LOCALIZATION OF CYCLOOXYGENASE-2 IN EXPERIMENTALLY-INDUCED CARCINOGENESIS FOLLOWING TREATMENT WITH THYMOQUINONE LOADED ON NANO-GOLD PARTICLES. (A RETROSPECTIVE STUDY)

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ABSTRACT

Introduction: Thymoquinone is one of the phytochemicals used as chemopreventive agents for oral cancer control. TQ loaded on nanoparticles had shown higher anti-proliferative and anti-inflammatory effects than free TQ. Natural products that can inhibit Cyclooxygenase-2 (COX-2) would be a better choice in the prevention of tumor development with fewer side effects than pharmaceutical agents.

Objective: To study the expression of Cox-2 in HBP/DMBA carcinogenesis model following combined topical application of TQ loaded on gold nanoparticles on alternative days.

Material and Methods: This research was carried out on archival paraffin blocks of a previous thesis. The paraffin blocks represented hamster buccal pouches (HBPs) from 85 male Syrian golden hamsters were divided into 2 groups. The control groups included: I: negative control group, scarified at day zero then after 7 and 14 weeks, and II: positive control, treated with 7, 12-Dimethylbenz [a]-anthracene (DMBA) for 7 and 14 weeks. The experimental groups included: (III, IV and V) which were treated with the chemopreventive agents for 2 weeks then combined DMBA and the chemopreventive agents on alternative days for 7 and 14 weeks. Sections of 5µm were cut and processed for H&E and IHC stains for light microscopic study.

Results: Negative and self-control groups showed negative COX-2 immunoreactivity. Group II showed intense Cox-2 immunoreactivity at both 7 and 14 weeks. Groups; III and IV showed moderate and intense Cox-2 immunoreactivity at 7 and 14 weeks, respectively. Group V showed mild and moderate Cox-2 staining reaction at 7 and 14 weeks, respectively.

Conclusion: Topical application of G-NPs-TQ (0.001) for 7 and 14 weeks was able to reduce Cox-2 expression and in turn retard the carcinogenesis process due to its anti-inflammatory effect.

Keywords: Thymoquinone, Gold-nanoparticles, Oral squamous cell carcinoma, Cyclooxygenase-2, and Dimethylbenz-(a) anthracene.

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INTRODUCTION

Oral cancer (OC) is the sixth common human cancer worldwide. Oral squamous cell carcinoma (OSCC) represents 90% of OC and may be preceded by epithelial dysplasia. Egypt has been found to have high incidence rate of OC and every year about 4.500 patients are diagnosed with OSCC and half of them will die of the disease (1). Extensive use of tobacco, betel quid and alcohol consumption are recognized as the major risk factors of OC (2).

One of the most important approaches for cancer control is chemoprevention. Phytochemicals have been shown to have important chemopreventive effects with a good safety profile (3). Thymoquinone (TQ), which is the major active constituent of *Nigella sativa* Linnaeus (NS.L), is one of

these phytochemicals. TQ exhibits effective antioxidant, anti-inflammatory, anti-cancer, anti-microbial, and immunomodulatory effects (4). TQ was found to inhibit 5-lipoxygenase products during inflammation and enhance free radical scavenging activity (antioxidative effect) (5). The main limitations for the use of TQ in humans are its poor bioavailability and the lack of knowledge about its toxicity in humans (6). TQ loaded on nanoparticles (NPs) has shown higher anti-proliferative, anti-inflammatory and chemosensitizing effects than free TQ. As G-NPs are smaller in size than proteins, they can selectively disturb and modify cellular processes, i.e. as intrinsic drug agents. G-NPs of about 1 nm diameter are able to cross the cell membrane and nucleus to interact with DNA (7). G-NPs were used as a cancer drug delivery, cell-specific targeting,

and controlled drug release. They also have the ability to bind to various molecules which make them potential candidates for chemical and biological applications with non-cytotoxic effect to normal cells (8).

Cyclooxygenase, a known prostaglandin-endoperoxide synthase (PTGS), is the key enzyme in prostaglandin biosynthesis and acts both as a dioxygenase and a peroxidase (9). Three isoforms of COX have been identified: COX-1, COX-2, and COX-3, where COX-2 is the most important regulator in response to inflammation and many types of cancers. COX-2 overexpression is known to be associated with inhibition of apoptosis, immune surveillance, promotion of angiogenesis, and increase of cancer invasiveness and metastasis (10). It was found that COX-2 expression in oral precancerous and cancerous lesions has increased from ED to SCCs with the elevation of cell proliferating activity. Patients with overexpression of COX-2 have poor prognosis and their overall 5-year survival rate was decreased (11). Selective Cox-2 inhibitors or antisense RNA have resulted in the suppression of tumor growth and invasion in vitro, as well as the prevention of OC caused by chemical carcinogens in animal models (12). The overexpression of NF- κ B and COX-2 was reported in several tumors including OC. COX-2 is one of the downstream targets of NF- κ B. There are two NF- κ B sites on the 5-flanking region of COX-2 gene (13). NS and TQ were found to be able to suppress the expression of COX-2 in the pancreatic tissue of streptozotocin (STZ)-induced diabetic rats (14). COX-2-inhibitors could be used at the premalignant stage for the prevention of OC through topical application or oral administration (15).

MATERIAL AND METHODS

The study was carried out on archival paraffin blocks of (a previous thesis by Shata et al.) which compared the effect of different TQ preparations as chemopreventive agents on the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) in the HBP/DMBA experimental model (16). The paraffin blocks represented buccal pouches from 85 male Syrian golden hamsters (*Mesocricetus auratus*)* which weighed 100-120 grams. (* The animals were purchased from Tiedor Blhars Research Institute, Cairo, Egypt.). The DMBA (cat. no: D3254) was dissolved in heavy mineral oil (cat.No: M 3516) to get 0.5% solution. Both were purchased from Sigma Chemical Company, St. Louis, Mo, USA.

The blocks were divided into 2 groups: A: Control groups and B: Experimental groups. The control groups included I and II. I: negative control group (15 animals), sacrificed at day zero then after 7 and 14 weeks (5 animals each). II: positive control (10 animals) painted topically with DMBA on left pouches 3 times / week for 7 and 14 weeks (5 animals each). The experimental groups included III, IV and V (10 animals each) which were treated with the chemopreventive agents. The group III was painted daily

and topically with TQ (0.001mg/kg) for 2 weeks then with both TQ (0.001mg/kg) and DMBA on alternative days for 7 and 14 weeks. The group IV was painted daily and topically with G-NPs for 2 weeks then with both G-NPs and DMBA as group III. The group V was painted daily and topically with TQ (0.001mg/kg) loaded on G-NPs for 2 weeks then with both TQ (0.001mg/kg) loaded on G-NPs and DMBA as group III.

All pouches were surgically excised, fixed in 10% neutral formalin, and embedded in soft paraffin wax. Sections of 5 μ m were cut and processed for H&E and IHC stains for light microscopic study. The slides were diagnosed by two pathologists and photographed by Olympus E-330 Evolt Digital Photography camera attached to an Image Analyzing System (Olympus BX50 Microscope). The oral epithelial dysplasia (OED) was graded, according to El-Dakhakhny et al. (2009) modified from Banoczy and Csiba (1976), into: Mild: when an average of less than 3 dysplastic criteria was found (for each group); Moderate: when an average of 3-7 criteria was found (for each group) Severe: when an average of more than 7 or more criteria was found (for each group); and Carcinoma in situ: when the criteria were distributed from top to bottom with intact basement membrane (17,18). The SCCs were classified, according to WHO (1977), into well, moderate, and poorly differentiated (19).

Immunohistochemical (IHC) stain for detection of Cox-2 expression was conducted following the manufacturer's instructions using Cox-2 antibody (rabbit polyclonal antibody) Cat #RB-9072-R7 (Thermo Fisher Scientific, Anatomical Pathology, UK). Staining was detected using Ultra vision detection system (cat. No. TP-015-HD). Three high-power fields were selected from each slide and at least 70 epithelial cells were evaluated and considered positive when the nuclei and/or the cytoplasm were stained. The semi-quantitative method was used to determine the intensity of immunohistochemical stain using the recent version of ImageJ software with IHC profiler plugin (1.48 version) (NIH, Bethesda, Maryland) (Java 1.8.9_66). The IHC-profiler generates IHC analysis using color deconvolution and computerized pixel profiling leading to automated scoring of the respective image. The final score is shown in semi-quantitative way (high positive, positive, low positive or negative). This semi-quantitative scoring method was classified into four categories: negative =0, low positive =1, positive =2 and high positive =3 according to the intensity of staining (20).

The mean percentage of stained cells was classified into 4 categories as follows: (0) No detectable immunostaining or basal immunostaining<10% cells, (+1) Mild immunostaining when 10–30% cells are positively stained, (+2) Moderate immunostaining when 30–50% cells are positively stained, and (+3) Intense immunostaining when>50% of the cells showed positive staining (21).

Statistical analysis: the Software Statistical Package for Social Sciences (SPSS) (version 12.0) was used. The mean percentage of stained cells was analyzed using Mann-Whitney test. The mean difference is significant at $p \leq 0.05$ level.

RESULTS

For the negative control group (group I), the right and left pouches showed normal appearing HBP mucosa and negative Cox-2 immunoreactivity (score 0) and negative intensity of IHC stain (score 0) (Figure 1).

Figure 1: Photomicrograph of the negative control group (group I) at 7 and 14 weeks, shows no detectable Cox-2 immunostaining (score 0) as the hamster buccal pouch mucosa was normal (IHC $\times 40$).

On the other hand, the positive control group (group II) showed severe dysplasia at 7 weeks, well to moderate SCC at 14 weeks, and intense Cox-2 immunoreactivity in all cases (score +3) and high positive intensity of stain (score 3) (Figures 2 and 3).

Figure 2: Photomicrograph of the positive control group (group II), shows squamous cell carcinoma where connective tissue was invaded with keratin pearls and dysplastic epithelial nests (H&E $\times 40$).

Figure 3: Photomicrograph of the positive control group (group II), shows intense Cox-2 immunostaining (score +3) in the tumor cells invading the connective tissue (IHC $\times 20$).

Animals treated with TQ 0.001/DMBA (group III) showed moderate dysplasia with moderate Cox-2 immunoreactivity at 7 weeks (score +2) with positive intensity of stain (score 2), while at 14 weeks, they showed CIS and superficial invasion with intense Cox-2 reaction (score +3) and positive intensity of stain (score 2). Hamsters treated with G-NPs and DMBA (group IV) showed moderate to severe dysplasia (at 7 weeks), and moderate Cox-2 stain (score +2) with positive intensity of stain (score 2), while at 14 weeks, they showed CIS and superficial invasion with intense COX-2 stain (score +3) and high positive intensity of stain (score 3) (Figure 4).

Figure 4: Photomicrograph of group III (TQ 0.001 /DMBA) and group IV (G-NPs/DMBA) at 7 weeks, shows moderate Cox-2 immunostaining limited to the lower 2/3 of epithelium (score +2) (IHC $\times 40$).

The best results were obtained from group V (G-NPs-TQ 0.001 and DMBA) which showed mild dysplasia with mild Cox-2 stain at 7 weeks (score +1) with low positive intensity of stain (score 1), while at 14 weeks, they showed mild to moderate dysplasia with moderate COX-2 staining reaction (score +2) and positive intensity of stain (score 2) (Figure 5).

Figure 5: Photomicrograph of group V (G-NPs-TQ 0.001 / DMBA 7 weeks), shows mild Cox-2 immunostaining of lower epithelial layers (score +1) and mild epithelial dysplasia (IHC $\times$ 40).

All H&E and IHC results were summarized in table 1.

Table 1: The histopathological and immunohistochemical results of all groups:

Group	Treatment	Histopathological results (H&E stain)	The mean percentage of IHC stained cells	Intensity of IHC stain
I	Untreated (-ve control)	Normal HBP mucosa.	Negative / low immun-reactivity (Score Zero)	Negative (Score Zero)
II.7	DMBA for 7 weeks (+ve control)	Severe epithelial dysplasia	Intense immun-reactivity (score +3)	High positive (Score 3)
II.14	DMBA for 14 weeks (+ve control)	Well to moderate SCC	Intense immun-reactivity (score +3)	High positive (Score 3)
III.7	TQ 0.001/DMBA for 7 weeks	Moderate epithelial dysplasia	Moderate immun-reactivity (Score +2)	Positive (Score 2)
III.14	TQ 0.001/DMBA for 14 weeks	Severe dysplasia, CIS and superficial epithelial invasion.	Intense immun-reactivity (Score +3)	Positive (Score 2)
IV.7	GNPs/DMBA for 7 weeks	Moderate / severe dysplasia.	Moderate immun-reactivity (score +2)	Positive (Score 2)
IV.14	GNPs/DMBA for 14 weeks	Severe epithelial dysplasia, CIS and superficial invasion.	Intense immun-reactivity (score +3)	High positive (Score 3)
V.7	G-NPs-TQ 0.001/DMBA for 7 weeks	Mild epithelial dysplasia.	Mild immun-reactivity (score +1)	Low positive (Score 1)
V.14	G-NPs-TQ 0.001/DMBA for 14 weeks	Mild / moderate epithelial dysplasia with focal areas of CIS	Moderate immun-reactivity (score +2)	Positive (Score 2)

Statistical results of Cox-2 IHC:

There were statistically significant differences between the negative and positive control groups at both 7 and 14 weeks. Also, there were statistically significant difference between negative control group and group III at both 7 and 14 weeks. Comparable finding was found between groups II and both III and IV at 7 weeks. While at 14 weeks, there

was no statistically significant difference between groups II and both group III and IV. There were statistically significant differences between groups II and V at both 7 and 14 weeks, as well as between group V and both III and IV at both 7 and 14 weeks (Tables 2, 3 and figure 6).

Table 2: The statistical analysis of Cox-2 IHC results of all groups at 7 weeks. (Mann-Whitney test results).

	M&SD	The mean difference (<i>p</i> values)				
		I	II	III	IV	V
I	5 $\pm$ 1.7		.050*	.050*	.050*	.050*
II	95.19 $\pm$ 2.8	.050*		.050*	.050*	.050*
III	37.22 $\pm$ 4.2	.050*	.050*		.827	.050*
IV	40.42 $\pm$ 8.1	.050*	.050*	.827		.050*
V	21.91 $\pm$ 10.14	.050*	.050*	.050*	.050*	

Table 3: The statistical analysis of Cox-2 positive cells IHC results of all groups at 14 weeks (Mann-Whitney test results).

	M&SD	The mean difference (<i>p</i> values)				
		I	II	III	IV	V
I	5 $\pm$ 1.7		.050*	.050*	.050*	.050*
II	90.95 $\pm$ 14.45	.050*		.184	.275	.050*
III	67.62 $\pm$ 22.19	.050*	.184		.376	.050*
IV	80.95 $\pm$ 10.82	.050*	.275	.376		.050*
V	38.29 $\pm$ 2.79	.050*	.050*	.050*	.050*

Figure 6: A graph showing comparison of Cox-2 positive cells' percentage between negative control group and all experimental groups at 7 and 14 weeks. Results presented as mean $\pm$ standard deviation (SD).

DISCUSSION

HBP carcinogenesis model has been used successfully for studying the induction and prevention of OC using different phytochemicals and classic chemotherapy (22-24).

Tumor prevention using 0.1 mg/kg TQ versus different concentrations of TQ loaded on G-NPs was demonstrated in the previous study performed by Shata et al (2016) (16). In their study, the best used concentration was (0.001mg/kg / body weight) of TQ loaded on G-NPs as it showed mild dysplasia at 7 weeks and CIS at 14 weeks associated with mild NF- κ B immunoreactivity. While severe dysplasia / CIS (at 7 weeks) and invasive carcinoma (at 14 weeks) associated with intense NF- κ B immunoreactivity when DMBA only was painted.

This study aimed to investigate COX-2 modulation in response to 0.001 mg/kg TQ loaded on G-NPs. G-NPs were been used as a drug carrier in that work due to their ability to cross cellular membranes. Topical application of G-NPs loaded by TQ (0.001 mg/kg) appeared to allow selective permission of TQ to penetrate inside the cancerous cells at higher levels compared to normal cells.

The results of group I (negative control) were consistent with results of another study that observed that the Cox-2 was barely detectable in normal epithelium (15). Cox-2 was over-expressed in group II (DMBA) where the epithelial lining of the pouch mucosa at 7 weeks showed severe dysplasia with focal areas of CIS. While after 14 weeks, it showed well to moderately differentiated SCCs in the form of papillomatous lesions and epithelial islands invading the connective tissue. These findings were documented by several authors (23,25,26). In this work, DMBA only has resulted in overexpression of Cox-2, which was in parallel with the increasing activity of NF- κ B reported by Shata et al. (2016) (16). It was documented that PAHs as DMBA, found in cigarette smoke, can increase NF- κ B activity causing overexpression of its downstream target COX-2 (27). Confirming this finding, COX-2 was up-regulated by exposure to tobacco and areca nut constituents (28). Other studies revealed that cigarette smoke condensate, as well as 4-methylnitrosamino-(1-3-pyridyl)-1-butanone treatment of oral keratinocytes can increase NF- κ B activity and expression of its downstream target COX-2 (27,29).

Overexpression of COX-2 during tumorigenesis was reported to cause excess production of PGs and induction of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), increase in cell proliferation and decrease in apoptosis. All these factors are mostly mediated by PG-E2 and its receptor EP1-4 that stimulate angiogenesis and tumor growth. PG-E2 also inhibits TNF- α and induces IL-10 (a cytokine with immunosuppressive effect). COX-2 up-regulation results in inactivation of tumor suppressor genes as p53, and activation of the proto-oncogenes. These activities will lead to the progression of the carcinogenic process (13).

The present study revealed a higher Cox-2 expression in epithelial dysplasia (ED) than its expression in SCC with no statistically significant difference. Shibata et al. (2005) examined COX-1 and -2 expressions by Western blot and IHC analysis in 65 dysplasias and 50 SCCs. They reported that COX-1 and -2 were higher in dysplasias than in SCCs (30). The reason for this finding was explained by Minter et al. and Sudbo et al. (2003) (15,31). They concluded that COX-2 overexpression was an early event in oral carcinogenesis, where COX-2 expression is correlated to DNA content (DNA aneuploidy) as a genetic risk marker for OC. On the other hand, Renkonen et al. (2002) results revealed that there is no significant difference of COX-2 expression in different grades of epithelial dysplasia (32). This contradicts with Shibata et al.'s study (2005) which reported that COX-2 expression correlated with the histological grade of dysplasias was at the highest level in severe dysplasia (30) as previously reported by Shamma et al. (2000) who considered COX-2 as a sensitive marker for high grade dysplasia of the esophagus (33).

In the present work, COX-2 expression was correlated with the advancement of dysplastic changes as in group III (TQ 0.001/DMBA). This finding indicated that TQ alone had moderate protective effect and this is mostly due to its known poor bioavailability when used topically. Although 14 weeks of combined 0.001mg/kg TQ and DMBA treatment showed lower Cox-2 level of expression than in group II, it was not statistically significant. The H&E finding is consistent with El-Dakhkhny et al. (2009) results who reported that TQ and polythymoquinone (PTQ) can modify the carcinogenic effect of DMBA through minimizing the advancement of dysplastic changes and decreasing the incidence of OSCC (17). In their work, they applied TQ and PTQ intraperitoneally rather than the topical painting of TQ which is used in the present study. It appears clearly that TQ is a promising phytochemical possessing a critical effect on COX-2 inhibition and in turn prostaglandin (PGs) production (6).

It appears that TQ could possess comparable effect, as that of selective COX-2 inhibitors, on tumor growth, metastasis, and the enhancement of the anticancer effects of radiotherapy and chemotherapy (in experimental animals and human models) (13).

In group IV (G-NPs/DMBA), Cox-2 IHC results were positively correlated with the histopathologic changes. These observations were comparable to groups given TQ only with DMBA, i.e. G-NPs had comparable effect as TQ against the carcinogenesis process. Although the intensity of IHC stain results of group III (TQ 0.001/DMBA) was better than group IV (G-NPs/DMBA) at 14 weeks, i.e. group III showed positive IHC stain intensity (score 2) while group IV showed high positive stain intensity (score 3) at 14 weeks, the percentage of stained cells was nearly the same, i.e. both showed intense immun-reactivity (score +3). These results indicated that continuous DMBA painting is a stressful stimulus that was hard to be aborted by TQ or G-NPs in the specified dose and route of administration, i.e. the continuous toxic and mutagenic effect of DMBA overcomes the proposed effect of either TQ alone or G-NPs alone.

The best result was observed in group V (G-NPs-TQ 0.001 with DMBA). These results indicated the TQ protective effect facilitated by its loading on G-NPs for intracellular internalization to exert its anti-inflammatory, apoptotic, and free radical scavenging effects. These results could be due to little TQ molecules loaded with GNPs resulted in proper distribution of GNP-TQ molecules on the cell membrane without crowding, and might resulted in proper GNP-TQ cellular uptake in this chemopreventive group. Thereby, G-NPs, in the present work, appear to be a good drug carrier for delivering TQ inside malignant cells.

The anti-inflammatory effect of TQ through Cox-2 inhibition was comparable to the results noted with NSAIDs (as ibuprofen, indomethacin, and aspirin), zileuton, celecoxib, curcumin, and ferulic acid (27,34-36). This protective effect could be through its suppression of chronic inflammatory response resulting from the continuous DMBA painting. Collectively, their anti-inflammatory effect could result in the suppression of both proliferation and invasiveness of tumor cells, as well as the induction of apoptosis (15). The COX-2 activation contributes to carcinogenesis via the direct effects on tumor cells by promoting mitotic activity and the conversion of pro-carcinogens to carcinogens, and indirectly on non-tumoral cells by increasing blood vessels, as well as modulating immune cells. So, its inhibition can prevent cancer (37).

In summary, the current results proved that the topical application of (0.001mg/kg) TQ loaded on G-NPs is effective in delaying and/or regressing the malignant progression along the experimental period (14 weeks). These effects are mostly mediated through the inhibition of chronic inflammation exerted by continuous DMBA painting, i.e. the suppression of the NF- κ B-Cox-2 pathway.

CONCLUSION

From the results of this study, it can be concluded that:

1. Topical application of TQ only was not effective in modulating the carcinogenesis process and this is mostly due to its hydrophobic nature and in turn, the weak bioavailability was unable to penetrate the cell membrane.
2. Gold nanoparticles in that model appear to be a good drug-carrier for weak bioavailable agents.
3. Topical application of (0.001 mg/kg) TQ loaded on G-NPs for 7 and 14 weeks was able to reduce Cox-2 expression compared to DMBA-only treated group, and in turn retard/regress the carcinogenesis process due to its anti-inflammatory and free radicle's scavenging effects.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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