THERAPEUTIC EFFECT OF PACLITAXEL LOADED ON GOLD NANOPARTICLES IN TREATMENT OF INDUCED ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

INTRODUCTION: Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide with high mortality rate. Conventional treatment strategies have improved but remain far from optimal. Cancer research is focused on improving cancer diagnosis and treatment methods using nanotechnology, by the production and application of nanoscale drug delivery systems. In medicine, several types of nanoparticles have evolved, gold nanoparticle (AuNPs) are an excellent candidate as drug delivery vehicle due to their favorable chemical and optical properties. Paclitaxel (PTX) is an effective antineoplastic drug that has a wide spectrum of antitumor activity, against head and neck malignancies. The encapsulation of PTX in nanodelivery systems can protect the drug from degradation during circulation and protect the body from its toxic side effects.

OBJECTIVES: To study the therapeutic efficacy of PTX-functionalized AuNPs versus the free form of the drug. Also the study will evaluate the treatment, by the use of proliferative immune-histochemical marker (PCNA).

MATERIAL AND METHODS: Squamous cell carcinoma will be chemically induced in sixty Syrian hamsters. Then they will be divided into three groups, 20 in each. One group will be treated with free PTX, another group will be treated with PTX-AuNPs, and the last group will be given saline as negative control group.

RESULTS: Group treated by PTX loaded on AuNPs showed significant results over group treated by free PTX.

CONCLUSIONS: The unique AuNPs properties in combination to the chemotherapeutic drug target cancer cells while maintaining no adverse effects on the surrounding normal cells.

KEYWORDS: OSCC, AuNPs, PTX, PCNA.

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide that invades local tissue, metastasizes and has high mortality rate with overall 5 year survival rate (1,2). Conventional treatment strategies, such as surgery, radio and chemotherapy, have improved over the past few decades; however, they remain far from optimal. Currently, nanotechnology is focused on improving cancer diagnosis and treatment methods (3).

The nanotechnology-based drug delivery systems (DDS) has been heavily investigated in the past few years, to increase the effectiveness of treatment and reduce side effects. Gold nanoparticles (AuNPs), are particularly promising tools to improve cancer treatment, due to their unique optical properties, non-toxic nature, relatively simple preparation and functionalization (4,5).

Polyethylene glycol (PEG) is commonly used to functionalize the surface of AuNPs in order to improve their in vivo stability and to avoid uptake by the reticular endothelial system. PEGylation of AuNPs also extends circulation time and concentrations of drug in tumors by 10-100 fold compared with the use of free drugs (6). Moreover, peptides functionalized nanoparticles enhance the targeting efficacy of drug delivery. In this respect Arginylglycylaspartic acid (Arg-Gly-Asp) (RGD) is the peptide of choice to functionalize the AuNPs surface. They target $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins that are expressed in the cancer endosystem, increasing nanoparticle uptake by the cancer cells (7,8).

Paclitaxel (PTX) is one of the most widely used and effective antineoplastic agents derived from natural sources from the bark of Pacific Yew (Taxus brevifolia) (9). It has a wide spectrum of antitumor activity, particularly against head and neck malignancies, Kaposi's sarcoma, ovarian cancer, breast cancer and non-small cell lung cancer. The mechanism of action of PTX is to promote and stabilize microtubules and inhibit late G2 or M phases of cell cycle, causing the cell death (10).

Paclitaxel is formulated in a mixture of Cremophor EL and dehydrated ethanol a combination known as Taxol,

due to its low water solubility. However, Taxol has some severe side effects related to Cremophor EL and ethanol. As a result, there is an urgent need to develop an alternative formulation (11). The encapsulation of paclitaxel in biodegradable and non-toxic nanodelivery systems can protect the drug from degradation during circulation and in-turn protect the body from toxic side effects of the drug. Thereby, it reduces its toxicity, increases its circulation half-life, improves pharmacokinetic drug, and demonstrates better patient compliance (12).

The aim of the present work was to study the therapeutic efficacy of PTX-functionalized AuNPs versus the free form of the drug. Also to evaluate the treatment, by the use of proliferative immune-histochemical marker.

MATERIALS AND METHODS

The study was performed in the Faculty of Dentistry, Alexandria University after gaining the approval of the Research Ethics Committee. Sixty Syrian golden male hamsters (Five weeks old), weighting 80-129g were obtained from Tedor Billhars Institute, Cairo, Egypt. They were housed in show box cages (Technoplast, Italy) at the experimental animal unit in the Medical Technology Center (Medical Research Institute, Alexandria University, Egypt).

a- Synthesis and Characterization of AuNPs

Gold nanospheres were prepared by the reduction of gold salts chloroauric acid (HAuCl4) in the presence of reducing agent as appropriate stabilizing agents that prevent particle agglomeration. Particle size was determined by Transmission electron microscope (TEM, JOEL, JSM-6360LA, Japan). This was performed in the Faculty of Science, Alexandria University.

Gold nanospheres were conjugated with PEG polymer to stabilize the nanospheres and inhibit their aggregation during the drug-peptide conjugation. After which, RGD peptide was conjugated to the AuNPs. Paclitaxel chemotherapeutic drug was conjugated to the particles to attain the desired percent surface coverage. This was performed in the Medical research Institute, Alexandria University.

b- Cancer Induction

The animals' left buccal pouches were painted with 0.5% 7, 12-dimethyl 1, 2-benzanthracane (DMBA) (Sigma-Aldrich) dissolved in liquid paraffin using a number 4 brush. Following a standard carcinogenesis protocol, this procedure was repeated 3 times per week for 18 weeks or upon developing oral cancers.

c- Treatment Plan

After cancer induction, the hamsters were injected with the desired treatment according to the planned protocol using an insulin syringe. During the treatment procedure they were lightly sedated using ketamine hydrochloride. The control group received saline of 0.9% concentration. The free PTX group was injected with a dose of 10 mg/kg (9). The hamsters in the other study groups received PTX-AuNPs, with dose of 150 µl of 5 nM concentration. Each group received the proposed treatment three times per week for one week.

After treatment two biopsies were extracted from the sacrificed animals in the three study groups. They were fixed in 10% neutralized formaldehyde solution and embedded in paraffin. Sections 4µm thick, stained with hematoxylin and eosin to confirm and grade the type of induced carcinoma and count apoptotic bodies after treatment. Other 3-4µm thick sections were mounted on super frost plus-coated glass slides for immunohistochemical evaluation of the proliferation activity using monoclonal proliferation cell nucleur antigen (PCNA). The immunostaining results were scored using an image analyzer (Surgical Pathology Department, Faculty of Medicine, Alexandria University).

d- Evaluation of the Proposed Treatment

i- Evaluation of Tumor Volume

Tumor volume was measured in all study groups animals just prior to treatment using a caliber. The same measurements were performed after treatment over a period of 4 weeks. Tumor volume percent change were calculated as follows: [(volume by the end of the 6thweek - volume before treatment) /volume before treatment] x100.

ii- Survival Time and Increased Life Span

Animal survival time and increased life span were recorded as well (mean, SD) and compared to the values for the control groups.

e- Statistical analysis

The data were collected, tabulated and statistically analyzed using the SPSS system (release 11.0 software). All results were expressed as mean±standard deviation (SD). Tumor volume changes before and after treatment was analyzed statistically using paired t-test.

Survival curves were estimated using the Kaplan-Meier method. The influences of the numerical variables on the survival were examined by univariate analysis and multivariate regression analysis (Cox proportional hazards model). One-way ANOVA was used to analyze the AI between study groups. Also it was used to analyze the MA% and MOD of immunohistochmical results. In all statistical results, a p value < 0.05 was considered significant.

RESULTS

a. Sequential Clinical Changes during Period of Carcinogenesis

The hamsters in the study groups were observed for the clinical changes during the period of carcinogenesis. After few weeks non-scrapable white lesions (leukoplakia) in different forms with sloughing of the mucosa started to be seen. Afterwards, some lesions turned into mixed white and red lesions (erythroleukoplakia). Meanwhile by the end of the carcinogenesis process, well developed oral tumors were observed, some were exophytic masses while others were endophytic ulcers with necrotic floor and raised margins.

b- Sequential Histological Changes during Period of Carcinogenesis

Hamsters were sacrificed periodically during the carcinogenesis period to evaluate and grade the histological changes of the tumor. After few weeks of

cancer induction, the epithelial dysplastic changes started to appear, where the hamster buccal pouch (HBP) showed mild epithelial dysplasia, then moderately then the dysplastic changes turned into severe epithelial dysplasia, and carcinoma in situ. (Figure1, 2).

At the end of cancer induction, some tissues revealed early invasive OSCC with evident points of invasion of the malignant epithelial cells into the underlying lamina propria. By the end of carcinogenesis, different grades of differentiation, well, moderately and poorly differentiated OSCC was observed (Figure 3).

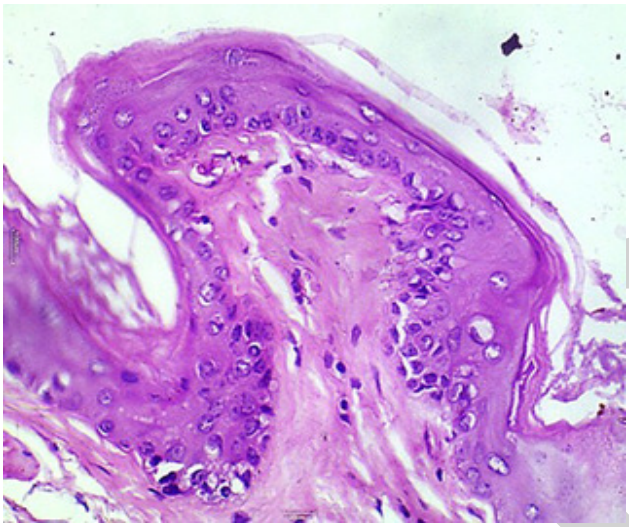

Figure 1: Moderate Epithelial Dysplasia Involving Half of the Epithelium with Exophytic Epithelial Growth Pattern (H&E X 400).

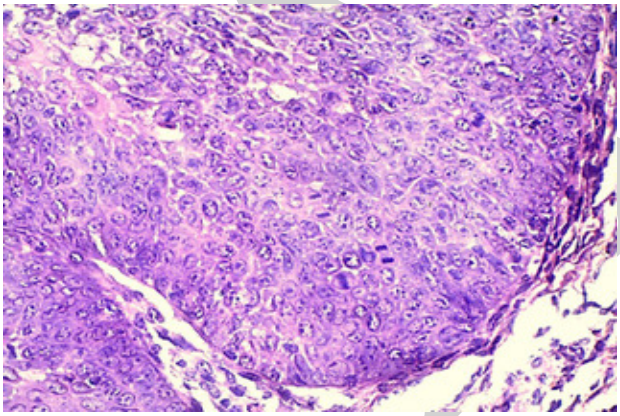

Figure 2: Carcinoma in situ; Top to Bottom Changes with bulbous rete pegs with numerous abnormal mitotic figures (H&E X 400).

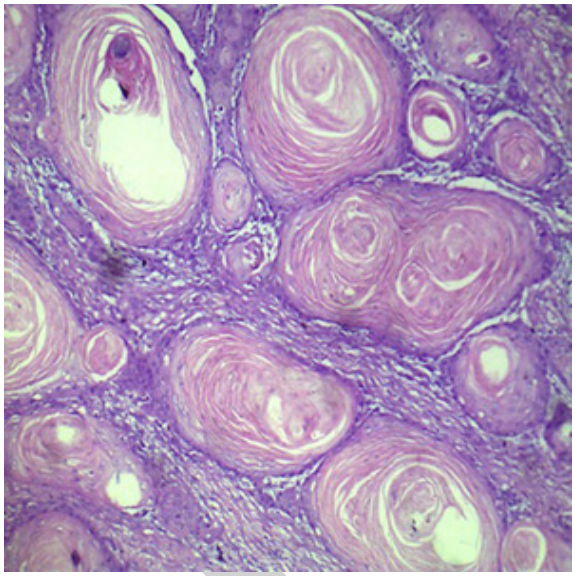

Figure 3: Well Differentiated SCC Showing Keratin pearls and Epithelial pearls (H&E X 400).

c- Grouping and Treatment Plan

At the end of carcinogenesis process, seven hamsters died during the induction process and the remaining hamsters developed tumor masses with incident rate of 100% and tumor multiplicity of 2.4 ± 1.53 tumors per hamsters. They were divided randomly into 2 main study groups and 1 control group, according to the type of the proposed treatment.

d- Evaluation of the Proposed Treatment

i- Evaluation of Tumor Volume

The tumor volumes in the study groups recorded changes at the 4th week after treatment. They were measured before and after treatment and the results was as follows: In the control saline group, some tumors continued to increase in their volume by a percent change of 6.8 %. In the free PTX group, the treated tumors recorded significant decrease in the mean tumor volume by -10.3% ($p<0.05$). While in the group treated with PTX-AuNPs, they recorded the highest decrease in the mean tumor volume by -29 %, which was significant ($p<0.05$) as shown in Table 1.

Table 1: Tumor volume* in mm^3 (mean ± SD) before and after treatment, percentage of volume change (mean ± SD).

Group (n=120)	Treatment	Mean tumor volume before treatment	Mean tumor volume after treatment	Percentage of volume change	P value
Group I (n=20)	Saline	141.0 (±90.1)	148.2 (±91.2)	6.8 (±2.4)	0.008**
Group II (n=20)	Free PTX	134.0 (±52.4)	123.7 (±50.6)	-10.3 (±1.6)	0.010**
Group III (n=20)	PTX-AuNPs	315.7 (±267.0)	286.7(±230.5)	-29.0(±18.1)	0.040**

*Tumor volume in mm^3 = D max X (D min) ^{2} / 2
** Statistically significant (p<0.05) in comparison between mean tumor volume before and after treatment in each group.

ii- Survival Rate

The control saline group, showed mean survival rate of 12 (±1.4). The free PTX group, revealed significant increase in the mean survival rate which was 21 (±2.9). While the group treated with PTX-AuNPs, recorded the highest mean of survival rate 29 (±4.2). The mean survival rate (± SD) and increased life span (ILS %) are summarized in Table 2.

Table 2: Survival rate in days (mean ± SD) after treatment and increased life span (ILF %).

Group (n=120)	Treatment	Mean survival time	ILF %	P value
Group I (n=20)	Saline	12 (±1.4)		0.001
Group II (n=20)	Free PTX	21 (±2.9) **	75 %	0.001
Group III (n=20)	PTX-AuNPs	29 (±4.2) **	141.6 %	0.001

$$*ILS\% = \left(\left(\frac{\text{mean survival time of treated group}}{\text{mean survival time of control group}}\right) - 1\right) x\ 100$$

** Statistically significant (p<0.05) in comparison to the mean survival rate of the control subgroups.

e- Microscopical Evaluation

I- Apoptotic Index (AI) Analysis

In the saline control group the quantitative AI analysis revealed mean values of 1.54 (±1.47) and in the free PTX group, the mean AI was 10.5 (±1.05) and both were not statistically significant of (p>0.05). The quantitative AI analysis revealed the highest mean value of 14.17 (±1.92) for the group treated with PTX-AuNPs which was statistically significant of (p<0.05). Apoptotic Index in All Groups after Treatment (mean ± SD) are summarized in Table 3.

Table 3: Apoptotic Index in All Groups after Treatment (mean ± SD).

Group (n=120)	Treatment	Apoptotic Index
Group I (n=20)	Saline	1.54 (±1.47)
Group II (n=20)	Free PTX	10.5 (±1.05)
Group III (n=20)	PTX-AuNPs	14.17 (±1.92) **

** Statistically significant (p<0.05).

II- Immunohistochemical Results

The immunohistochemical proliferation assay was done among the study groups, using PCNA proliferative marker. The immunoreaction in the three study groups showed variations in both, mean area percent (MA %) and mean optical density (MOD).

In the control saline group, the malignant epithelial cells showed intense nuclear immunoreactivity to PCNA. Most of the cells showed positive reaction, indicating high proliferative index in this group, it also showed the highest MA % of 83.2 ± 2.7 and the highest MOD of 73.9 ± 58.1 (Figure 4).

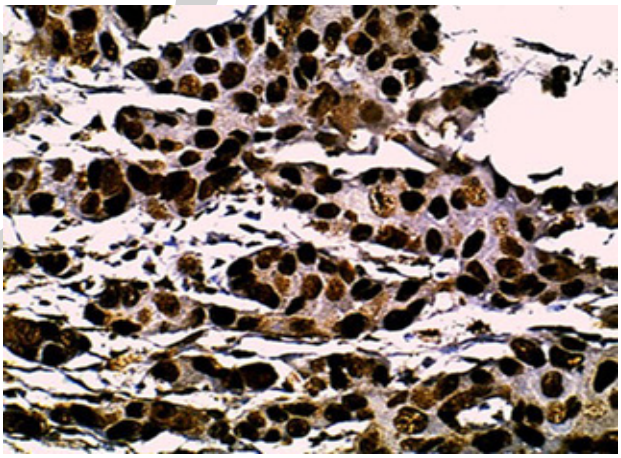

Figure 4: Intense Nuclear Immunoreaction to PCNA throughout Malignant Epithelial Cells in Control Saline Group (PCNA X 400).

In the free PTX group, the immunoreactivity of malignant epithelial cells to PCNA was decreased. Intense nuclear immunoreaction was noted at the peripheral cells, lining the cell nests. The MA% decreased in comparison to the control saline group to 71.4 ± 3.2. The MOD also, decreased to 67.0 ± 59.3. The MA was of significant value (p<0.05) (Figure 5).

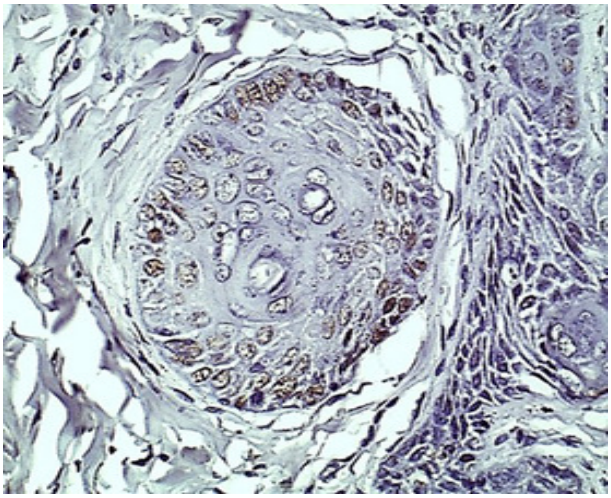

Figure 5: Intense Nuclear Immunoreaction at the Peripheral Malignant Epithelial Cells with some Cells Showing Negative Immunoreaction in the Centre in the Free PTX Group (PCNA X400).

In the PTX-AuNPs group, the immunreactivity to PCNA was almost negative throughout the tumoral tissue. The MA% decreased significantly to 10.0 ± 2.6 and the decrease of the MOD was of significant value 10.2 ±5.1 (Figure 6).

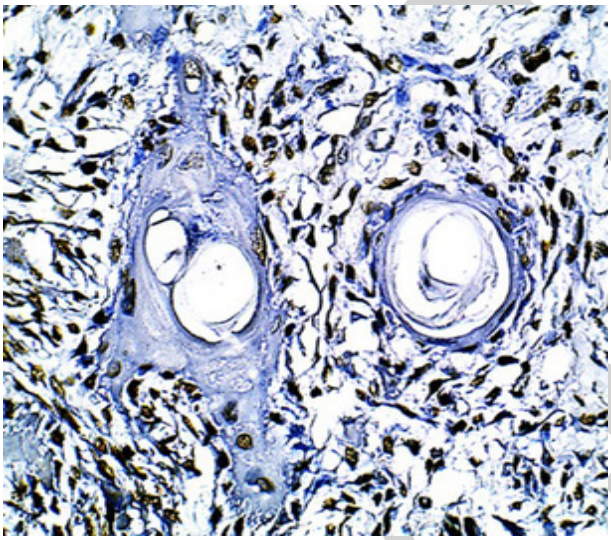

Figure 6: Negative Immunoreaction to PCNA with some Nuclear Immunoreaction in PEG-RGD-PTX-AuNPs Group (PCNA X 400).

DISCUSSION

Nanotechnology is in the spotlight of therapeutic innovation, and AuNPs are particularly promising tools to improve cancer treatment (4). In the current study, AuNPs were synthesized by the citrate reduction method, using sodium citrate as a reducing agent. This method was developed by Turkevich and modified by Frens (13, 14). They reported that the citrate acts as a loosely bound capping agent that stabilizes the particles. Moreover, Mackey and El-Sayed and Afifi et al. followed the citrate reduction method in their researches of cancer therapy using AuNPs (15, 16).

Targeting the tumor site is a barrier in designing AuNPs based-drug delivery system. These challenges can be achieved by functionalization of AuNPs by surface ligands, each with specific function (8). In the present study, the PEG was added to functionalize the synthesized AuNPs in order to improve their in vivo stability (6).

In a study done by Prencipe et al., they mentioned that PEGylated AuNPs were stable over a wide pH range and at high temperatures, with the most remarkable result was the long circulation time of PEG-AuNPs (17). Furthermore, Akimya et al. found that PEGylated gold nanorods were able to avoid reticuloendothelial system (RES) clearance and to circulate longer (18).

The present research functionalized the AuNPs with RGD peptide ligand to target the induced OSCC, since the peptide ligands have shown significant targeting potential. It targets $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins that are overexpressed on both cancerous cells and endothelial cells of tumor angiogenic vessels (7). Similarly, Mackey and El-Sayed and Afifi et al. used RGD to functionalize the PEGylated AuNPs in their studies on squamous cell cancer cell line (15, 16). They confirmed the successful active targeting of the cancer cell lines with the AuNPs by the help of RGD and reported an enhanced delivery of the loaded drugs to the cancer cells by the RGD-AuNPs compared to the passive cellular diffusion of the free drug forms. Moreover, Yin et al. demonstrated that RGD-AuNPs can specifically target the melanoma cell line better than the breast cancer cell line, which express the integrin $\alpha_v\beta_3$ (19).

Many chemotherapeutic drugs are used in treating different types of cancers, among them Paclitaxel (PTX), it has a prominent role in anticancer treatment (20). Chen et al. in their studies showed that paclitaxel has been applied to many anticancer treatment regimens and used it for the treatment of ovarian and breast cancer (21). However, the therapeutic efficacy of PTX has been found to induce multidrug resistance and reduce function of important proteins involved in apoptosis (20).

Abraxane® the PTX albumin bound nanoparticle formulation, was developed to overcome the toxicity and low water solubility of free PTX. It was approved by the food and drug administration (FDA) in 2005 for the treatment of lung cancer and metastatic breast cancer (22). Since then, many nanoparticle formulations were investigated to be loaded with PTX for the treatment of wide range of cancers. The current study therefore, used PTX to be loaded on PEG-RGD-AuNPs to target chemically induced OSCC in Syrian hamsters.

In the present study, the clinical results in terms of survival rate and tumor volume revealed the effectiveness of treating the induced OSCC with PTX loaded on AuNPs. The survival rate of hamsters treated with PTX-AuNPs showed prolonged life span with significant decrease in the mean tumor volume. This reveals that the minimal dose of PTX loaded on AuNPs has effective antitumor action with minimal side effects. This was in accordance with Au et al.

in their work, they loaded PTX on AuNPs to treat pancreatic cancer (23). They revealed higher cellular uptake of the loaded PTX with less cytotoxic effects. Similarly, Gibson et al. examined the anti-tumor effect of using AuNPs size 20 nm loaded on it PTX and offers a new alternative for the design of nanosized drug-delivery systems (24).

Comparing to the studies treated induced OSCC, the clinical results of the present study goes with Essawy et al. (25). They reported significant increase of mean survival rate and decrease in the tumor volume in the group treated with actively targeted AuNPs loaded with Doxorubicin. However, these results revealed more tumor inhibition and longer life span. This can be attributed to the combined photothermal therapy and the intralesional injection (IL) of the AuNPs rather than the intraperitoneal IP injection. These results also are in line with Afifi et al., they used only AuNPs as photothermal therapy in induced OSCC in hamster's buccal pouch. They revealed significant tumor volume decrease and increase survival time when injected IL rather than IP. However, they didn't use AuNPs as drug delivery system (26).

In the current study, the histological results in terms of apoptotic index (AI) confirmed that the minimal dose of PTX loaded on AuNPs had an effective cytotoxicity that could be used in the treatment of OSCC. The AI recorded their higher values in the group PTX-AuNPs, with the presence of different stages of apoptosis. This suggested that the success in targeting the tumor site with PTX and the effectiveness of PTX loaded on AuNPs in cancer therapy without affecting normal cells and overcoming the PTX side effects. This is in accordance to Heo et al., they revealed apoptotic tissue reaction to the treatment of breast cancer with PTX loaded on AuNPs and has found that the signaling process of AuNPs on cancer cells (27). This also goes with Manivasagan et al. who used PTX loaded AuNPs in the treatment of breast and ovarian cancer and exhibited strong cytotoxic effect through the induction of apoptosis (28).

The use of different types of microscopical assessments to characterize various tissue changes is essential to report the proposed treatment. In the present study, the therapeutic efficacy of the proposed treatment was done by assessing the AI by light microscope, whereas, the immunhistochemical analysis was done to assess the proliferative activity by PCNA marker. The histological results confirmed the therapeutic anti-cancer effect of loading PTX on AuNPs in the treatment of induced OSCC in HBP. The positive nuclear immunoreactivity showed its highest significant values in the group treated with AuNPs and the saline control group. On the contrary decreased to diminished PCNA immunoexpression was strongly noted in the group treated with PTX-AuNPs. Tumor regression is noted with an inverse correlation between PCNA as a proliferative marker and tumor apoptotic index. These results are in accordance with Afifi et al., who used PCNA and with Essawy et al., who used MCM3 as a proliferative marker (25,26).

CONCLUSIONS

The highest decrease in the mean tumor volume, the highest survival rate and the highest mean AI were recorded in the group treated with PTX-AuNPs.

The conjugation of the chemotherapeutic drug to the AuNPs targeted cancer cells while maintaining no adverse effects on the surrounding normal cells, with reducing the dose of the used chemotherapeutic, and preserving its anticancer effect.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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