EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 71, 2185:2194, JULY, 2025
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY
Submit Date : 26-04-2025 • Accept Date : 28-05-2025 • Available online: 10-07-2025 • DOI : 10.21608/edj.2025.379202.3454

ANTI-CANCEROUS IMPACT OF TOPICAL CHITOSAN NANOPARTICLES IN DMBA-INDUCED ORAL PRECANCEROUS LESIONS

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ABSTRACT

Objective: This study aimed to evaluate the effect of chitosan nanoparticles as a topical treatment of oral epithelial dysplasia that was induced chemically in the hamster buccal pouch.

Methods: Thirty-five male Syrian golden hamsters were distributed into four groups. Group A: served as a negative control group, group B: served as positive control group where left buccal pouches of the hamsters were painted 3 times/week/6 weeks with DMBA carcinogen, group C: hamsters were painted 3 times/week/6 weeks with DMBA then treated topically by chitosan -nanoparticles (0.235 mg/kg) for another six weeks and group D: hamsters were painted 3 times/week/6 weeks with DMBA then treated topically by chitosan -nanoparticles (2.53 mg/kg) for another six weeks. After euthanasia, the pouches were dissected and prepared for histological and immunohistochemical examination with BAX and PCNA.

Results: Histopathological evaluation showed prevention of oral cancer proceeding in hamster buccal pouches when treated with high dose of chitosan nanoparticles (2.53 mg/kg). These observations were online with increased BAX expression and decreased PCNA expression.

Conclusion: High concentrations of chitosan nanoparticles may have the potential to prevent oral carcinogenesis processes in those who are at high-risk for developing OSCC as well as act as auxiliary therapy for patients undergoing conventional treatments.

KEYWORDS: Dysplasia, Chitosan-nanoparticles, Hamster, Bax, PCNA

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INTRODUCTION

Oral precancerous lesions (OPL) show a higher risk of developing oral squamous cell carcinoma (OSCC) that accounts for more than 90% of all malignant lesions in the oral cavity with varied clinical presentations. Oral epithelial dysplasia (OED) is a sequence of histological alterations in cells and architecture of epithelium that is seen in OPL.[1] Treating OPL and stopping its malignant transformation is one of the greatest ways to prevent oral cancer.[2]

For many cancer patients, chemotherapy remains one of the most crucial treatment options among the various anticancer medications. It is regarded as one of the primary treatment strategies to stop the growth of cancer cells. However, because both cancer and healthy cells are exposed to cytotoxic chemotherapy drugs, which restrict their therapeutic effect and increase the likelihood of serious side responses, the active chemicals used to treat cancer usually do not distinguish between the two types of cells.[3]

Nanomedicine is the medical application of nanotechnology especially in treatment of cancer. It aims to selectively and effectively internalize nanotherapeutics into tumor cells thus increasing their accumulation within tumors and thereby reducing its effects in the surrounding normal tissues.[4]

Chitosan (CS) is a mucoadhesive biocompatible polymer and one of the common natural polysaccharides, that can be found in many shellfish, including shrimp, crab, and crayfish. CS has an abundance of active free protonated amino groups, and because of this, it is more soluble in acidic environments. These groups can be chemically changed in a variety of ways to increase their solubility, biocompatibility, and targeting activity.[5] Chitosan-based nanoparticles show promise for cancer treatment in laboratory and animal studies.[6]

At different stages of OSCC, cellular apoptosis has exhibited irregular dysregulation. Cancer cells evade apoptosis through the disruption of pro-apoptotic and anti-apoptotic protein functions.[7] The anticancer action of CS in several cell types is attributed to apoptosis. It inhibits cellular growth, which is launched by the activation of procaspase triggered from outside the cell to expedite the cleavage of the cascade and intensify the death signals.[8]. Bax and Bcl-2 are prominent proteins that regulate programmed cell death. When Bax is predominated and Bcl-2 is suppressed, there is evidence of accelerated apoptosis.[9]

Proliferating Cell Nuclear Antigen (PCNA) is a valuable marker for determining the state of proliferation of tumor tissue (i.e., prognostic). PCNA is a marker of proliferative cells in the early G1 and S phases that is present in the nucleus as well as it is a cofactor of DNA polymerase delta. During DNA replication, it enhances the processing of leading strand.[10]

The present study investigated the effect of different concentrations of chitosan nanoparticles as a topical treatment for oral epithelial dysplasia that is induced in the hamster buccal pouch by evaluating the apoptotic and proliferation activities through detecting the level of Bax and PCNA expression.

Material and methods

Chemicals: CS with degree of deacetylation of 92% and Molecular weight: 161.61 KDa was purchased from Oxford Lab Fine Chem, Vasai, Maharashtra. Glacial acetic acid was supplied from Chemajet Chemical Company, Alexandria, Egypt. Tripolyphosphate (TPP) was delivered from Sigma Aldrich Chemical Company, Saint Louis, USA. 7,12-Dimethylbenz-(a)-anthracene (DMBA) was delivered from Sigma Aldrich Chemical Company, Saint Louis, USA. Mineral oil was delivered from Sigma Loba Chemie, India. Rabbit polyclonal antibody to BAX was delivered from Diagnostic Biosystems 6616 Owens Drive Pleasanton. PCNA

immunohistochemical kit Rabbit poly-colonal Mouse antibody to PCNA was delivered from Gene Tex International Corporation. All aqueous solutions were prepared with distilled water. Every reagent was used exactly as it was delivered.

Synthesis and characterization of the chitosan-nanoparticles (CS-NPs): The ionic gelation process was used to fabricate the CS-NPs.[11] Briefly, 35 ml distilled water with 2% glacial acetic acid and 0.5 gm of CS were mixed for 30 minutes using a magnetic stirrer, then sodium tripolyphosphate (STPP) solution (0.167 gm in 15 ml distilled water) was dropped wisely into the acidic CS solution at room temperature. The mixture was left to stir for another 45 minutes to obtain CS-NPs. The CS-NPs were centrifuged and washed, reaching neutralization by centrifuging for 20 minutes at 4 C to collect nanoparticles.

The synthesized nanoparticles were morphologically characterized using a transmission electron microscope (TEM) (JEOL, model JEM-2010) operating at 200 kV with an accelerating voltage. The microscope was connected to a Gatan digital camera (Model Erlangshen ES500). The zeta-potential and zeta size of the synthesized CS-NPs were measured using a Malvern Zetasizer Nano ZS90 analyser set to 25C^0. UV–VIS double-beam spectrophotometer (model: Shimadzu UV-2450 spectrophotometer, one cm quartz cells) was used to record the sample's optical absorbance.

Carcinogen: To prepare a 0.5% DMBA solution, one gram of 7,12-Dimethylbenz-(a)-anthracene (DMBA) was dissolved in 200 ml of heavy mineral oil (USP). To induce oral dysplasia, 0.5% DMBA was applied onto the left hamster buccal pouches (HBPs), using camel brushing number 4, three times a week for six weeks.

Animals: According to sample size calculation test (G*Power version 3.1.9.2), thirty-five male *Syrian* golden hamsters (*Mesocrietus auratus*) were sufficient to be used in the current experiment. The hamsters ordered from VACSERA (Holding Company for Biological Products and Vaccines), located in Helwan, Cairo, Egypt. They weighed 90-100 grams and given water and recommended nutrients, ad libitum. The animals were kept in the animal house at the Faculty of Dentistry, Suez Canal University. All protocols for animal procedures adhered to the Guide for the Care and Use of Laboratory Animals and were approved by the Experimental Animal Research Ethics Committee of Suez Canal University, protocol number 833/2024.

Experimental Design: Thirty-five animals randomly allocated into four groups as follows: **Group A** (negative control group): 5 animals did not receive any treatment and were euthanized at the end of the 12th week. **Group B (positive control group**): 10 animals were painted with DMBA 3 times/week in 6 weeks. At the end of the sixth week, five of them were sacrificed to confirm the onset of oral dysplasia (group B1). The other five animals were left without treatment till the end of the 12th week (group B2). **Group C (CS-NPs 0.235 mg/kg group):** 10 animals were painted with DMBA 3 times/week for 6 weeks, then with CS-NPs (0.235 mg/kg) 3/week for another 6 weeks. **Group D (CS-NPs 2.53 mg/kg group):** 10 animals were painted with DMBA 3 times/week for 6 weeks, then with CS-NPs (2.53 mg/kg) 3/week for another 6 weeks.

Euthanasia and tissue sample collection: After the completion of the 12th week, animals were anesthetized and then euthanized using carbon dioxide. The left HBPs were separated, cleaned, and fixed for 24 hours in a solution of 10% buffered formalin. Afterward, the specimen was embedded in soft paraffin wax, and sections measuring 5µm were cut with a rotary microtome and mounted on glass slides for staining.

Histopathological Evaluation: Slides were stained with Hematoxylin and Eosin (H&E) for light microscopy investigation. Photos were taken with an E-330 Digital Photography camera and an

Olympus BX50 microscope. As a result of the thin buccal pouch epithelial layer of hamsters, OED was graded according to El-Dakhakhny et al. (2009).[12] Mild dysplasia is diagnosed when there are fewer than three dysplastic criteria. Moderate dysplasia is defined as having 3-7 dysplastic criteria. Severe dysplasia occurs when there are above seven dysplastic criteria.

Immunohistochemical evaluation (IHC):

Slides were de-paraffinized, rehydrated, and kept in phosphate buffers containing 3% hydrogen peroxide. In a 10% aqueous EDTA buffer with a pH of 7.4, sections were heated for 20 minutes. Subsequently, rabbit polyclonal anti-BAX and PCNA antibodies (50 µl at a dilution of 1:50) were added and incubated at 4°C overnight. Following the incubation, 50 µl of a working solution of biotinylated goat anti-rabbit immunoglobulin G secondary antibody was introduced and incubated at 37°C for 30 minutes, then developed with diaminobenzidine. The growth of positive immunoreactivity in BAX and PCNA tissue samples was analyzed using a conventional light microscope. Subsequently, the optical density of cells positive for BAX and PCNA, along with the intensity of immunostaining, was examined with a computerized device known as the J Image analyzer.

Statistical analysis:

It was performed using SPSS software for Windows version 22.0 (Statistical Package for Social Science, Armonk, NY: IBM Corp) at significant levels < 0.05 (P- Value < 0.05). Descriptive statistics were calculated as Mean ± Standard deviation (SD), and range (Max-Min), and One-way ANOVA (Analysis of variance) was used according to the types of data to compare the four groups under study. Tukey's post hoc test was performed for the evaluation of statistical significance among the groups. P value <0.05 is considered statistically significant.

RESULTS

Characterization of CS-NPs

The TEM imaging was used to examine the morphological properties of the prepared CS, illustrating the formation of CS-NPs with sizes ranging from 80 to 150nm, the particles possess irregular sheet-like particles (Figure 1: A&B). The absorption spectrum showed that the recorded UV-visible spectra of CS-NPs displayed a small absorption peak centered at 306nm, which corresponds to the π-π^* transition of the CS-NPs (Figure 1: C). The zeta potential value obtained for prepared CS-NPs with surface charge of about 16.52 mV (Figure 1:D).

Histopathological findings:

Group A (negative control): The left HBPs showed normal buccal mucosal epithelial lining. Thin stratified squamous epithelium composed of two to four cell layers, with thin surface keratinization and no rete ridges. The underlying lamina propria had non-inflamed loose collagenous connective tissue (c.t.) with thin vascular spaces (Figure 2: A1)

Group B1 (positive control / 6w): The left HBPs treated with DMBA for six weeks revealed severe dysplastic epithelial changes in the form of epithelial hyperplasia, hyper-keratinization, drop-shaped rete ridges, loss of polarity, and basal cell layer hyperplasia, individual cell keratinization, cellular and nuclear pleomorphism, prominent nucleoli, with increased normal and abnormal mitosis. The underlying c.t. revealed moderate chronic inflammatory cellular infiltration with dilated blood capillaries (Figure 2: B1:1).

Group B2 (positive control group/12w) & Group C (CS-NPs 0.235 mg/kg group): The left HBPs showed the same results in both groups; the group was painted with DMBA for 6 weeks then left for another 6 weeks without any treatment or the group that was treated with small concentration of CS-NPs 0.235 mg/kg. Both revealed areas of well-to-moderate differentiated OSCC, papillomatous

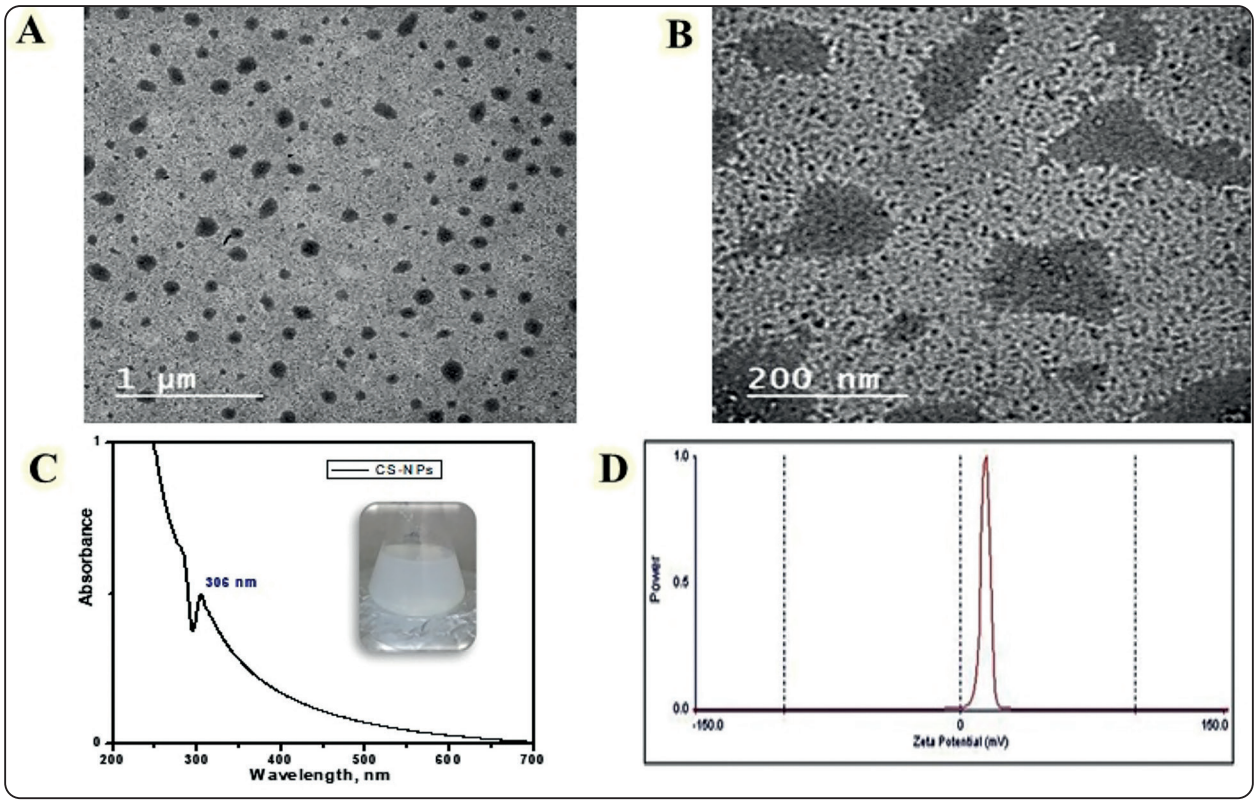

Fig. (1) (A&B) TEM images of CS-NPs taken from different spots. (C) UV–Visible spectrum of CS-NPs, and its photo image (the inset). (D) Zeta potential value obtained for prepared CS-NPs with surface charge of about 16.52 mV.

overgrowths, superficial malignant cellular invasion, and areas of severe dysplasia. The underlying c.t. revealed intense infiltration of chronic inflammatory cells (Figure 2: B2:1 & C1).

Group D (CS-NPs 2.53 mg/kg group): The left HBPs showed mild dysplastic changes in the group that was treated with high concentration of CS-NPs 2.53 mg/kg. There was loss of polarity of basal cell layer with few nuclear pleomorphism and hyperchromatism. The underlying connective tissue showed mild chronic inflammatory cellular infiltrate (Figure 2: D1).

Immunohistochemical Finding (BAX & PCNA staining)

The immunoreactivity of BAX could be noticed positive as brown cytoplasmic expression within epithelial cells. The quantitative analysis in group A showed strong expression within normal epithelium (101.8±2.3). In groups B1, B2 &C, there was a statistically significant decrease in their expression (47.6 ± 2.7, 55.1 ± 1.4 and 48.1 ± 2.1) in comparison to the negative control group (101.8±2.3). While in Group D, there was a statistically significant increase in its expression (132.5±3.9) in comparison to the positive control group (47.6±2.7). (Figure 2: A2, B1:2, B2:2, C2 & D2) and (Figure 3)

The immunoreactivity of PCNA could be observed positive as brown nuclear expression within epithelial cells. Weak expression was represented in group A throughout the basal layer (21.2±2.2). In group B1, there was a marked increase in the expression all over epithelial thickness (34.8±3.7). Regarding groups B2& C, there was a significant increase in their expression (49.2±2.5 and 56.8±3.1). While in group D, there was a significant decrease in its expression (25.9±1.9) that was limited to a lower third of epithelium. (Figure 2: A3, B1:3, B2:3, C3 & D3 and Figure 3)

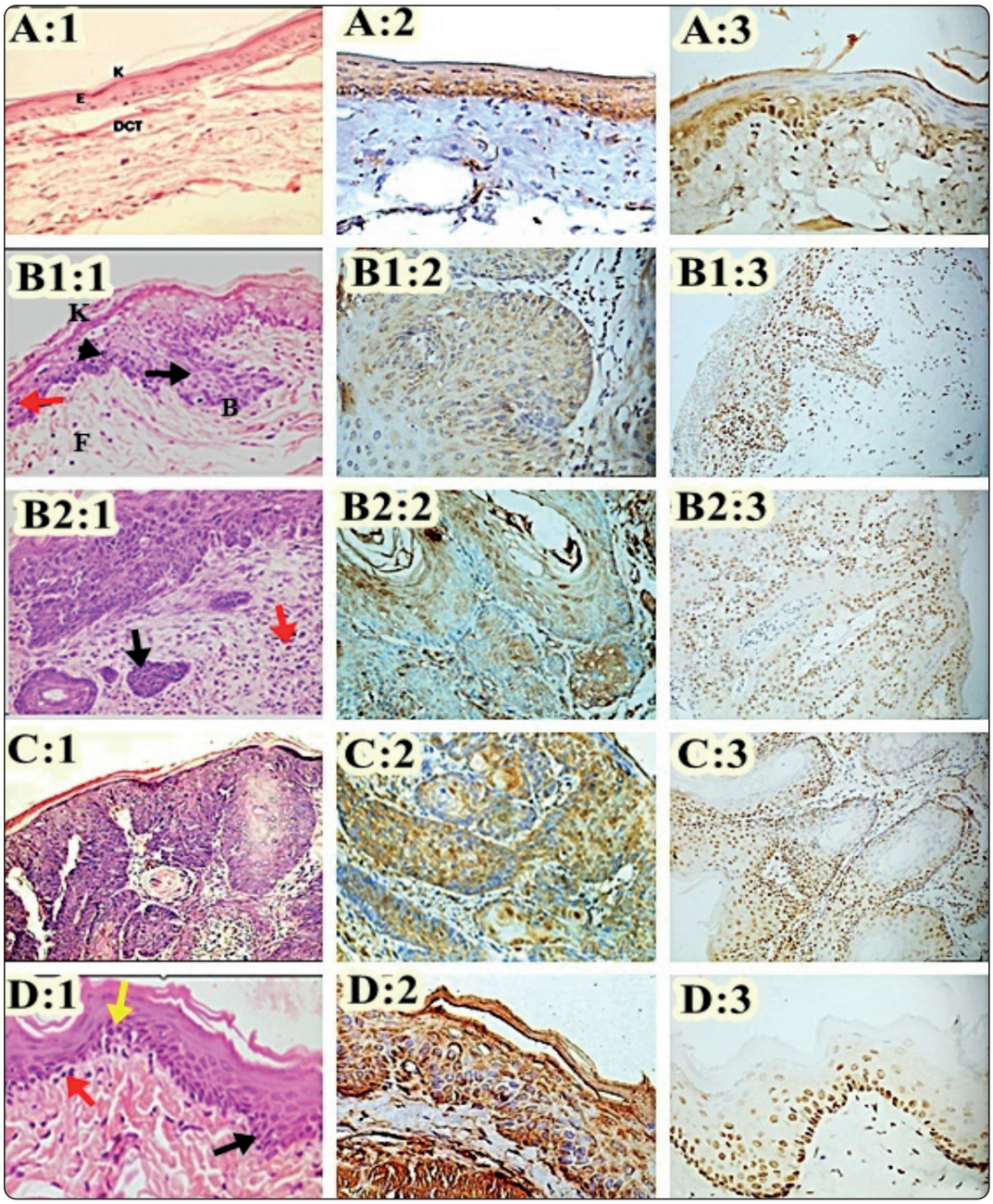

Fig. (2) H&E staining and immunohistochemical expression of BAX, and PCNA. **Group A** (negative control) (A1) shows normal buccal mucosal epithelial lining (H&E X200), (A2 & A3) shows strong cytoplasmic expression of BAX within all epithelial thickness and mild nuclear expression of PCNA limited to basal layer (IHC X400). **Group B1** (DMBA / 6w) (B1:1) shows severe dysplasia with moderate chronic inflammatory infiltrate (H&E×400). (B1:2&3) shows moderate cytoplasmic expression of BAX and strong nuclear expression of PCNA all over epithelial thickness. **Group B2** (DMBA / 6w, served for other 6) (B2:1) shows well/moderate differentiated SCC with invading islands in the connective tissue, and intense chronic inflammatory cell infiltrate (H&E X400). (B2:2&3) shows moderate cytoplasmic expression of BAX and strong nuclear expression of PCNA (IHC X400). **Group C** (DMBA / 6 weeks, CS 0.235 / 6w) (C:1&2&3) shows the same findings as in group B2 (H&E X200) (IHC X400). **Group D** (DMBA / 6w, CS 2.35 / 6w) (D:1) H&E section shows mild to moderate epithelial dysplasia with basal cell layer hyperplasia, cellular &nuclear pleomorphism, and mild chronic inflammatory cells in the CT (H&E X400). (D:2&3) shows strong cytoplasmic expression of BAX with moderate nuclear expression of PCNA (IHC X400).

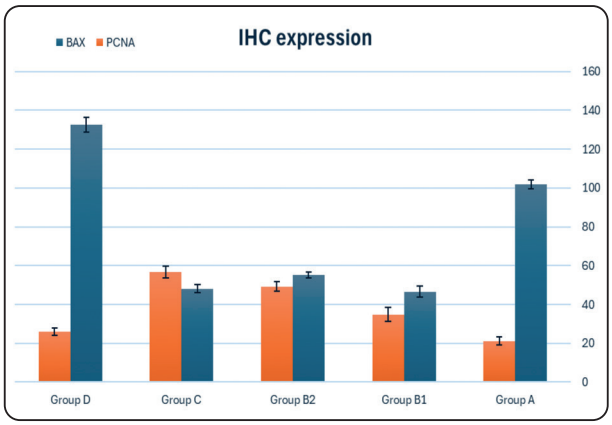

Fig. (3) Quantitative analysis of Bax and PCNA expressions in different groups

DISCUSSION

Early detection and treatment of oral precancerous lesions has a great interest in lowering morbidity and death that occur with OSCC. Oral cancer has a serious health impact on individuals and a severe economic impact on their families. Despite advances in treatment options, the 5-year survival rate and the quality of these people remain extremely low.[2] As a result, looking for natural products with less adverse effects and more effective anticancer efficacy may improve patients' overall quality of life and survival outcomes.[13]

Many studies have investigated the anticancerous effect of CS in many types of cancer.[14] Therefore, current study has evaluated the effect of CS-NPs, in two different concentrations, as a topical treatment that may help in eliminating the systemic toxicities or side effects. It was found that the best results were achieved with a high concentration of CS-NPs (2.53 mg/kg). On contrary, CS-NPs with low concentration (0.235 mg/kg) gave poor results that are like DMDA group without treatment. This may be due to the very low concentration, keeping in mind that the treatment was topical, which reduced its absorption by the tissues.

Carcinogenesis model induced chemically by painting DMBA in the HBP has been intensively researched for the assessment of histochemical, genetic, and biomolecular changes during the disease. Given the similarities in the carcinogenesis process between people and hamsters, this animal model is a valuable tool for study in OSCC.[15]

In the current study, CS-NPs solution was prepared, and HR-TEM imaging revealed that sizes ranging from 80 to 150 nm. These observations were consistent with those found in the literature.[16] Also, as is typical of CS-NPs, the recorded UV-visible spectra of CS-NPs displayed a small absorption peak centered at 306 nm, which corresponds to the π-π^* transition of the CS-NPs.[17] It was reported previously that the degree of interaction between the cationic groups of CS ($-NH^{3+}$) determines the surface charge of CS-NPs and TPP's polyanionic groups. A higher CS concentration indicates more $-NH^{3+}$ groups on the surface of CS-NPs that have not interacted with TPP molecules.[18] As a result, the zeta potential should rise as the CS concentration does. The present zeta potential value obtained suggests good conjugation between TPP and CS to form CS-NPs.

In the present study, high concentration of CS-NPs group (2.53 mg/kg) showed mild dysplastic changes while low concentration of CS-NPs and DMBA groups showed formation of OSCC. Our results are parallel to Ezzat & ELsherbini [19] as well as Wimardhani et al.[8] who confirmed that only cancer cells had a cytotoxic effect by the action of CS. Chitosan molecules have high positive charged amino groups that are attracted to the membrane of cancer cells. Whereas the cancer cells have a greater negative charge than that of normal cells. So that, CS interacts with the tumor cell extracellularly via a specific receptor, or via endocytosis.

The current study observed an improvement in the expression of Bax and decreased levels of PCNA with CS-NPs group (2.53 mg/kg). This suggested that it controlled the expression of pro-apoptotic markers in a way that inhibited tumors. The findings observed suggest that CS-NPs have intensified the apoptotic cascade in cancerous cells. Expressions of

PCNA were lower, further supporting the findings mentioned above. According to Wu et al.[20] research findings, seleno-short chain CS causes apoptosis and has a cytotoxic effect on BT-20 and MCF-7 breast cancer cells. By down-regulating Bcl-2 expression and up-regulating BAX expression, seleno-short-chain CS causes apoptosis in cancer cells through the mitochondrial route, according to gene expression study. Another study of Dou et al.[21] reported that, in the HL-60 acute leukemia cell line, CS markedly reduced the expression of anti-apoptotic Bcl-2 while up-regulating the expression of proapoptotic components Fas, FADD, and Bax.

Shen et al.[22] investigated the anticancer and antimetastatic effects of CS oligosaccharides in HepG2 hepatocellular carcinoma cell lines. It inhibited cell proliferation and decreased the rate of DNA synthesis. According to gene expression study, PCNA, cyclin A, and CDK-2 were down-modulated and p21 was up-modulated.

In agreement with CS-NPs group results, li et al.[23] explained the anti-tumor activity of CS to dendritic cell activation, increased NK cell activity, INF-γ production, cytotoxic activity, and cell survival in B16 melanoma mouse model. Conversely, Yeh et al.[24] reported that there was no significant effect on T- and B-cell proliferation. Moreover, cytotoxic activity of NK cells remained unchanged in WEHI-3 cell-generated leukemia mice.

The molecules of CS act as a penetration enhancer by opening epithelial tight junctions. It interacts with mucus to produce a complex via hydrogen or ionic bonding, as well as hydrophobic interactions with amino groups.[25] CS nanoparticles aggregate in the tumor site, polarize M1 macrophages, and convert the immunosuppressive tumor environment to an immunosupportive state, creating an anticancer effect and increasing the therapeutic effectiveness of cancer immunotherapy. Additionally, CS may also decrease tumor cell proliferation, angiogenesis, and metastasis.[5]

CONCLUSION

Topical administration of CS-NPs with 2.53mg/kg concentration was capable of inhibiting progression of oral carcinogenesis process as well as upregulation of BAX and downregulation of PCNA. However, more research is required for the clinical application of CS-NPs, including determining the mechanisms of anticancer actions and improving drug loading with releasing characteristics.

Declarations

Ethics approval: The present study was carried out after the approval of the Research Ethics Committee (REC), Faculty of Dentistry, Suez Canal University (833/2024). The authors followed the ARRIVE guidelines regarding the care and use of animals for experimental procedures as well as the guidelines for the euthanasia of animals.

- **Competing interest:** The authors declare no conflict of interest.
- **Data availability:** All data included in this study are available from the corresponding author upon request.
- **Funding:** This research was not funded by any specific grants from governmental, private, or public organizations.

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