TOPICAL CHEMOPREVENTIVE EFFECT OF THYMOQUINONE VERSUS THYMOQUINONE LOADED ON GOLD NANOPARTICLES ON DMBA-INDUCED HAMSTER BUCCAL POUCH CARCINOGENESIS (IMMUNOHISTOCHEMICAL STUDY)

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ABSTRACT

**Background:** Oral cancer is the third most common cancer in developing countries. Thymoquinone has a powerful anticancer, antioxidant, and anti-inflammatory properties.

**Aim:** To compare the topical chemopreventive effect of thymoquinone (TQ) versus thymoquinone loaded on gold nanoparticles (GNPs-TQ) on DMBA-induced oral carcinogenesis through histological and immunohistochemical expression of p53.

**Material & methods:** Characterization of the prepared drugs was done through transmission electron microscope, and Ultraviolet-Visible spectroscope. Experimental design: The study was carried out on forty male Syrian golden hamsters, weighing 90-100 grams. Negative control group A (n=10) didn’t receive any type of treatment till the end of experiment. Positive control group B (n=10) was painted with DMBA, 3 times/week for 14 weeks. Group C (n=10) was daily painted by topical application of TQ for two weeks, and then painted with both TQ and DMBA on alternative days for 14 weeks. Group D (n=10) had been managed as group C but TQ was replaced by GNPs-TQ. These groups was examined histologically and immunohistochemically through expression of p53.

**Results:** Proper loading of TQ on GNPs was confirmed. Histopathological evaluation showed superior effect of GNPs-TQ in retardation of carcinogenesis compared to free TQ. Immunohistochemical evaluation revealed significant decrease in P53 expression in the group treated with GNPs-TQ than TQ in comparison with positive control group.

**Conclusions:** GNPs-TQ is a promising chemo-preventive agent of oral cancer through topical application.

**KEY WORDS:** Oral cancer, DMBA, Thymoquinone, GNPs-TQ, and p53

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck.\(^1\) Every year in Egypt, approximately 4,500 people are diagnosed with oral cancer; about half of them would die because of the disease.\(^2\) Once the cell damage become irreversible due to various risk factors, it would be manifested as dead cell or neoplastic transformation.\(^3\) The neoplastic transformation may lead to genetic alterations and altered cell cycle regulation.\(^4\) 7,12-dimethylbenz(a)-anthracene (DMBA) is widely used carcinogen in experimental oral carcinogenesis through formation of DNA adducts, DNA damage, inhibition of DNA repair enzymes, generation of excess reactive oxygen species and production of chronic inflammation.\(^5\) Cancer chemoprevention uses natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression.\(^6\)

Thymoquinone is considered the main active constituent of *Nigella sativa* L.\(^7\) It was documented that TQ inhibits various cancer hallmarks as tumor cell proliferation, inflammation, cancer cell death, tumor angiogenesis, invasion and metastasis.\(^8\) Majdalawieh et al represented the anti-tumor effect of TQ alone *in vitro* and *in vivo* studies.\(^9\) Poor bioavailability of TQ is the main complication. So, nano-carriers were prepared to modify the drug’s bioavailability, efficacy, stability and toxicity associated with high doses.\(^10\) GNP s could be easily synthesized with different shapes and characterized due to the characteristic Surface Plasmon Resonance (SPR) bands.\(^12\) P53 is considered the guardian of the genome because of the adaptive and protective cellular responses which prevent cellular proliferation.\(^13\) Any mutation in p53 is considered an early event of carcinogenesis; its accumulation is associated with poorer differentiation and malignancy.\(^14\) The present study attempted to compare the topical chemo-preventive effect of TQ versus GNP s-TQ in DMBA-induced hamster buccal pouch carcinogenesis through histological examination and immunohistochemical detection of p53.

MATERIAL AND METHODS

Preparation of thymoquinone loaded on gold nanoparticles: Tetrachloroauric acid (HAuCl\(_4\)), trisodium citrate (Na\(_3\)C\(_6\)H\(_5\)O\(_7\)), thymoquinone (C\(_{10}\)H\(_{12}\)O\(_2\)), and propylene glycol (C\(_3\)H\(_8\)O\(_2\)) were purchased from Sigma Chemical Company, St. Louis, Mo, USA. GNPs were prepared by Turkevich method.\(^15\) Tetrachloroauric acid was heated until boiling with stirring, and then trisodium citrate was injected. The gold nanoparticles were gradually formed as indicated by the change in color from pale yellow to deep red color. Thymoquinone solution of 0.01 mg/kg was dissolved in propylene glycol with the aid of a magnetic stirrer for 2 hours. The loading of thymoquinone on GNPs was achieved by mixing equal amount of both, stirred for 2 hours then kept in 5°C.

High resolution transmission electron microscope (TEM) was used to study the morphology of GNPs, GNPs-TQ using JEOL JEM 2100 (Japan). Pictures of nanoparticles were taken in the Egyptian Petroleum Research Institute. TEM operating at a magnification of 80 kV with 1K × 1K digital images captured using an AMT CCD camera (Danvers, MA). For GNPs, a drop was placed on a paraffin sheet; a copper grid was placed on the sample and left for 1 minute to allow NPs to adhere. For GNPs-TQ, the grid was placed on a drop of phosphotungstate for 5 minutes. The remaining solution was removed by absorbing the liquid with a piece of filter paper, samples were air dried, then examined by TEM. Ultraviolet-Visible (UV-vis) spectroscope was used to determine the maximum absorption of ultraviot for GNPs, TQ, and GNPs-TQ at room temperature in a quartz cuvette using T90+UV-VIS Spectrometer (PG Instruments Ltd). Its wavelength ranges from 250-850 nm.
Experimental design:

The chemical carcinogen DMBA was dissolved in heavy mineral oil to get solution of 0.5% concentration. Both of them were purchased from Sigma Chemical Company, St. Louis, Mo, USA. The carcinogen was topically applied to hamster buccal pouch by using number (4) camel hair brush. The experiment was done at the animal house of Faculty of Dentistry, Suez Canal University, Ismailia. The study was carried out on forty Syrian male golden hamsters, weighing 90-100 grams. The animals were purchased from, Tiedor Blhars Research Institute, Cairo, Egypt. The animals were housed (five per cage) at controlled temperature; all animals were given water and recommended nutrient, ad libitum, and were divided as follow:

Negative control group A (n=10) didn’t receive any type of treatment till the end of experiment. Five animals were sacrificed at day zero and five animals were sacrificed at 14th week. Positive control group B (n=10) the left cheek pouch was painted with DMBA, thrice/week for 14 weeks. Group C (n=10) the left cheek pouch was daily painted by topical application of TQ 0.01mg/kg for two weeks, and then painted with both TQ and DMBA on alternative days for 14 weeks. Group D (n=10) the left cheek pouch was daily painted by topical application of GNPs-TQ 0.01 mg/kg for two weeks, and then painted with both GNPs-TQ and DMBA on alternative days for 14 weeks.

Histopathological evaluation:

The left pouches were surgically removed after euthanization, fixed in 10% neutral formalin solution, processed, embedded in paraffin, sectioned into 5μm, mounted on glass slides and stained with hematoxylin & eosin for light microscopic study, then photographed by E-330 Olympus digital camera. Grading of epithelial dysplasia was carried out according to Banoczy and Sciba (1976)\textsuperscript{16} and modified by El-Dakhakhny et al (2010)\textsuperscript{17} due to thin nature of hamsters’ epithelial lining. Grading of squamous cell carcinoma was done according to Broder’s classification.\textsuperscript{18}

Immunohistochemical evaluation (IHC):

From each paraffin block, 5 μm sections were cut and mounted on positively-charged slides. The antibody used was p53; rabbit polyclonal antibody which purchased from Gene Tex International Corporation, Cat. No. GTX100629, with brown nuclear expression. The steps of IHC followed according to manufacturer’s instructions. Image analyzer software (image J / figi 1.46) was used to count the number of immunopositive cells as well as the number of the remaining unstained ones. The percent of the positive cells was calculated.

Statistical analysis:

Microsoft excel 2013 was used for data entry and the statistical package for social science (SPSS) version 24 was used for data analysis. All values were expressed as mean ± standard deviation. Comparisons between groups were performed using one-way analysis of variance (ANOVA). Probability value less than 0.001 was considered significant.

RESULTS

I- Characterization of GNPs-TQ:

Citrate ions acted as both a reducing agent and a capping agent. Once they settled on the particle surface, GNPs were stabilized trough electrostatic repulsion. Color change from pale yellow color to deep red color confirmed GNPs formation. TEM micrographs revealed that GNPs were spherical and well dispersed without agglomeration, with average size 25-30 nm in size (figure 1-A). The loading of TQ on GNPs was represented in figure 1-B. UV-visible spectrometer results showed sharp peak was given by UV-visible spectrum for GNPs at λ_{max}=526nm (figure 1-C) which confirmed the nanoparticles formation. GNPs-TQ gave maximum absorption peak λ_{max} =532nm (figure 1-D). This
deviation in the maximum peak confirmed loading of TQ on GNP.

**II-Clinical findings:** Animals in negative control group (A) showed no gross changes throughout the experimental period. Their buccal pouch lengths were about 5cm for all hamsters. Animals in the positive control group (B) showed marked hair loss, pouch depth decrease, skin ulcers and marked debilitation of all animals. Animal’s pouches showed large exophytic growths with pronounced vascularity and the pouch length ranged from 1.5-2cm. The animals in the group D painted with GNP-TQ showed significant improvement in comparison with animals in the group C painted with TQ only. There was marked decrease in the size of the lesions and the pouch length reached 3.5cm. (figure 2).

**III-Histopathological findings:** Group A (negative control) revealed normal thin stratified squamous epithelium formed of two to four cell layers, lacking rete ridges and thin keratin surface layer. The underlying connective tissue (c.t.) showed non inflamed loose connective tissue, with thin vascular spaces. Deeper c.t. layer was formed of larger vessels and longitudinal striated muscle layer (figure 3-A). Group B (positive control) showed well to moderate differentiated squamous cell carcinomas in the form of papillomatous lesions with invading islands of epithelium into underlying connective tissue. The invading epithelial cells showed basal cell hyperplasia, loss of basal cells polarity, hyperchromatism, prominent nucleoli, altered N/C ratio, swirling of spinous layer and cellular & nuclear pleomorphism. Connective tissue was

![Fig. (1) Showing characterization of GNPs- TQ. (A) GNPs appear spherical, well dispersed without agglomeration, (B) the proper loading of TQ on GNPsl (C) sharp peak of UV -visible spectrum for GNPs at 526nm, (D) sharp peak of UV-visible spectrum for GNPs-TQ at 532nm.](image-url)
Fig. (2) Showing different sizes of the lesions (A) normal pouch length within 5cm in untreated group, (B) large exophytic masses with marked shortening of the pouch to about 1.5cm in positive control group, (C) moderate-sized exophytic masses when painted with TQ alone, (D) small exophytic mass when painted with GNPs-TQ with marked increase in pouch length.

Fig. (3) Histopathological photomicrographs showing (A) normal hamster buccal pouch lining X40. (B) Well to moderate differentiated sec from the group painted with DMBA for 14w X4. (C) High power of figure B showing different dysplastic criteria X20. (D) Superficial invasion in chemopreventive group with TQ X10. (E) High power of figure D showing long rete ridges with significant dysplastic criteria X20. (F) Carcinoma in situ in chemopreventive group with GNPs-TQ X20. (G) High power of figure F showing multiple dysplastic changes without any signs of invasion X40.
invaded with keratin pearl and dysplastic epithelial nests (figure 3 B&C). Group C (painted with TQ only) showed superficial invasive carcinoma with multiple signs of dysplasia. The invasive islands were located below the papillomatous lesion directly (figure 3 D&E). Group D (painted with GNPs-TQ) revealed carcinoma in situ only without any signs of invasion. The epithelium showed severe cellular and architectural changes as basal cell hyperplasia and loss of polarity, hyperchromatism, altered N/C ratio and cellular/nuclear pleomorphism (figure 3 F&G).

IV-Immunohistochemical (IHC) assessment:

By examining the negative control group, p53 nuclear expression was not detectable (figure 4-A). Examining the DMBA-painted group revealed marked increase in the nuclear p53 expression suprabasally, extended allover epithelium thickness (figure 4-B&C). In the group painted with TQ only, there was a slight dwindle in the nuclear expression of p53 (figure 4-D&E). Noteworthy, there was a significant decrease of p53 nuclear expression in the group painted with GNPs-TQ (figure 4-F&G).

V-Statistical results evaluation: (table 1) regarding p53, there was a high statistical significant difference between whole groups (P-value < 0.001) through using Annova - one way test (figure 5). The most statistically significant result was associated with the group painted with GNPs-TQ [15.82 ± 1.65] in comparison with the group painted with TQ only [25.48 ± 3.84]. Also, both of chemopreventive drugs showed statistically significant result versus positive control group [47.01 ± 6.79].

Fig. (4) Showing photomicrographs of p53 immunohistochemical expressions: (A) untreated group reveals undetected immune reactivity, (B&C) the group painted with BMBA reveals extensive increase in immune reactivity extended suprabasally, (D&E) the group painted with TQ only reveals slight decrease in immune reactivity, (F&G) the group painted with Glu-p-s- TQ reveals noticeable decrease in immune reactivity.
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DISCUSSION

This study aimed to compare the topical chemopreventive effect of TQ only versus GNPs-TQ through immunohistochemical detection of p53 in DMBA-induced oral carcinogenesis. In this work, the prepared GNPs were spherical, well dispersed, its particle size ranged between 25-30nm. Chithrani et al\textsuperscript{19} concluded that the cellular uptake of spherical GNPs is higher than rod-shaped counterparts. Several studies confirmed that GNPs cytotoxicity depends on their sizes. Particles larger than 15nm were nontoxic\textsuperscript{20,21} Other studies documented the biosafety of GNPs in experimental models after intraperitoneal injection through liver histology and blood markers analysis.\textsuperscript{22,23} Ultraviolet-Visible spectra for the prepared gold nanoparticles gave a sharp peak at 526 nm which confirmed gold nanoparticles formation. This result was in line with several studies reported the maximum peak at 529nm and 522nm respectively.\textsuperscript{24,25} Sharp peaks shifting to longer wavelength were noted with GNPs-TQ which indicated proper loading of TQ on GNPs. On line with the present result Daduang \textit{et al}\textsuperscript{26} reported comparable deviation in the maximum peak after loading of gallic acid on GNPs from 525nm to 560nm.

Topical application of GNPs-TQ 0.01mg/kg gave promising results in this study. This concentration was 10-folds lower than a comparable study used TQ 0.1 mg/kg by intra-peritoneal route.\textsuperscript{17} A comparable dose reduction was observed in case of polylactic acid-polyethylene-glycol (PLA-PEG) encapsulated epigallocatechin-3-gallate (EGCG).\textsuperscript{27} Another study documented the efficacy of GNPs-TQ 0.01mg/kg in regression of malignancy through inhibition of NF-\textit{kB} expression during treatment of OSCC by intraperitoneal injection. It might be due to proper distribution of GNPs-TQ on the cell membrane due to use of little amount of TQ that led to improvement of the cellular uptake.\textsuperscript{28} Soni \textit{et al}\textsuperscript{29} reported that the nano-particles release the drug within the cells, not outside to increase the drug intracellular concentration. Hence, lower concentration would result in higher efficacy.

To ensure the topical chemopreventive effect of the used drugs; evaluation of the clinical, histopathological, and p53 immunohistochemical expression was done.

Clinically, in this study gross remarks were detected in DMBA-painted pouches for 14w, like hair loss, skin ulcers, large exophytic masses, and significant shortening in pouchs’ length. These observations were on line with other researchers.\textsuperscript{17,28,30} The buccal necrosis might be due to local toxic effect of DMBA or local infection that led to severe inflammation.\textsuperscript{31,32} The group painted with TQ only gave slight decrease in the size of the lesions due to use of low concentration of TQ

<p>| TABLE (1) Annova one way test |</p>
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<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>P value</th>
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<tr>
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<td>1.80</td>
<td>.40</td>
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<tr>
<td>Positive group</td>
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<tr>
<td>GNPs-TQ</td>
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Fig. 5: representing as mean ± standard deviations of p53 expression between negative group (red bar), DMBA group (green bar), TQ only (blue bar), and GNPs-TQ (orange bar)
(0.01 mg/kg). The animals painted with GNPs-TQ showed the smallest masses in comparison to other groups with significant improvement in general health. These findings indicated the beneficial effect of loading GNPs with TQ to achieve favorable results. Significant pouch elongation was noted in this group which confirmed the direct effect on muscle regeneration rather than fibrosis. Myofibers regeneration begins with activation of myogenic precursor cells which proliferate and differentiate into multinucleated myotubes and myofibres. Fibroblasts begin to produce extracellular matrix (ECM) to restore the framework of the connective tissue surrounding the muscle fibers. Resident or infiltrated macrophages play prominent role in muscle repair through pro-inflammatory cytokines as tumor necrosis factor-α (TNF-α), interleukins (ILs), and cyclo-oxygenase-2 (COX-2). The histopathological findings in the present work in DMBA-treated group for 14w showed well to moderately differentiated squamous cell carcinoma with invading malignant epithelial islands and keratin pearls. These findings were supported by several authors. Topical application of TQ only and GNPs-TQ resulted in delaying the malignancy. The best result was noted with GNPs-TQ. The chemopreventive effect of TQ was related to its anti-inflammatory, anti-apoptotic & cell cycle arrest, anti angiogenic properties in addition to prevention of invasion and metastasis. To enhance the effect of TQ due to its hydrophobic nature, attempts were carried out to encapsulate TQ in different nano-formulations for better targeting of the cancer hallmarks. Nanoparticles can passively target tumor cells by an accumulation and entrapment process through enhanced permeation and retention (EPR) effect. It can be imposed by angiogenic vessels through leaky blood vessel (gaps~600nm). This concept allows selective penetration of the nanoformulations at higher levels than to normal cells.

Immunohistochemical evaluation of p53 in the present study in normal tissues of negative control group revealed negative nuclear expression. Due to the short half life time of wild p53 protein it is hard to be detected by immunohistochemical evaluation. In the present work, significant increase in the nuclear expression of p53 in all layers in DMBA-painted group was detected. A close relation between development of OSCC and alterations in the expression of tumor suppressor genes and oncogene was reported. These results were on line with several studies. Mutant p53 was reported as the most frequent alterations in OSCCs resulted in stabilization of the protein within the nuclei of malignant cells. Several studies reported that there was a strong relationship between increasing the positivity of p53 expression and the degree of tumors differentiation. Baweja et al detected that suprabasal nuclear expression of p53 is an early event in oral carcinogenesis. Stronger staining had greater risk of malignancy progression. Only benign hyperplastic lesions didn‘t reveal any immune reaction. In the present study, there was marked decrease in the nuclear expression of p53 when the pouches were painted with TQ, which is explained by the chemoprevention effect of thymoquinone. Other researchers reported the effect of thymoquinone on a number of carcinogenic signaling pathways or signaling molecules. The decrease in p53 expression was more significant in GNPs-TQ treated group. This finding could be explained by delivering more particles to the targeted cells and the longer exposure to the anticancer drug when dealing with nanosized preparations. In connection with the above findings, Montazeri et al reported that p53 plays an important role in mediating the survival by dendrosomal nanocurcumin through antiproliferative effects and induction of apoptosis. Sulfikkarali and his colleagues studied the chemopreventive efficacy of free naringenin (NAR) and naringenin-loaded nanoparticles (NARNPs) in DMBA hamster buccal pouch. There was marked decrease in the expression...
of PCNA and p53 after oral administration of NARNPs rather than free NAR. Mirunalini et al examined the effect of Ellagic acid-chitosan nanoparticle on DMBA-induced hamster buccal pouch tumorigenesis against free Ellagic acid which had superior therapeutic activity.

CONCLUSION

TQ loaded on GNPs (0.01mg/kg) gave promising results in delaying of carcinogenesis process and improving the general health of the animals through significant decrease of p53 nuclear expression in comparison with free TQ.

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