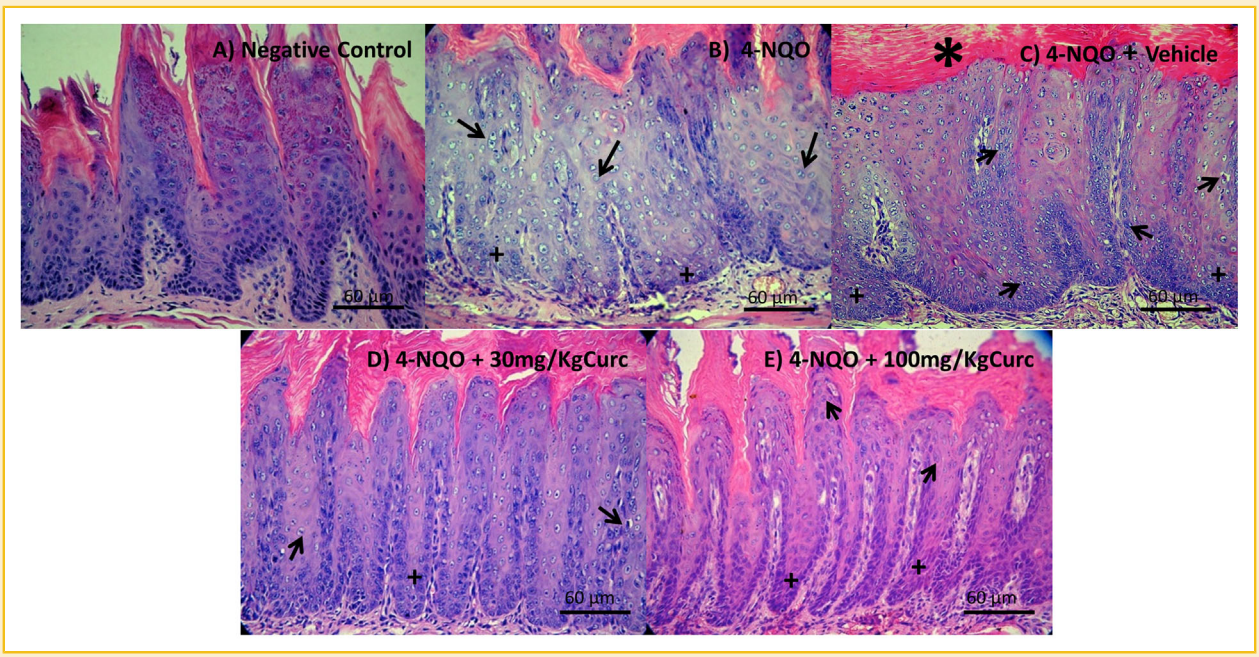

Figure 2. Histopathological analysis (H&E). Groups of experimental period of 8 weeks. Can be observed in figures B and C pronounced epithelial changes, severe dysplasia, characterized by atypical mitoses extending into the granular layer, and nucleus/cytoplasmic ratio increased (arrows) and disruption of the basal cell layer (+), hyperkeratosis (*), among others; and in the figures D and E also can be observed alterations (arrows), however, lesser degree, smaller amount of mitoses and juxtaposed basal cells (+), showing the strong reduction of these atypical cells. H&E staining.

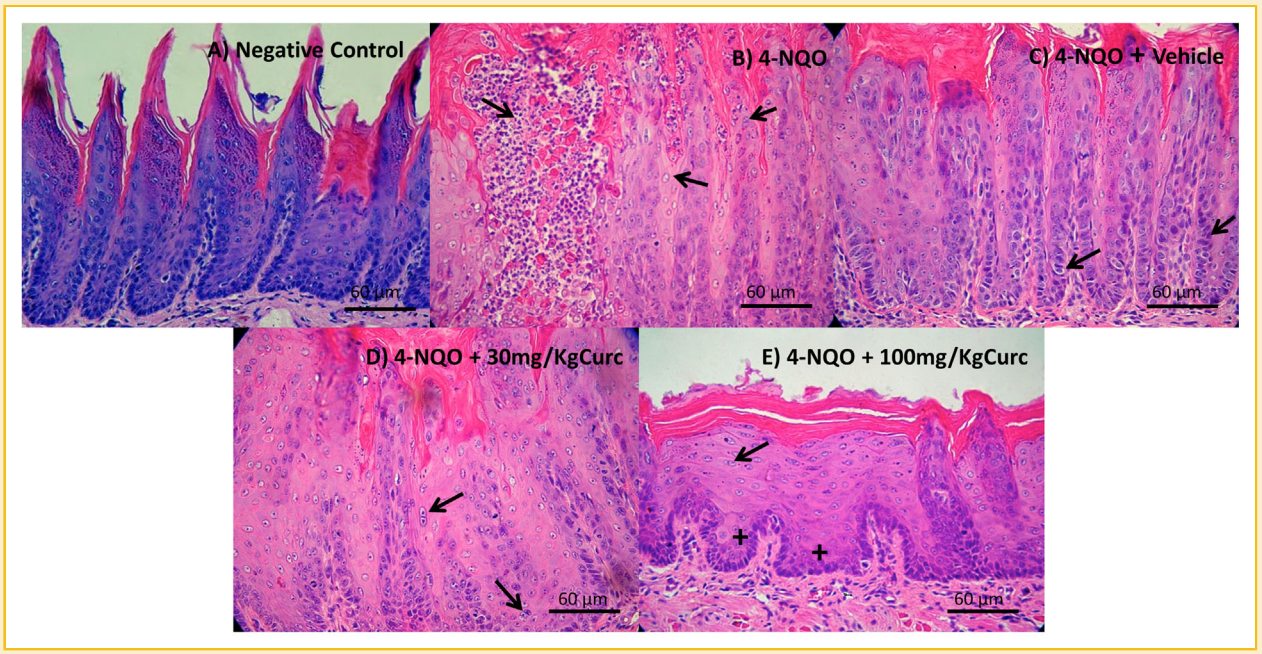

Figure 3. Histopathological analysis (H&E). Groups of experimental period of 12 weeks. In fifure B and C, cellular and structural changes more pronounced than in the period of 8 weeks (atypical mitoses, hypherchromatic nucleus, nucleus/cytoplasm ratio increased–arrows). Can be observed in figures D and E, epithelial changes and atypical cells (arrows), however with evident and accentuated reduction of them when compared to other groups. In this group (4NQO + 100 mg/KgCurc) cells of the basal layer already demonstrated organized similarly to the control group (+). H&E staining.

cells were distributed equally in all layers of the tongue epithelium (H-score = 146). Bcl-2 expression increased after 8 weeks of treatment with 4-NQO or 4-NQO + Vehicle (226% and 265%, respectively); after 12 weeks, this increase in Bcl-2 expression was sustained in both groups in comparison to the negative control group, though it was slightly reduced to 210% and 174%, respectively. Curcumin administration for 8 weeks increased the expression of Bcl-2 compared with the negative control group by 312% and 332% at 30 and 100 mg/kg, respectively ($P < 0.05$). At the 12-week period, the highest dose of curcumin caused a discrete and non-statistically significant reduction of Bcl-2 expression compared with the other experimental groups ($P > 0.05$).

All samples presented SOCS1 expression in the negative control group, but at low-intensity staining (H-score = 45) and occurring in different strata of the oral epithelium. After 8 weeks, there was a significant increase of SOCS1 following treatment with 4-NQO (251% increase, H-score = 113) and 4-NQO + Vehicle (70% increase, H-score = 103) ($P < 0.05$). At 12 weeks, the expression of SOCS1 remained significantly higher in the group treated with 4-NQO (177% increase, H-score = 80) than in the negative control group ($P < 0,05$). Treatment with 30 mg/kg of curcumin for 8 weeks increased SOCS1 expression by 344% (H-score = 155) compared with the negative control ($P < 0.05$). This increase remained after 12 weeks in both groups treated with 30 mg/kg curcumin (291% increase, H-score = 131) or 100 mg/kg of curcumin (411% increase, H-score = 185) ($P < 0.05$).

All samples in the negative control group were also positive for SOCS3 expression. After 8 weeks of exposure to 4-NQO, SOCS3 expression increased by 505% (H-score = 197) and, interestingly, even further in the 4-NQO + Vehicle group (748% increase, H-score = 292%) ($P < 0.05$). This initial increase was maintained at the 12-week period of exposure to 4-NQO and treatment with vehicle (274% increase, H-score = 107). SOCS3 expression was massively increased by 656% compared with the negative control group at 30 mg/kg of curcumin for 8 weeks (H-score = 256) ($P < 0.05$) and slightly decreased (H-score = 181) by treatment with 100 mg/kg of curcumin ($P > 0.05$). After 12 weeks of exposure to 4-NQO and treatment with 100 mg/kg of curcumin, SOCS3 expression was markedly reduced (approximately 80% decrease, H-score = 19) compared with the groups treated with 4-NQO + Vehicle (H-score = 107) and the groups treated with 30 mg/kg of curcumin (H-score = 93) ($P < 0.05$).

STAT3 immunostaining was positive in the membrane and cytoplasm of oral epithelial cells. The immunoexpression was present and distributed in all epithelial layers in all samples of the negative control group, but with low intensity (H-score = 20). After 8 weeks, the expression of STAT3 was reduced in the groups exposed to 4-NQO (70% decrease, H-score = 6) and 4-NQO + Vehicle (95% decrease, H-score = 1) ($P < 0.05$). At 12 weeks, there was a significant reduction in the expression of STAT3 in the group treated with 4-NQO (95% decrease H-score = 1) compared with the negative control group. The treatment with 100 mg/kg of curcumin for 8 weeks (H-score = 5) was associated with a reduction of 75% in STAT3 expression in comparison with the negative control group and 17% in comparison with 4-NQO ($P < 0.05$). After 12 weeks, the expression of STAT3 in both groups treated with curcumin was

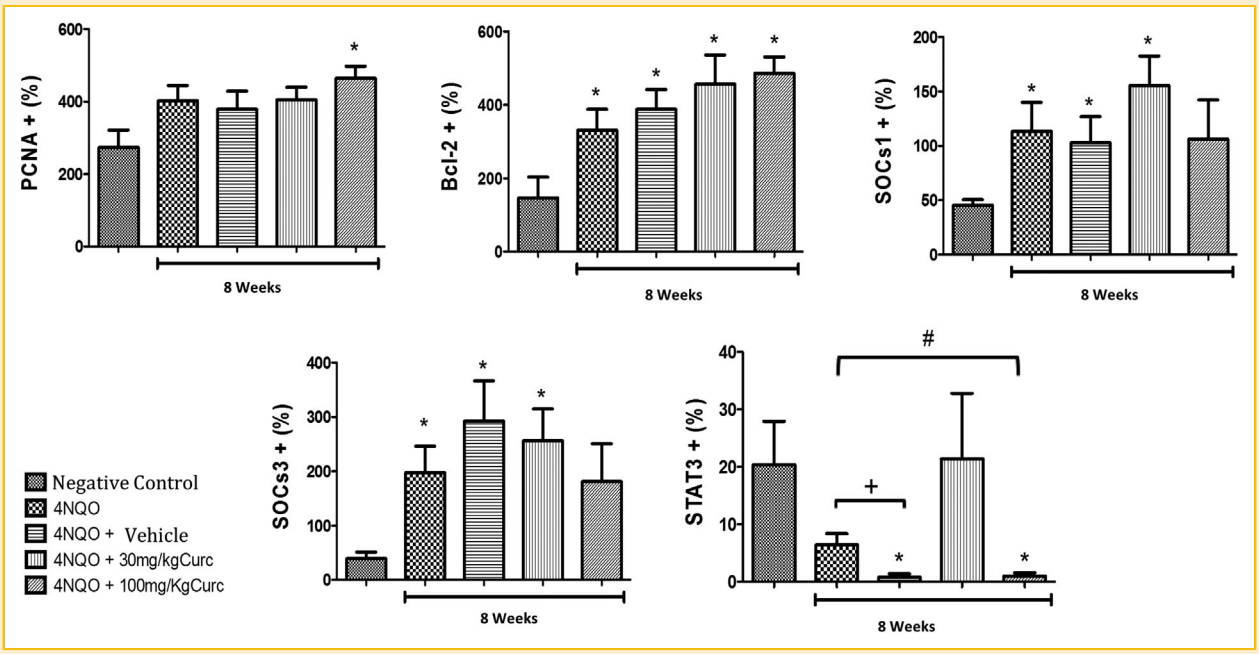

Figure 4. Immunohistochemical analysis for the samples related to experiment period of 8 weeks. The bars indicate the means and vertical lines the standard deviations. (*$P < 0.05$ compared to the negative control group, +$P < 0.05$ compared to 4-NQO + vehicle group, #$P < 0.05$ compared to 100 mg/kg body weight of curcumin group).

reduced compared with negative control group, but not significantly ($P > 0.05$). Conversely, the expression of STAT3 in the curcumin-treated groups at 12 weeks was higher than that of the groups treated with 4-NQO and vehicle (H-score = 56 and 4 in the 30 and 100 mg/kg groups, respectively; H-score = 1 in the 4-NQO-vehicle group; $P < 0.05$). (Figs. 4 and 5)

EXPRESSION OF EMT-ASSOCIATED GENES IN THE 4NQO MODEL OF ORAL CARCINOGENESIS IS ALSO SIGNIFICANTLY REDUCED BY CURCUMIN

Gene expression analysis was performed only on samples from the 12-week experimental period, which showed more prominent histological changes that were consistent with the oncogenic process. The expression of Cdh2 (N-Cadherin) was increased following treatment with 4-NQO + Vehicle and 4-NQO, presenting significant differences compared with the negative control group ($P < 0.05$). Treatment with both doses of curcumin significantly reduced the expression of Cdh2 compared with 4-NQO ($P < 0.05$).

The expression of Cdh1 (E-cadherin) was also increased after treatment with 4-NQO and 4-NQO + vehicle in comparison with the negative control group ($P < 0.05$). Treatment with both doses of curcumin reduced the expression of Cdh1 ($P > 0.05$). Vimentin and TWIST1 expression were slightly increased by the treatment of 4-NQO and 4-NQO + Vehicle compared with the negative control ($P > 0.05$). Treatment with 30 and 100 mg/kg significantly reduced the expression of Vimentin in comparison with either group exposed to 4-NQO ($P < 0.05$), whereas a non-statistically significant trend for TWIST1 expression to decrease was observed following curcumin treatment ($P > 0.05$). (Fig. 6)

DISCUSSION

Because the systemic administration of curcumin does not cause adverse effects when administered experimentally in animals [Gulcubuk et al., 2006; Fu et al., 2008; Guimarães et al., 2011] or humans [Goel et al., 2007; Anand et al., 2008] and because of its potent biological activities, curcumin is a potential sole or adjunct therapeutic agent in a variety of conditions. Evidence obtained from in vitro and in vivo studies indicated that curcumin has a therapeutic potential in treating and/or preventing several chronic diseases, including cancer [Gulcubuk et al., 2006; Fu et al., 2008; Lin et al., 2010; Zong et al., 2012]. The doses of 30 and 100 mg/kg of curcumin used in this study were based on previous studies [Guimarães et al., 2011], which showed that these doses of curcumin administered intragastrically were well tolerated by the animals and showed notable biological effects in attenuating the immune and inflammatory response in an experimental model of periodontal disease. The experimental periods of 8 and 12 weeks adopted in this study represent the initial stages of the carcinogenic process induced by 4-NQO [Tanaka et al., 1994; Noguti et al., 2012]. Therefore, to evaluate the effects of curcumin in the early stages of the carcinogenesis process, curcumin was administered concomitantly with the experimental periods mentioned above in a ′preventive model′. The 4-NQO induced notable changes in the tongue epithelium after 8 weeks of exposure, and these changes were even more defined and severe after 12 weeks. Systemic treatment with curcumin was associated with decreased cellular atypia and marked attenuation of dysplastic and other pre-oncogenic morphological changes, particularly at 100 mg/kg dose in the 12-week period. These findings are in

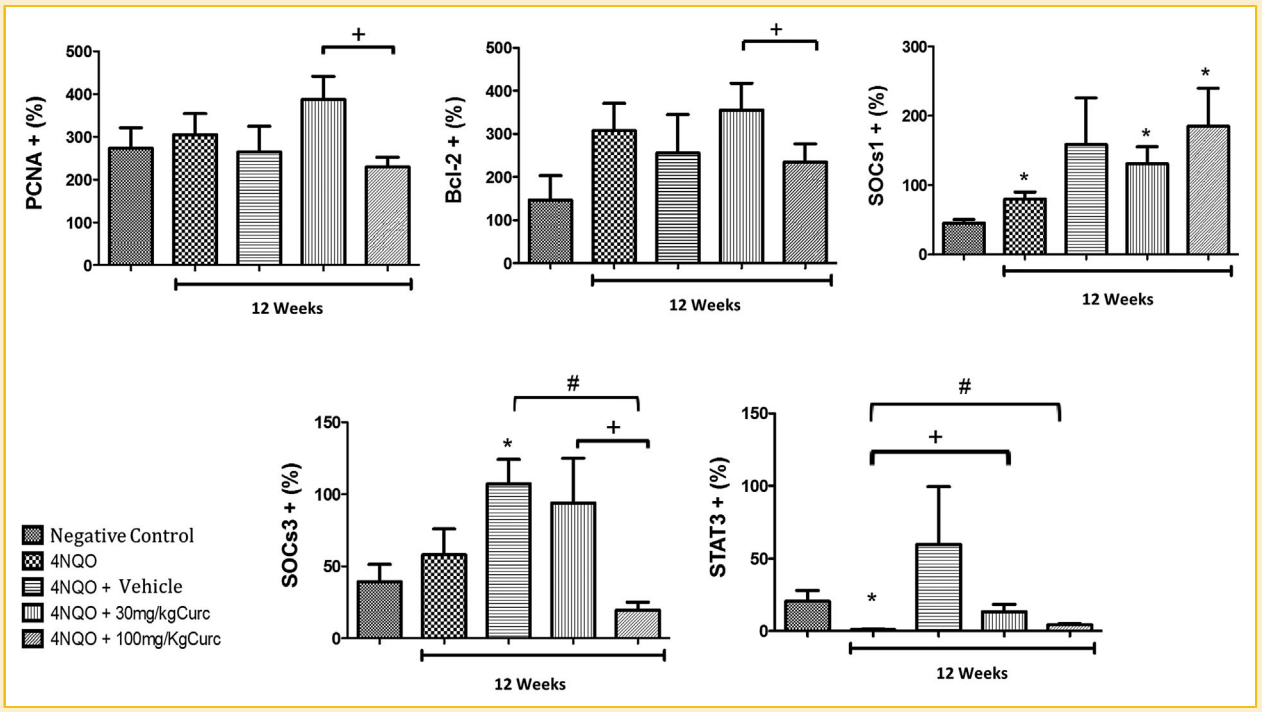

Figure 5. Representative graphics of the immunohistochemical analysis for detection of PCNA, Bcl-2, SOCS1, and 3, STAT3, for the samples related to experimental period of 12 weeks. The bars indicate the means and vertical lines the standard deviations. (*$P < 0.05$ compared to negative control group).

line with the literature [Tanaka et al., 1994], with a reported reduction in premalignant lesions in rats treated with curcumin when using the 4-NQO experimental protocol to examine the development of oral carcinogenesis.

To obtain further insight into the biological mechanisms involved in the chemopreventive effects of curcumin in the 4-NQO oral cancer model, we assessed the expression of candidate genes associated with tumor activity and growth, including proliferation, apoptosis, endogenous regulatory mechanisms of cytokine signaling, and expression of genes associated directly with EMT process. The proliferating cell nuclear antigen (PCNA) [Zhao et al., 2012], apoptosis regulatory gene (Bcl-2) [Placzek et al., 2010], suppressor of cytokine signaling 1 and 3 (SOCS1 and SOCS3) [Molavi et al., 2013; Ohara et al., 2013], and signal transducer and activator of gene transcription 3 (STAT3) [Buettner et al., 2002; Pardoll et al., 2009] are all involved in oral cancer activity and progression. Vimentin, Cdh1, Cdh2, and TWIST1 are the candidate genes chosen to be representatives of carcinogenesis and neoplastic transformation (EMT) [Nijkamp et al., 2011; Silva et al., 2012; Zhang et al., 2013], possibly acting in the malignant transformation of oral premalignant lesions. The immunopositivity for PCNA indicating active proliferation was found only in the basal and parabasal cell layers of normal epithelium. In dysplastic areas, PCNA positivity was evident in cells from the spinous and granular layers. This is consistent with other studies [Faria, 2006; Silva et al., 2007]. Furthermore, treatment with 4-NQO increased PCNA immunostaining after 12 weeks. These results were consistent with previous studies using the 4-NQO model to induce squamous cell carcinoma (SCC) in mice [Silva et al., 2007; Moon et al., 2012]. We observed that Bcl-2 was also elevated after treatment with 4-NQO after 8 and 12 weeks, as reported in other studies [Nishimura, 1999] that used the same methodology as the present work. This finding supports the theory of an increased resistance of these cells to apoptotic cell death, which is characteristic of cancerous lesions. The administration of curcumin for 8 weeks did not change the proliferative response and did not reduce the increase in Bcl-2 induced by exposure to 4-NQO; however, there was a significant reduction in PCNA expression after 12 weeks. There was a non-significant, but noticeable, decrease in Bcl-2 expression, which has been reported by other authors [Dahmke et al., 2013] who have investigated the impact of the oral administration of curcumin on murine melanoma. Furthermore, the decrease in Bcl-2 by curcumin was found when the mechanism of action of curcumin administration was studied in small-cell lung cancer [Li et al., 2013]. Our in vivo results indicate that systemically administered curcumin at 100 mg/kg decreased cell proliferation and attenuated the resistance to apoptosis in pre-cancerous lesions.

The expression of other target proteins analyzed, with the exception of STAT3, was also increased by treatment with 4-NQO in both experimental periods. Interestingly, the data indicates that the vehicle (corn oil) had a modulatory effect on some of the genes evaluated; the effect included a reduction of SOCS1 and increase of SOCS3, which may be related to the possible influence of the vehicle on lipid metabolism. Therefore, subsequent studies that are currently in progress will use a non-lipid, nano-structured vehicles for entrainment of curcumin. The increase of SOCS3 and the reduction of STAT3, verified after treatment with 4-NQO in both periods,

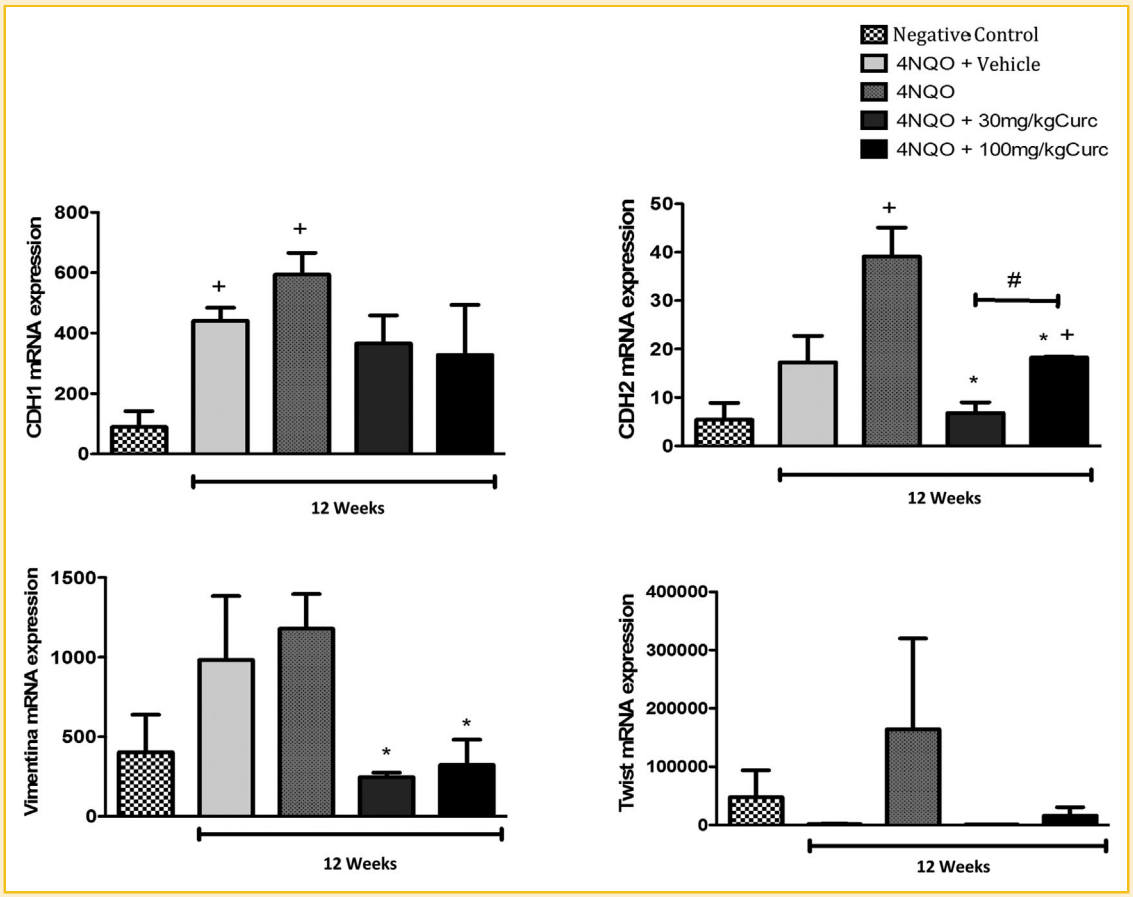

Figure 6. Representative graphics of the gene expression by RT-qPCR for samples related to the experimental period of 12 weeks. The bars in the graphs indicate the means and vertical lines the standard deviations. (+$P < 0.05$ compared to the negative control group, *$P < 0.05$ compared to 4-NQO group, #$P < 0.05$ between groups 30 and 100 mg/KgCurc).

contradict the well-documented events of STAT3 increased activity and decreased SOCS3 expression in oral cancer and other carcinomas [Buettner et al., 2002; Isomoto, 2009; Pardoll et al., 2009; Molavi et al., 2013). We think that the experimental periods used in this study represent early stages of the carcinogenesis process; these contradictory findings regarding STAT3 and SOCS3 may be typical of early stages in the tumorigenesis process. This rationale is supported by our histological findings of increased dysplasia and initial/in situ lesions, instead of 'full-fledged', locally invading typical oral cancer lesions. In support of these findings, a study analyzing the immunostaining of SOCS3 in several types of cancer reported an increase during the early events of the carcinogenic process [Ohara et al., 2013]. The changes in the expression of EMT-related genes further support the interpretation of an early stage of tumorigenesis in our model because the E-cadherin expression was increased in the experimental groups, whereas the expressions of TWIST1 and Vimentin were only slightly increased.

Interestingly, SOCS3 immunopositivity at 12 weeks was reduced compared with the immunopositivity at 8 weeks, which may be the result of the progress of the tumorigenesis process. The persistent low levels of STAT3 immunopositivity may be due to the remaining, but slightly reduced, SOCS3 expression. We should also consider the limitations of not being able to determine the subcellular localization of SOCS3 because some HNSCC cells still present SOCS3 expression, but with increased nuclear localization; this may affect the role of SOCS3in inhibiting STAT3 activation [Rossa et al., 2012].

SOCS1 expression was also increased and remained increased after 12 weeks of treatment with 4-NQO. These results are similar to the findings reported by a previous study [Raccurt et al., 2003]. We speculate that this increased expression of SOCS1 is related to a resistance of the epithelial cells to the malignant transformation, as reported in the literature [Cooper et al., 2010].

Treatment with the 100 mg/kg dose of curcumin concomitant with exposure to 4-NQO reduced the immunostaining of both SOCS3 and STAT3 at 8 weeks, with a more marked decrease in STAT3 expression at 12 weeks. This result suggests that curcumin's inhibition of STAT3 activity is not mediated by an induction in the expression of its endogenous inhibitor, SOCS3. Curcumin was able to reduce STAT3 immunoexpression, as noted when evaluating the role of xenobiotics in a rat glioma cell line [Weissenberger et al., 2010]. It is important to stress that the marked reduction of SOCS3 by curcumin may not represent a favorable mechanism in suppressing the progression of carcinogenesis. Conversely, SOCS1 was significantly increased by both doses of curcumin, particularly after treatment with 100 mg/kg for 12 weeks. This event has been previously described [Chen et al.,

2013]. Recent studies [Park et al., 2003; Strebovsky et al., 2011] have suggested that SOCS1 regulates NF-kB signaling. Our research group [Guimarães et al., 2013] has recently reported curcumin-induced SOCS1 expression increased in macrophages, which may represent an additional mechanism involved in the inhibition of NF-kB by curcumin as part of its anti-inflammatory effects. This curcumin-mediated increase in the expression of the endogenous negative regulator of NF-kB may have favorable effects in inhibiting the oncogenic process and/or tumor growth and progression.

The expression of Vimentin was increased by exposure to 4-NQO and significantly reduced by curcumin treatment, suggesting a direct effect of this compound in interfering with EMT. These findings have also been described by other authors, who found a reduced expression of vimentin in pancreatic cancer cell lines after treatment with curcumin [Sun et al., 2013]. Therefore, the decreased vimentin expression modulates the carcinogenesis process, particularly the process involved in cell invasion and metastasis [Liu et al., 2010; Nijkamp et al., 2011]. Interestingly, the expression of Cdh1 (E-cadherin) was increased by exposure to 4-NQO and was also attenuated by treatment with curcumin. Because this is a gene involved in cell-cell adhesion and the proliferation of epithelial cells (and in the inhibition of an invasive phenotype) the modulation observed is not clear in terms of pro- or anti-oncogenic outcomes. These results are similar to the results of other studies, which reported elevated expression of this gene after the administration of curcumin in pancreatic [Sun et al., 2013] and hepatic cancer cell lines [Chang et al., 2013]. However, exposure to 4-NQO also increased the expression of N-cadherin (Cdh2), which is associated with EMT; this increase was significantly attenuated by curcumin treatment. The TWIST1 gene is involved in the later phases of tumor development; it plays an important role in the progression of oral squamous cell carcinoma [Ou et al., 2008] and acts as a predictor of distant metastasis [Xie et al., 2009]. Curcumin treatment also reduced (though non-significantly) its expression. Considering the contribution of each of these genes in the course of oral carcinogenesis, the systemic administration of curcumin may have an inhibitory effect on EMT events.

In summary, curcumin was effective in reversing the severity and intensity of occurrence of cellular dysplasia and atypia; it also modulated the expression of candidate genes associated with EMT, tumor growth, and progression in a manner consistent with a chemopreventive effect of the oncogenic changes induced by the chemical carcinogen 4-NQO. It is important to highlight that curcumin was used as the sole therapeutic agent. As such, this study provides evidence for the potential use of this compound, which should be further explored as an adjunctive therapy; different dosages and vehicle presentations should also be explored. Additional studies will improve the understanding of the molecular mechanisms involved in the chemopreventive effect of curcumin in oral cancer.

CONCLUSION

This study demonstrates the beneficial effects of curcumin in modulating the oral carcinogenesis process by attenuating changes induced by the chemical carcinogen 4-NQO, including changes in the expression of genes associated with cell proliferation and EMT.

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REFERENCES

Aggarwal S, Takada Y, Singh S, Myers JN, Aggarwal BB. 2004. Inhibition of growth and survival of human head and neck squamous cell carcinoma cells by curcumin via modulation of nuclear factor-kappaB signaling. Int J Cancer 111(5):679–692.

Anand P, Sundaram C, Jhurani S, Kunnumakkara AB, Aggarwal BB. 2008. Curcumin and cancer: An "old-age" disease with an "age-old" solution. Cancer Lett 267(1):133–164.

Buettner R, Mora LB, Jove R. 2002. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin Cancer Res 8(4):945–954.

Chang YH, Jiang M, Liu KG, Li XQ. 2013. Curcumin inhibited hypoxia induced epithelial-mesenchymal transition in hepatic carcinoma cell line HepG2 in vitro. Zhongguo Zhong Xi Yi Jie He Za Zhi 33(8):1102–1106.

Chen CC, Sureshbabul M, Chen HW, Lin YS, Lee JY, Hong QS, et al. 2013. Curcumin suppresses metastasis via Sp-1, FAK inhibition, and E-Cadherin upregulation in colorectal cancer. Evid Based Complement Alternat Med 541695. DOI: 10.1155/2013/541695

Chen HW, Lee JY, Huang JY, Wang CC, Chen WJ, Su SF, et al. 2008. Curcumin inhibits lung cancer cell invasion and metastasis through the tumor suppressor HLJ1. Cancer Res 68(18):7428–7438.

Cooper JC, Shi M, Chueh FY, Venkitachalam S, Yu CL. 2010. Enforced SOCS1 and SOCS3 expression attenuates Lck mediated cellular transformation. Int J Oncol 36(5):1201–1208.

Dahmke IN, Backes C, Rudzitis-Auth J, Laschke MW, Leidinger P, Menger MD. 2013. Curcumin intake affects miRNA signature in murine melanoma with mmu-miR-205–5p most significantly altered. PLoS One 8(12):DOI: 10.1371/journal.pone.0081122

Faria PR. 2006. Oral carcinogenesis induced by 4-NQO in tongue Knockout mice for the galectin-3 gene [dissertation]. Uberaba: Federal University of Triangulo Mineiro.

Fu Y, Zheng S, Lin J, Ryerse J, Chen A. 2008. Curcumin protects the rat liver from CCl4-caused injury and fibrogenesis by attenuating oxidative stress and suppressing inflammation. Mol Pharmacol 73(2):399–409.

Goel A, Kunnumakkara AB, Aggarwal BB. 2007. Curcumin as Curecumin: from kitchen to clinic. Biochem Pharmacol 75(4):787–809.

Guimarães MR, Coimbra LS, de Aquino SG, Spolidorio LC, Kirkwood KL, Rossa C, Jr.. 2011. Potent anti-inflammatory effects of systemically administered curcumin modulate periodontal disease in vivo. J Periodontal Res 46(2):269–279.

Guimarães MR, de Aquino SG, Coimbra LS, Spolidorio LC, Kirkwood KL, Rossa C, Jr.. 2012. Curcumin modulates the immune response associated with LPS-induced periodontal disease in rats. Innate Immun 18(1):155–163.

Guimarães MR, Leite FR, Spolidorio LC, Kirkwood KL, Rossa C, Jr.. 2013. Curcumin abrogates LPS-induced pro-inflammatory cytokines in RAW 264.7 macrophages. Evidence for novel mechanisms involving SOCS-1, -3 and p38 MAPK. Arch Oral Biol 58(10):1309–1317.

Gulcubuk A, Altunatmaz K, Sonmez K, Haktanir-Yatkin D, Uzun H, Gurel A, et al. 2006. Effects of curcumin on tumour necrosis factor-alpha and interleukin-6 in the late phase of experimental acute pancreatitis. J Vet Med A Phisiol Pathol Clin Med 53(1):49–54.

Isomoto H. 2009. Epigenetic alterations in cholangiocarcinoma- sustained IL-6/STAT3 signaling in cholangio- carcinoma due to SOCS3 epigenetic silencing. Digestion 79(1):2–8.

Kamat AM, Sethi G, Aggarwal BB. 2007. Curcumin potentiates the apoptotic effects of chemotherapeutic agents and cytokines through down-regulation of nuclear factor-kappaB and nuclear factor-kappaB-regulated gene products in IFN-alpha-sensitive and IFN-alpha-resistant human bladder cancer cells. Mol Cancer Ther 6(3):1022–1030.

Kanojia D, Vaidya MM. 2006. 4–Nitroquinoline–1- oxide induced experimental oral carcinogenesis. Oral Oncol 42(7):655–667.

Li Y, Zhang S, Geng JX, Hu XY. 2013. Curcumin inhibits human non-small cell lung cancer A549 cell proliferation through regulation of Bcl-2/Bax and cytochrome C. Asian Pac J Cancer Prev 14(8):4599–4602.

Lin YC, Chen HW, Kuo YC, Chang YF, Lee YJ, Hwang JJ. 2010. Therapeutic efficacy evaluation of curcumin on human oral squamous cell carcinoma xenograft using multimodalities of molecular imaging. Am J Chin Med 38(2):343–358.

Liu LK, Jiang XY, Zhou XX, Wang DM, Song XL, Jiang HB. 2010. Upregulation of vimentin and aberrant expression of E-cadherin/beta-catenin complex in oral squamous cell carcinomas: Correlation with the clinicopathological features and patient outcome. Mod Pathol 23(2):213–224.

Molavi O, Wang P, Zak Z, Gelebart P, Belch A, Lai R. 2013. Gene methylation and silencing of SOCS3 in mantle cell lymphoma. Br J Haematol 161(3):348–356.

Moon SM, Ahn MY, Kwon SM, Kim SA, Ahn SG, Yoon JH. 2012. Homeobox C5 expression is associated with the progression of 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. J Oral Pathol Med 41(6):470–476.

Nijkamp MM, Span PN, Hoogsteen JI, Van der Kogel JA, Kaanders HAMJ, Bussink J. 2011. Expression of E-cadherin and vimentin correlates with metastasis formationin head and neck squamous cell carcinoma patients. Radiother Oncol 99(3):344–348.

Nishimura A. 1999. Changes in Bcl-2 and Bax expression in rat tongue during 4-nitroquinoline-1-oxide-induced carcinogenesis. J Dent Res 78(6):1264–1269.

Noguti J, Carvalho JG, da Silva VH, Dedivitis RA, Franco M, Ribeiro DA. 2012. Alkylation-induced genotoxicity as a predictor of DNA repair deficiency following experimental oral carcinogenesis. J Mol Histol 43(2):145–150.

Ohara KI, Kondo T, Ito M, Yoshimura A. 2013. SOCS, inflammation, and cancer. JAKSTAT 2(3):e24053.

Ou DL, Chien HF, Chen CL, Lin TC, Lin LI. 2008. Role of Twist in head and neck carcinoma with lymph node metastasis. Anticancer Res 28(2):1355–1359.

Pardoll D, Yu H, Jove R. 2009. STATs in cancer, inflammation and immunity: A leading role of STAT3. Nat Rev Cancer 9(11):798–809.

Park SH, Kim KE, Hwang HY, Kim TY. 2003. Regulatory effect of SOCS on NF-kappaB activity in murine monocytes/macrophages. DNA Cell Biol 22-(2):131–139.

Placzek WJ, Wei J, Kitada S, Zhai D, Reed JD, Pellecchia M. 2010. A survey of the anti-apoptotic Bcl-2 subfamily expression in cancer types provides a platform to predict the efficacy of Bcl-2 antagonists in cancer therapy. Cell Death Dis 1(5):e40.

Raccurt M, Tam SP, Lau P, Mertani HC, Lambert A, Garcia-Caballero T. 2003. Suppressor of cytokine signalling gene expression is elevated in breast carcinoma. Br J Cancer 89(3):524–532.

Ribeiro DA, Salvadori DM. 2007. Gingival changes in wistar rats after oral treatment with 4-nitroquinoline 1-oxide. Eur J Dent 1(3):152–157.

Rossa C, Jr, Sommer G, Spolidorio LC, Rosenzweig SA, Watson DK, Kirkwood KL. 2012. Loss of expression and function of SOCS3 is an early event in HNSCC: Altered subcellular localization as a possible mechanism involved in proliferation, migration and invasion. PLoS One 7:(9).DOI: 10.1371/journal.pone.0045197

Scully C, Kirby J. 2014. Statement on mouth cancer diagnosis and prevention. Br Dent J 216(1):37–38.

Sharma C, Kaur J, Shishodia S, Aggarwal BB, Ralhan R. 2006. Curcumin down regulates smokeless tobacco-induced NF-kappaB activation and COX-2 expression in human oral premalignant and cancer cells. Toxicology 228(1):1–15.

Smith CJ, Pindiborg JJ. 1969. Histological grading of oral epitelial atypia by the use of photografics standards. Copenhagen: C. Hamburger.

Silva BSF, Yamamoto FP, Pontes FSC, Cury SEV, Fonseca FP, Pontes HAR, et al. 2012. TWIST and p-Akt immunoexpression in normal oral epithelium, oral dysplasia and in oral squamous cell carcinoma. Med Oral Patol Oral Cir Bucal 17(1):29–34.

Silva RN, Ribeiro DA, Salvadori DM, Marques ME. 2007. Placental glutathione Stransferase correlates with cellular proliferation during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. Exp Toxicol Pathol 59(1):61–68.

Strebovsky J, Walker P, Lang R, Dalpke AH. 2011. Suppressor of cytokine signaling 1 (SOCS1) limits NFkappaB signaling by decreasing p65 stability within the cell nucleus. FASEB J 25(3):863–874.

Sun XD, Liu XE, Huang DS. 2013. Curcumin reverses the epithelial-mesenchymal transition of pancreatic cancer cells by inhibiting the Hedgehog signaling pathway. Oncol Rep 29(6):2401–2407.

Tanaka T, Makita H, Ohnishi M, Hirose Y, Wang A, Mori H, et al. 1994. Chemoprevention of 4-Nitroquinoline 1-Oxide-induced oral carcinogenesis by dietary curcumin and hesperidin: Comparison with the protective effect of B-Carotene. Cancer Res 54(17):4653–4659.

Van Der Waal I, Schepman KP, Van Der Meij EH, Smeele LE. 1997. Oral leukoplakia: A clinicopathological review. Oral Oncol 33(5):291–301.

Vered M, Allon I, Buchner A, Dayan D. 2007. Stromal myofibroblasts and malignant transformation in a 4NQO rat tongue carcinogenesis model. Oral Oncol 43(10):999–1006.

Warnakulasuriya S. 2009. Causes of oral cancer–an appraisal of controversies. Br Dent J 207(10):471–475.

Weissenberger J, Priester M, Bernreuther C, Rakel S, Glatzel M, Seifert V, et al. 2010. Dietary curcumin attenuates glioma growth in a syngeneic mouse model by inhibition of the JAK1,2 /STAT3 signaling pathway. Clin Cancer Res 16(23):5781–5795.

Xie F, Li K, Ouyang X. 2009. Twist, an independent prognostic marker for predicting distant metastasis and survival rates of esophageal squamous cell carcinomapatients. Clin Exp Metastasis 26(8):1025–1032.

Zhang X, Liu G, Kang Y, Dong Z, Qian Q, Ma X. 2013. N-Cadherin expression is associated with acquisition of EMT phenotype and with enhanced invasion in erlotinib-resistant lung cancer cell lines. PLoS One 8:(3).DOI: 10.1371/journal.pone.0057692

Zhao H, Ho PC, Lo YH, Espejo A, Bedford MT, Hung MC, et al. 2012. Interaction of proliferation cell nuclear antigen (PCNA) with c-Abl in cell proliferation and response to DNA damages in breast cancer. PLoS One 7(1):2941–2946.

Zong H, Wang F, Fan QX, Wang LX. 2012. Curcumin inhibits metastatic progression of breast cancer cell through suppression of urokinase-type plasminogen activator by NF-kappa B signaling pathways. Mol Biol Rep 39(4):4803–4808.