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The antineoplastic potential of crotoxin isolated from *Crotalus durissus terrificus* snake venom on oral squamous cell carcinoma

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ABSTRACT

This study investigated the antineoplastic effects of crotoxin isolated from snake venom of the South American *Crotalus durissus terrificus* in oral cancer cell lines and in an animal model of chemically induced oral cancer. We analyzed cell viability and death, clonogenic formation, DNA fragmentation, migration assay, and gene expression of MMP2, MMP9, COL1A1, and CASP3. In the animal model, after induction of oral cancer by 4-nitroquinoline-1-oxide carcinogen, mice were treated with crotoxin to investigate its effects on tumor development in tongue and oral mucosa. Crotoxin inhibited cell proliferation, viability, colony formation, and migration, favoring cell death. Furthermore, crotoxin increased caspase-3 expression, decreased Ki-67 protein and mRNA expression of MMP2, MMP9, and COL1A1. Mice treated with crotoxin at 10 μg/kg did not alter biochemical parameters total cholesterol, very-low-density lipoprotein, high-density lipoprotein, liver transaminases, glycemia, creatinine, and urea. Crotoxin treatment significantly reduced the frequency of oral squamous cell carcinoma lesions by 50%. Thus, this study highlights crotoxin as a promising chemotherapeutic substance, considering its effects on controlling the neoplastic cell population, reducing cell migration, and inhibiting tumor development. Clinical studies are necessary to understand better the impact of crotoxin as a potential adjuvant therapeutic agent for oral cancer patients.

1. Introduction

Crotoxin is the main toxic component isolated from the South American rattlesnake venom *Crotalus durissus terrificus* It is the most abundant toxin present in venom, representing about 60% of its dry weight (Slotta and Fraenkel-Conrat, 1938). The historical background of crotoxin reports that it was first isolated and crystallized by Slotta and Fraenkel-Conrat, in 1938 (Slotta and Fraenkel-Conrat, 1938/39). The amino-terminal analysis showed two distinct subunits in the crotoxin (Fraenkel-Conrat and Singer, 1956). Posteriorly, in 1971, ion-exchange chromatography identified the two different proteins from crotoxin, a basic subunit with phospholipase A_2 activity, and a non-enzymatic, non-toxic acidic subunit, named crotapotin (Rubsamen et al., 1971).

Crotoxin exerts biological activities such as neurotoxicity, myotoxicity, nephrotoxicity, cardiotoxicity, immunomodulatory, antimicrobial, antitumor, and analgesic effects (de Araujo Pimenta et al., 2019; Muller et al., 2018; Sampaio et al., 2010). Many studies have investigated the antitumor profile of crotoxin in various cancers, including analysis in cell lines of lung, colon, kidney, ovary, breast, esophagus, brain, and melanoma, indicating antineoplastic effects and inhibition of aggressive

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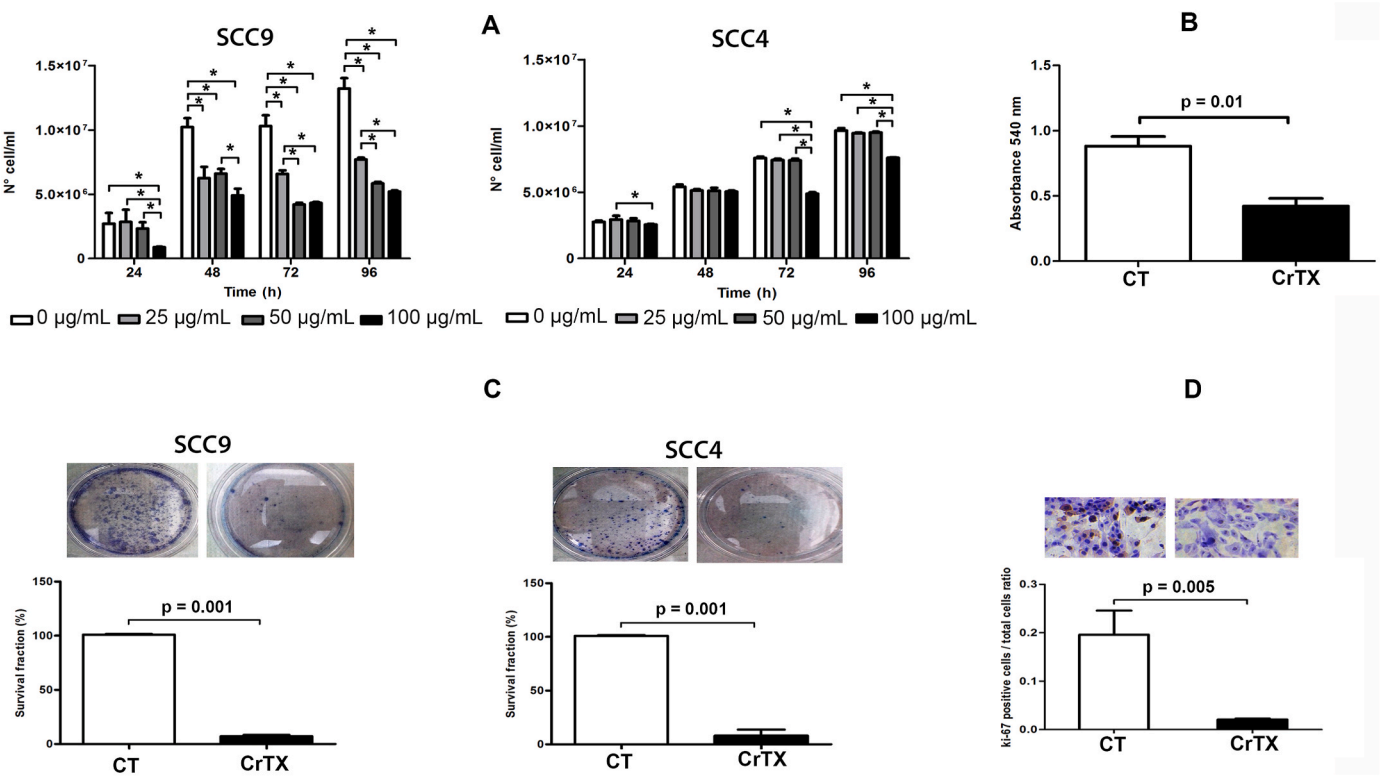

Fig. 1. Effects of crotoxin on proliferative behavior of oral cancer cells. A) Assay of dose-response curve. The cell lines SCC-9 and SCC-4 were treated at 25, 50, and 100 µg/ml of crotoxin for 24, 48, 72, and 96 h. B) MTT cell viability assay. SCC-9 cells were treated with 100 µg/ml of crotoxin, for 72 h. C) Clonogenic formation assay. D) Immunohistochemical expression of Ki-67 in SCC-9 cells. Experimental groups: CT: control; CrTX: crotoxin treatment. Asterisks indicate statistical significance: $p < 0.05$. Anova Two-Way Test was applied to the dose-response assay, and the T-Test to the other analyzes.

neoplastic behaviour (Brigatte et al., 2016; Han et al., 2014). These effects have been associated with its antiproliferative activity and cell cycle inhibition through S phase arrest (Han et al., 2014). Cell death induction through apoptosis and autophagy have also been demonstrated (Yan et al., 2007). In brief, crotoxin promotes collapse of the mitochondrial membrane potential in chronic myeloid leukemia cells (Yan et al., 2006). It also increases p38MAPK phosphorylation, activation of caspase-3, and also promotes a high expression of autophagy-related proteins LC3-II and beclin 1 in lung squamous carcinoma. The c-Jun N-terminal kinase pathway is also upregulated in crotoxin-induced apoptosis (Han et al., 2014).

Head and neck cancer represents an overall public health problem with a poor outcome in advanced stages (Johnson et al., 2020). Oral squamous cell carcinoma (OSCC) is the most common type in the oral cavity, making it the sixth most common human cancer worldwide (Johnson et al., 2020; Sung et al., 2021). Despite the significant progress in recent years in anticancer treatments, limited improvement in the 5-year survival rate of OSCC patients has been reported (Weckx et al., 2020). Therefore, there is an enormous scientific effort towards the discovery of new therapeutic approaches targeting unresponsive tumors. Although there is a wide spectrum of antineoplastic drugs, causes severe toxicity and thus are discontinued (Ketabat et al., 2019). Thus, it is necessary to investigate new antineoplastic therapeutic approaches leading to tumor cell death and cell growth control to improve the success rate of cancer treatment.

In this sense, due to the biological effects of crotoxin previously reported for crotoxin, we hypothesized that this substance could modulate the neoplastic behavior of OSCC cells and inhibit tumor growth. Therefore, through phenotypic, molecular assays, and *in vivo* analysis, we investigated the antineoplastic potential of crotoxin isolated from *Crotalus durissus terrificus* snake venom in oral squamous cell carcinoma.

2. Material and methods

2.1. Cell culture and crotoxin

Two human OSCC cell lines, SCC-9 and SCC-4 (BCRJ Cat# 0196, RRID:CVCL_1685; BCRJ Cat# 0195, RRID:CVCL_1684), were cultured in DMEM/Ham's F-12 (Gibco, Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum and 0.4 µg/mL hydrocortisone (Gibco, Invitrogen, Carlsbad, CA), according to ATCC protocol. All *in vitro* experiments were performed in triplicate and are at least the result of three independent experiment.

Crotoxin used for cell treatment was supplied by Instituto Butantan (São Paulo, Brazil). It was purified from *Crotalus durissus terrificus* venom by a combination of size exclusion and anion exchange chromatography. The mass spectrometry analyzed the molecular weight of proteins, showing averaged signals at 9500 Da and 14,500 Da, in addition to the presence of isoforms. As determined by PAGE and size exclusion, the purity was confirmed to be >99%.

2.2. Dose-response curve and cell viability assay

Firstly, we performed a dose-response curve to establish crotoxin concentration and time for experimental treatment (Fig. 1A). For this, cells were treated at 25, 50, and 100 µg/ml of crotoxin for 24, 48, 72, and 96 h. Trypan-blue exclusion determined the number of viable cells per mL of culture medium. From the analysis of the dose-response curve, we defined a concentration of 100 µg/ml of crotoxin and a time of 72 h for treatment, as a standard for the other assays.

The cell viability analysis by MTT assay was also performed. Cells were plated in 96-well plates at a density of 5×10^4 cells per well in 100 µL of DMEM and Ham's F-12 medium and allowed to grow in a CO_2 incubator for 24 h. The medium was then removed and replaced with a

fresh medium containing 100 μg/mL of crotoxin for 72 h. The cultures were then incubated in 100 μL of medium with 10 μL of 5 mg/mL MTT solution for 3 h at 37 °C. Finally, the medium with MTT was removed, and 100 μL of DMSO was added to each well to dissolve the formazan. The absorbance was read at 570 nm on a microplate reader (BIORAD-Benchmark, California, USA).

2.3. Clonogenic assay

The OSCC cells were seeded in six-well plates at a density of 5.0×10^2 cells. After treatment with 100 μg/mL of crotoxin for 72 h, the cells were trypsinized and then cultured in the crotoxin-free medium for 2 weeks. The culture medium was changed every 2–3 days. The colonies were fixed in methanol for 30 min and then stained with Giemsa. The number of colonies was defined as a cluster containing ≥50 cells. Survival fraction (SF) was calculated according to the formula: SF = (number of colonies in the treated cells/Number of colonies in control cells) X 100 (Franken et al., 2006). The ImageJ software (NIH; Bethesda, MD; http://imagej.nih.gov/ij/) was used to quantify cell clusters.

2.4. Cell death/viability assay

Acridine orange/ethidium bromide (AO/EB) staining was used to visualize dead and viable cells. The experimental groups were stained with a solution containing Acridine Orange l00 μg/ml (AO, Sigma, St. Louis, MO, USA) and Ethidium Bromide 100 μg/ml (EB, Sigma, St. Louis, MO, USA) (Kasibhatla et al., 2006). The intense EB staining (Ex360-370, Em420-460, DM400 filter) demonstrates cell death, while intense AO (Ex460-495, Em510-550, DM505 filter) live cells. The FSX100 microscope (Olympus, Center Valley, PA, USA) was used for image analysis. Results were expressed as dead cells/total cells ratio.

2.5. DNA fragmentation assay

After treatment, the cells were lysed with lysis buffer (10 mM Tris pH7.5, 400 mM NaCl, 1 mM EDTA, 1% Triton X-100), incubated on ice for 15 min, and centrifuged at 4 °C for 15 min at the speed of 13600×*g*. The supernatant was incubated with RNase (0.2 mg/mL) with proteinase K (0.1 mg/mL) for 2 h at 37 °C. The DNA was extracted using phenol/chloroform (1:1, v/v) and precipitated in 96% ethanol overnight at −80 °C. The precipitated DNA was centrifuged (13600×*g*, 4 °C, 15 min), and the dried pellet and dissolved in 20 μl of Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The DNA obtained was analyzed on 1.5% agarose gel containing 0.3 μg/ml ethidium bromide in 75 V Tris-borate-EDTA buffer for 1.5 h. The bands were visualized in ultraviolet transilluminator and photographed.

2.6. Migration assay

Cell migration was assayed by wound healing method (Liang et al., 2007). Briefly, at the full confluence, OSCC cells were scraped away horizontally using a 200 μl tip; we marked the place and measured the area. The culture medium was then replaced by a serum-free medium, adding 100 μg/mL of crotoxin for 72 h. We measured the area again by subtracting from the previous area. In order to measure the wound-covered area by migrating cells, images of the wounded cell monolayers were taken using an Olympus IX81 inverted microscope (Olympus, Center Valley, PA, USA) coupled to a camera SC30 (Olympus, Center Valley, PA, USA).

2.7. Isolation of RNA and qRT-PCR

To verify the effect of crotoxin on mRNA expression in OSCC cells, quantitative real-time PCR was performed to analyze the expression levels of matrix metalloproteinases (MMP2, MMP9, COL1A1), and Caspase-3, related to the tumor migration, and apoptosis. After treatment with 100 μg/ml of crotoxin for 72 h, the cells were lysed and the RNA was isolated using Trizol (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol (Invitrogen, USA). Reverse transcription was conducted with 1.5 μg of total RNA using a Reverse transcription kit (Invitrogen, Life Technology, USA). The RT-synthesized complementary DNA (cDNA) was amplified using specific primer sequences (Sobrinho Santos et al., 2017) and SYBR Green reagent (Applied Biosystems, USA). The analysis was performed through the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), on a QuantStudio 6 Flex real-time PCR system (Applied Biosystems, Life Technologies, USA).

2.8. Immunocytochemical assay

An amount of 2×10^4 OSCC cells was plated on coverslips and submitted to the crotoxin treatment. After, the cells were fixed with 70% ethanol for 30 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. The polyclonal antibody to ki67 (clone MIB-1, DAKO, USA, 1:50) was detected using the LSAB kit (LSAB-Kit Plus Peroxidase, Dako, California, USA). Signals were developed with 3′3-diaminobenzidine-tetrahydrochloride and counterstained with Mayer's hematoxylin. For staining quantification, ten fields were photographed at 400 × magnification in a microscope FS×100 (Olympus, Center Valley, PA, USA). Positive and negative cells were summed, and the proportions of positive cells in neoplastic parenchyma were used to quantify the staining. Cell counting was then performed in the software ImageJ (http://rsbweb.nih.gov/ij/).

2.9. Assay in animal model: chemically induced oral carcinogenesis and crotoxin treatment

The study was carried out according to the National Council for the Control of Animal Experimentation (CONCEA, Brazil), and it was approved by the Ethics Committee for Experimentation and Animal Welfare of State University of Montes Claros, Brazil (CEEBEA; Protocol number 195/2019). Male Swiss mice, 12 weeks of age, approximately 30 g, were kept under suitable conditions of temperature 22 ± 2 °C, relative humidity of 60 ± 5%, 12 h of light/dark cycles, and fed with Purina-Labina® diet *ad libitum*.

Previously, we carried out a pilot assay to evaluate crotoxin effect on the liver, biochemical markers, and body weight. Crotoxin was reconstituted in 0.9% sterile saline solution at concentrations of 0, 10, and 100 μg/kg (six animals per group) and administered by intraperitoneal route, twice a week, for four weeks. This treatment assayed was previously established in tests with crotoxin (Ye et al., 2011). In this assay, we longitudinally investigated for four weeks the crotoxin effect on body weight, and after euthanasia, blood samples were collected for plasma analysis of the following parameters: glycemia, total cholesterol (TC), high-density lipoprotein (HDL), very-low-density lipoprotein cholesterol (VLDL-cholesterol), triglycerides, creatinine, urea, and the liver transaminases, glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT). Enzymatic specific kits (Labtest Diagnostica, Brazil) and equipment LabMax 240 (ABX Diagnostic, France) were used. Histopathological analysis of the liver was also investigated to verify the effects of crotoxin on hepatocytes.

In order to better understand the crotoxin effect on OSCC, we induced oral carcinogenesis using 4-nitroquinoline-1-oxide (4NQO; Sigma-Aldrich, USA), as described previously (Sobrinho Santos et al., 2017). Briefly, experimental groups of male mice have been exposed to carcinogen 4NQO 50 μg/mL for 16 weeks, available in water *ad libitum*. Once identified 4NQO-induced oral lesions, after 26 weeks, the animals were treated with crotoxin (CrTX) 10 μg/kg by intraperitoneal injection, twice a week for four weeks. Through sample size calculation, a total of 24 animals were randomly distributed into four treatment groups (n = 6 each): Group 1 (untreated control), Group 2 (4NQO-induced oral cancer), and Group 3 (4NQO-induced oral cancer + CrTX treatment), and Group 4 (only CrTX treatment, without 4NQO). In the control group,

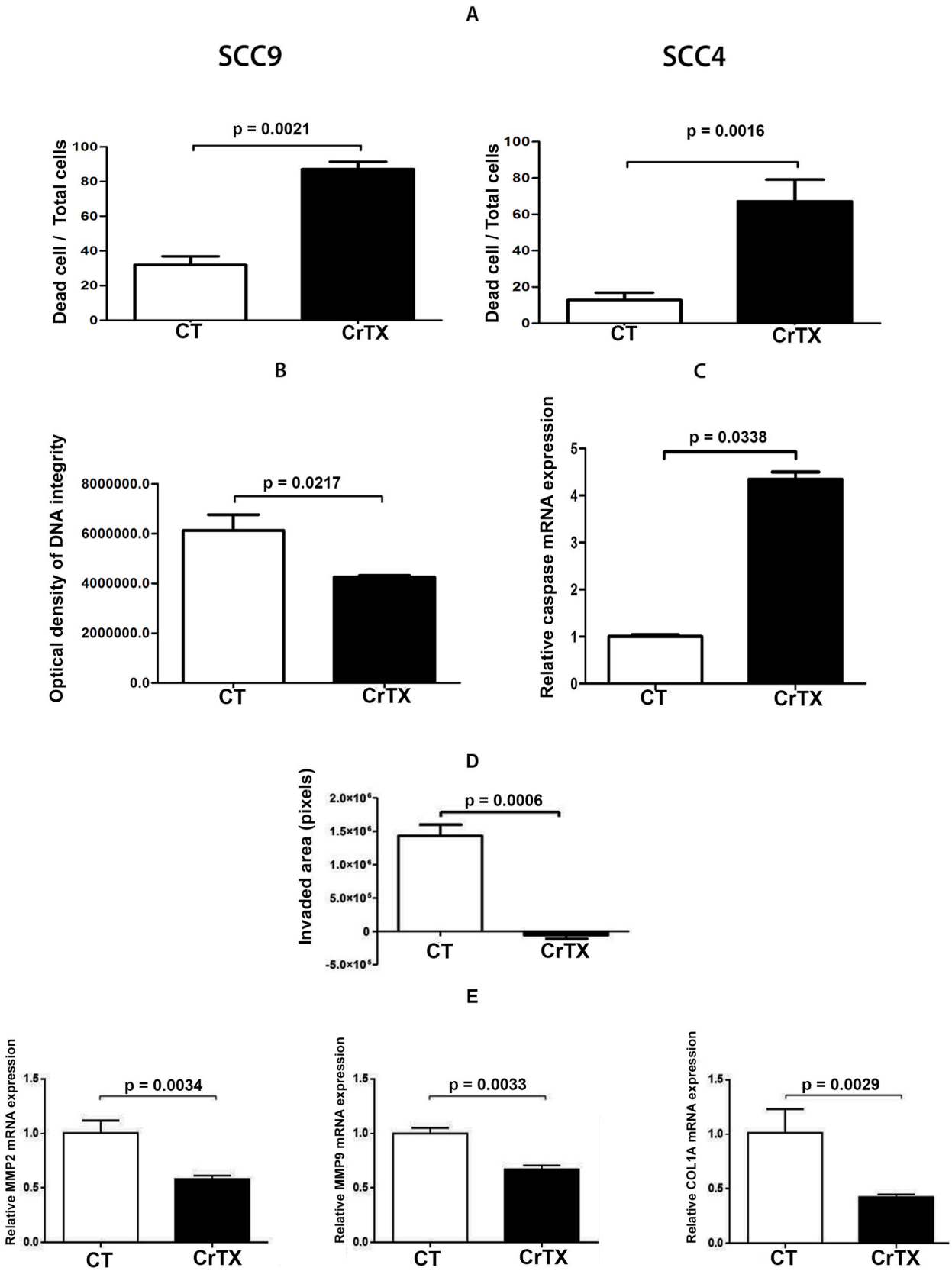

Fig. 2. Effects of crotoxin on cell death and migration behavior of oral cancer cells. A) Cell death/viability assay by colorimetry with Acridine Orange/Ethidium Bromide. AO penetrates into viable cells emitting green fluorescence. EB emits red fluorescence in dead cells. The cell lines SCC-9 and SCC-4 were treated with 100 μg/ml of crotoxin, for 72 h. B) DNA Fragmentation assay. The lower optical density indicates a loss of DNA integrity. C) mRNA expression of Caspase-3 by qPCR. D) Migration test. It was measured through the area invaded by migrating cells. E) mRNA expression of genes associated with cell migration, including MMP-2, MMP-9, and COL1A1. Experimental groups: CT: control; CrTX: crotoxin treatment; T-Test.

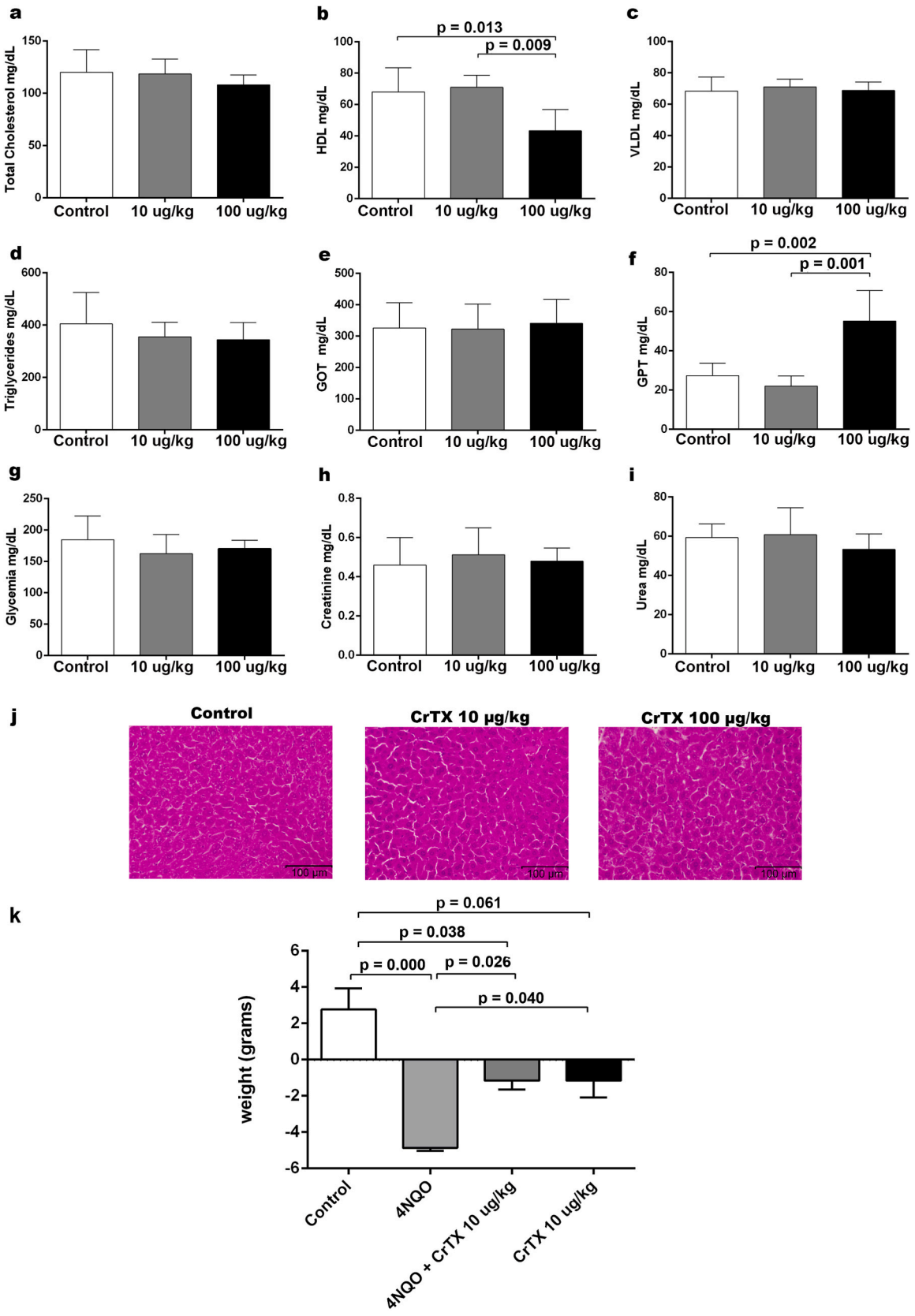

Fig. 3. Effects of crotoxin on the biochemical parameters, liver tissue, and body weight in mice treated with crotoxin for 30 days. a) total cholesterol, b) HDL c), VLDL d), triglycerides, e) GOT, f) GPT, g), Glycemia, h) Creatinine, i) Urea, j) Liver photomicrographs of animals treated with crotoxin at 10 µg/kg and 100 µg/kg, in pilot assay for dose standardization (H&E staining), k) Effect of crotoxin on weight loss in an animal model of chemically induced oral cancer. Anova one-Way Test. CrTX: crotoxin treatment; 4NQO: carcinogen used to induce oral cancer in mice.

animals were not treated with 4NQO and received injections of 0.9% sterile saline instead of crotoxin.

At the end of 30 weeks from the beginning of the experiments, the animals were killed by guillotine decapitation, and the oral lesions were removed by biopsy. The tissues were fixed in 10% formalin, embedded in paraffin, and histological sections were stained with Hematoxylin and Eosin for the definition of histopathological diagnosis.

2.10. Statistical analysis

All data were analyzed in SPSS statistics software, version 22.0, and GraphPad Prism software (Version 6.0, GraphPad Software Inc., San Diego, CA, USA). Shapiro–Wilk test was performed to analyze the normality of data distribution. Data were expressed as the mean ± SEM. Statistical significance between cell groups was assessed by One-way ANOVA, followed by LSD Post-Hoc comparisons, Two-way ANOVA, Student's t-test, or Fisher's exact test when applicable. Statistical significance was established at $p < 0.05$. The association strength between the frequency of oral lesions (OSCC, epithelial dysplasia) and crotoxin treatment was measured by Cramer's V.

3. Results

3.1. Crotoxin inhibits proliferative behavior of oral cancer cells and promotes cell death

Firstly, to verify the crotoxin effect on cell proliferation, we performed an initial assay of dose-response curve that demonstrated that crotoxin promoted a decrease in cell counting at all concentrations and experimental times. We selected treatment with crotoxin 100 μg/ml for 72 h as reference for *in vitro* assays since this treatment promoted a similar effect in both cell lines, SCC-4 and SCC-9, during a shorter time (72 h), reducing cell proliferation (Fig. 1A).

Then, to assess the crotoxin effects on population control of OSCC cells, the phenotype assays of cell viability, colony formation, and cell death were performed. Crotoxin treatment was able to significantly reduce cell viability (Fig. 1B), and clonogenic formation, as represented by the cell survival fraction (Fig. 1C). Compared with the control group, crotoxin led to a reduction of 92.8% in the number of colonies formed by OSCC cells. According with these results, in the crotoxin-treated group, we identified lower protein expression of Ki-67, in immunohistochemistry analysis (Fig. 1D).

Supporting this crotoxin effect on cell population control, cell death/viability assay and DNA fragmentation analysis revealed that this substance significantly increased the percentage of dead cells (Fig. 2A), and decreased the intact DNA, as demonstrated by the lower optical density of DNA integrity in cells treated with crotoxin (Fig. 2B). Furthermore, the mRNA caspase-3 expression was increased by treatment (Fig. 2C).

3.2. Crotoxin decreases the migration of oral cancer cells and mRNA expression of matrix metalloproteinases and collagen

We identified a significant decrease in migration in cells treated with crotoxin (Fig. 2D). This substance also promoted the downregulation of MMP2, MMP9, and COL1A1 expression, genes involved in the molecular pathways associated with neoplastic cell migration. (Fig. 2E).

3.3. Crotoxin does not impair biochemical parameters and body weight of mice

Before investigating the antineoplastic effect of crotoxin in the animal model, we performed a pilot assay to verify crotoxin effect on the liver tissue, and its influence on biochemical markers. When compared to control mice, animals treated with 10 μg/mL crotoxin did not show significant changes in biochemical parameters, including total cholesterol, VLDL, HDL, GOT, GPT, Glycemia, Creatinine, and Urea (Fig. 3A to I). In contrast, crotoxin 100 μg/ml was able to decrease HDL cholesterol (Fig. 3B) and increase GPT (Fig. 3F). Liver cells did not suffer histological changes by crotoxin treatment (Fig. 3J). All animals treated with 10 μg/kg crotoxin remained alive during treatment, and only one animal died when treated with the dose of 100 μg/kg. Therefore, crotoxin 10 μg/kg was selected to perform animal experiments.

Table 1
Frequency of oral squamous cell carcinoma and epithelial dysplasia in an animal model of chemically-induced oral cancer followed by crotoxin treatment.

Groups	Histological Diagnosis*		
	Normal Mucosa	Mild epithelial dysplasia	OSCC
Control (n = 6)	6 (100%)	0 (0%)	0 (0%)
4NQO (n = 6)	1 (16.7)	1 (16.7%)	4 (66.7%)
4NQO + Crotoxin (n = 6)	2 (33.3%)	3 (50%)	1 (16.7%)
Crotoxin (n = 6)	6 (100%)	0 (0%)	0 (0%)

* Fisher's exact test; $p = 0.002$.
Cramer's $V = 0.648$; $p = 0.001$.

3.4. Crotoxin was able to reduce lesions of oral squamous cell carcinoma lesions in mice

We monitored the development of 4NQO-induced oral lesions, which usually start after the twentieth week of treatment initiation (four weeks after suspending exposure to the carcinogen). After the development of lesions in the tongue or oral mucosa, the animals were exposed to the crotoxin treatment. In the follow-up, we verified significant weight loss in the 4NQO-treated groups. Crotoxin did not significantly alter the weight loss of mice compared to the control group ($p = 0.061$). The toxin significantly minimized the weight loss in mice exposed to the carcinogen ($p = 0.026$), as demonstrated by a comparison between Group 2 (4NQO-induced oral cancer), and Group 3 (4NQO-induced oral cancer + CrTX treatment) (Fig. 3K).

A strong association between the histological diagnosis of the oral mucosa (OSCC, epithelial dysplasia, normal Mucosa) and the treatment groups (Control, 4NQO, 4NQO + CrTX, and CrTX) was identified (Cramer's $V = 0.648$; $p = 0.001$). Histological analysis demonstrated the presence of epithelial dysplasia (16.7%) or squamous cell carcinoma (66.7%) in the tongue in the groups exposed to the carcinogen 4NQO. On the other hand, in the group that received the crotoxin, the number of animals with the diagnosis of squamous cell carcinoma was reduced by 50%, and it was identified an increased percentage of animals with normal epithelium, and mild epithelial dysplasia by 16.6% and 33.3%, respectively (Table 1 and Fig. 4).

4. Discussion

The deregulation of migration and proliferation of neoplastic cells are key events to oral cancer progression (Rivera and Venegas, 2014). Consequently, antineoplastic drugs inhibit cell cycle pathways and invasiveness while promote cell death. Several chemotherapy drugs act on the signal transduction of these molecular pathways. However, neoplastic cell clones can confer tumor resistance and impair the therapeutic effectiveness of chemotherapy (Brigatte et al., 2016; Feitelson et al., 2015).

There is an enormous scientific effort to discover new therapeutics for oral cancer patients. Studies have investigated the antineoplastic

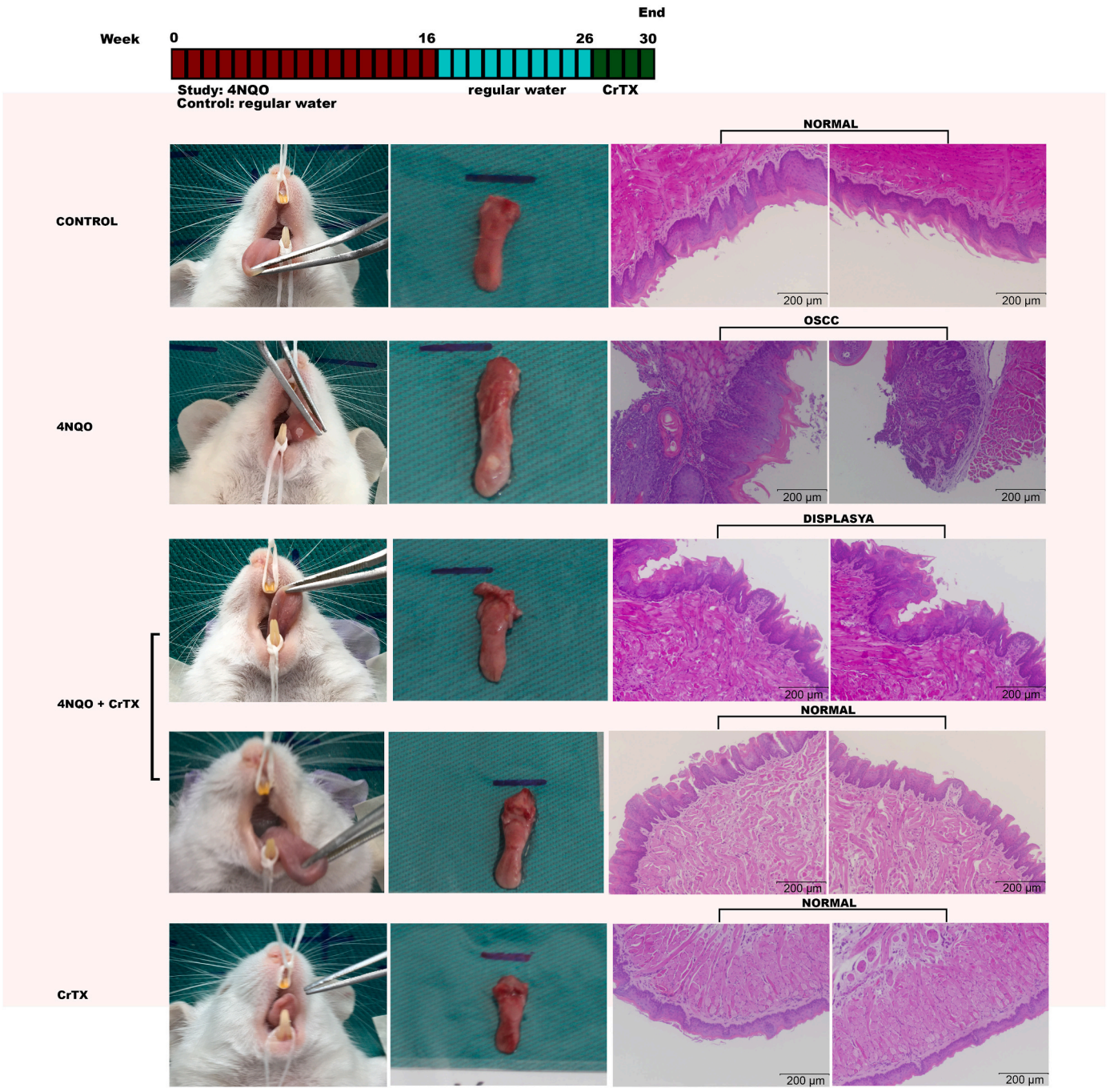

Fig. 4. Effect of crotoxin on development of oral cancer lesions in mice. Experimental scheme of induction of oral cancer, followed by crotoxin treatment, and representative images of oral lesions and histological images. The mice were exposed to the carcinogen 4NQO for induction of lesions, and treated with CrTX 10 µg/kg by intraperitoneal injection, twice a week for four weeks Crotoxin reduced the lesion number diagnosed as squamous cell carcinoma, and increased the percentage of animals with normal epithelium, and mild epithelial dysplasia.

potential of substances isolated from plants on oral cancer cells, including curcumin, epigallocatechin-3-gallate from green tea extract, polyphenols, and resveratrol from grapes (Giordano and Tommonaro, 2019; Wang et al., 2012). However, few studies exist on the antineoplastic effect of substances from animal sources, such as snake venom. Some studies have shown a promising antineoplastic effect of crotoxin, a phospholipase A_2 isolated from the snake venom *Crotalus durissus terrificus*, to inhibit aggressive behavior of cancer cell lines of lung, colon, kidney, ovary, breast, esophagus, brain, and melanoma (Han et al., 2014; He et al., 2013; Muller et al., 2018).

Crotoxin, besides its cytotoxicity, also causes autophagy in lung cancer cells (Han et al., 2014), promotes macrophage reprogramming towards an antiangiogenic phenotype, and decrease the capillary formation (de Araujo Pimenta et al., 2019). In this study, we investigated crotoxin effects on oral cancer cells and in an animal model of chemically induced oral cancer. Crotoxin decreased viability and proliferation in human oral cancer cells and promoted cell death by caspase-3 upregulation. Similar observations have previously been conducted in pancreas, esophageal, cervical and glioma cancers (He et al., 2013; Muller et al., 2018).

Our results showed that crotoxin was also able to decrease cell clone proliferation and the Ki-67 expression, an important marker associated

with proliferation and tumor progression in individuals with oral squamous cell carcinoma (Jing et al., 2019). Despite the controversies about the real clinical significance of Ki-67 in oral cancer, a meta-analysis showed high Ki-67 expression is a negative prognostic marker in OSCC patients, and it can still compromise the therapeutic response (Xie et al., 2016).

The matrix metalloproteinases (MMP) play a pivotal role in the tumor invasive phenotype in oral squamous cell carcinoma (Celentano et al., 2021). The increased levels of active MMP2 and MMP9 are associated with invasion and metastasis, degrading of various components of the extracellular matrix, and inducing epithelial to mesenchymal transition in carcinoma. Downregulation of this pathway affects the survival and invasiveness of oral cancer cells (Celentano et al., 2021; Pang et al., 2016). Our data showed that crotoxin impaired migration of oral cancer cells, associated with lower expression of MMP2 and MMP9.

The collagen COL1A1 is an important constitutive component of the tumoral microenvironment in the carcinogenesis pathway being considered a pro-metastatic factor (Dong et al., 2020). It was identified that downregulation of COL1A1 expression by glycosaminoglycan chondroitin sulfate-E promotes anti-migratory effects in breast cancer (Willis and Kluppel, 2014). Similarly, in oral cancer cells, the downregulation of COL1A1 suppressed cell proliferation and invasion (He et al., 2018). In our study, crotoxin significantly decreased COL1A1 expression.

The antineoplastic effect of the crotoxin was also demonstrated in this study by an animal model of 4NQO-induced oral cancer. Our data revealed a significant reduction of 50% in the frequency of animals developing OSCC and an increase of mice with normal epithelium or mild epithelial dysplasia. Furthermore, crotoxin at 10 μg/ml did not impair biochemical parameters and body weight of animals, as evidenced in the toxicity assay. In a similar study using a lung cancer animal model, crotoxin significantly decreased the growth of the tumor xenograft and led to important antiangiogenic effects, such as decreased levels of microvessel density and vascular endothelial growth factor (Ye et al., 2011). In esophageal carcinoma, crotoxin can exert an anti-proliferative effect as well as inhibit tumor growth in nude mice. These effects were accompanied by an increase in p15 and caspase-3 p17 proteins, and a reduction in the level of Bcl-2 protein (He et al., 2013).

5. Conclusion

In summary, this study highlights crotoxin as a promising chemotherapeutic substance, considering its effects on controlling the neoplastic cell population, reducing cell migration, and inhibiting tumor development. Clinical studies are necessary to understand better the potential effects of crotoxin as a potential adjuvant therapeutic agent for oral cancer patients.

Credit author statement

Conceptualization and Methodology: RGR, EMSS, LCF, ALSG; Investigation: EMSS, RGR, MGS, ESBG, FADG, LHS; Validation: EMSS, RGR, ESBG; Data Curation and Investigation: LCF, RGR, EMSS, RSG, AMTA, CSS, AMBP, SHSS; Formal analysis: LCF, RGR, EMSS; Funding acquisition: LCF, AMTA, ALSG; Supervision: EMSS, RGS; Project administration: LCF, ALSG; Manuscript preparation: RGR, LCF, EMSS, RSG, AMTA; Manuscript editing: LCF, EMSS, RSG, AMTA, CSS; All authors read and approved the final manuscript.

Ethical statement

The study was carried out according to the National Council for the Control of Animal Experimentation (CONCEA, Brazil), and it was approved by the Ethics Committee for Experimentation and Animal Welfare of State University of Montes Claros, Brazil (Protocol number 195/2019).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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