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XRCC1 IMMUNOHISTOCHEMICAL EXPRESSION IN DMBA – INDUCED ORAL SQUAMOUS CELL CARCINOMA TREATED WITH DIFFERENT THYMOQUINONE PREPARATIONS

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ABSTRACT

Introduction: The X-ray repair cross-complementing 1 (XRCC1) enzyme plays an important role in the DNA repair pathway. XRCC1 polymorphism increased the risk of human oral squamous cell carcinoma. Loading of thymoquinone on gold nanoparticles as a drug carrier, had revealed a superior anti-cancer effect as a chemotherapeutic agent in DMBA-induced OSCC. **Aim of the work:** This study aimed to evaluate the expression of DNA repair enzyme X-ray repair cross-complementing 1 (XRCC1) following treatment of induced oral cancer in hamster buccal pouch with thymoquinone (TQ) only and loaded on gold nanoparticles (AuNps). **Materials and methods:** Sixty male Syrian golden hamsters were divided into 4 groups: Group A: (negative control). The left pouches of the rest of animals were painted with the carcinogen DMBA (3times / week/ 12 weeks), then: Group B: (positive control), Group C: painted and injected intraperitoneally (i.p) with TQ only (3 times/week for 6 and 12 weeks). Group D: painted with TQ loaded on AuNps (3 times/week for 6 and 12 weeks). After euthanization, all pouches were surgically excised, fixed and processed for H&E and XRCC1 immunohistochemical stains. **Results:** Groups B, C1, C2, and D1 showed well-differentiated squamous cell carcinoma with low intensity of immune staining**.** Groups D2 showed remarkable regression of tumors both clinically and histologically with high intensity of IHC staining. **Conclusion:** Loading of TQ at low concentration (0.001 mg/kg) on AuNps /12 weeks was a promising chemotherapeutic combination, through enhancing XRCC1 expression to regress the carcinogenesis process. This effect could be due to the anti-oxidant, free-radicle scavenging effect and enhancing apoptosis by TQ.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide, with approximately 700,00 new cases per year and an overall 5- year survival rate between 40-50%(1). The development of OSCC is multifactorial and a multistep process that involves several genetic and epigenetic mutations(2). Although several animal models for the development of oral cancer have been generated, the best-characterized model was the hamster cheek pouch system that was the classically introduced model for oral carcinogenesis through topical application of 7,12-dimethylbenz-[a]-anthracene (DMBA) for 12-14 weeks(3).

It is estimated that approximately 10^{13} cells within the human body incur tens of thousands of DNA-damaging events per day. Mutations that may result from the daily DNA damage would cause the development of cancer. Thus, cells have evolved a network of DNA repair mechanisms to remove different types of DNA damage(4) .

The X-ray repair cross-complementing 1 (XRCC1) enzyme plays an important role in the base excision repair (BER) pathway(5), and has an effect on the base excision repair of genomic damage caused by exposure to carcinogens such as tobacco and alcohol(6). Curioni *et al*(7), reported that XRCC1 polymorphism increased the risk of human OSSC, with reduced capacity for removal of DNA damage. This indicates an association between the XRCC1 polymorphism and initiation of head and neck carcinogenesis.

Thymoquinone (TQ) is the most abundant constituent and biologically active compound of Nigella sativa oil(8). TQ is a potent antioxidant, anti-cancer, anti-inflammatory, and immunomodulatory agents(9). Gold nanoparticles (AuNPs) have unique physical and chemical properties; and are relatively safe, stable and easy to prepare. They have many unique characteristics, such as the small size, surface effects, quantum size effects, electrical, and optical effects(10). Loading of thymoquinone on gold nanoparticles as a drug carrier, had revealed a superior anti-cancer effect as a chemotherapeutic agent in DMBA-induced OSCC, through interaction of NF-k B(11).

The current study aims to detect the immuno-histochemical expression of XRCC1 following application of different preparations of thymoquinone as a chemotherapeutic agent in the DMBA-induced OSCC model.

MATERIALS 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.
* XRCC1 antibody (mouse monoclonal) [cat# GTX23133] was purchased from Genetex, Incorporation, California, USA.

2.Animals:

Sixty male Syrian golden hamsters were held at the animal house of Faculty of Dentistry, Suez Canal University and were divided as follow:

Group A: Ten animals; negative control group (untreated group), they were euthanized at day zero.

All remaining animals were painted with DMBA on the left pouches 3 times/week/ twelve weeks.

Group B: Ten animals; a positive control group were euthanized by the end of the 12th week.

Group C1: Ten animals; painted and injected intraperitoneally (i.p) with TQ (0.1 mg/kg) only (3 times/week / 6 weeks).

Group C2: Ten animals; painted and injected intraperitoneally (i.p) with TQ (0.1 mg/kg) only (3 times/week / 12 weeks).

Group D1: Ten animals; painted and injected i.p with 0.001 mg/kg TQ loaded on GNps (3 times/ week / 6 weeks).

Group D2: Ten animals; painted and injected i.p with 0.001 mg/kg TQ loaded on GNps (3 times/ week / 12 weeks).

3. Treatment protocol:

3.a. DMBA preparation and application:

The chemical carcinogen 7,12 dimethylbenz-[a]-anthracene (DMBA) was dissolved in heavy mineral oil to get a 0.5% solution. The carcinogen was topically applied to hamster left buccal pouches (HBP) of hamsters using number (4) camel brush. The painting was carried out 3 times/week/ 12 weeks.

3.b. Animal euthanization:

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.[12]

4. Immunohistochemical analysis:

From each paraffin block, 5 μm sections were cut and stained for H&E stain. Another 5 μm sections were mounted on positively-charged slides for IHC staining. The antibody used was Anti-XRCC1. Five slides from each group were stained. Three fields from each slide were subjected to counting by two pathologists from the Oral Pathology Department, Faculty of Dentistry, Suez Canal University.

The intensity of nuclear stain for both XRCC1 was performed using the image j Fiji program following the protocol of Varghese *et al*. by adding the number of positively stained nuclei to the surface area of the examined slide.[13] The sum of three fields added to those of the five slides (mean reading) in each group was compared with the readings in each group, then subjected to statistical analysis.

5. Statistics:

Statistical analysis of the nuclear stain intensity of XRCC1 results was performed by Statistical Package for the Social Sciences (SPSS) 21 program for windows.

RESULTS

1. Gross observation of animals:

Group A animals didn't show any gross changes, with healthy and active behavior; and both buccal pouches' length were about 4 cm..**Group B** animals revealed a bad smell and whitish debris coming out with the brush from the third painting and disappeared one week later. Starting from the third week of the experiment, marked perioral hair loss in all hamsters was noted up to the abdomen in some animals. From the fourth week, the pouch depth began to decrease up to 2 cm and remained fixed until end of the experiment. Skin ulcers or abscesses were marked mainly in areas of hair loss which continued to increase till end of the experiment. The animals were debilitated and skinny.

Groups C1 and **C2,** animals showed no improvement in the animals' general health. The length of painted pouches was approximately 2 cm in all animals and multiple different sizes of exophytic masses appeared.

Groups D1 and **D2,** animals showed marked improvement in the general health. There was a significant increase in the pouches' length to about 3.5 cm, especially after 12 weeks of treatment. There was a marked decrease in the size and number of exophytic masses as compared to animals treated with DMBA or TQ only.

2. **Histopathological (H&E) & immunohistochemical (IHC) results:**

Group A sections did not show any change from normal HBP architecture. It revealed normal lining, composed of four distinct layers: flat stratified squamous epithelium of two to four cell layers without rete ridges and a thin keratin layer, lamina propria of dense fibrous connective tissue, submucosa contains a layer of striated muscle fibers; and a deeper layer of loose areolar connective tissue. Immuno-histochemically, **group A** revealed high-intensity expression for XRCC1 immune-staining along with the whole epithelial thickness (Fig. 1a).

Group B sections 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 is 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. Immuno-histochemically, **group B** revealed the low intensity of expression of XRCC1 scattered through the malignant lesion (Fig. 1b).

Group C1 sections revealed well-differentiated squamous cell carcinomatous lesions with invading islands to the underlying connective tissue. Different dysplastic criteria were also seen along the rest of the epithelial lining, with hyperplastic / keratinized epithelium. The dysplastic criteria noted were basilar hyperplasia, loss of basal cells polarity, cellular & nuclear pleomorphism, altered nuclear/cytoplasmic ratio, nuclear hyperchromatism, swirling of spinous layer, individual and group cells keratinization. Immuno-histochemically, **group C1** revealed the low intensity of expression of XRCC1 scattered through the epithelium (Fig. 1c).

Group C2 revealed hyperplastic and hyperkeratinized stratified squamous surface epithelium with well-differentiated squamous cell carcinoma. Marked dysplastic criteria as basilar hyperplasia, loss of polarity of basal cells, cellular & nuclear pleomorphism, altered nuclear/cytoplasmic ratio, nuclear hyperchromatism and prominent nucleoli. Immuno-histochemically, revealed the low intensity of expression of XRCC1 along with the superficial epithelial layers (Fig. 1d).

Group D1 showed well-differentiated squamous cell carcinoma. Invading epithelial islands into the underlying connective tissue, with marked dysplastic criteria in the rest of surface epithelium. These criteria were in the form of basilar hyperplasia, loss of basal cells polarity, cellular & nuclear pleomorphism, altered nuclear/cytoplasmic ratio, nuclear hyperchromatism and prominent nucleoli. Immuno-histochemically, revealed moderate intensity of expression of XRCC1 (Fig. 1e).

Group D2 showed no apparent endophytic or exophytic lesions, but rather hyperplastic, hyperkeratinized stratified squamous epithelium with severe dysplastic criteria up to carcinoma in situ. Basilar hyperplasia, loss of basal cell polarity, altered nuclear/cytoplasmic ratio, prominent nucleoli, and nuclear hyperchromatism are the main dysplastic features. Immuno-histochemically, revealed high intensity of expression of XRCC1along the whole epithelium (Fig. 1f).

3. **Statistical analysis of immunohistochemical staining:**

The intensity of nuclear staining of XRCC1 was performed using the Image j Fiji program, and the results were statistically analyzed using Statistical Package for the Social Sciences (SPSS) 21 program for windows.

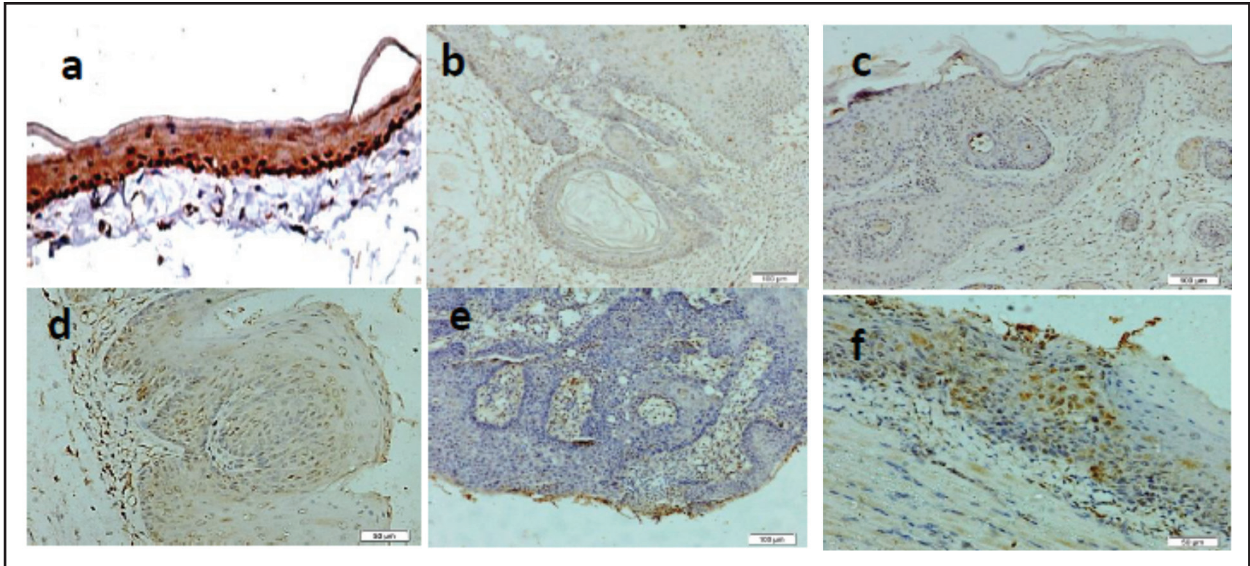

Fig. (1) (a-f): Photomicrographs of XRCC1 expression showing; (a) group A shows intense expression along the whole epithelial thickness (x20), (b) group B shows scattered low expression along the malignant lesion (x10), (c) group C1 shows scattered low expression through the epithelium (x20), (d) group C2 shows low expression along the superficial epithelial layers (x40), (e) group D1 shows moderate intensity of expression at basal and suprabasal epithelial layers (x20); and (f) group D2 shows high intensity of expression along the whole epithelial thickness (x40)

The mean levels and ranges of the nuclear staining intensity among the study groups revealed decreased intensity (from the normal level of group A), in groups B and C1, while were improved in groups C2 and D1. MeD2, normal level of nuclear staining intensity was comparable to that seen in group A (Table 1).

A comparison of the mean nuclear stain intensity levels between group A and other experimental groups revealed a high significant difference in all groups except group D2 that showed approximated levels with group A. However, a high significant difference was obtained between group B and the different study groups.

Moreover, high significant difference was observed after 6 weeks of treatment between group C1 and D1; as well as after 12 weeks of treatment between group C2 and D2 (Table 2).

Table 1: *The mean levels and ranges of XRCC1 enzyme nuclear stain intensity among different groups*

Group	N	Mean ± standard deviation	Range
(A) Negative control	10	28.49 ± 1.36	26.22 – 30.6
(B) DMBA positive control	10	13.69 ± 1.17	12.58 – 16.77
(C1) DMBA + TQ (6 weeks)	10	15.57 ± 0.39	14.98 – 16.1
(C2) DMBA + TQ (12 weeks)	10	18.73 ± 0.53	17.52 – 19.55
(D1) DMBA + AuNps- TQ 0.001 (6 weeks)	10	25.74 ± 0.52	24.88 – 26.47
(D2) DMBA + AuNps- TQ 0.001 (12 weeks)	10	29.54 ± 0.96	27.48 – 30.81

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

Table 2: Comparison of nuclear stain intensity of XRCC1 enzyme after 6 weeks and 12 weeks of treatment

(I) Subgroup	(J) Subgroup	Mean difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C1 (mean ± standard deviation 5.8 ± 0.32)	D1	-10.17220	.37875	.000**	-10.9247	-9.4197
C2 (mean ± standard deviation 5.8 ± 0.32)	D2	-10.81200	.37875	.000**	-11.5645	-10.0595

*** p value is high significant at ≤ 0.01*

DISCUSSION

The present study aimed to detect the expression of XRCC1 repair enzyme in dimethylbenz-[a]-anthracene (DMBA) – induced squamous cell carcinoma (SCC) in hamster buccal pouch (HBP); after the application of thymoquinone (TQ) alone and TQ (0.001 mg/kg) loaded on gold nanoparticles (AuNPs – TQ) as chemotherapeutic agents.

Results of the present work revealed normal-appearing pouch mucosa of the negative control group (group A), as well as the right pouches of all experimental groups. Immunohistochemical (IHC) expression of the XRCC1 enzyme, revealed intense diffuse cytoplasmic and nuclear staining that was expressed in all layers. This indicates the effectiveness of this enzyme in repairing the continuous local insults to normal epithelial cells from daily stresses that may affect the DNA. Caldecott[14] revealed increased XRCC1 expression in normal human cells, Chinese hamsters with decreased expression in cancerous cells.

Although epithelial thickness is very thin compared to all groups, the area percentage (according to image j results) revealed highly statistically significant results. This could be due to the diffuse nuclear/cytoplasmic reaction of the XRCC1 enzyme. While in the other groups the scoring was evaluated according to the nuclear reaction only, that was scattered along with the epithelial thickness. The reaction in group A confirms the success of the staining technique.

Induction of cancer in the HBP/DMBA model requires multiple local paintings of 0.5 % DMBA dissolved in mineral oil. Different studies reported that clinical tumors were apparent from 8-14 weeks (from the first application of the carcinogen)[17], depending mainly on the used solvent (mineral oil or acetone)[18].

In the present work, the application of 0.5% DMBA (dissolved in mineral oil) for 12 weeks, resulted in multiple, different size exophytic tumors as well as endophytic well-differentiated squamous cell carcinomas (histologically). Corresponding results were reported by Shklar[16] and Vinoth and Kowsalya[19]. The present clinical findings revealed general debilitation of the DMBA - treated animals (group B), significant reduction of the pouches' length, large exophytic masses on the painted pouches, hair loss, and skin lesions, corresponding with results of studies using the same model[18, 20-22]. Furthermore, necrosis and shortening of the painted pouches was a constant finding following the early DMBA paintings, as reported by other studies[20,22].

These observations are mainly due to the strong toxic DMBA effect.

Moreover, a marked decrease in the expression of the XRCC1 repair enzyme was noted in DMBA – treated animals than in control group. A finding that was in line with Kavitha et al.[(23)] who reported downregulation of XRCC1 following the twelve weeks of DMBA application to the right HBP.

The chemotherapeutic role of TQ[(24)] is halted in the present study due to the topical application of TQ, which is a hydrophobic agent. The painting of this material could mask complete internalization of TQ given intraperitoneally, at least for the first 6 weeks. Furthermore, animals treated with TQ only showed similar results as DMBA-only treated animals. This finding may be attributed to the nature of TQ, as a hydrophobic molecule. That would affect its bioavailability and activity as it leads to limitations in drug internalization to epithelial cells[(25)]. Another explanation is due to its irritating nature [as declared by the manufacturer's label (Sigma Aldrich)].

In group C when TQ only (0.1mg/kg) was given after cancer induction, the histologic finding was well-differentiated SCC (both exophytic and endophytic) at both 6 and 12 weeks. IHC showed low intensity of staining for the XRCC1 enzyme, i.e. comparable results to the DMBA-only group. 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[(20-22, 24, 26)], taking into consideration that most of these studies had applied TQ i.p, whereas, the present work used TQ both topically and i.p. It appears that retention of the topical hydrophobic TQ, in the pouch, did not allow internalization of systemic TQ to perform its full function. However, when loaded on AuNps (groups D), TQ was internalized to perform its anti-oxidant, and apoptotic-induction effects, as revealed by the remarkable reduction of the tumors' number and size, as well as a better expression of the DNA repair enzyme. This finding emphasizes that AuNps, at the prepared size and shape was helpful as a drug carrier to a poor bioavailable agent (TQ) to be internalized into target cells.

In group C, although the IHC scoring showed low intensity as in group B, numerically the readings were higher in group C than in group B. It was even more in group C2 (12 weeks) than in group C1 (6 weeks). Histologically, there was regression of SCC to carcinoma in situ (after 12 weeks of TQ treatment), which matches the higher activity of XRCC1 at that time point. A research reported that with the treatment of SCC, the repair enzymes are more active than in untreated tumors[(27)].

Loading TQ 0.001 mg/kg on AuNps (group D1 6-weeks treated animals) was much effective in the regression of clinically visible lesions, with the histopathologic sections showing superficial invading islands rather than deeply invading islands in the DMBA- treated group. The reason for best results obtained at this concentration could be due to its lower concentration allowing more molecules to be loaded on each AuNp i.e. more TQ could be internalized into malignant cells and thus induce its full activities. Similar results were obtained from this combination in previous studies carried out in the same model.[15, 21] These studies reported inactivation of the Nf-ϰB protein, in the groups treated with TQ, or AuNps-TQ (in different concentrations), whether used topically, [21] or given i.p. after DMBA.[15] The best results from both studies were obtained with 0.001 mg/kg TQ loaded on AuNps. Group D2 (0.001 mg/kg TQ loaded on GNps for 12 weeks) in the present study, showed IHC intense stain (score 3) along with the epithelial layers. However, 12 weeks of treatment showed more enzymes' expression compared to 6 weeks of treatment. This matches with regression of the SCC lesions to severe dysplasia in group D2,

and to superficial invasion in group D1. Moreover, the thinner epithelial thickness in group D2 resulted in a higher score, compared to scattered positive cells along with the bulky epithelial thickness in case of carcinoma and invading islands (group D1).

Collectively, it can be proposed that TQ loaded on AuNps had resulted in regression of the carcinogenesis process, possibly through two main mechanisms. TQ as a potent antioxidant can antagonize elevated ROS levels in malignant cells[28,29]. So in that model, the antioxidative activity was mediated when TQ was internalized by AuNps. Therefore, allowing the unrestricted ability of TQ to pass from physiologic barriers and easily get access to subcellular compartments, to perform its radical scavenging effects. The reduction of the intracellular reactive oxygen species (ROS) would allow the repair enzymes to fulfill their main target[30]. Second: TQ through inactivation of Nf-ϰB, which in turn induced *p*53 activation, i.e. stimulated apoptosis of malignant cells. This effect would result in reducing the tumor size/number, therefore facilitating DNA repair mechanisms.

Finally, the statistically insignificant results between the treated groups could be attributed to the timing of euthanization (at 6- and 12- weeks intervals). The early therapeutic effect of TQ on that model, was evident within one day to two weeks after one or two intraperitoneal (i.p.) TQ injections. This study aimed to determine the sequence of Myo D expression with i.p. injection of TQ (following 6 weeks of DMBA painting) a highly significant increase of serum TNF-α was reported from the second day of the first injection, as compared to untreated and DMBA only-treated groups. Following the second injection, a statistically significant increase of the TNF-α was achieved, which was decreasing up to near the DMBA only group after 2 weeks. Myo D expression was reported after 4 days of either one or two injections in non-myogenic cells,[31] where the short pouches regained their normal length in 2 weeks only.

CONCLUSION

Loading of TQ at low concentration (0.001 mg/kg) on AuNps /12 weeks was a promising chemotherapeutic combination, through enhancing XRCC1 expression to regress the carcinogenesis process. This effect could be due to the anti-oxidant, free-radicle scavenging effect and enhancing apoptosis by TQ.

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