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MOLECULAR EVALUATION OF THE CHEMOTHERAPEUTIC EFFECT OF THYMOQUINONE LOADED ON GOLD NANOPARTICLES THROUGH EXPRESSION OF DNA REPAIR ENZYMES IN INDUCED ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

Introduction: Different types of DNA damages occur during the induced oral carcinogenesis, which can be eliminated through several DNA repair pathways. XRCC1 and ERCC1 are the main repair enzymes involved in repair of oral squamous cell carcinoma. A combination of thymoquinone with gold nanoparticles as a novel chemotherapeutic modality is the aim of the present work against chemically induced SCC in the classic model of HBP/DMBA carcinogenesis. **Materials and methods:** One hundred male Syrian golden hamsters were divided into: Group A: Ten animals (negative control), group B: Ten animals (positive control) painted with DMBA only 3times / week/ 12weeks. The rest of animals were painted with DMBA (3times / week/ 12weeks) then painted and injected intraperitoneal with TQ only, 0.01mg/kg TQ-GNps, 0.001mg/kg TQ-GNps or GNps only for 6- and 12- weeks, intervals. By end of the experiment, both pouches from all groups were surgically excised, fresh samples from each pouch were processed for RT-PCR technique. The rest of the pouches were fixed and processed for H&E evaluation. **Results:** Loading of thymoquinone on gold nanoparticles was promising chemotherapeutic combination, through regression of well-differentiated SCC (positive control) to dysplasia and enhanced expression of the studied DNA repair enzymes compared to either thymoquinone or gold nanoparticles groups. **Conclusion:** Loading of TQ on GNps revealed superior effect over the use of TQ only or GNps only in regression of tumors and increased expression of DNA repair enzymes.

INTRODUCTION

Oral cancer is a lethal disease worldwide with a 5-year survival rate of around 50%. Oral squamous cell carcinoma (OSCC) accounts for 90% of all oral cancers found in the mouth, tongue, and lips.(1)

Several morphological and physiological similarities were observed between the development of human OSCC and chemically-induced hamster buccal pouch carcinogenesis. Moreover, they show close resemblance in the histologic and molecular changes, therefor this model is widely accepted in the study of pathogenesis, prevention and treatment of OSCC.(2)

Thymoquinone (TQ), the active ingredient of black cumin, has a wide range of beneficial biological and pharmacological properties. TQ possesses potent antioxidant, anti-inflammatory, and anticancer effects. It is involved in the adjustment of several biological pathways as cellular proliferation, cell cycle regulation, apoptosis, angiogenesis, carcinogenesis, and cancer metastasis.[3]

In recent years, nanomaterials and nanotechnology have increasingly used in clinical application. Gold nanoparticles (GNps) have unique physical and chemical properties and are relatively safe, stable and easy to prepare. GNps, as drug delivery agents, can increase the pharmacokinetics of the drug, thereby reducing non-specific side effects and achieving higher doses of targeted drug delivery.[4]

DNA repair system is essential to maintain genomic stability against endogenous and exogenous factors through various pathways.[5] The X-ray repair cross-complementing 1 (XRCC1) enzyme plays an important role in the base excision repair (BER) pathway.[6] XRCC1 polymorphism increases the risk of cancer development in humans and animals,[7] especially in head and neck squamous cell carcinoma.[8]

Excision repair cross-complementing enzyme (ERCC1) is a core protein in the nucleotide excision repair, that is responsible for DNA- and protein-protein interactions and further activation of the NER pathway.[9] Cell lines deficient in ERCC1 were found more sensitive to radiation under hypoxic conditions.[10]

The development of real-time polymerase chain reaction (RT-PCR) added great advantages to traditional PCR and proved to be valuable in the study of human tumors and diseases.

The present study was concerned about the expression of XRCC1 and ERCC1 repair enzymes following treatment of chemically - induced oral cancer using thymoquinone loaded on gold nanoparticles in 2 different concentrations and 2 treatment periods.

MATERIAL AND METHODS

1. Chemicals:

- Tetrachloroauric acid (HAuCI4) [cat# (27988-77-8)], trisodium citrate (Na3C6H5O7) [cat# 6132-04-3] to get GNps solution.
- Thymoquinone (C10H12O2) [cat# 490-91-5] and propylene gylcol (C3H8O2) [cat# 57-55-6] to get thymoquinone solution.
- The chemical carcinogen 7, 12 Dimethylbenz-[a]-anthracene (DMBA) [cat# D3254], and heavy mineral oil [cat# M 3516] to get DMBA solution.
- All the previous chemicals were purchased from Sigma Chemicals Company, USA.
- Total RNA extraction kit, SYBR Green PCR amplification kit and primers for XRCC1 (5'-CAGCCCTACAGCAAGGACTC-3') and ERCC1 (5'-TTGTCCAGGTGGATGTGAAAGATC-3') were purchased from Qiagen Company, Netherlands

2. Animals:

One hundered male Syrian golden hamsters were held at the animal house of Faculty of Dentistry, Suez Canal University, were divided into:

Group A: Ten animals served as the negative control group (untreated group), and were euthanized at day zero.

Group B: Ten animals were painted with DMBA on the left pouches, 3 times/week/ twelve weeks;

served as the positive control group and were euthanized by end of the 12th week.

Group C1: Ten animals were painted with DMBA for 12 weeks, then painted and injected intraperitoneally (i.p) with TQ (0.1 mg/kg) only (3 times/week / 6 weeks).

Group C2: Ten animals were painted with DMBA for 12 weeks, then painted and injected i.p. with TQ (0.1 mg/kg) only (3 times/week / 12 weeks).

Group D1: Ten animals painted with DMBA for 12 weeks, then painted and injected i.p with 0.01 mg/kg TQ loaded on GNps (3 times/week / 6 weeks).

Group D2: Ten animals painted with DMBA for 12 weeks, then painted and injected i.p with 0.01 mg/kg TQ loaded on GNps (3 times/week / 12 weeks).

Group E1: Ten animals painted with DMBA for 12 weeks, then painted and injected i.p with 0.001 mg/kg TQ loaded on GNps (3 times/week / 6 weeks).

Group E2: Ten animals painted with DMBA for 12 weeks, then painted and injected i.p with 0.001 mg/kg TQ loaded on GNps (3 times/week / 12 weeks).

Group F1: Ten animals painted with DMBA for 12 weeks, then painted and injected i.p with GNps only (3 times/week / 6 weeks).

Group F2: Ten animals painted with DMBA for 12 weeks, then painted and injected i.p with GNps only (3 times/week / 12 weeks).

3. DMBA preparation and application:

One gram of DMBA was dissolved in 200ml of heavy mineral oil, to get 0.5% DMBA solution, was painted to left buccal pouches of the experimental animals using number 4 camel hairbrush 3 times/ week / 12 weeks.

4. Preparation of gold nanoparticles:

Turkevich (1951) method was used to prepare GNps, where tertachloroauric acid (HAuCl4) solution was considered as a source of gold ions and trisodium citrate as a reducing and stabilizing agent. HAuCl4was heated with stirring until boiling, then trisodium citrate solution was injected. A gradual change in color of the solution from pale yellow to deep red, indicates gold nano-size formation.[12]

5. Preparation of thymoquinone working solution:

One gram of TQ was dissolved in 100ml propylene glycol to get 0.1mg/ml solution of TQ, then a working TQ solution of 0.01 and 0.001mole/L was prepared (with the aid of magnetic stirrer for 2 hours).

6. Loading of TQ on GNps:

The solution of GNps with thymoquinone was prepared by mixing equal amounts (1:1). The mixture was stirred using a magnetic stirrer for 2 hours and kept at 5° C.

7. Characterization of loaded TQ on GNps:

Loading of TQ on GNps was tested using High-resolution transmission electron microscope (TEM), Ultraviolet-visible (UV-vis) spectroscopy and Fourier transform infrared spectroscopy (FT-IR)

8.Animal euthanization and biopsy extraction:

Animal euthanasia was carried out using inhalation of an ether-soaked cotton piece, in a tightly closed container. Immediately after euthanization,

both pouches from all groups were excised. A thin longitudinal strip from each pouch was taken and preserved in an alcohol-based fixative for PCR processing.[13]

9. Polymerase chain reaction procedure:

Total RNA was isolated from the biopsies of all groups, then purified using RNAeasy columns (Qiagen GmbH, Hilden, Germany) and treated with ribonuclease free. A single-stranded cDNA was synthesized from total RNA using a high capacity cDNA reverse transcription kit. A quantitative real-time PCR technique was performed following the manufacturer's instructions.[14]

10. H&E evaluation:

The rest of the pouches were formalin-fixed, then paraffin-emmbeded and sections of 5 μm thickness were cut and evaluated through the examination of 3 serial sections of each pouch.

11. Statistics:

Statistical analysis of the PCR results was performed by Statistical Package for the Social Sciences (SPSS) 21 program for windows.

RESULTS

1. Characterization of GNps and TQ -GNps:

1.1.Color variation:

The formation of GNps was preliminarily confirmed by the visual observation of color change from pale yellow to deep red color.

1.2. Transmission electron microscope (TEM) results:

TEM micrographs revealed GNps as spherical and well dispersed without agglomeration. Most particles were between 25-30 nm in size with an average size of 27 nm (Fig. 1).

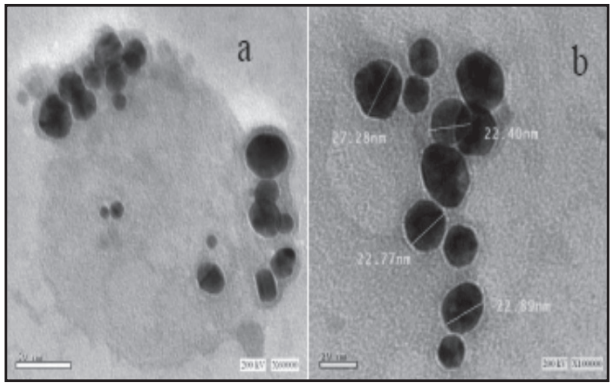

Fig. (1) TEM micrographs of GNps at a magnification of (a) 1:60.000 and (b) 1:100.000

1.3. UV-visible spectrometer (UV-vis):

The produced solutions of GNps, TQ, and TQ-GNps were subjected to characterization by UV-visible spectroscopy. The sharp peak was given by the UV-visible spectrum for GNps at λmax=526nm which confirmed the formation of the nanoparticles. The maximum absorption peak for TQ was recognized at λmax= 316 nm, while TQ-GNps gave maximum absorption peak λmax= 532 nm. This deviation in the maximum peak confirmed loading of TQ on GNps.

1.4. Fluorescence spectroscopy (FT-IR):

In the formation of TQ-GNps, one band of C=O was shifted to 1750 cm-1 which might confirm the attachment of TQ with GNps through one C=O group forming TQ-GNp. The other C=O group was not involved in the interaction with GNps as it appeared at 850 cm-1. Another shift in the O-H band was recognized at 3420 cm-1 which also confirmed the formation of TQ-GNps (Fig.2).

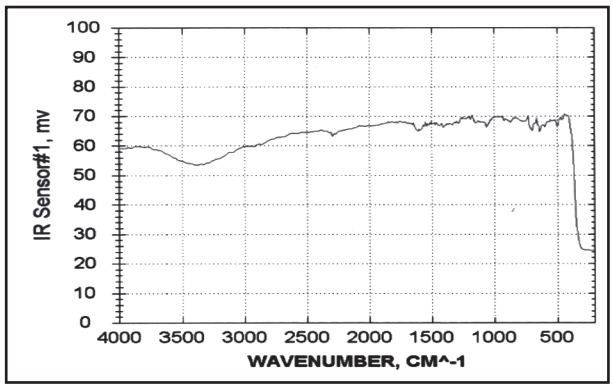

Fig. (2) FT-IR results confirming the formation of GNps-TQ

2. H&E results:

Group A did not show any change from normal HBP architecture. It revealed normal HBP lining mucosa, composed of four distinct layers: a- flat stratified squamous epithelium of two to four cell layers without rete ridges and a thin keratin layer. b- lamina propria of dense fibrous connective tissue. c- submucosa contains a layer of striated muscle fibers. d- a deeper layer of loose areolar connective tissue (Fig. 3.a).

Group B (DMBA-painted left pouches for 12 weeks) revealed multiple papillary lesions of well-differentiated squamous cell carcinoma with invading epithelial islands into the underlying connective tissue. The rest of the surface epithelium is hyperplastic and hyperkeratinized with elongated rete ridges. Variable degrees of epithelial dysplasia are noted in the form of basilar hyperplasia, loss of basal cells polarity, cellular and nuclear pleomorphism, altered nuclear/cytoplasmic ratio, prominent nucleoli, nuclear hyperchromatism, and keratin pearl formation. The connective tissue shows variable degrees of inflammatory infiltration (Fig. 3.b).

Group C1 (TQ for 6 weeks) revealed well-differentiated squamous cell carcinomatous lesions with invading islands to the underlying connective tissue, with variable dysplastic criteria along the rest of epithelial linning (Fig. 3.c).

Group C2 (TQ for 12 weeks) revealed hyperplastic and hyperkeratinized stratified squamous epithelium with well-differentiated squamous cell carcinoma, and marked dysplastic criteria along the rest of epithelial linning(Fig. 3.c).

Group D1 (0.01mg/kg TQ-GNps for 6 weeks) revealed well-differentiated squamous cell carcinoma. Invading epithelial islands were more superficial than seen in group B (Fig. 3.c).

Group D2 (0.01mg/kg TQ-GNps for 12 weeks) showed hyperplastic, hyperkeratinized surface epithelium with marked dysplastic criteria involving the whole epithelial thickness (carcinoma in situ) (Fig. 3.d).

Group E1 (0.001mg/kg TQ-GNps for 6 weeks) showed well-differentiated squamous cell carcinoma. Invading epithelial islands, with marked dysplastic criteria in the rest of surface epithelium (Fig. 3.e).

Group E2 (0.001mg/kg TQ-GNps for 12 weeks) showed no apparent endophytic or exophytic lesions, but rather hyperplastic, hyperkeratinized stratified squamous epithelium with severe dysplasia and/or carcinoma in situ (Fig. 3.f).

Group F1 revealed both endophytic and exophytic well-differentiated squamous cell carcinomas with marked dysplastic criteria in the rest of the epithelial lining (Fig. 3.c).

Group F2 revealed both endophytic and exophytic well-differentiated squamous cell carcinomas with marked dysplastic criteria in the rest of the epithelial lining (Fig. 3.c).

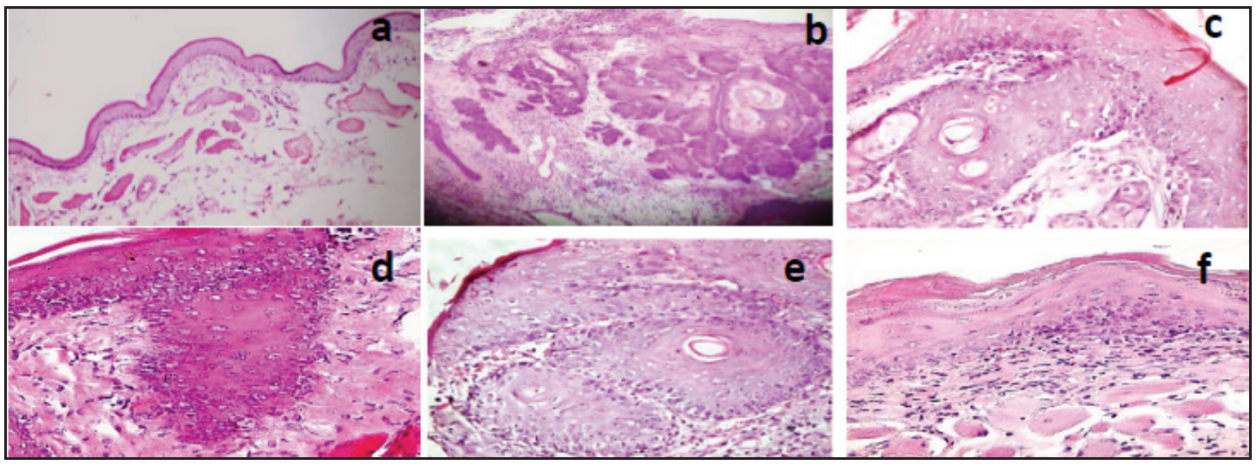

Fig. (3) (a-f): H&E sections showing; (a) group A shows normal hamster buccal pouch (x10), (b) group b shows well-differentiated squamous cell carcinoma with multiple invading islands into the underlying connective tissue (x20), (c) group C1, C2, D1, F1 and F2 show invading epithelial islands into the underlying connective tissue(x20), (d) group D2 shows variable dysplastic criteria in the surface epithelium (x40), (e) group E1 shows well-differentiated squamous cell carcinoma with invading epithelial islands and variable dysplastic features (x20); and (f) group E2 shows hyperplastic, hyperkeratinized stratified squamous epithelium with severe dysplasia and/or carcinoma in situ (x20).

3. Expression of repair enzymes:

The levels of mRNA expression for both XRCC1 and ERCC1 were measured in relation to the level of expression of Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) (control marker). Accordingly, the low expression had score from 0-1, normal expression 1-2 and high expression above 2.

3.1. mRNA ERCC1 expression:

TQ (0.001 mg/kg) loaded on GNps at both 6 and 12 weeks of treatment revealed the highest levels of expression among the experimental groups (B-F).

The mean ± standard deviation and range of mRNA ERCC1 expression in different study groups are shown in ***Table 1***.

Quantitative comparison of ERCC1 mRNA expression between the negative control group (A) and all experimental groups; showed a statistically significant difference, except for group E1.

Quantitative comparison of ERCC1 mRNA expression between the positive control group (B) (low expression) and all experimental groups: showing a statistically significant difference in all experimental groups.

Comparison of ERCC1 mRNA expression of the 6 weeks - treated groups, revealing a statistically significant difference between groups C1 and E1, groups D1 and E1 and between groups E1 and F1.

Comparison of ERCC1 mRNA expression of the 12 weeks - treated groups, revealing a statistically significant difference between groups C2 and E2, groups C2 and F2, groups D2 and E2, as well as between groups E2 and F2.

3.2. mRNA XRCC1 expression:

The highest expression of that enzyme was in the negative control group as well as the groups treated with 0.001 mg/kg TQ loaded on GNps for both

6 and 12 weeks. This finding was not statistically significant.

On the other hand, the lowest expression was recorded for the positive control group. The other experimental groups showed variable lower expression that was statistically significant as compared with the negative control group. However, a comparison between the positive control and other experimental groups (C, D, E, and F) showed statistically significant difference of lower expression, and not statistically significant with group (E) for its higher expression.

The mean ± standard deviation and range of XRCC1 mRNA expression in different groups are summarized in ***Table 2***.

Quantitative comparison of XRCC1 mRNA expression between the negative control group (A) and all experimental groups: showing statistically significant differences except for group E1 and E2.

Quantitative comparison of XRCC1 mRNA expression between the positive control group (B) (low expression) and all experimental groups: showing a highly statistically significant difference in all experimental groups, except group (D1) that was statisticaly significant.

Comparison of XRCC1 mRNA expression of the 6 weeks - treated groups revealed a statistically significant difference between groups C1 and E1, groups D1 and E1 and between groups E1 and F1.

Comparison of XRCC1 mRNA expression of the 12 weeks - treated groups, revealing a statistically significant difference between groups C2 and E2, groups C2 and F2, groups D2 and E2, as well as between groups E2 and F2.

Table 1: *Mean levels of ERCC1 mRNA expression in different groups*

Group	N	Mean ± standard deviation	Range
(A) Negative control	10	6.1± 0.1	5.50 - 7.00
(B) DMBA positive control	10	0.22± 0.13	0.10 - 0.40
(C1)TQ (6 weeks)	10	0.64 ± 0.2	0.40 - 0.90
(C2)TQ (12 weeks)	10	0.7 ± 0.15	0.5 - 0.9
(D1) GNps + TQ 0.01 (6weeks)	10	0.7 ± 0.14	0.5 - 0.9
(D2) GNps + TQ 0.01 (12 weeks)	10	1.02 ± 0 .25	0.8 - 1.3
(E1) GNps + TQ 0.001 (6 weeks)	10	6.74 ± 5.01	1.2 - 11.1
(E2) GNps + TQ 0.001 (12 weeks	10	8.3 ± 1.6	6.5 - 10.5
(F1) GNps (6 weeks)	10	0.9 ± 0.22	0.6 - 1.2
(F2) GNps (12 weeks)	10	1.36 ± 0 .16	1.1 - 1.5

N = Number of animals in each group
TQ (Thymoquinone), GNps (Gold nanoparticles).

Table 2: *Mean levels of XRCC1 mRNA expression in different groups*

Group	N	Mean ± standard deviation	Range
(A) Negative control	10	5.8 ± 0.32	5.5 - 6.30
(B) DMBA Positive control	10	0.22 ± 0.13	0.10 - 0.40
(C1)TQ (6 weeks)	10	0.7 ± 0.15	0.5 - 0.90
(C2)TQ 12 weeks	10	0.76 ± 0 .11	0.6 - 0.90
(D1) GNps+ TQ 0.01 (6 weeks)	10	0.66 ± 0.2	0.4 - 0.90
(D2) GNps + TQ 0.01 (12 weeks)	10	1.1 ± 0. 3	0.85 - 1.50
(E1) GNps + TQ 0.001 (6 weeks)	10	6.48 ± 4.9	1 - 11
(E2) GNps + TQ 0.001 (12 weeks)	10	7.7 ± 2.2	4.5 - 10
(F1) GNps (6 weeks)	10	0.9 ± 0.1	0.8 - 1
(F2) GNps (12 weeks)	10	1.3 ± 0.2	1 - 1.5

N = Number of animals in each group
TQ (Thymoquinone), GNps (Gold nanoparticles).

DISCUSSION

The present study was concerned about the chemotherapeutic effect of thymoquinone (TQ) alone, thymoquinone loaded on gold nanoparticles (TQ-GNps) in different concentrations and gold nanoparticles (GNps) alone, on induced oral cancer, following the classic protocol of hamster buccal pouch / dimethylbenz-[a]-anthracene (HBP/DMBA) model.[15]

In human head and neck cancer (HHNC), the most prevalent mutations are repaired by XRCC1[16] and ERCC1.[17, 18] As the HBP / DMBA –induced cancer model mimics HHNC biologically, these enzymes were the target of this study. Left buccal pouches were DMBA- painted for twelve weeks, then experimental animals were treated with the chemotherapeutics for 6- and 12-weeks intervals.

GNps were used as drug carrier due to their ability to cross cellular membrane,[19] ease of fabrication, high surface and high binding capacity to target cancer cells.[20]

In this work, the technique used to prepare GNps produced spherical, well-dispersed without agglomeration and of 25-30 nm particle size (average size 27nm). These criteria were comparable with Afifi et al[21] who used GNps for PTT in an average size 30 nm to treat DMBA-induced HBPs carcinoma through intra-lesional injection, without any sign of toxicity. Moreover, Rieznichenko et al[22] demonstrated that the intravenous injection of GNps to rats at size between 30-45 nm didn't cause DNA damage in normal organs of the experimental animals.

Ultraviolet-visible spectra (UV-vis) were used to ensure characterization of the prepared nanoparticles. GNps showed sharp peak at 526 nm that confirmed the proper formation. This result corresponds to El-Sayed et al.[23] who reported a maximum peak at 530 nm, Afifi et al[21] with maximum peak at 529nm, and Mahmoud and Bayoumi[24] with maximum peak at 522 nm.

Deviation of the maximum peak to a longer wavelength at 532 nm verified the loading of TQ on GNps. The same maximum peak was confirmed by Hassan et al[25], who showed the same result of TQ loaded on GNps. Daduang et al[26] reported comparable deviation in the maximum peak from 525 nm to 560 nm after loading of gallic acid on GNps.

However, apart from external factors that induce cellular mutations, it was estimated that approximately 10^{13} cells, in the human body, are subjected to DNA damage, daily.[27] The effectiveness of DNA repair mechanisms prevents/aborts spontaneous tumor development.[28]

Group A (negative control) revealed elevated levels of mRNA expression for both XRCC1 and ERCC1 repair enzymes, indicating the effectiveness of these enzymes in preserving normal epithelial cells from daily stresses that may affect the DNA. Similar results were obtained by Sakano et al[29] study, on bladder cancer patients.

Group B treated with DMBA for 12 weeks, showed low expression of both enzymes reported by the RT-PCR levels. In this group, the painted pouches showed clinically and histologically well-differentiated SCC, this result is consistent with Vaezi et al[30] where both enzymes were expressed at lower levels, in human lung cancer and HHNC cells. As the function of these enzymes is to repair damaged DNA, their lower expression indicates either their mutation by the carcinogen, or the tumor cells' mutation does not involve the type of mutation for these enzymes to work upon, or the potent effect of the carcinogen (for 12 weeks of DMBA application) overcomes the amount of cellular repair enzymes. Furthermore, the oxidative stress,

associated with the carcinogenesis process,[31] was not relieved by the natural intracellular anti-oxidant mechanisms, i.e. DNA is continuously subjected to mutation, that its repair is aborted.

Group C when TQ only (0.1mg/kg) was given, after DMBA cessation, revealed low expression of both repair enzymes, i.e. comparable results to the DMBA-only group. Same SCC findings were also repoted. This finding indicates that TQ-only was ineffective in inducing DNA repair, most probably due to its poor bioavailability. However, the anticancer effect of TQ was reported in many studies,[32-36] taking into consideration that most of these studies had applied TQ i.p, whereas, the present work used TQ both topically and i.p. Retention of topical TQ in the pouches (as an oily material) would impede internalization of TQ given systemically (i.p). The present work address that animals treated with TQ only, for 6 week, showed similar results as DMBA-only or GNps –only treated animals both histologically and in RT-PCR results. This finding may be attributed to the nature of TQ, as a hydrophobic molecule, i.e. affecting its bioavailability and activity through to limitations in drug internalization to epithelial cells of buccal pouches.[37] Another explanation is due to its irritating nature [as declared by the manufacturer's label (Sigma Aldrich)], that accentuated the inflammatory reaction associated with carcinogenesis process. However, after 12 weeks, TQ group showed carcinoma in situ only.

Loading of TQ on GNps (TQ-GNps) at both concentrations (0.01 mg/kg and 0.001 mg/kg) for 6- and 12- weeks intervals (groups D and E), revealed improvement in the levels of expression of DNA repair enzymes, with the best result in group E2 (0.001 mg/kg TQ-GNps for 12 weeks). Regression of tumors in these groups up to superficial invasion that could be related to lesser TQ concentration allowing more molecules to be loaded on each GNp i.e. more TQ could be internalized into malignant cells and thus induce its full activities. These results are in accordance with results of Hassan et al,[25] that treated hamsters with a similar concentration for prolonged period. Confirming this significant expression is the clinical and histopathologic findings, that showed only severe dysplasia in 0.001mg/kg TQ-GNps for 12 weeks.

While the histopathologic sections, in 0.001 mg/kg TQ-GNps for 6 weks, showing superficial invading islands rather than deeply invading islands in the DMBA- treated group. Similar results were obtained from this combination in previous studies carried out in the same model.[35, 38] These studies reported inactivation of the Nf-κB protein, in the groups treated with TQ, or TQ- GNps (in different concentrations), whether used topically, [35] or given i.p. after DMBA.[38] The best results from both studies were obtained with 0.001 mg/kg TQ loaded on GNps.

However, GNps by itself (group F), revealed similar low levels of DNA repair enzymes expression as group C, after 6 weeks. Meanwhile, after 12 weeks, a moderate expression for both enzymes was noted, with regression of the tumors' size and marked dysplasia. A finding that indicates that more GNps had penetrated tumor cells and either induced apoptosis,[39] or stimulated the repair mechanism.

CONCLUSION

It is apparent that application of TQ, even at low concentrations, loaded on GNps was promising chemotherapeutic combination, through enhancing expression of the studied DNA repair enzymes, parallel with regression of SCC to severe dysplasia, when compared to results obtained with the application of TQ only or GNps only.

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