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A TRIAL OF THYMOQUINON LOADED ON GOLD NANOPARTICLE AS A THERAPY FOR INDUCED SQUAMOUS CELL CARCINOMA IN BUCCAL POUCH OF HAMSTER

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ABSTRACT

Objectives: Combining the anti - tumor effect of thymoquinone (TQ) with the efficient penetration of gold nanoparticles (GNPs) into cells and nuclei as an attempt to treat the induced oral squamous cell carcinoma in buccal pouch of hamster.

Materials and methods: This study was carried out on forty two male Syrian golden hamsters (n=42), its age ranged from 6 to 7 weeks, weighting 90-110 gm. Animals were housed with controlled temperature and were given pellets formed of seeds, grain, cracked corn and tap water ad libitum. The hamsters were divided into: Group A: (Control groups) n=24, Group A1: eighteen animals (n=18) were served as "negative control" . Sub-group A1a: Six animals were sacrificed at day zero. Sub-group A1b: Six animals were sacrificed at 14th week. Sub-group A1c: Six animals were sacrificed at 20th week. Group A2: Six animals (n=6) were considered as "positive control". DMBA was painted to the left cheek pouches, three times /week for 14weeks. Group B: Eighteen animals (n=18) were painted with DMBA to left cheek pouches three times/week for 14weeks, and subdivided into: Sub-group B1: Six animals were injected intra-peritoneal with GNPs/3 times/week for six weeks. Sub-group B2: Six animals were injected intra-peritoneal with TQ/3 times/week for six weeks.Sub-group B3: Six animals were injected intra-peritoneal with GNPs-TQ/3 times/week for six weeks. All pouches were extracted and prepared to examined through histological examination for any structural changes and immunohistological detection of COX2.

Results: Improve superior anti-inflammatory role of TQ when loaded with GNPS in COX-2 retardation and tumor size regression.

Conclusion: Thymoquinon loaded on nanogold particle showed protective role in the oral cancer.

KEY WORD: Thymoquinon, Nano gold particles, oral cancer, , COX

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INTRODUCTION

Oral squamous - cell carcinoma (OSCC), is the sixth most prevalent cancer in the world..[1] In 2018, the United States estimated 51.540 new cases of oral and pharynx (throat) cancer. The incidence for men is more than double for females with an age of 5 years survival[2].. OSCC estimated of 90.1% of all oral cancers in Egypt [3], the delayed diagnosis of potentially malignant symptoms is considered responsible for the late diagnosis of possibly malignant disorders due to an lack of adequate awareness of indications, disorders or risk factors, are considered to be responsible for late diagnosis of potentially malignant disorders. Surgical solution of oral cancer is considered among the most life threatening or distorting treatment of oral cancer. It leads to dysfunction, speech distortion, chewing and swallowing problems and affects the patient's ability to interact socially. Despite considerable progress in oral cancer treatment, it remains a major cause of mortality in humans.[4]

Chronic inflammation has been shown to contribute not only to inflammation and repair but also to the progression of cancer. Chronic inflammation was also shown to play a role in oesophageal, pancreas and gall bladder cancer pathogenesis[5]. In some people, the development of OSCC is also associated with long - term inflammation or irritation of the oral cavity caused by dental cavities and periodontitis.[6]

Prostaglandin E2 (PGE2) mediates many biological and active inflammation functions in which the recruitment and activation of inflammatory cells which is activated by local vasodilation.[7] The cyclooxygenase pathway (generating PGE2 and other prostaglandins) and the lipoxygenase pathway (producing a variety of leukotrienes and anti - inflammatory lipoxins), both products may be included in inflammation control, but PGE2 is related to inflammation and cancer, including OSCC.[8]

There are at least two forms of isoenzyme cyclooxygenase (COX), COX-1 is expressed constitutively in most tissues and appears to be a household enzyme responsible for various physiological functions, For example, stomach cytoprotection, kidney vasodilation and platelet production of pro - aggregatory prostanoid thromboxane. Conjuncture 2, In contrast, it is not constitutively expressed in most tissues, but is induced in pathophysiological states by a wide range of growth factors and cytokines. In both premalignant and malignant tissues, increased expression of COX-2 is commonly found.[9] It has been shown that over - expression of COX-2 in epithelial cells inhibits apoptosis and increases tumor cell invasiveness.[10]

Thymoquinone (TQ) is a specific chemical entity found in Nigella Sativa (NS). Studies have shown that the biological activity of NS seeds is mainly due to its predominantly thymoquinone essential oil component (30 - 48 percent).[11] TQ's anti - inflammatory effect is one of the main roles against a wide variety of human diseases. The primary constraints of the translation of TQ into the clinic are its poor bioavailability and lack of knowledge of its toxic effects in humans..[12] Unfortunately, TQ has not added therapeutic value, which may be due to its failure to exhibit anticancer effects in recurrent aggressive tumors. It is also possible that, in addition to its high ability to bind to plasma proteins, TQ's poor dissolution and bioavailability actually prevented it from reaching the tumor site.[13]

Nanotechnology is seen as an emerging field that can revolutionize contemporary cancer medicine.[14] Nanoparticles can be used to aid drugs directly into tumor cells without affecting healthy cells as drug carriers for chemotherapy regimes. They can protect drugs against body degradation before reaching their destination, improve drugs in cancer cells and prevent drug interaction with normal cells with decreasing side effects.[15]

Gold is known to be a little reactive inert noble metal. Gold is a desirable metal for medical use due to its properties. It is resistant to corrosion and has low poisoning.[16] GNPs can act as chemical carriers to deliver drugs to a target location for the destruction of a cancer cell or can be used in conjunction with photothermal treatment. In nanoparticles, Water encapsulation - insoluble drugs as TQ increased their bioavailability, reduced their diffusion to ordinary tissues and prevented their rapid metabolism in nanoparticles. Because of the leaky nature of the tumor vasculature and poor lymphatic drainage, nanoparticles often accumulate near the tumors, leading to better targeting and reduced exposure to non - specific drug sites. Thus, TQ and different nano - formulations (TQ - Nps) are believed to have a greater promise than free TQ. [17]

MATERIALS AND METHODS

Chemicals

- Tetra chloroauric acid (HAuCl4) cat. No: (27988-77-8), trisodium citrate (Na3C6H5O7) (cat. No: 6132-04-3) to get GNPs solution.
- Thymoquinone (C10H12O2) (cat. No: 490-91-5) and propylenegylcol (C3H8O2) (cat. No: 57-55-6) to get thymoquinone solution.
- The chemical carcinogen 7, 12 Dimethylbenz-[a]-anthracene (DMBA) (cat. no: D3254), and heavy mineral oil (cat. No: M 3516) to get DMBA solution.
- All the previous chemicals were purchased from Sigma Chemical Company, USA.

Preparation of gold nanoparticles (GNPs) Turkevich method192 was used to prepare GNPs in which tetra-chloroauric acid solution was considered as a source of gold ions and trisodium citrate as a reducing and stabilizing agent. Briefly tetrachlorauric acid was heated until boiling with stirring, and then trisodium citrate solution was injected. The gold nanoparticles were gradually formed as the trisodium citrate reduced the Au (III) to Au (0) as indicated by change in color from pale yellow to red color. Heating continued till the color became deep red suspension. The size of produced particles was controlled by changing the amount of available gold in the solution.[18]

Loading of thymoquinone on gold nanoparticles. The solution GNPs with thymoquinone solution of 0.001mg/100gm was prepared by mixing equal amounts of both(1:1). The mixture was stirred on a magnetic stirrer for 2 hours and the formed solution was kept in 5˚c.

Characterization of the prepared solutions

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 Institute1. Ultraviolet-Visible (UV-vis) spectroscope was used to determine the maximum absorption of ultraviolet for GNPs, TQ, and GNPs-TQ that was recorded at room temperature with samples in a quartz cuvette using T90+UV-VIS Spectrometer (PG Instruments Ltd) wavelength range from 250-850 nm.

GNPs, TQ, and GNP-TQ were studied by Ultra Violet-Visible spectra (UV-Vis) that were recorded at room temperature with samples in a quartz cuvette using T90+UV-Vis Spectrometer, PG Instruments Ltd (wavelength range 250-850 nm) to determine the wavelength of the highest absorption

Experimental Design

Carcinogen The chemical carcinogen 7,12 dimethylbenz-[a]-anthracene (DMBA) was dissolved in heavy mineral oil to get 0.5% solution. The carcinogen was topically applied to hamster left buccal pouches (HBP) of Syrian golden hamster (*Mesocricetus auratus*) by using number (4) camel hair brush.

Animals The experiment was held at the animal house in Pharmacology and Toxicology Department, Faculty of Pharmacy, Suez Canal University,

Ismailia, Egypt. This study was carried out on forty two male Syrian golden hamsters (n=42), its age ranged from 6 to 7 weeks, weighting 90-110 gm, purchased from Tiedor Blhars Research Institute, Cairo, Egypt. Animals were housed, five per cage, with controlled temperature and were given pellets formed of seeds, grain, cracked corn and tap water ad libitum. The hamsters were divided into:

Group A: (Control groups) n= 24

Group A1: Eighteen animals (n=18) were served as "negative control"

- Sub-group A1a: Six animals were sacrificed at day zero.
- Sub-group A1b: Six animals were sacrificed at 14th week.
- Sub-group A1c: Six animals were sacrificed at 20th week.
- Group A2: Six animals were considered as "positive control".
- DMBA was painted to the left cheek pouches, three times /week for 14 weeks.

Group B: Eighteen animals (n=18) were painted with DMBA to left cheek pouches three times/week for 14weeks, and subdivided into

- Sub-group B1: Six animals were injected intra-peritoneal with GNPs/3 times/week for six weeks.
- Sub-group B2: Six animals were injected intra-peritoneal with 0.001 TQ/3 times/week for six weeks.
- Sub-group B3: Six animals were injected intra-peritoneal with 0.001 GNPs-TQ/3 times/ week for six weeks[19].

The animals were sacrificed by a cotton-soaked with a lethal dose of diethyl ether inside a closed glass container. After animals sacrificing all pouches were fixed in 10% neutral formalin solution, processed, embedded in soft paraffin, left to be hardened and sectioned into 5μm, sections were cut in a rotary microtome, mounted on glass slides and stained with hematoxylin and eosin for light microscopic study and the ultravesion mouse tissue detection system using antimouse monoclonal antibody for demonstration of COX2, with brown cytoplasmic expression. The slides were diagnosed by two pathologists, and photographed by E-330 Olympus digital camera.

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

Characterization of GNPs, and GNPs-TQ

A) Transmission electron microscope (TEM) results

TEM micrograph revealed that GNPs were spherical and well dispersed without agglomeration. Most of particles were between 25-30 nm in size with an average size of 27 nm as shown in Histogram 1. Figure 1

B) UV-visible spectrometer (UV-vis) results

The produced solutions of GNPs, TQ, and GNPs-TQ were subjected to characterization by UV-visible spectroscope. Sharp peak was given by UV-visible spectrum for GNPs at λmax=526 nm which confirmed the nanoparticles formation. The maximum absorption peak for TQ was recognized at λmax = 316 nm, while GNPs-TQ gave maximum absorption peak λmax =532 nm. This deviation in the maximum peak confirmed loading of TQ on GNPs. Figure 1

Clinical findings

Group A1 "negative control" showed no gross changes, after sacrificing, the buccal pouches length was about 5cm. **Group A2** Debilitation of all animals treated with DMBA was a noticeable remark. Pronounced blood capillaries appeared on the left pouches surface. Different exophytic papillary and smooth surfaced overgrowths were observed in all painted-pouches, which have 2cm length in general. Figure 1

Groups B1 showed no improvement in general health of animals. Length of the pouches was about 2cm and multiple different sizes of exophytic masses appeared. **Groups B**2 showed slight improvement in the general health. The length of the pouches was about 2.5cm. Slight decrease in size of the papillomatous lesions was observed rather than the lesions treated with GNPs only. **Groups B3** showed marked improvement in general health of animals. There was a significant increase of the pouches length to about 3.5cm. There was marked

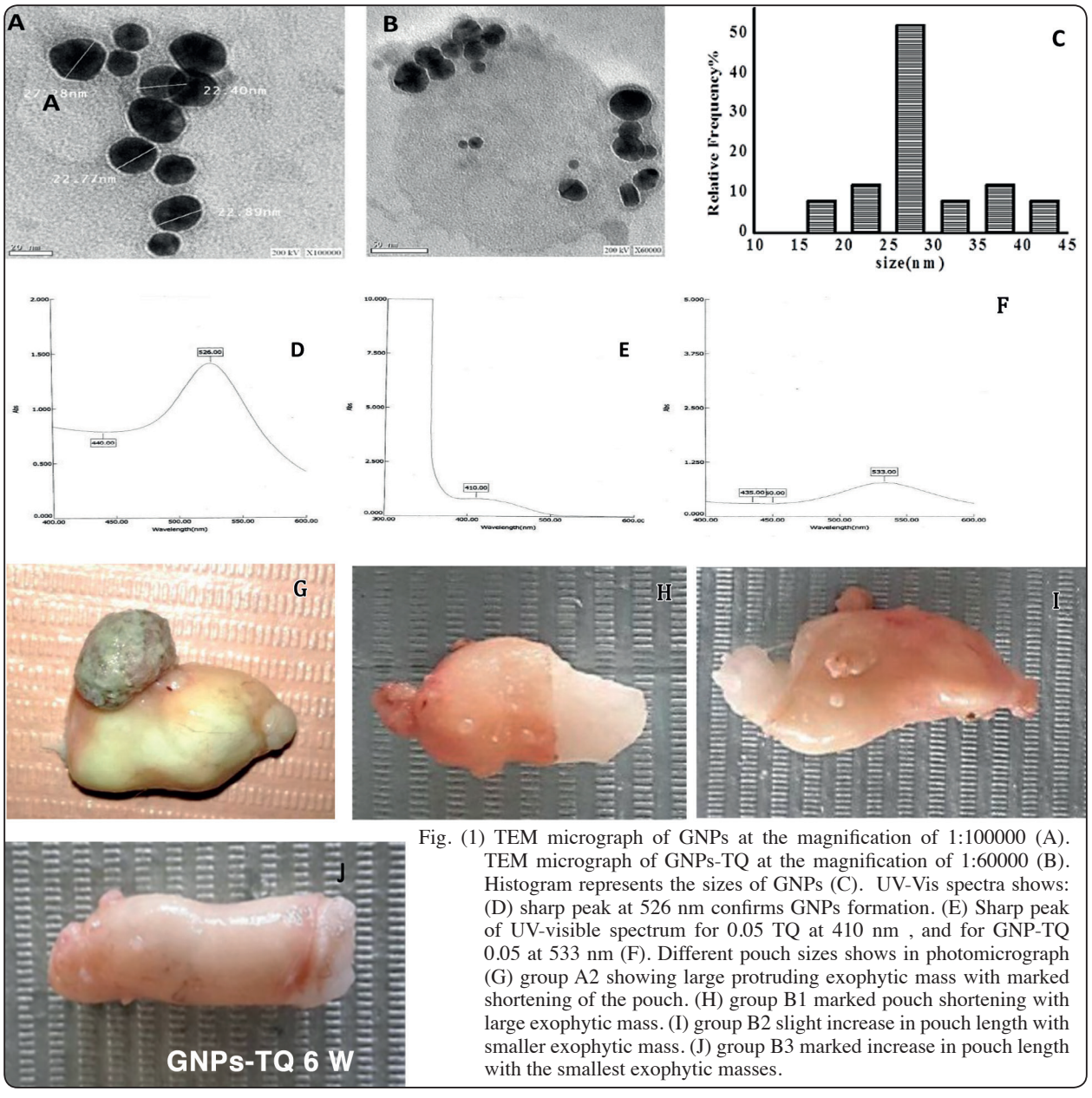

Fig. (1) TEM micrograph of GNPs at the magnification of 1:100000 (A). TEM micrograph of GNPs-TQ at the magnification of 1:60000 (B). Histogram represents the sizes of GNPs (C). UV-Vis spectra shows: (D) sharp peak at 526 nm confirms GNPs formation. (E) Sharp peak of UV-visible spectrum for 0.05 TQ at 410 nm , and for GNP-TQ 0.05 at 533 nm (F). Different pouch sizes shows in photomicrograph (G) group A2 showing large protruding exophytic mass with marked shortening of the pouch. (H) group B1 marked pouch shortening with large exophytic mass. (I) group B2 slight increase in pouch length with smaller exophytic mass. (J) group B3 marked increase in pouch length with the smallest exophytic masses.

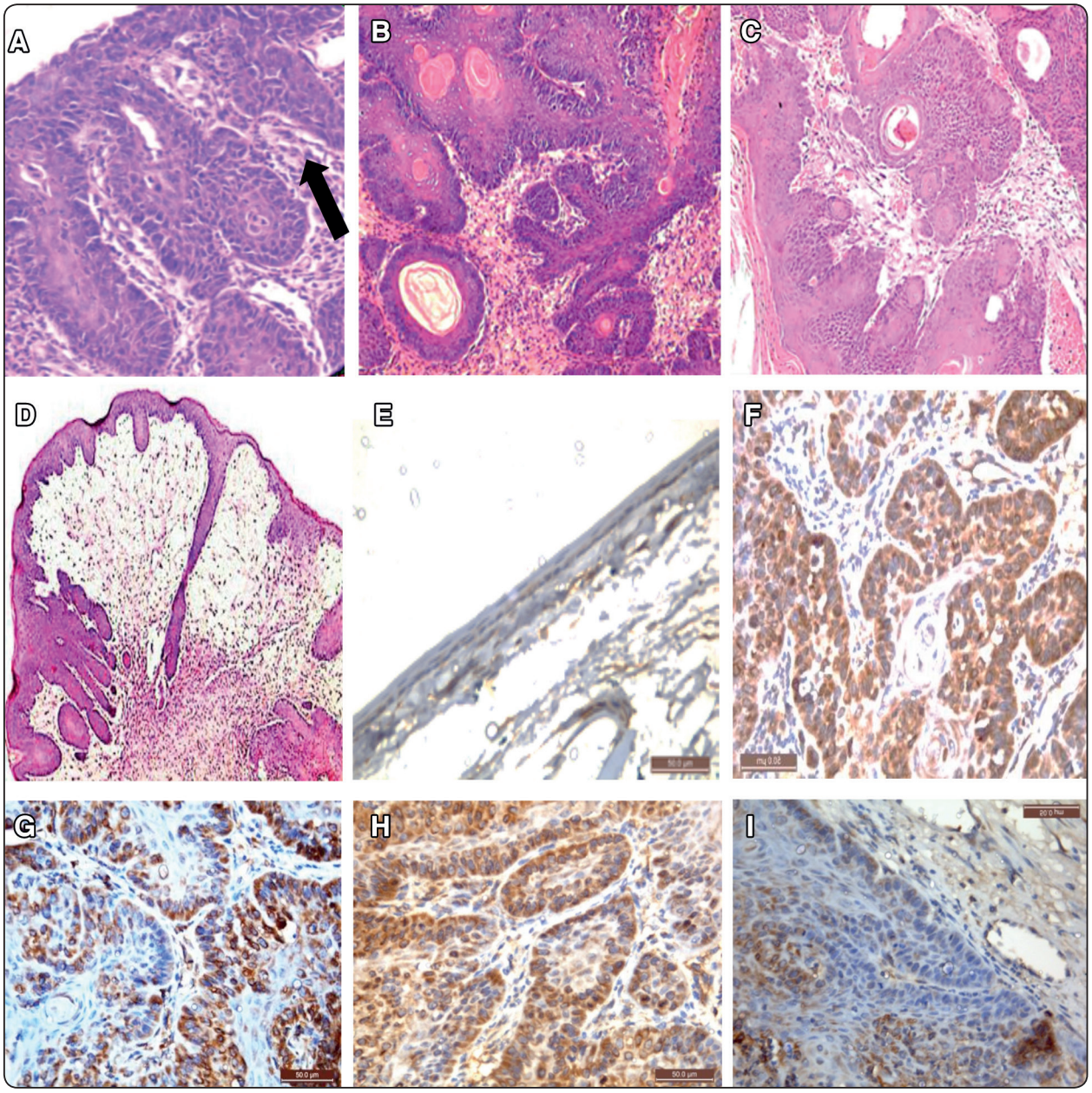

Fig. (2) **Histopathological results** (A) group A2 (DMBA 14 wks) shows well to moderate differentiated SCC. Note intense chronic inflammatory cells (arrow) (H&E × 20). (B) group B1 (GNPs) shows invasive SCC in the form of papillomatous overgrowth with multiple invading epithelial islands. (H&E ×10). (C) group B2 (TQ) shows superficial invasive squamous cell carcinoma limited to the nodule. (H&E ×10). (D) group B3 (GNPs-TQ) shows CIS in nodular mass. (H&E ×4). **Immunohistochemical results** (E) group A1 negative control group shows low COX-2 immuno-reactivity (IHC ×40)**.** (F) group A2 (DMBA) group shows intense COX-2 immuno-reactivity (IHC ×40)**.** (G) group B1 (GNPS) shows intense COX-2 immuno-reactivity (IHC ×40)**.** (F) group B2 (TQ) shows intense COX-2 immuno-reactivity (IHC ×40)**.** (F) group B3 (GNPS-TQ) shows moderate COX-2 immuno-reactivity (IHC ×40)**.**

decrease in the size of exophytic masses in these groups when compared to animals treated with GNPs or TQ only. Figure 1

Histopathological & immunohistochemical results:

Group A1 revealed normal hamster buccal pouch lining. Immunohistochemical results revealed singnificant decrease in COX2 immune reactivity Figure 2

Group A2 revealed well to moderate differentiated squamous cell carcinoma in the form of large papillomatous lesions with deeply invading islands of epithelium into underlying connective tissue with marked chronic inflammatory cells. Immunohistochemical results revealed singnificant increase in COX2 immune reactivity. Figure 2

Group B1, revealed well differentiated SCC in the form of papillomatous overgrowth with multiple deeply invading dysplastic epithelial islands into underlying connective tissue, not limited to the nodules. Immunohistochemical results revealed significant increase in COX2 immune reactivity. Figure 2

Group B2, showed superficial invasion of malignant cells in the form of well differentiated squamous cell carcinoma which was limited to the nodules only, not extended to deeper areas. Other areas in the pouch showed nodular elevation exhibiting carcinoma in situ. Immunohistochemical results revealed significant increase in COX2 immune reactivity. Figure 2

Group B3, revealed smaller size of papillomatous lesions without any sign of invasion. Epithelium had focal areas of variable grades of dysplasia i.e. from moderate dysplasia to carcinoma in situ which represented as hyperchromatism, altered N/C ratio; cellular & nuclear pleomorphism, prominent nucleoli, as well as multiple group cell keratinizations. The connective tissue showed marked increase thickness of striated muscle layer. Immunohistochemical results revealed significant decrease in COX2 immune reactivity. Figure 2

Statistical results evaluation: (table 1) regarding COX2, there was a high statistical significant difference between whole groups (P-value < 0.001) through using Annova - one way test (figure 3). The most statistically significant result was associated with the group treated with GNPs-TQ (B3) [55.49± 13.14] in comparison with other groups. Also, both of chemopreventive drugs showed statistically significant result versus positive control group DMBA (A2) [107.58± 17.75]. Figure 3.

TABLE (1) One Way Annova test

Groups	N	Mean	Std. Deviation	P value
Negative controls (A1)	6	25.08	2.92	< 0.001
Positive controls (A2)	6	112.92	10.52	
TQ (B1)	6	108.02	16.8	
GNPS (B2)	6	88.06	23.67	
GNPS-TQ (B3)	6	55.49	13.14	

There was a high statistical significant difference regarding the Mean between whole groups (P-value < 0.001).

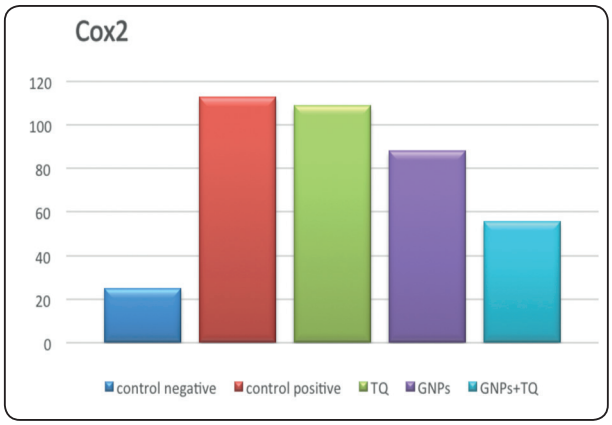

Fig. (3) A histogram showing the mean ± standard deviations of COX2 expression between negative group, DMBA group, TQ only, GNPs, and GNPs-TQ.

DISCUSSION

This study aimed to combine the anti - tumor effect of thymoquinone (TQ) with the efficient penetration of gold nanoparticles (GNPs) into cells and nuclei.[20'21] This combined formulation was used to investigate a possible therapeutic effect of TQ loaded on GNPs when given through intra-peritoneal injection in the hamster buccal pouch carcinogenesis (HBP/DMBA) model. The best result was associated with GNPs-TQ when compared to either GNPs or TQ alone. The possible explanation of different outcomes between different agents used in the present study could be attributed to the fact that nanoparticles can passively target tumor cells through the accumulation and trapping process known as enhanced permeation and retention (EPR) impact. An effect mediated through angiogenic vessels, and extravasation through leaky blood vessels (gaps~600nm), and improper lymphatic flow. [22]

Many nanoparticles and nanomaterials have emerged from various bulk elements such as gold, silver, iron, copper, cobalt, platinum, etc. with recent advances in cancer treatment..[23] Gold nanoparticles can be prepared in a wide range of core sizes (1 to 150 nm), and their biocompatibility and non - toxicity make them an excellent candidate for use.[24]

In our study, the prepared GNPs were spherical, well dispersed without agglomeration with the particle sizes between 25-30 nm and possess an average size of 27 nm. **Pan *et al.,* (2007)**[25] observed that GNPs of 1–2 nm were highly toxic, while particles larger than 15-nm were comparatively non - toxic. In addition, the cellular absorption of spherical GNPs has been documented to be higher than counterparts in rod form. Deviation of the maximum ultraviolet spectrometer peak between GNP, TQ and GNP - TQ confirmed the loading of TQ on GNPs, which improved in parallel work.[26]

Gross clinical findings were confirmed in other studies, almost the same model following DMBA painting for 14 weeks.[27'28'29'30] After treatment with intraperitoneal injection of GNPs, TQ and GNPs - TQ, different sizes of exophytic masses were observed in relation to other groups. The worst result associated with GNPs was attributed to animals treated with GNPs - TQ, which showed a dramatic reduction in these exophytic masses compared to other groups with massive improvements in general health. These findings showed the beneficial effect of combining GNPs with TQ in order to achieve these positive results. These results can be compared Hsieh et al in (2011)[31], who confirmed inhibition of bladder tumor growing in a mice model. There was significant decrease in size of the lesion when EGCG-GNP combination rather than GNP alone. On line with these findings, Afifi et al (2013)[32] reported reduced exophytic growth of HBP by almost 80 percent with increased survival rate of treated animals using both GNPs and plasmon photothermal therapy (PPTT). The authors found that GNPs affected tumor growth alone and showed unfavorable survival rates for hamsters. Histopathological findings of the present study were hopeful. Hamsters exposed to DMBA for 14w showed good to moderate differentiated squamous cell carcinoma (SCC) with marked chronic inflammation, these findings had been documented by several authors.[31'25] The improvement or regression of the histopathological findings confirmed the proposed therapeutic effect of the used agents. Intra-peritoneal injection of GNPs, and TQ, were resulted in smaller papillomatous growth with well differentiated SCC. The present findings showed that GNPs alone had no therapeutic effect on DMBA-painted animals. This result was in line with Mukerjee et al (2007)[33] who noted that induction of apoptosis, in the treatment of chronic lymphocytic leukemia cell lines, with gold-VEGF was significantly higher than those treated with GNPs only. Afifi et al (2013)[31] had documented the GNPs only has no anti-cancerous effect without application of laser. The best results were given with loading GNPs and TQ, which ranged from moderate dysplasia to carcinoma in situ in 10-fold lower dose than a comparable study used TQ 0.01 mg/100gm by intra-peritoneal route.[27]

Immune-histochemistry findings of the present work improve superior anti-inflammatory role of TQ when loaded with GNPS in COX-2 retardation and tumor size regression. On line with our results, Erovic et al. (2003)[34] found that COX-2 is expressed in OSCC tumors and the surrounding lymphocytic infiltrates, the authors suggested that COX-2 is an important link between chronic inflammation and carcinogenesis. Further support of COX-2 in OSCC comes from Pontes et al. (2013)[35] who found increased levels of COX-2 in oral dysplastic lesions and in OSCC, when compared with oral hyperplastic epithelium, suggesting that COX-2 is involved in the early stages of oral carcinogenesis. Another study found that COX-2 is rarely expressed in normal epithelium, but it is highly expressed in dysplastic cells and carcinoma cells, and only to a variable degree in a few inflammatory cells, fibroblasts and vascular endothelial cells.[36] Authors improved that TQ had inhibited Cox-2 protein expression and prostaglandin E-2 (PGE-2) production in HPAC pancreatic cancer cells. TQ was used to reduce the adverse effects arising from elevated levels of free radicals in inflammatory disorders, drug detoxification. In addition, it could prevent damage caused by reactive oxygen species (ROS) that provoke cell death by mitochondrial membrne disruption.[37]

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