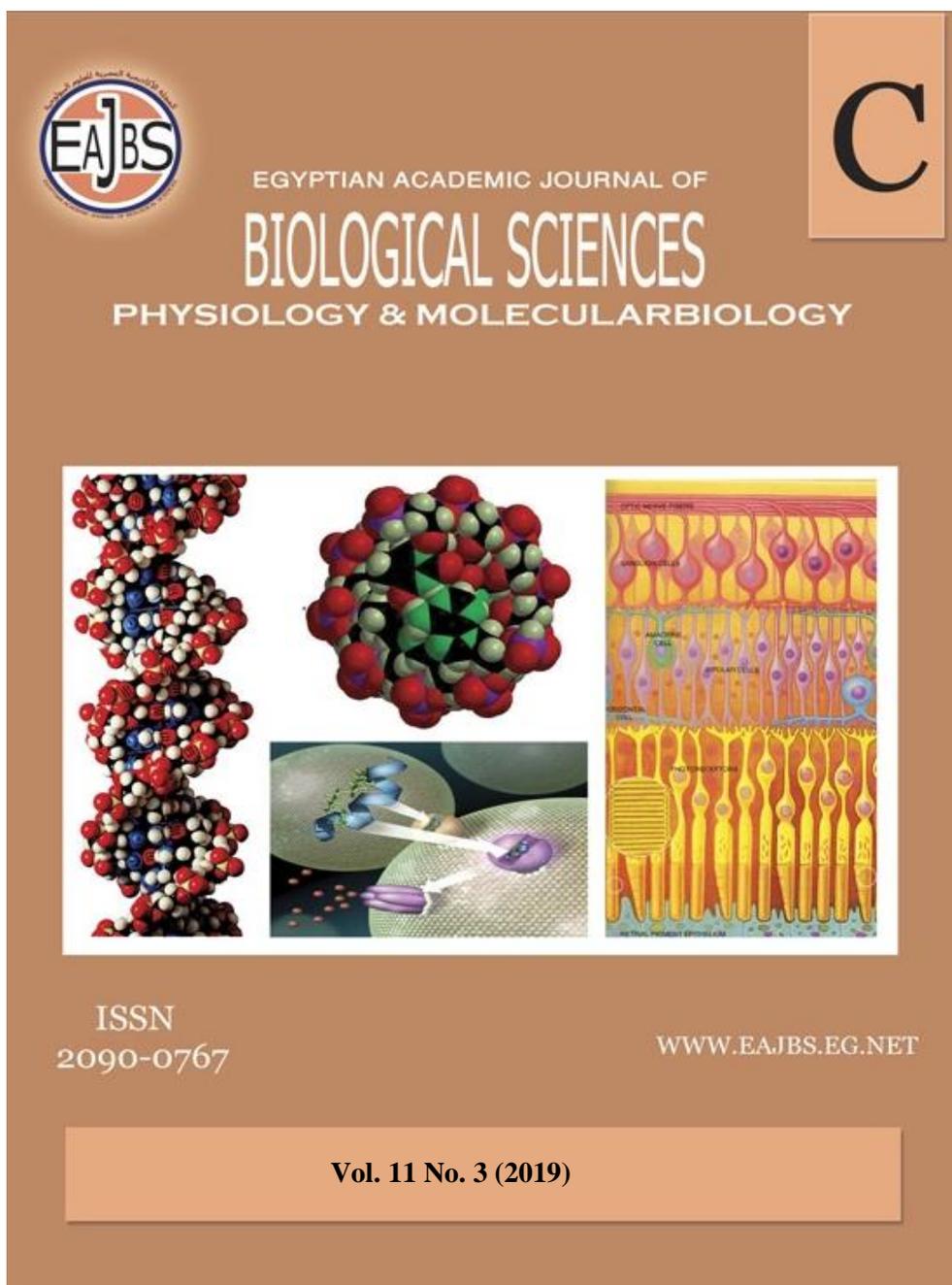


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DNA Damage in Ehrlich Carcinoma induced by Gold Nanorods Mediated Photothermal Therapy

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ABSTRACT

Gold nanorods (AuNRs) are promising nanoplatforms for cancer photothermal therapy due to their capability to absorb light in the near-infrared (NIR) window. This work was conducted to evaluate the DNA damage in Ehrlich tumor tissues associated with photothermal therapy mediated with very small sized pegylated gold nanorods (PEG-AuNRs). The accumulated gold nanorods inside tumor tissues were photo-excited with 300 mW NIR laser for 50 min. The physical properties of PEG-AuNRs were investigated using transmission electron microscopy, size distribution measurement, and UV-vis absorption spectroscopy. The efficacy of the in-vivo photothermal therapy of intravenously injected PEG-AuNRs was assessed using tumor size measurements. Also, oxidative stress and comet assay were performed to measure the percentage of DNA damage. The results showed that PEG-AuNRs were successfully prepared with a length of 8 nm and a longitudinal absorption peak at 800 nm. FTIR study confirmed the successful coating of polyethylene glycol on the surface of the gold nanorods. The in vivo experiment showed that PEG-AuNRs elicited tumoral growth arrest by producing free radicals in addition to its photo heat conversion which was sufficient to induce cellular damage by causing DNA damage.

INTRODUCTION

Nanomedicine is the medical application of nanoparticles to diagnose and treat human diseases such as infectious diseases (Li *et al.*, 2019; Yang *et al.*, 2018) and cancer (Wang *et al.*, 2017; Ocsoy *et al.*, 2017). Several types of nanomaterials can be used in cancer therapy such as gold nanoparticles (Singh *et al.*, 2018), silver nanoparticles (Rageh *et al.*, 2018), magnetic nanoparticles (Wu & Huang, 2017) and copper oxide nanoparticles (EG& AA, 2018).

Among different nanomaterials, gold nanoparticles have drawn considerable attention due to their localized surface plasmon resonance (Huang & El-Sayed, 2010; Tong *et al.*, 2009; Amendola *et al.*, 2017). When the light of resonant frequency interacts with free electrons on the surface of nanoparticles, it causes collective oscillation of the conduction band electrons resulting in localized surface Plasmon resonance (Cao *et al.*, 2014; Huang & El-Sayed, 2010).

Presently, significant concern has been paid for using gold nanorods in plasmonic photothermal therapy (PPTT) as a cancer ablation technique. This promising rod-shaped gold nanoparticles not only have unique physicochemical properties, good biocompatibility and chemical stability (Tong *et al.*, 2009) but also have the capability of transforming absorbed near-infrared (NIR) light into heat which is efficient to ablate cancer cells (Chen *et al.*, 2018). The absorption can be tailored to absorb a wide range of light wavelengths by adjusting their aspect ratio (Alekseeva *et al.*, 2006). More importantly, NIR laser irradiation is the most suitable for the clinical application due to its deep penetration (Castillo-Martínez *et al.*, 2015).

In this study, the aim is to evaluate the mechanism of gold nanorods inducing damage to tumor cells by assessing oxidative stress induction which leads to the genotoxic effect. This genotoxic effect induced by gold nanorods mediated photothermal cancer ablation was evaluated by comet assay.

MATERIALS AND METHODS

Silver nitrate (AgNO₃), Tetrachloroauric acid (HAuCl₄), sodium borohydride (NaBH₄), cetyltrimethyl ammonium bromide (CTAB), Ascorbic acid, hydrochloric acid (HCl), methoxy-PEG-thiol (molecular weight 6000) m PEG-SH were obtained from Sigma Aldrich The Superoxide dimutase assay kit, lipid peroxide kit and phosphate buffer pH 7.4 were obtained from Biodiagnostic company, Egypt.

1. Synthesis of Small Gold Nanorods (AuNRs):

Gold nanorods were synthesized according to a previously reported

seed-mediated method with slight modifications (Jia *et al.*, 2015).

For PEG coating of AuNRs, (0.5ml, 1mM) of PEG solution was added to 10 ml AuNRs. Then the mixture was kept under vigorous stirring for at least 4h. Then the excess mPEG-SH molecules were removed by centrifugation (10000g, 20 min). Finally, the precipitate was redispersed into 10 ml deionized water (Zhu *et al.*, 2014).

2. Characterization of the Prepared AuNRs:

The size and morphology of AuNRs and PEG-AuNRs were assessed using high-resolution transmission electron microscopy (TEM) (Jeol JEM1230). The absorption spectrum of PEG-AuNRs was measured in the wavelength range from 400 to 900 nm using UV-Vis spectrophotometer (Jenway, Barloworld scientific (UV-6420), UK). PEG-AuNRs size distribution was measured using dynamic light scattering (Malvern panalytical, Zetasizer Nano ZS90).

3. In Vivo Photothermal Therapy:

Male albino mice around 25 gm body weights were injected with Ehrlich ascites fluid subcutaneously in their right flanks. Ten days later tumors developed into a single solid form. Thirty mice were initially used and divided into three groups. Mice of control group A which received no injections. Mice of Laser group B were subjected to NIR irradiation for 50 mins. Mice of PEG-AuNRs treated group C were injected intravenously with two hundred microliters of PEG-AuNRs via the tail vein, after 24 hrs the mice were subjected to NIR irradiation for 50 mins. All animal studies carried out in accordance with the institutional animal care and use committee (IACUC), Cairo University.

4. Measurements of the Change In Tumor Size:

The tumor size measurements were performed over a 12-day period for the three groups. The change in tumor volume (V) was calculated as previously described in our previously published work (Monem *et al.*, 2014). The data were represented as the mean \pm standard deviation (SD). The statistical evaluation of the tumor size data was analyzed using SPSS v.19. Significant differences between groups were assessed using one-way analysis of variance (one-way ANOVA). P-values less than 0.05 were considered statistically significant.

5. Oxidative Stress Analysis:

Tumor tissues from different experimental groups were excised 2 hours after laser irradiation homogenized in cold phosphate-buffer. Then centrifuged at 4000 rpm for 10 mins, the supernatants were used for measurement of MDA level and SOD activity. The SOD assay relies on the ability of the enzyme to prevent the reduction of nitroblue tetrazolium (NBT) dye by phenazine methosulfate (PMS). The change in the absorbance was measured at 560 nm for 5 min. SOD activity can be expressed as a function of tissue weight in gm (Nishikimi *et al.*, 1972).

$$\text{u/gm tissue} = \% \text{ inhibition} \times 3.75 \times (1/\text{gm tissue used})$$

Malondialdehyde (MDA) is a colorimetric assay used in measuring the amount of lipid peroxidation. The assay measures the reaction of thiobarbituric acid with MDA which gives a pink complex that can be detected at 532 nm (Uchiyama and Mihara 1978).

6. Histopathological examination of tumor tissues.

To obtain a complete picture of the therapeutic effect of PEG-AuNRs, the morphology of tumor

tissues was examined under light microscopy. After 24 hrs. of treatment mice were sacrificed by sudden decapitation method. Then tumors were removed, fixed in neutral formalin (10%), embedded in paraffin, sectioned and then stained with hematoxylin and eosin (H&E). Tissue sections were imaged using a light microscope connected with a camera (CX31 Olympus microscope, Canon).

7. Single Cell Gel Electrophoresis of Tumor Tissues:

Comet assay is a simple, sensitive, rapid and visual technique to evaluate the early apoptosis stage and percentage of DNA damage (Moller, 2006; Moller *et al.*, 2000; Awara *et al.*, 1998). The amount of DNA damage in tumor cells can be evaluated by measuring the percentage of migrated DNA and its length.

Then, the Olive moment was computed using (Comet 5 image analysis software developed by Kinetic Imaging, UK) linked to CCD camera.

RESULTS AND DISCUSSION

The physical properties of pegylated gold nanorods were investigated by TEM, size distribution measurements, FTIR and UV-vis absorption spectroscopy. TEM Images showed the successful preparation of rod shape PEG-AuNRs (Fig. 1a). The absorption spectrum of PEG-AuNRs showed two absorption peaks one at 550 nm and the other in 800 nm. The strong absorption peak at 800 indicates that PEG-AuNRs can efficiently absorb NIR laser at 800 nm so it's a very suitable and promising candidate for cancer photothermal therapy (Fig. 1b). Water and hemoglobin have very low absorption in the NIR region, which in turn allows NIR light to penetrate several centimeters into tumor tissues.

The average diameter of gold nanorods was measured by dynamic

light scattering. In the case of rod-shaped particles, the device gives information about the diameter of the nearest sphere. The average diameter

of the nearest sphere to PEG-AuNRs was 8.12 ± 2.9 nm with PDI of 0.683(Fig. 1c).

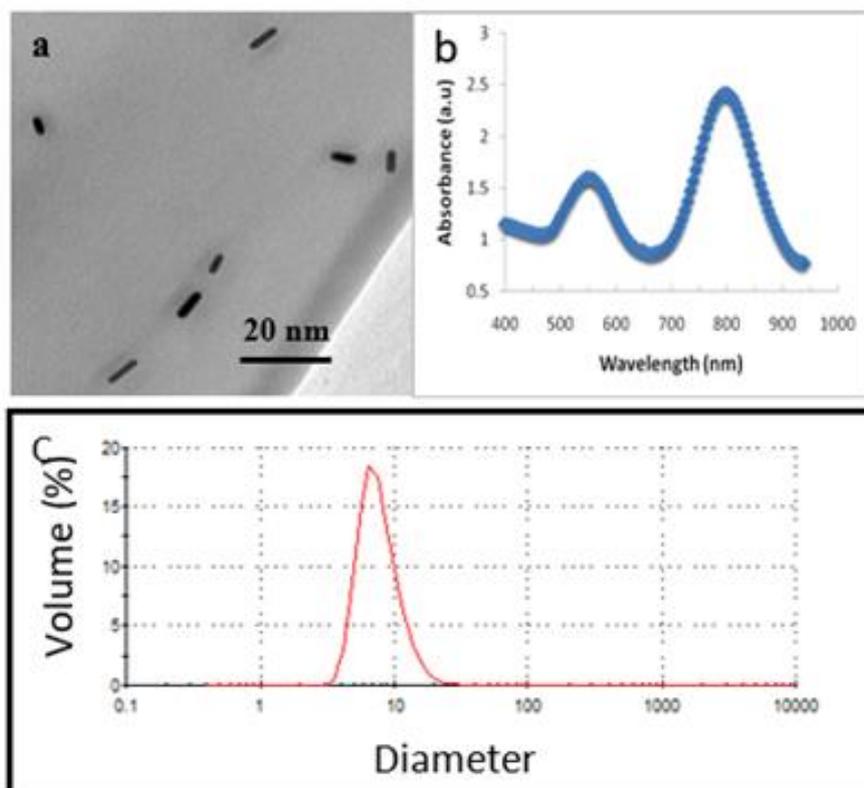


Fig. 1: a: TEM image of the PEG-AuNRs b: UV-Vis absorption spectrum (b) and c: Dynamic light scattering size distribution

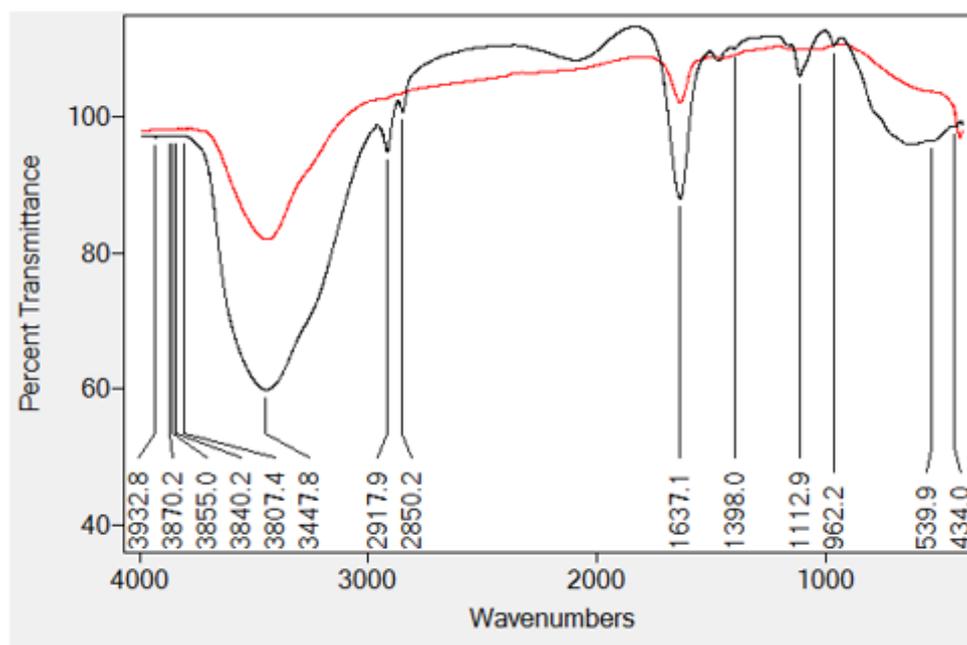


Fig.2: The FTIR spectra of PEG-AuNRs and AuNRs

FTIR of AuNRs and PEG-AuNRs was performed to investigate the successful replacement of CTAB molecules with PEG layer and formation of PEG-AuNRs (Fig.2). A characteristic absorption peaks at 2917.9 and 2850.2 cm which is due to C–H stretch vibration were noticed in the AuNRs spectrum. While this peak can't be observed in the spectrum of PEG-AuNRs, which proved the replacement of CTAB surfactant with PEG-SH.

This result agreed with previously reported data. These results confirmed the successful modification of AuNRs surface with PEG.

The effect of PEG-AuNRs mediated photothermal therapy (PTT) for inhibiting tumor growth was investigated using tumor size measurements, and comet assay in addition, measurement of oxidative stress induction. The Change in tumor volume was monitored over 12 days period. A significant difference ($P < 0.001$) was observed for PEG-AuNRs treated group C which subjected to photo-excitation with NIR laser compared to group A and B (Table 1). These results indicated that PEG-AuNRs mediated photothermal therapy was more effective than the NIR

treated group. No significant differences can be observed in tumor volumes for the control group and the laser-irradiated group. The above results indicate that intravenous administrated PEG-AuNRs elicited tumoral growth arrest when associated with NIR laser photo excitation.

Histopathological examination of Ehrlich tumor sections shows the presence of some necrotic regions in NIR treated group (Figs. 3a & b). This means that NIR penetrated the tissues and induced damage in some regions. For PEG-AuNRs treated group c, Ehrlich tumor sections show loss of the major cancer cell details with the presence of some apoptotic bodies (Figs. 3 c & d). The presence of apoptotic bodies may be attributed to the special effect of smaller size pegylated gold nanorods which passively accumulate in tumor tissues. Then internalized and produce ROS which induced apoptotic damage to the cancer cells. In addition, when the tumor region photo excited with NIR laser the passively accumulated PEG-AuNRs absorb NIR laser and convert it into localized heat which selectively causes cancer cell damage by protein denaturation and membrane damage.

Table 1: the change in Ehrlich tumor volume for 12 days period

Group	Δv_3	Δv_6	Δv_9	Δv_{12}
Group A	0.0429±0.021	0.062 ±0.019	0.069 ±0.025	0.0978 ± 0.0302
Group B	0.026 ±0.017	0.048±0.023	0.0542 ±0.0216	0.0785 ±0.029
Group C	-0.0066 ±0.0037	-0.0225 ±0.028	-0.0305 ±0.0317	±0.0198 ±0.014

The above results motivated us to further evaluate the degree of lipid peroxidation and SOD activity. In addition to the amount of DNA strand break was measured using single-cell gel electrophoresis. The results showed a significant decrease ($P < 0.0001$) in the SOD enzyme level for PEG-AuNRs treated group C compared to groups A and B (Table 2). In addition

to the significant increase in MDA level in tumor tissues excised from PEG-AuNRs treated group C by approximately two folds compared to groups A and B. this means that treatment with small sized PEG-AuNRs produces ROS which inhibit the antioxidant system and increase the level of lipid peroxidation.

Table 2: SOD enzyme level, MDA level, and different comet parameters. Data present as mean \pm standard deviation

Group	SOD (u/gm)	MDA (nmol/ μ l)	Comet %	Tail Length (px)	%DNA in Tail	Tail Moment	Olive Moment
Group A	628.71 \pm 97.41	59.8 \pm 5.86	10.4 \pm 1.15	5.6 \pm 1.03	4.3 \pm 0.73	0.202 \pm 0.059	0.631 \pm 0.066
Group B	575.63 \pm 86.85	61.9 \pm 3.43	10.5 \pm 0.503	5.9 \pm 0.442	4.56 \pm 0.105	0.223 \pm 0.016	0.73 \pm 0.06
Group C	121.4 \pm 29.48	112.7 \pm 7.98	15.726 \pm 1.26	7.47 \pm 0.904	6.72 \pm 1.25	0.443 \pm 0.079	1.155 \pm 0.14

ROS produced are very reactive and caused oxidative damage that is the major source of DNA damage (Rageh *et al.*, 2018). Comet assay for Ehrlich solid tumor tissues showed the formation of the comet in PEG-AuNRs groups. DNA damage was measured as percent DNA in tail and the olive moment was computed for the three groups. These parameters reflected the number of DNA breaks.

PEG-AuNRs treated group C exhibited a significant increase in all comet parameters compared to groups A and B (Table 2). In addition to comet images for PEG-AuNRs treated group C (Fig. 4c) shows a DNA break that lost its supercoiling structure then became free and extended forming tail structure compared to the coiled structure that appeared for group A and B (Figs. 4a and b) respectively.

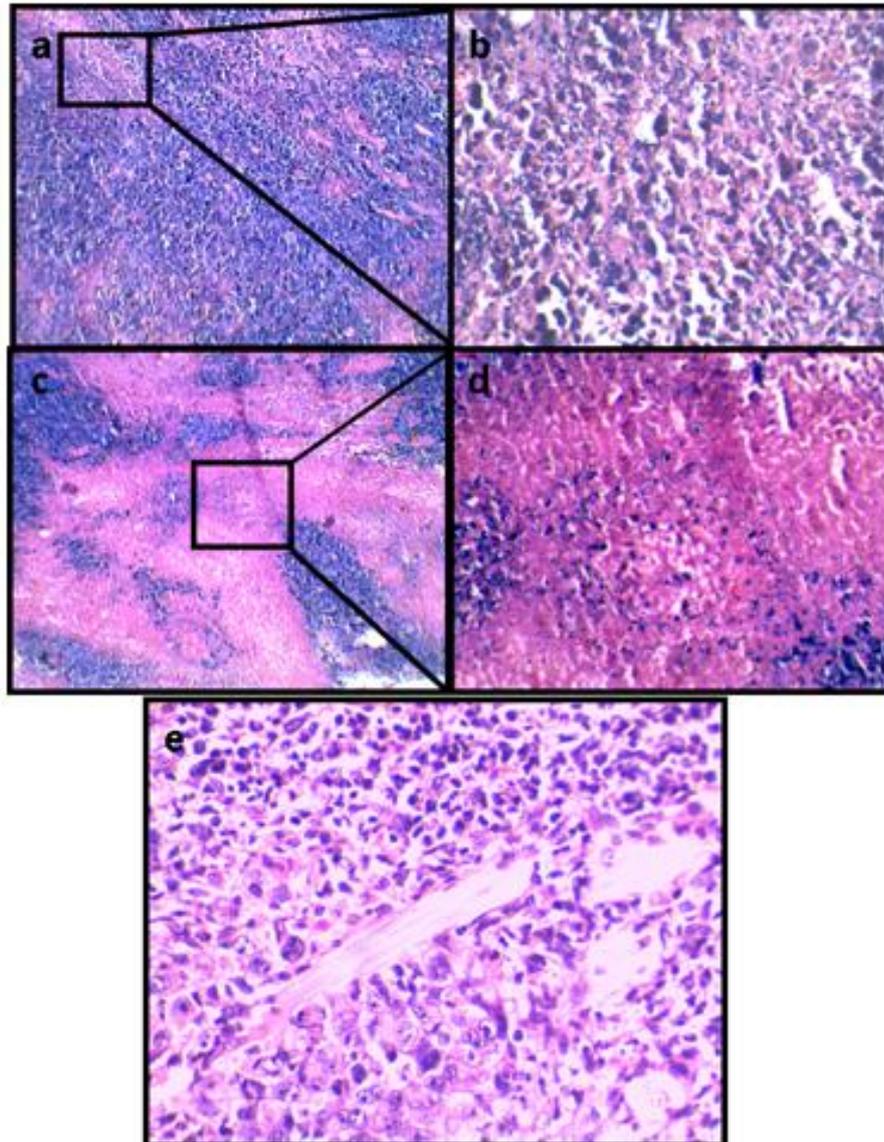


Fig. 3 : Histopathological examination of Ehrlich tumor cells of group B (a,b), Group C (c,d) and control group A(d). a, c x100, b,d,e x400.

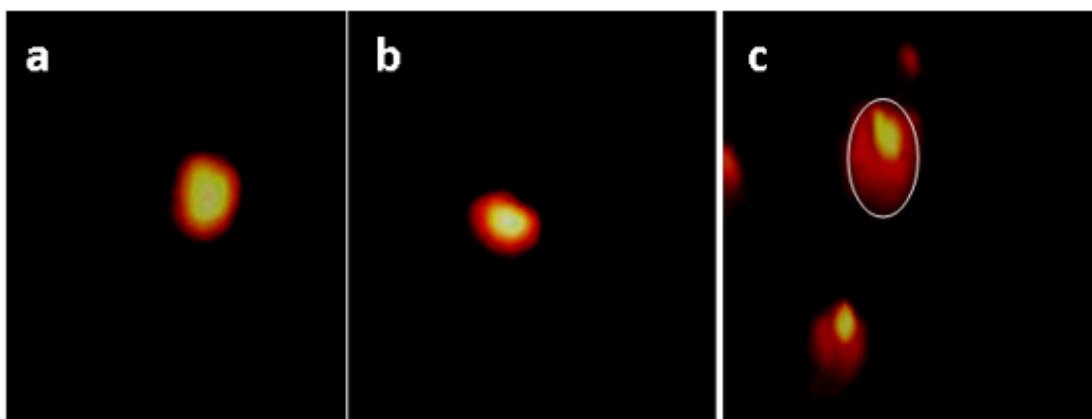


Fig. 4: comet images of tumor excised from group A (a), group B (b) and group C (c)

Conclusion:

Very small sized pegylated gold nanorods were successfully prepared with an average diameter 8nm. These nanoparticles exhibited a strong NIR absorption which made them ideal candidates for use in both NIR imaging and photo thermal therapy. The prepared nanoparticles have several advantages as photo thermal contrast agents because of their biocompatibility, long-circulating time, passive accumulation and its capability to convert NIR laser into heat which lead to cancer cell damage. Treatment of Ehrlich carcinoma with PEG-AuNRs induced cancer cell growth arrest which was due to the formation of free radicals and the exposure to localized heat that in-turn induced damage on the molecular level.

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ARABIC SUMMARY

العلاج الضوئي الحراري باستخدام جزيئات الذهب الاسطوانية المغطاة بالبولي ايثيلين جليكول المسببة لتلف الحمض النووي لورم الايرلش

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تعتبر جزيئات الذهب النانومترية جزيئات واعدة في العلاج الضوئي الحراري للسرطان لقدرتها على امتصاص الضوء في منطقة الأشعة تحت الحمراء. يهدف هذا العمل الى تقييم تلف الحمض النووي في ورم الايرلش نتيجة العلاج الضوئي الحراري باستخدام جزيئات الذهب النانومترية صغيرة الحجم. تم تعريض جزيئات الذهب النانومترية بداخل الورم الى شعاع الليزر بقوة 300 مللي وات لمدة خمسين دقيقة. تم قياس الخصائص الفيزيائية لجزيئات الذهب باستخدام الميكروسكوب الالكتروني والحجم وطيف الامتصاص. وتم قياس نجاح العلاج الكهروضوئي بمتابعة حجم الورم. كذلك تم قياس ضغط الاكسدة وجزيئات الكومت لقياس نسبة تلف الحمض النووي. لقد أثبتت النتائج نجاح تحضير جزيئات الذهب النانومترية وطولها 8 نانومتر وموجة امتصاص طولية 800 نانومتر. ولقد أثبت قياس الاشعة تحت الحمراء نجاح تغطية جزيئات الذهب بالبولي ايثيلين جليكول. وقد أثبتت التجربة نجاح الجزيئات النانومترية في تقليص حجم الورم بانتاج الشوارد الحرة بالاضافة الى قدرتها على تحويل الضوء الى حرارة التي كانت كافية لاحداث تلف الخلايا باتلاف الحمض النووي.