Antioxidative stress of Chitosan-Gallium Nanoparticles and low doses of γ -irradiation on mice bearing Ehrlich carcinoma

Riham M Mohammad 1, Neamat H Ahmed 2, Eman I Kandil 3 and Abdelfattah M Badawi4.

1 Department of Biochemistry, Faculty of Science, Ain Shams University, Cairo, Egypt.
rihamabdelmoula@gmail.com, Tel. +201145029005

2 Radiation biology department, national center for radiation research and Technology,
Atomic Authority. neamathanafi@gmail.com, Tel. +201001731786

3 Department of Biochemistry, Faculty of Science, Ain Shams University, Cairo, Egypt.
kandil.eman@yahoo.com, Tel. +201271174112

4 Petrochemical Department, Egyptian Petroleum Research Institute, Cairo, Egypt.
badawi8@yahoo.com, Tel. +20111749248

Abstract

Guided treatments with nanoparticles and low doses of radiation are a new approach in cancer therapy. This study evaluated the ability of Chitosan-Gallium nanoparticles (Ch.GaNP) and low dose of γ -radiation to suppress oxidative stress of female mice bearing solid Ehrlich carcinoma (EC) in the neck region. Begins from 7th day of EC cells inoculation Ch.GaNP (0.5 mg/kg body weight) were orally administered via gavages for a period of 15 consecutive days and Whole-body γ -irradiation was carried out at a single dose of 0.25Gy/twice a week for 2 weeks. Tumor size was monitored. Oxidative stress markers were assessed, histopathological changes in tumor and liver tissues were studied. In addition, the angiogenic markers concentrations were evaluated. Treatment of EC-bearing mice with Ch.GaNP and/or low dose of γ -radiation exposure significantly reduced tumor volume, increased lipid peroxidation and decreased glutathione content as well as glutathione peroxidase activity in tumor and liver tissues. Moreover, the dual treatment significantly reduced serum VEGF, PDGF and TNF- α levels, and significantly elevated Cas-3 activity. These results were well appreciated with the histopathological findings in the tumor and liver tissue. In conclusion, the combined treatment of Ch.GaNP and low dose of γ -radiation have a role in suppressing oxidative stress of female mice bearing solid Ehrlich carcinoma.

Key words: Chitosan-Gallium Nanoparticles, low doses of γ -irradiation, Ehrlich carcinoma

Introduction

There has been a huge interest in experimental tumor for the purposes of modeling, and Ehrlich ascites carcinoma (EAC) is one of the most common. EAC is mentioned as an undifferentiated carcinoma, and is originally hyper diploid, rapid proliferation, has high transplantable capability, no-regression, shorter life span, 100% malignancy and does not have tumor specific transplantation antigen (TSTA) [1].

Radiotherapy is one of the most significant processes for tumor regression [2]. Lately, scientists have revealed that low-dose radiation can grant beneficial biological effects indicating that low doses of radiation could combat cancer formation and reduce tumor prevalence and metastasis [3,4]. Researchers have inspected the influences of low-dose

radiation, given as a single dose or fractionated or prolonged over time, with respect to modifying cancer development or elimination while others have concentrated on the effects of low-dose radiation on the systemic immune system[4,5].

Nanobiotechnology, an emergent field of nanoscience, uses nano based-systems for diverse biomedical applications. This speedily emerging field of nanoscience has extended the prospect of utilizing therapeutic nanoparticles in the diagnosis and treatment of human cancers. Nanoscale particles and molecules are a probable alternate for cancer treatment because they hold unique biologic effects based on their size and structure, which differ from traditional small-molecule drugs[6].

There has been a revival in the development of natural products as anticancer agents[7]. Chitosan is proven to have numerous biological actions involving immune enhancing effects, anti-inflammatory, antitumoral, antimicrobial, antifungal activities and drug carrier[8]. The small size of ChNPs makes them able to pass through biological barriers in vivo (such as the blood–brain barrier) and makes them not only proper for parenteral route, but also applicable for mucosal routes of administration, i.e., nasal, oral, and ocular mucosa, which are non-invasive route[9].

Gallium (Ga) is the second metal ion to be used in cancer treatment[10]. A constant concern in Ga compounds is due to the recognized ability of Ga cations to delay tumor growth, and increased bioavailability and efficacy supported by the conversion of Ga into chelate complexes. The mechanism of action of Ga does not seem to affect DNA, but more because of its effect on ribonucleotide reductase, with a competitive effect with iron. Numerous biological effects have been noticed on membrane permeability, mitochondria, the cytoskeleton, the activity of various enzymes involved in the development of cancer cells and proteasome activity[11]. The curative properties of Ga led to the development of innovative formulations for treatment of diverse disorders in humans, including autoimmune diseases, cancer, accelerated bone resorption and infectious diseases[12]. Our study was carried out to evaluate the antioxidative effect of Chitosan-Gallium Nanoparticles and/or low-dose gamma radiation on mice suffering from Ehrlich carcinoma.

Material and methods

Chemicals

Chemicals and reagents used in the study were of high analytical grade. Chemicals and reagents used in the study were of high analytical grade. Propidium Iodide with Mwt of 668.4 was purchased from MP Biomedicals Co. (France) and Acridine Orange was purchased from LOBAL chimie Co. (France).

Cell line

The EAC cell line used in this study was supplied by The Egyptian National Cancer Institute (NCI), Cairo University (Giza, Egypt). The cell line was maintained by weekly intraperitoneal injection of 2.5 million cells in a volume of 0.2 mL per mouse in female Swiss albino mice. The EAC cells were diluted using physiological sterile saline solution, and cells were counted using the Bright-LineTM hemocytometer.

Animals

Adult female Swiss albino mice of 8 weeks old and 22-25g weight purchased from the breeding unit of the Egyptian Organization for Biological Products and Vaccines were used in this study. The animals were maintained on a commercial standard pellet diet and tap water. Animal maintenance and treatments were conducted in accordance with the National Institute of Health Guide for Animal, as approved by Institutional Animal Care and Use Committee (IACUC).

Radiation facility

Irradiation was performed at the National Center for Radiation Research and Technology (NCRRT), Nasr city, Cairo, Egypt. The source of radiation was (^{137}Cs) which ensured a homogenous dose distribution all over the irradiation ray. The experimental animals were exposed to 0.25Gyx2/week for two weeks of γ - radiation.

Methods (work flow design)

Chemical studies

Preparation of nanoparticles

The preparation took place in Egyptian Petroleum Research Institute), Nasr city, Cairo, Egypt. Gallium metal was mixed mechanically very well with Chitosan (1:3) using vortex. Then both were grind to nano-sized particles using ball mill at 200 rpm for 10 hours. The Chitosan-Gallium nanoparticles (Ch.GaNP) were obtained.

Characterization of Ch.GaNP

Size and morphology of Ch.GaNP were done by using transmission electron microscopy (TEM). Samples for TEM were prepared using the clear solution of nanoparticles. The sample solution was put on a formvar coated grid. On this grid, a drop of the sample solution (containing dispersed nanoparticles) was placed and allowed to air-dry. A TEM picture was taken by a JOEL JEM 2000 EX200 microscope.

Biochemical studies

Evaluation of therapeutic antitumor efficacy of Ch.GaNP.

This study was designed to comprise a series of in vitro and in vivo investigations as follows.

In vitro study (Cytotoxicity of nanoparticles):

Antitumor effect of Ch.GaNP was assessed by observation of changes with respect to viable and nonviable Ehrlich ascites tumor cell count. Cytotoxicity effects of the nanoparticles on tumor cells were determined according to the method of **El- Merzabani et al., [13]**. In order to detect the cytotoxicity of nanoparticles, EACs were treated with Ch.GaNP at the concentrations of 0, 10, 20, 30, 40, 50, 100, 110, 120, 130, 140, 150 and 160 $\mu\text{g/ml}$. The EACs were obtained by needle aspiration of ascites fluid from the pre-inoculated mice under aseptic condition using ultra violet laminar air flow system. The percentages of non-viable cells were determined by counting dead and viable EACs. To differentiate between dead and viable EAC cells, trypan blue stain was used. Then the percentages of non-viable cells (NVC) were calculated according to the following equations $\% \text{ NVC} = \frac{C}{T} \times 100$, where (C) is number of non-viable cells and (T) is total number of viable cells.

In vivo study.

Experimental design: The animals were allowed 7 days for adaptation. 50 mice were then randomly distributed into 5 equal groups, 10 mice for each. The animal groups were recognized as follows:

1. Group (1): Normal Control group (N.C): the mice in this group served as controls and were neither treated nor irradiated.
2. Group (2): Ehrlich Carcinoma-bearing group (E.C), 10 mice were subcutaneously injected with 0.2ml of $2.5 \times 10^6/\text{ml}$ /mouse viable EAC cells in the neck region.
3. Group (3): Ehrlich carcinoma irradiated group (E.IR): 10 mice bearing EC were subjected to 0.25Gy x2/week for 2 weeks begins on the 7th day after EAC inoculation.

4. Group (4): Ehrlich carcinoma Gallium-Chitosan nanoparticles treated group (**E.Ch.Ga**): 10 mice bearing EC were gavages with 0.5mg/kg/day for 15 successive days Ch.GaNPs starting from the 7th day after EAC inoculation.
5. Group (5): Ehrlich carcinoma Gallium-Chitosan nanoparticles irradiated group (**E.Ch.Ga.IR**): 10 mice bearing EC treated with Ch.GaNPs and subjected to γ – irradiation starting from the 7th day after EAC inoculation.

Monitoring the tumor size

Tumor size was monitored twice or thrice weekly throughout the experiment. The tumor size being measured regularly using Vernier calipers and represented in terms of tumor size. The tumor size was estimated using the following formula: Tumor size (mm³)= 0.52 AB², where A is the major axis and B is the minor axis[14]. The mean tumor size with the corresponding standard error was calculated in each experimental group.

After 24 hours from the last dose of Ch.GaNPs treatment, all experimental mice were sacrificed and immediately, blood was collected via heart puncture. The liver and tumor tissues were quickly excised from each animal and washed with saline. Small parts from liver and tumor were placed in 10 % phosphate-buffered formalin to be used in histopathological and apoptotic and necrotic examinations. The rest of the liver and tumor tissues were homogenate in 0.25 M ice-cold isotonic sucrose to be used for the estimation of the biochemical assessed parameters.

Biochemical analysis

Serum(TNF- α , VEGF, PDGF and Casp-3) were assayed by the standard sandwich enzyme-linked immune-sorbent assay (ELISA) technique using ELISA kit purchased from MyBioSource, California, USA. In tumor and liver homogenate all biochemical analysis was performed. The level of malondialdehyde (MDA), the end product of lipid peroxidation (LPO) was measured according to the method of Yoshioka *et al.*[15]. Glutathione (GSH) concentration was determined by the method of Beutler *et al.*[16]. Glutathione peroxidase (GSHPx) concentration was determined by the method of Grosset *et al.*[17].

Histopathological examination

At the time of sacrifice, the tumors were excised from the animals. Samples from each tumor tissues were fixed in 10 % formalin and embedded in paraffin. Sections of 5- μ m thickness were cut and stained with hematoxylin and eosin[18]. and examined by light microscope for histopathological investigation.

Statistical analysis

The obtained data were expressed as mean $\pm$ standard error (SE). All data were analyzed statistically using one-way analysis of variance (ANOVA) followed by Student's t-test. Statistical significance was considered at $P < 0.05$. Statistical Package for Social Sciences (SPSS) for Windows version 20.0 software was used for this analysis.

Results

1. Chemical studies

1. Characterization of nanoparticles

Figure(1) represents the morphology and size of Ch.GaNPs. Ch.GaNPs, as shown by the TEM images, are crystal shaped uniform solid dense structure and have nearly uniform particle size distribution, which is very important for drug delivery. Average particle size of Ch.GaNPs is 7.5 nm $\pm$ 0.5 nm.

Figure (1): Morphology and size of nanoparticles**2. Cytotoxicity of Ch.GaNP on Ehrlich ascite carcinoma cells (EAC)**

The tumoricidal effect of different concentrations of Ch.GaNP on Ehrlich ascite carcinoma cells viability was evaluated in table (1) and figure (2). The low concentration (10 $\mu\text{g/ml}$) of Ch.GaNP decreases the tumor cells viability by 10%. For concentration of 50 $\mu\text{g/ml}$ led to the death of 50% of EAC cells and 30% of these cells appeared to be burst. At concentration of 140 $\mu\text{g/ml}$ Ch.GaNP, all Ehrlich carcinoma cells appeared to be burst.

Table (1): The effect of Ch.GaNP on the viability of EAC cells.

Ch.GaNP			
Ch.GaNP($\mu\text{g/mL}$)	% of viable cells	% of dead cells	% of burst cells
0	99	1	-
10	90	10	-
20	80	20	-
30	60	40	-
40	60	40	10
50	50	50	30
100	40	60	40
110	30	80	50
120	10	90	70
130	0	100	90
140	0	100	100
150	0	100	100
160	0	100	100

Figure (2): The effect of Ch.GaNP on the viability of Ehrlich ascite carcinoma cells. A) Photomicrograph of Ehrlich carcinoma cells line representing 100 % of viable cells ($\uparrow$). B) Photomicrograph of Ehrlich carcinoma cells line as affected by Ch.GaNP.

Notice: underwent apoptotic ascite carcinoma cells (↑), rupture ascite carcinoma cells (▲) and dead cells (curved arrow).

3. In vivo study.

1. Monitoring the tumor size

Monitoring of Ehrlich solid tumor size cleared that Ch.GaNP's have anti-proliferation effect on solid tumor (Figure 3). The inoculation of EC cells in the neck region of female mice produced a tumor with a mean size of $137.8 \pm 3.2 \text{ mm}^3$ on the 7th day after tumor inoculation (ATI). Tumor size proceeds with time reaching $3051 \pm 7.6 \text{ mm}^3$ at the 21st ATI. Exposure of the tumor-bearing animals to low dose γ -radiation produced significant tumor size regression, which reached $1020 \pm 11.8 \text{ mm}^3$ on the 21st day ATI. When mice bearing EC were treated with 15 successive doses of 0.5 mg/kg/day Ch.GaNP's, significant reduction in tumor size occurred and recorded $472 \pm 4.3 \text{ mm}^3$ on the 21st days ATI. While treatments of tumor-bearing mice with Ch.GaNP's and γ -radiation caused a significant delay on EC progression, and tumor size recorded $107.5 \pm 6.3 \text{ mm}^3$ on the 21st day ATI.

Figure (3): Effect of Ch.GaNP's and/or γ -radiation on tumor volume of mice bearing EC.

2. Biochemical analysis

1. LPO level and antioxidant status in tumor tissues

The results presented in Table 2 showed that the mean value of tumor LPO, GSH levels and GSH-Px activity were 107.4 ± 2.9 , 3.3 ± 0.09 and 0.82 ± 0.03 , respectively. The results revealed that the treatment of EC-bearing mice with Ch.GaNP's and/or exposure to low dose of gamma radiation caused a significant increase in LPO level. While the activity of tumor GSH and GSH-Px decreased upon treatment with Ch.GaNP's and/or exposure to low dose of γ -radiation and showed a significant decrease in E.Ch.Ga and E. Ch.Ga.IR groups when compared to EC tumor group.

Table (2): Effect of Ch.GaNP's and/or γ -irradiation on Tumor LPO levels and antioxidant status in different groups in mice bearing E.C

Parameters Groups	TBARS ($\mu\text{mol/gm}$)	GSH (mg/gm)	GSH-Px (mg GSH/min/gm)
EC	107.4 ± 2.9	3.3 ± 0.09	0.82 ± 0.03
E.IR	$125 \pm 1.6^{b^{\dagger}}$	$2.5 \pm 0.09^{b^{\dagger}}$	$0.79 \pm 0.01^{N.S}$
E.Ch.Ga	$197.5 \pm 3.6^{b^{\dagger}}$	$1.7 \pm 0.1^{b^{\dagger}}$	$0.73 \pm 0.009^{b^{\dagger}}$
E. Ch.Ga.IR	$138.6 \pm 3.2^{b^{\dagger}}$	$1.2 \pm 0.09^{b^{\dagger}}$	$0.71 \pm 0.006^{b^{\dagger}}$

Values are expressed as Means of 10 records $\pm$ standard Error (M $\pm$ SE).

b[†]: highly significant against EC at ($P \leq 0.01$)

b[‡]: very highly significant against EC at ($P \leq 0.001$).

N.S: non-significant.

1. LPO level and antioxidant status in Liver tissues

The results in Table 3 showed that the mean value of normal control group of Liver LPO & GSH levels and GSH-Px activity were 167 ± 2.9 , 7.8 ± 0.3 and 1.66 ± 0.005 , respectively. The obtained results revealed that EC-bearing mice experienced a very highly significant increase in liver LPO level in comparison with control level. Meanwhile, a very highly significant decrease in liver GSH and GSH-Px content were observed in comparison with the NC values. Whole body γ -irradiation of experimental mice-bearing solid EC revealed a very highly significant decrease in liver content of LPO in comparison with EC-bearing mice. Meanwhile, a very highly significant increase in GSH and GSH-Px content was observed in comparison with EC-bearing mice. Treatment of the experimental mice-bearing solid Ehrlich with Ch.GaNPs induced a very highly significant reduction in liver LPO content compared to EC-bearing group. Meanwhile, a significant increase in the liver content of GSH and a very highly significant increase in GSH-Px activity were observed in comparison with Ehrlich-bearing group. Treatment of experimental mice-bearing EC with Ch.GaNPs and exposed to γ -radiation revealed a highly significant increase in liver GSH content and a very highly significant increase in GSH-Px activity in comparison with EC-bearing group. Whereas, a significant decrease in liver LPO content was observed in comparison with EC-bearing mice.

Table (3): Effect of Ch.GaNPs and/or γ -irradiation on Liver LPO levels and antioxidant status in different groups in mice bearing E.C.

Parameters Groups	TBARS ($\mu\text{mol/gm}$)	GSH (mg/gm)	GSH-Px (mg GSH/min/gm)
N.C	167 ± 2.9	7.8 ± 0.3	1.66 ± 0.005
EC	$228.5 \pm 8.9^{a\ddagger}$	$2.2 \pm 0.1^{a\ddagger}$	$1.43 \pm 0.01^{a\ddagger}$
E.IR	$190 \pm 1.4^{a\ddagger b\ddagger}$	$3.1 \pm 0.2^{a\ddagger b\ddagger}$	$1.56 \pm 0.01^{a\ddagger b\ddagger}$
E.Ch.Ga	$179 \pm 9.5^{a\ddagger b\ddagger}$	$2.6 \pm 0.1^{a\ddagger b\ddagger}$	$1.58 \pm 0.01^{a\ddagger b\ddagger}$
E. Ch.Ga.IR	$202.2 \pm 4.2^{a\ddagger b\ddagger}$	$2.7 \pm 0.1^{a\ddagger b\ddagger}$	$1.59 \pm 0.01^{a\ddagger b\ddagger}$

Values are expressed as Means of 10 records $\pm$ standard Error (M $\pm$ SE).

a^* : significant against NC at ($P \leq 0.05$),

$a^{\ddagger}$: very highly significant against NC at ($P \leq 0.001$).

b^* : significant against EC at ($P \leq 0.05$),

$b^{\ddagger}$: highly significant against EC at ($P \leq 0.01$),

$b^{\ddagger\ddagger}$: very highly significant against EC at ($P \leq 0.001$).

2. Casp-3, Angiogenic and Anti-Angiogenic markers

Table 4 revealed that mean level values of EC group of Casp-3, TNF- α , PDGF and VEGF were 2.3 ± 0.2 , 177.7 ± 5.3 , 138.5 ± 5.0 and 207.9 ± 7.7 , respectively. Whole body γ -irradiation of experimental mice bearing EC revealed a very highly significant increase in the Caspase-3, very highly significant decrease in the VEGF level and significant decrease in the TNF- α level and PDGF level compared to EC-bearing group. Treatment of the experimental mice-bearing EC with Ch.GaNPs produced a very highly significant decrease in the VEGF level and TNF- α level, significant decrease in the PDGF and a very highly significant increase in the Caspase-3 level against EC -bearing group. Treatment of experimental mice-bearing EC with Ch.GaNPs and exposed to 0.25 Gy twice a week x 2 weeks a very highly revealed significant decrease in the VEGF level, TNF- α level and PDGF level and a very highly significant increase in the Caspase-3 level compared to EC -bearing group.

Table (4): Effect of Ch.GaNP sand/or γ -irradiation on serum VEGF level(pg/ml), PDGF level (pg/ml), TNF- level(pg/ml) and Casp-3 level(ng/ml) in different groups in mice bearing E.C.

Parameters Groups	VEGF (pg/ml)	PDGF (pg/ml)	TNF- α (pg/ml)	Casp-3 (ng/ml)
EC	207.9 $\pm$ 7.7	138.5 $\pm$ 5.0	117.7 $\pm$ 5.3	2.3 $\pm$ 0.2
E.IR	115.2 $\pm$ 2.9 ^{b†}	104.8 $\pm$ 3.9 ^{b*}	108.2 $\pm$ 3.1 ^{b*}	8.4 $\pm$ 0.5 ^{b†}
E.Ch.Ga	107 $\pm$ 6.2 ^{b†}	91.3 $\pm$ 5.7 ^{b*}	76 $\pm$ 7.6 ^{b†}	10.7 $\pm$ 1.5 ^{b†}
E. Ch.Ga.IR	71.9 $\pm$ 5.6 ^{b†}	62.5 $\pm$ 1.9 ^{b†}	51.7 $\pm$ 4.9 ^{b†}	17.5 $\pm$ 1.6 ^{b†}

Values are expressed as Means of 6 records $\pm$ standard Error (M $\pm$ SE).

b*: significant against EC at (P $\leq$ 0.05).

b[†]: very highly significant against EC at (P $\leq$ 0.001).

3. Histopathological examinations

1. EC Tumor tissue

Histopathological examination of the Ehrlich carcinoma tumor under light microscope showed compactness and aggregation of the tumor tissue cells spread subcutaneously within the soft tissues in the neck region. Ehrlich carcinoma tumor showed groups of large, round and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei and binucleation. Several degrees of cellular and nuclear pleomorphism were seen (Figure4 A).

Treatment of female mice bearing Ehrlich carcinoma tumor by Ch.GaNP recorded great destruction of tumor tissue represented by the appearance of dead ,remnants and necrotic cells (star) ($\uparrow$) (Figure4 B).

Exposure of female mice bearing Ehrlich carcinoma to fractionated low doses of γ -radiation showed the presence of necrotic cells and remnant tumor cells encircled by the soft tissue ($\leftrightarrow$) (Figure4C).

Combined treatment of female mice bearing Ehrlich carcinoma by Ch.GaNP with low doses of γ -irradiation represented more necrotic cells and remnant tumor cells ($\rightarrow$) (Figure 4 D).

Figure (4): Photomicrograph of Ehrlich carcinoma bearing mice represents (A): control Ehrlich carcinoma in mice. (B): treated with Chitosan Gallium Nanoparticles C: exposed to low doses of γ - radiation. D: treated with Chitosan Gallium Nanoparticles and exposed to low doses of γ - radiation.(H & E x 100).

2. Liver tissue

Normal histological pattern of the control liver of young mice is shown in (Figure 5A&B). Normal central vein (CV) is surrounded by radiating cords of hepatocytes with prominent Kupffer cells. Normal sinusoidal spaces, branches of the hepatic portal veins (PV) and branches of the hepatic arteries and bile ducts could also be noticed. The liver sections of mice bearing Ehrlich carcinoma showed accumulation of Ehrlich carcinoma cells (ECs) around congested portal blood vessels with completely haemolysed red blood cells (RBCs) in the portal vein (Figure 5 C&D). However, the exposure to low doses of γ -radiation produced a spongy structure in most hepatocytes and activation of Kupffer cells (arrow head). Meanwhile, liver tissue showed some appearance of metastatic Ehrlich cells around the portal blood vessel in Ehrlich carcinoma irradiated group (Figure 6 A&B). Treatment of female mice with Ch.GaNPS represented appearance of less metastatic ECs around the portal blood vessel of liver tissue section (Figure 6 C&D). On the other hand, treatment of the experimental mice-bearing solid Ehrlich with Ch.GaNPS and exposed to low doses of γ -radiation revealed disappearance of metastatic ECs and normalization of liver tissue (Figure 6 E&F).

Figure (5): Photographs of sections in liver of mice. **A, B:** Control sections showing the normal appearance of hepatocytes and the central vein (CV) in A and portal vein (PV) in B. **C&D:** Liver sections of mice bearing Ehrlich carcinoma represented accumulation of ECs (blocked arrow) around a congested portal blood vessel (star) with completely haemolysed RBCs. (H&E stain X 400).

Figure (6): Photographs of sections in liver of mice bearing Ehrlich carcinoma (ECs). A, B: Liver sections of mice bearing Ehrlich carcinoma represents some appearance of metastatic ECs (blocked arrow) around the portal blood vessel (PV) in irradiated group C, D: Liver tissue represents some appearance of less metastatic ECs (▲) around the portal blood vessel (PV) in Chitosan Gallium Nanoparticles treated group. E, F: Liver tissue represents disappearance of metastatic ECs and normalization of liver tissue section in Chitosan Gallium Nanoparticles and γ -irradiation treated. group. (H&E stain X 400).

Discussion

The effects of chemotherapeutics on normal cells are described as the side effects of therapy. In addition, some chemotherapy drugs may have a direct effect on organ(s) in the body and cause toxicity over time. In parallel with the widespread cellular/tissue damage that results in site-specific side effects, the child undergoing chemotherapy may also experience generalized effects, such as fatigue, anorexia, taste changes, nausea and vomiting, and pain[19].

Nanoparticles (NPs) have been of significant interest over the last decade as they offer great benefits for drug delivery to overcome limitations in conventional chemotherapy. They can be formed in a range of sizes (1-100nm) and made using a variety of materials like polymers, lipids, inorganic materials and biological materials. Furthermore, they can be modified to concurrently carry both drugs and imaging probes and designed to precisely target molecules of diseased tissues[20]. Besides that, using targeted NPs to deliver chemotherapeutic agents in cancer therapy offers many advantages to improve drug delivery and to overcome many problems associated with conventional chemotherapy[21].

Radiotherapy is a commonly used local treatment for malignant tumors, characterized by uncontrolled growth and the capability of invading neighboring tissues and metastasize[22]. Researchers have considered the impacts of low-dose radiation, given as a single dose or fractionated or prolonged over time, with regard to altering cancer progression

or resolution as well as their impacts on the systemic immune system[23]. It was found that low-dose radiation increases cellular antioxidant activity; facilitates DNA damage repair; reduces malignant transformation and mutagenesis, and stimulates immune surveillance[24].

The cell nucleus and DNA are susceptible to several stressors[25,26,27,28] and can be greatly harmed with relatively low doses of ionizing radiation[29,30]. As adverse effects of ionizing radiation on (cancer) cells are mainly mediated by fragmentation of nuclear chromatin via inserting double strand breaks (DSBs) into the DNA molecule[31]. Also, NPs have been attributed to an amplified number and/or complexity of DSBs caused by radiation in existence of nanoparticles[32].

In the present work, the cytotoxicity of Chitosan-Gallium Nanoparticles (Ch.GaNPs) on Ehrlich carcinoma (EC) cell line was detected. The present study demonstrated that chitosan nanoparticles could exert a high cytotoxicity against Ehrlich ascite carcinoma cell line. The median lethal concentration of Ch.GaNPs was 50 µg/ml and at concentration of 140 µg / ml Ch.GaNPs all Ehrlich carcinoma cells appeared to be burst.

NPs less than 100 nm are useful for biological applications. Particle size is an important property, which may influence the biological activity of NPs and has been suggested as a key factor through the interaction with charged surfaces. NPs with different particle size may have different mechanisms of inhibition[12]. In the recent study TEM images showed that Ch.GaNPs size didn't exceed 16.79 nm.

Several studies have shown that chitosan nanoparticles (ChNPs) could exert a high cytotoxicity against EAC cell line, this is due to their ability to adhere to the cell membrane and degrade products in the cell culture medium or inside the cells[33,34]. ChNPs can be first adsorbed onto the negatively charged tumor cell membrane by electron interaction, then exhibit antitumor effects by damaging membrane and disrupting organelle, and finally lead to cell death with the structure breakdown[35]. The reactive hydroxyl and amine groups of chitosan facilitate the adherence to negatively charged surfaces, thereby enhancing cellular transport. Biomedical application of Chitosan has been investigated extensively to exploit its cationic functional amine group to attach anionic molecules e.g. DNA, proteins[36]. Other studies found that chitosan nanoparticles are capable of changing the composition of the cell membrane of malignant cells. The investigators found that ChNPs also neutralized the surface charge of human liver tumor cells and altered the charge of the membrane surrounding the cells' mitochondria[37].

On the other hand, gallium (Ga) has shown a great role in the modulation of cell membrane permeability which could be related to several mechanisms including alteration of the cell membrane potential, modification of electric charges at the surface of the cell membrane, a modification of ATPase activity and a decrease of the membrane pore diameter[38]. Ga induces an efflux of calcium from mitochondria in a dose related manner. This release is more significant when the calcium content of mitochondria is elevated. It should be remembered that calcium efflux is a preliminary step in apoptosis[10]. Several researches have reported that Ga is taken up by various malignant cells in vitro through cell surface transferrin receptor (TFR)- mediated endocytosis of Ga-transferrin complexes[12].

In the present study, the inoculation of 2.5 million of EAC cells in the neck region of healthy mice produced a tumor size exceeds 3000 mm³ 21 days after tumor inoculation (ATI). The irregular mass of tissue that does not include cyst or liquid is stated as solid tumor and is mostly epithelial in nature[39].

The effect of ROS production, as a result of tumor growth, on other organs in the body can be explained by the fact that ROS cause activation in nuclear factor-κB (NF-κB) and phosphorylation of its inhibitor (IκB). Thus, they enable NF-κB to translocate to the cell nucleus in which it binds to DNA and regulates the transcription of various target genes (i.e., inducible nitric oxide synthase, cyclooxygenase II, cytokines, etc.), which contribute to cell

damage[40]. Interestingly, in tumor cells, cytokines activate NF- κ B which protects the tumor cells from TNF- α induced apoptotic cell death[41]. NF- κ B activation in cancer cells regulates transcription of genes involved in cell proliferation, anti-apoptosis, and invasion. Thus, activation of NF- κ B induces tumor growth and metastasis and reduces cytokines-induced apoptosis[42].

The results obtained in the present study showed that the exposure of the tumor-bearing animals to low dose of γ -radiation produced tumor size regression compared to tumor-bearing animals. Meanwhile, the data obtained also revealed that the administration of Ch.GaNpsto EC-bearing mice exposed to γ -radiation separately or in combination produced significant reduction in tumor size represented by the appearance of dead, remnants and necrotic cells that pronounced a synergistic effect of such combined treatment.

Ga is capable to inhibit tumor growth, mainly because of its competition to ferric ion. It affects cellular acquisition of iron by binding to transferrin besides its interaction with the iron-dependent enzyme ribonucleotide reductase, deactivating the enzyme through a conformational change resulting in reduced dNTP pools and inhibition of DNA synthesis. Upregulation of ribonucleotide reductase and the abundance of TFRs render tumor cells susceptible to the cytotoxicity of gallium[43]. Ga may interact with DNA by acting as a competitor with magnesium for DNA binding because Ga's affinity to DNA is 100 times higher than that of magnesium[44], the decrease of intracellular magnesium is able to decrease DNA synthesis, tubulin polymerization and enzymatic activities[45]. Ga may bind to DNA phosphate group and nucleic bases causing DNA structural modifications, which destabilizes DNA helix[46].

Mitra *et al.*, [47] studied the *in vivo* efficacy of using ChNPs as a drug carrier and suggest that encapsulation of the conjugate in nanoparticles not only reduces the side effects, but also improves its therapeutic efficacy in the treatment of solid tumors. On the other hand, context with the finding of Zhang *et al.* [48] and consensus with the role of ChNPs in tumor destruction, treatment of the experimental animals with ChNPs great destruction and appearance of dead and necrotic cells represent many zones of sporadic underwent apoptotic cells in addition to the presence of apoptotic zone were detected in EC.

The techniques of NPs in lowering the tumor size may be via the long-circulating nanoparticulate carriers. They can efficiently bring chemotherapeutics to solid tumors by using the enhanced permeability and retention (EPR) effect and thus can greatly increase the therapeutic index of the drug or enhance reducing adverse side effects[49].

Also, the exposure to low dose of γ -radiation caused a continuous delay of tumor size recorded in EC-bearing mice due to radiation oxidative stress induction which is reflected by the enhanced levels of peroxidative damage, DNA fragmentation[50]. Also, the decrease in tumor size might result from the fact that EC is an undifferentiated malignancy and is sensitive to radiation when compared with other tumor models[51]. On the other hand, there is evidence that low-dose radiation stimulates the immune functions and that it can induce some degree of tumor regression and suppression of metastases. Also, Previous mechanistic studies have revealed that low-dose radiation enhances immune responses such as mitogenic response, IL-1 production, IL-2 response, and plaque-forming cell reaction[52].

Angiogenesis is a process that literally means the formation of new blood vessels, usually through sprouting from the pre-existing vasculature, and it is a natural physiological process during the development of all mammalian life forms. Tumors arise due to abnormalities in cellular proliferation due to a variety of molecular mechanism(s) including angiogenesis. A tumor acquires the ability to form new blood vessels to ensure a constant supply of nutrition and oxygen. In a malignant tumor, the vasculature helps in tumor cell invasion and migration to distant site organs, which is known as tumor metastasis. Thus, the tumor angiogenesis represents an attractive target for tumor growth inhibition[53].

Vascular endothelial growth factor (VEGF) and its cognate receptor (VEGF receptor 2 [VEGFR-2]) are considered as the most noticeable regulators of angiogenesis. VEGF signaling activates cellular pathways that cause development and branching of new tumor blood vessels, stimulates rapid tumor growth, and accelerates metastasis[54]. Platelet-derived growth factor (PDGF) is another mediator of angiogenesis that regulates angiogenesis, tumor growth, and metastasis. PDGF signaling involves pericyte recruitment to vessels; proangiogenic factors secretion; prompting endothelial cell proliferation, migration, sprouting, and tube formation in tumors; and stimulation of lymphangiogenesis followed by lymphatic metastasis[55].

As significance of angiogenesis in tumor progress was noticed more than 3 decades ago, interest in the therapeutic possibility of antiangiogenic treatments has continued to develop. Moreover, since chemotherapy appears to have reached an efficacy plateau, there is an unfulfilled clinical need for patients with cancer immunity to conventional chemotherapy. It is possible that antiangiogenic agents may have an additive or synergistic effect when administered with chemotherapy, potentially by normalizing the permeability of existing tumor blood vessels which improves chemotherapy delivery to the tumor itself. As tumor growth and progress is operated by numerous pathways, there is also a clear basis for joining antiangiogenics to other targeted agents[56].

In the present study, EC-bearing mice was accompanied by a high serum concentration of VEGF and PDGF. The high serum level of VEGF in the EC-group could be attributed to hypoxia induced in the tumor and the subsequent induction of the expression of VEGF and its receptor. Similarly, it has been reported that hypoxia inducible factor 1 alpha (HIF-1 α) increased the expression of VEGF in experimental carcinogenesis[57]. On the other hand, the high serum level of PDGF in the EC-group could be attributed to a number of unrelated proteins that are involved in regulating PDGF signaling, and deregulation of these proteins will upregulate the PDGF pathway. One such regulatory protein is the tumor suppressor p53, which in its wild-type functional state can transactivate or transrepress a number of target genes. PDGFR is a target, which is transrepressed by p53 to regulate cellular proliferation[58]. Mutation of p53 is a common occurrence, observed in about 50% of all cancers, and this may likely be contributing to PDGF-dependent tumorigenesis[59].

Present study shows that the treatment of the experimental mice-bearing EC with Ch.GaNPs and/or γ -radiation exposure induced a significant decrease in VEGF and PDGF levels against EC-bearing group. The delay in tumor neovascularization could be attributed to Ga which modifies the three-dimensional structure of DNA and inhibits its synthesis, modulates protein synthesis, inhibits the activity of a number of enzymes, such as ATPases, DNA polymerases, ribonucleotide reductase and tyrosine-specific protein phosphatase[10]. Also, Moustafa *et al.*, [60] found that GaNPs may prevent brain metastasis via reducing levels of angiogenesis process markers (VEGF and VCAM-1), as a result of inhibition CYP450 and BSSP4 mRNA expression. Mechanistically, immuno-histochemical and quantitative real-time reverse transcription-polymerase reaction assays provided evidence that ChNP-mediated inhibition of tumor angiogenesis was linked to impaired levels of VEGFR2[61].

The significance of PDGF signaling in tumor angiogenesis is supported by numerous studies indicating that PDGFR inhibitors enhance the antitumor ability of VEGFR blocking agents[55].

Inflammation; part of the normal host response to infection and injury; is a component of the wound healing process that leads to the deposition of extracellular matrix and fibrosis in the liver. There is a considerable proof supporting a key role for pro-inflammatory cytokines, especially TNF- α and IL-6, in the progress of steatohepatitis[62].

Tumor necrosis factor alpha (TNF- α) is a 17 kilodalton cytokine that is synthesized by monocytes/macrophages, natural killer cells/large granular lymphocytes, and T lymphocytes subsets[63] implicated in the promotion or inhibition of tumor development. It is produced by tumor cells or inflammatory cells in the tumour microenvironment. The function of TNF- α in chronic inflammatory diseases and tumor-promoting effects is well known[64] and it is an essential factor implicated in initiation, proliferation, angiogenesis, and metastasis of different types of cancers.

In the present study, female Swiss albino was accompanied by a systemic inflammation as manifested biochemically by a high concentration of TNF- α serum level. The increase of proinflammatory cytokine in the present study might be attributed to the fact that EC can cause a series of deleterious side-effects, including oxidative stress that may triggers lipid peroxidation which in turn initiates release of malondialdehyde and combines with hepatocyte proteins starting a potentially dangerous immune response and excites neutrophil chemotaxis or stimulates transcriptional factor NF- κ B which in turn intensifies the production of proinflammatory cytokines[65].

However, the exposure of female mice bearing EC to low doses of γ - radiation showed a significant decrease in TNF- α serum level compared to EC-bearing group for the enhancement of the immune response. Due to its cytotoxic effect on tumor cells, radiation exposure can supply a source of antigen that is suitable for cross presentation by the host antigen-presenting cells (*i.e.*, dendritic cell) which in turn can produce an antigen-specific immune response. Furthermore, other immuno-potentiating features of radiation therapy may be detected by its impact on the tumor microenvironment to increase cell trafficking to tumor sites, its impact on altering changing antigen presentation itself and direct impacts on the immune effector cells[66,67].

Present study shows that the treatment of the experimental mice-bearing EC with Ch.GaNPs alone or with γ -radiation exposure induced a very highly significant decrease in TNF- α serum level against EC-bearing group. The obtained results could be attributed to Chitosan, which was confirmed to partially inhibit the secretion of both TNF- α and IL-8 from mast cells reducing the allergic inflammatory response, so chitosan nutraceuticals may help to prevent or alleviate some of these complications[68]. Furthermore, low molecular weight chitosan is capable of decreasing the production of the inflammatory cytokines TNF- α and IL-6[69].

It was reported that mitochondria play a crucial role in regulation of cell death[70]. Under extreme conditions (e.g., high Ca^{2+} , oxidative stress) mitochondria undergo drastic changes, accompanied with decrease of the mitochondrial potential, de-energization, swelling, and permeabilization of the inner membrane[71]. Furthermore, the occurrence of mitochondrial dysfunction is rapidly followed by or nearly coincident with the loss of plasma membrane integrity[72], thus leading to necrotic cell death at last. Qi *et al.*, [73] observed a drastic decrease of MMP in chitosan nanoparticles-treated human gastric carcinoma cells, indicating that the mitochondrial membrane was damaged.

Caspases are aspartate-directed cysteine proteases that have a major role in the initiation and execution of necrosis, apoptosis and inflammation, malfunction of which may cause tumor growth and numerous auto-immune diseases[74]. Once activated, they split cellular substrates, directing to morphological hallmarks of apoptosis[75,76].

The present study shows that the treatment of the experimental mice-bearing EC with Ch.GaNPs alone or with γ -radiation exposure induced a very highly significant decrease in Casp-3 serum level compared to EC-bearing group. These results are well agreed with previous investigations conducted in human leukemic/lymphoma cells showing that the pathways involved in Ga-induced cell death include the mitochondria. Human leukemia/lymphoma cells exposed to Ga salts display an initial translocation of inositol

phosphatidylserine to the cell surface, an early marker of apoptosis. This is followed by a loss of mitochondrial membrane potential leading to the release of cytochrome C from the mitochondria to the cytoplasm and the activation of caspase-3 and morphologic changes of apoptosis[46,77].

The unique mechanism of antineoplastic action of Ga that involves its ability to target and disrupt tumor cell iron homeostasis sets it apart from other chemotherapeutic drugs[78]. Cancer cells undergo intensive cell division and need a considerable amount of iron for their development[79]. Iron not only facilitates the appearance of oxidative stress, but also activates proinflammatory cytokines and, in the case of their excess, leads to intracellular hypoxia[80]. Ga concentrates in tissues having a high concentration of transferrin (TF), TF receptor, lactoferrin, or ferritin[81]. Ga is found to concentrate in proliferating tissue (including most tumors), where large amounts of TF receptor are expressed. When Ga reaches these sites, it will compete with iron and will interfere with its absorption, metabolism, and activity[82,83]. Cancer cells that take up Ga become iron-deprived, which causes upregulation of transferrin receptor 1 (TFR1). Increased TFR1 promotes increased Ga-TF uptake, leading to increased iron deprivation, and this cycle continues until apoptosis of the cell results[83]. Also, previous studies have shown that cellular uptake of Ga leads to activation of caspases and induction of apoptosis. In phase II trials in patients with relapsed or refractory lymphoma, the antitumor activity of Ga nitrate is similar to, or better than, that of other commonly used chemotherapeutic agents[82]. Activated caspase-3 is capable of autocatalysis as well as cleaving and activating other members of the caspase family, leading to rapid and irreversible apoptosis[84]. Hence, it is known as a central protein in the execution of apoptosis[85].

On the other hand, several studies have shown that chitosan induces apoptosis and activates caspase-3, as well as activating the extrinsic apoptosis pathway through activation of caspase-8. It has also been hypothesized that necrosis is the mechanism responsible for cell death when cancer cells are exposed to ChNPs[86]. Hasegawa *et al.* [87] reported the growth inhibitory effect of chitosan on bladder tumor cells. They observed DNA fragmentation, which is characteristic of apoptosis, and elevated caspase3-like activity in chitosan-treated cancer cells.

Tokoro *et al.*[88] indicated that hexameric chitosan oligomer had growth-inhibitory effect against Meth-A solid tumor transplanted into BALB/C mice. The antitumor process was thought to be involved in enhanced production of lymphokines including interleukins 1 and 2, sequentially, leading to occurrence of antitumor effect through proliferation of cytolytic T-lymphocytes[89].

Oxidative stress takes place due to the imbalance between the production of ROS and the efficacy of the antioxidant defense. In another words, oxidative stress occurs if disproportionate production of ROS overpowers the antioxidant protective system or when the antioxidant defense weakens[90]. Possible biological targets for free radical harm include proteins, lipids and nucleic acids[91]. The epoxides produced due to elevated oxidative stress may spontaneously act on nucleophilic centers in the cell and thus covalently bind to RNA, DNA and protein. Such reactions may cause cytotoxicity and carcinogenicity depending on the properties of the epoxides[92]. Furthermore, severe oxidative stress is not only known to cause DNA damage and mutations of tumor suppressor genes, which are primary events in carcinogenesis[90], but can also play a notable role in the stimulation of multistep carcinogenesis[93].

The results of the present study revealed that EC inoculation either alone or combined with low dose γ -radiation exposure induces oxidative stress in liver tissue. Metastases of tumor carcinoma cells to the liver tissue produced oxidative stress in them manifested by

increased lipid peroxidation (LPO) level along with reduction in GSH level and antioxidant enzyme glutathione peroxidase (GSH-Px) activity.

Oxidative stress in **tumor** tissue manifested by significant increase in LPO level concomitant with significant decrease in GSH content and GSH-Px activity upon treatment with Ch.GaNps and/or γ -irradiation. These findings were in harmony with other studies which reported that EC induces excessive production of intracellular free radicals resulting in oxidative stress causing antioxidant disturbances, acceleration in lipid peroxide, and decrease in GSH-Px activity [94,95].

Malondialdehyde, the end product of LPO, due to its high cytotoxicity and inhibitory action on defensive enzymes, is indicated to act as a tumor promoter and a cocarcinogenic agent [90]. In the present work, increased levels of LPO level in **tumor tissue** might be attributed to the deficiency of antioxidant defense mechanisms or probably due to the generation of ROS and altered redox statuses which are common biochemical aspects in tumor cells. ROS can act on the polyunsaturated fatty acids of lipid membranes and lead to lipid peroxidation [96].

The present study shows that the treatment of the experimental mice-bearing EC with Ch.GaNps alone or with γ -radiation exposure induced a significant decrease in GSH content and GSH-Px activity and a significant increase in LPO level in tumor tissue compared to EC-bearing group.

In this regard, our results could be appreciated by studies made by **Yang and Chitambar** [97] who have shown that Ga is implicated in intracellular oxidative stress through the generation of reactive oxygen species, with a decrease in the ratio of cellular glutathione reduced form (GSH) on glutathione oxidized form (GSSG). Cells exposed to inorganic gallium salts, such as gallium nitrate, displayed increased generation of ROS [98]. Also, the depletion of GSH has been reported to enhance cell death and apoptosis of the tumor cells along with the loss of essential sulfhydryl groups that result in an alteration of calcium homeostasis and eventually loss of cell viability [99]. In addition, **Chitambar** [78] documented that the new insights into the cellular response to Ga could suggest a model in which the initial exposure of cells to Ga results in an elevation of intracellular ROS which, in turn, invokes a cytoprotective response (i.e., an increase in MT2A and HO-1 gene expression). Thus, cell death occurs only when these cytoprotective responses are overwhelmed. It has been suggested that oxidant generation and antioxidant depletion are the common pathways through which anti-cancer drugs trigger apoptosis in cancer cells [100].

Furthermore, ROS have been closely linked to stimulation of the mitochondrial pathway of apoptosis [101]. ROS mainly activates the intrinsic apoptotic pathway, which provokes opening of the mitochondrial permeability transition pore, which may lead to the release of proapoptotic molecules, such as cytochrome c, to the cytosol. Mitochondrial release of cytochrome C is demanded for the formation of apoptosome and caspase activation, and several death-related signals may cause its release to the cytosol [102].

In the current study, the harmful effect of EC on the mice liver and the possible ameliorating effect of Ch.GaNps and γ -irradiation in those animals were studied. It was also reported that the presence of tumors in the human body or even in experimental laboratory animals were known to affect many functions of the vital organs, especially in the liver, even when the site of the tumor does not interfere directly with organs function [103].

Liver is the primary target for carcinogenic effect of more than two hundred chemicals tested (including pesticides, food additives, pharmaceuticals, and industrial intermediates) in long-term toxicity safety assessment assays [104]. Some of these are harmful agents for the liver as EC.

EC is an experimental model for breast cancer and is a well-known liver carcinogen which induces multiple cellular, molecular, and biochemical changes [105]. Also,

histopathological examination of liver tissue showed that the liver sections of mice bearing Ehrlich carcinoma showed accumulation of Ehrlich carcinoma cells (ECs) around congested portal blood vessels with completely hemolyzed red blood cells (RBCs) in the portal vein. Also, sporadic infiltrations of necrotic cells within the hepatocytes and between them. The results of the current study indicated a pronounced significant decrease of liver GSH-Px and GSH content parallel to an increase in LPO level in EC group. The results obtained support previous findings that EC has been suggested to cause the generation of ROS resulting in oxidative stress, to alter the antioxidant defense system in tissues and cellular injury, which may be one of the factors in the etiology of cancer[106,107]. Excess ROS can destroy hepatocytes and stimulate hepatic stellate cells[108], which play a key role in liver damage and fibrosis[109].

The decline in GSH has been correlated to an enhanced oxidation of GSH to oxidized glutathione (GSSG) as a result of increased generation of ROS[110]. Furthermore, the drop in the total antioxidant levels (enzymatic and non-enzymatic), due to EC inoculation, leads to rise of the circulating lipid peroxides which leads to the accumulation of superoxide anions, that can traverse membranes and cause damaging effects at sites beyond the tumor[111].

However, Human bodies have developed a highly sophisticated antioxidant protection system in order to protect the cells and organ systems against ROS. It includes a variety of components, both endogenous and exogenous in origin, that act interactively and synergistically to neutralize free radicals[112].

The results of the present study revealed that EC-bearing mice experienced an oxidative stress relief in liver tissue after being treated with Ch.GaNPs and/or γ -irradiation when compared to E.C group. The treatment resulted in decline in LPO level and a marked increase in GSH-Px activity and GSH levels as well as histopathological disappearance of metastatic Ehrlich cells from liver tissue with the presence of low amount of sporadic late apoptosis in liver hepatocytes and presence of green viable hepatocytes.

The previous results were context with the findings of **Qi et al., [37]** whom proposed that the antitumor effect of ChNPS was mediated by neutralization of cell surface charge, decrease of mitochondrial membrane potential and induction of lipid peroxidation. On the other hand, Previous researches reported that exposure to low doses of γ -radiation alters liver functions activity[113]. They owed that the enhancement effect of low dose of γ radiation on antioxidant system may modify damage effect of tumor induction on the liver.

From the previous data we can concluded that administration of Ch.GaNPS to mice bearing EC with or without subjection to low doses of γ -radiation, exhibited reduction in tumor size, reduced liver oxidative stress, inhibited angiogenesis and systemic inflammation and remarkably increased apoptosis.

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