Role of 5-Fluorouracil Chitosan Nanoparticles on Decreasing the Oxidative Stress in Mice Bearing Ehrlich Carcinoma

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ABSTRACT

5-Fluorouracil (5-FU) as anticancer drug has many side effects. Site-specific delivery of 5-FU would reduce the systemic side effects and provide effective and safe therapy. Chitosan nanoparticles (CNPs) are used in drug delivery systems. In the present study the effects of 5-fluorouracil chitosan nanoparticles (5FUCNPs) on decreasing the oxidative stress were investigating within a model of female mice bearing solid Ehrlich carcinoma (EC). 7 days After solid tumor induction, 5-FUCNPs were administrated by gavages (0.5 mg/kg body weight) to mice for 15 days. Tumor size was monitored; oxidative stress markers were assessed. In addition, the angiogenic markers concentrations were evaluated. In vitro, 5FUCNPs showed high cytotoxic effect on EC tumor cells. Gavages of EC-bearing mice with 5FUCNPs significantly reduced tumor size, increased MDA level, decreased GSH level and recorded great destruction in tumor tissues. Meanwhile, in liver tissue MDA level significantly decreased and GSH level increased. On the other hand, a significant decrease in the levels of angiogenic markers were recorded. In conclusion: CNPs as a drug carrier for 5-FU have a role in reducing tumor growth and may represent a novel class of anticancer drug.

KEYWORDS

5-fluorouracil chitosan nanoparticles (5FUCNPs), Ehrlich carcinoma, angiogenic markers.

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INTRODUCTION

Cancer, known medically as a malignant neoplasm, is a broad group of various diseases, all involving unregulated cell growth (Estanqueiro et al., 2015).

Experimental tumors have great importance in modelling of carcinogenesis studies. Ehrlich tumor is a transplantable neoplasia from a malignant epithelium, which corresponds to mammary adenocarcinoma in female mice (Ozaslan et al., 2011).

Nanoparticles have been shown to be delivered to specific sites by size-dependent passive targeting (Alvarez-Lorenzo et al., 2011). Chitosan (Cs) is considered one of the most valuable polymer for biomedical and pharmaceutical applications due to its biodegradability, biocompatibility, antimicrobial, non-toxicity, and anti-tumor properties. Nanoparticles, microspheres, hydrogels, films, and fibers are typical chitosan based forms for biomedical and pharmaceutical applications such as transdermal drug delivery (Ravi Kumar, 2000). 5-Fluorouracil (5-FU) is a cytotoxic drug, which interferes with nucleic acid synthesis, inhibits DNA synthesis, and eventually halts cell growth (Burns and Beland, 1983). It is extensively used to treat solid tumors such as liver, breast, colorectal and brain cancer. However, 5-FU is rapidly metabolized, associated with wide ranging side effects among which severe gastrointestinal toxicity, hematologic disturbance, and severe bone marrow deficiency (Wettergren et al., 2012). Yang and colleagues (Yang and Hon, 2009) investigated 5-FU-loaded chitosan nanoparticles whether they could be used as effective drug delivery systems and suggested that chitosan-drug conjugates are expected to overcome the problematic side effects, which were induced by 5-FU.

Our study was carried out to evaluate the anti-angiogenic efficacy of Chitosan nanoparticles as a drug carrier for 5-Fluorouracil on mice suffering from Ehrlich carcinoma.

MATERIAL AND METHODS

Ehrlich ascites carcinoma cells (EACs)

EACs were obtained from the Egyptian National Cancer Institute (NCI), Cairo University. The tumor line was maintained in female Swiss albino mice by intra-peritoneal inoculation of 2.5 million cells per mouse. The EACs were counted before intraperitoneal injection using the bright line hemocytometer and dilution were made by physiological sterile saline solution and desired numbers of cells were injected in a volume of 0.2 ml.

Experimental 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. All the experimental procedures were carried out according to the principles and guidelines of the Ethics Committee of the National Research Centre conformed to “Guide for the care and use of Laboratory Animals” for the use and welfare of experimental animals, published by the US National Institutes of Health (NIH publication No. 85–23, 1996).

Preparation of nanoparticles

Nanoparticles were prepared according to the method described by Banerjee et al. (2002). Briefly, the surfactant sodium bis (2-ethylhexyl) sulfosuccinate (AOT) was dissolved in n-hexane. 400 μl of 0.1% w/v chitosan solution dissolved in acetic acid was added to 40 ml of 0.04 M AOT solution with continuous stirring at room temperature. The solvent was evaporated off in a rotary evaporator and dry the mass in 20 ml of Tris–HCl buffer (pH 8.0) by sonication. 4 ml of 30% CaCl2 solution were added to precipitate the surfactant as calcium salt of diethylhexyl-sulpho-succinate. The precipitate was pelleted by centrifugation at 6000 rpm for 15 min at 4 °C. The cake of Ca was dissolved in 10 ml n-hexane and
washed two to three times with 1 ml of Tris-HCl buffer. The phase-separated aqueous layer was drained out and centrifuged. The total aqueous dispersion of nanoparticles was dialyzed and lyophilized.

**Mitra et al. (2001)** reverse micellar method was used to prepare 5FUCNPs. 200 μl of 5-Flourouracil (10 mg/ml) was added after addition of chitosan solution in 6% v/v acetic acid. Size and morphology of nanoparticles 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.

**In vitro study**

Cytotoxicity effects of the nanoparticles on tumor cells were determined according to the method of **El- Merzabani et al., (1979)**. In order to detect the cytotoxicity of 5FUCNPs, EACs were treated with nanoparticles at the concentrations of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μg/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 nonviable 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 nonviable cells (NVC) were calculated according to the following equations % NVC = C/T X 100, where (C) is number of nonviable cells and (T) is total number of viable cells.

**In vivo study**

To assess a solid mass of Ehrlich tumor, 0.2 ml EAC cells containing 2.5 x 10^6 cells/mouse were inoculated subcutaneously in the back of the neck region of female mouse. After 7 days of tumor inoculation the experimental animals were subdivided into 2 groups having 10 animals in each group. **T-group:** Female Swiss albino mice bearing Ehrlich carcinoma without any treatment and **5FUCNPs-group:** Swiss albino mice bearing Ehrlich carcinoma that given 5-FUCNPs at a dose level 0.5 mg/kg/day for 15 days.

**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) = 4 (A/2) (B/2)² = 0.52 AB², where A is the major axis and B is the minor axis **(Ghoneum et al., 2008)**. The mean tumor size with the corresponding standard error was calculated in each experimental group.

**Sample preparation**

21 days after tumor inoculation (ATI) of final treatment of each group, experimental animals were sacrificed. Immediately Ehrlich tumor and liver tissues were excised and rinsed in saline. Small part from tumor and liver tissues were placed in 10 % phosphate-buffered formalin to be used in histopathological and apoptotic and necrotic examinations. The rest of the Ehrlich tumor and liver tissues were homogenates in cold isotonic sucrose to be used for the estimation of the biochemical assessed parameters.

**Histopathological examination**

Paraffin slide sections from tumor and liver tissues were stained with Hematoxylin and eosin.

**Biochemical analysis**

All biochemical analysis was performed in Ehrlich tumor and liver homogenate. The level of malondialdehyde (MDA), the end-product of lipid peroxidation was measured according to the method of **Yoshioka et al. (1979)**. Glutathione concentration
(GSH) was determined by the method of Beulter et al. (1963). Also, serum (TNF-α, VEGF and PDGF) were assayed by the standard sandwich enzyme-linked immune-sorbent assay (ELISA) technique used for Quantitative Detection of Mouse TNF alpha, VEGF and PDGF Concentrations in Serum.

Statistical analysis

The obtained data were expressed as mean ± standard error (SE). All data were analyzed statistically using one-way analysis of variance (ANOVA) followed by Student’s t-test. Statistical Package for Social Sciences (SPSS) for Windows version 12.0 software was used for this analysis.

RESULTS

Figure 1. Represents the morphology and size of 5FUCNPs. TEM observations revealed that Chitosan have a spherical shape. However, 5FUCNPs of cross-linking (10%) appear as small aggregates. Ultrafine 5-FUCNPs, as shown by the TEM images, spherical shaped uniform solid dense structure and have nearly uniform particle size distribution, which is very important for drug delivery. Average particle size of 5FUCNPs is 60nm±19nm and the size for distribution ranged from 40 to 100 nm.

Table (1) : The effect of 5-Fluorouracil Chitosan nanoparticles (5FUCNPs) on the viability of Ehrlich ascites carcinoma cells.

| Nanoparticles concentration (µg/mL) | % of viable cells | % of dead cells | % of rupture dead cells |
|------------------------------------|-------------------|----------------|------------------------|
| 0                                  | 99                | 1              | -                      |
| 1                                  | 99                | 1              | -                      |
| 2                                  | 99                | 1              | -                      |
| 3                                  | 99                | 1              | -                      |
| 4                                  | 90                | 10             | -                      |
| 5                                  | 90                | 10             | -                      |
| 6                                  | 90                | 10             | -                      |
| 7                                  | 70                | 30             | -                      |
| 8                                  | 50                | 50             | -                      |
| 9                                  | 40                | 60             | 50                     |
| 10                                 | 30                | 70             | 20                     |
| 20                                 | 10                | 90             | 80                     |
| 30                                 | 0                 | 100            | 100                    |
| 40                                 | 0                 | 100            | 100                    |
| 50                                 | 0                 | 100            | 100                    |
| 60                                 | 0                 | 100            | 100                    |
| 70                                 | 0                 | 100            | 100                    |
| 80                                 | 0                 | 100            | 100                    |
| 90                                 | 0                 | 100            | 100                    |
In vitro studies

Chemosensitivity of nanoparticles on Ehrlich ascites carcinoma:

The cytotoxic effect of different concentrations of 5FUCNPs on Ehrlich cells viability was evaluated in Table 1 and Figure 2. The low concentration (10μg/ml) of 5FUCNPs decreases the tumor cells viability by 70%. The cytotoxicity of nanoparticle not only led to the death of Ehrlich carcinoma cells, but also led to burst of these dead cells at certain doses. The median lethal concentration of 5FUCNPs was 20μg/ml leading to the death of 90% Ehrlich carcinoma cells.

Fig. (2): Effect of 5FUCNPs on the viability of Ehrlich ascites carcinoma cell line. (A): Photomicrograph of Ehrlich carcinoma cells line representing 100% of viable cells (↑). (B) Photomicrograph of Ehrlich carcinoma cells line as affected by 5FUCNPs nanoparticles. Notice: viable ascites carcinoma cells ( ), underwent apoptotic ascites carcinoma cells (↑), rupture ascites carcinoma cells (▲) and dead cells (curved arrow).

Table (2): Effect of 5FUCNPs on TNF-α, PDGF and VEGF levels (pg/ml) of mice bearing Ehrlich carcinoma.

| Parameter | Groups | EC | E.5FUCNPs |
|-----------|--------|----|-----------|
| TNF-α     | Mean ± SE | 117.5±2.7 | 61.6±1.8 |
| PDGF      | Mean ± SE | 138.5±5.0 | 80.3±2.7 |
| VEGF      | Mean ± SE | 207.9±7.7 | 83.9±2.4 |

Values are expressed as Means of 6 records ± standard Error (M ± SE).

Monitoring of Ehrlich carcinoma tumor size:

Ehrlich tumor size is represented in Figure 3 it is clear that the inoculation of 2.5 million of Ehrlich ascites carcinoma (EAC) cells in 2 ml physiological saline in the back of the neck region of healthy normal mice produced a solid tumor with a mean size of 175±3.4 mm³ on the 7th day after tumor inoculation (ATI). EC size exceeds 300 mm³ on the 9th day ATI. The increase of EC size proceeds by days reaching 1733±2.0 mm³ on the 21st day ATI.

When mice bearing Ehrlich carcinoma received by gavages 0.5 mg/kg/day 5FUCNPs for 15 days beginning from the 7th day ATI, a significant reduction in tumor size was recorded 194±1.4 mm³ on the 9th day ATI and reaching 459±1.5 mm³ on the 21st day ATI.

Fig. (3): Effect of 5FUCNPs on Ehrlich Carcinoma (EC) tumor size.
**Oxidative stress markers level in tumor and liver tissues of tumor bearing mice**

As shown in Table 3, treatment of the experimental mice-bearing EC with 5FUCNPs produced a significant increase in tumor MDA by 29% and significant decrease in tumor GSH by -42.4% compared with EC group. Meanwhile, treatment of the experimental mice-bearing EC with 5FUCNPs produced a significant decrease in liver MDA by -16.9 and a high significant increase in liver GSH level by 77.2% against EC group.

**Table (3) : Effect of 5FUCNPs on TNF-α, PDGF and VEGF levels (pg/ml) of mice bearing Ehrlich carcinoma.**

| Parameters              | Groups       | EC       | E.5FUCNPs |
|-------------------------|--------------|----------|-----------|
|                         | Tumor       | Liver    | tumor     | Liver    |
| MDA (μ mol/gm wet tissue) | Mean ± SE   | 222.6±2.2| 109.8±1.3 | 141.6±1.9b† | 185±1.5b† |
|                         | % of change from EC | 0 | 0 | 29 | -16.9 |
| GSH (mg/gm fresh tissue) | Mean ± SE   | 2.2±0.09 | 3.3±0.06 | 1.9±0.04b† | 3.9±0.09 |
|                         | % of change from EC | 0 | 0 | -42.4 | 77.2 |

Values are expressed as Means of 6 records ± standard Error (M ± SE)

b†: very highly significant against EC at (P ≤ 0.001).

**Histopathological examination of the Ehrlich carcinoma:**

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 (Figure 4 A). Treatment of female mice bearing Ehrlich carcinoma tumor by 5FUCNPs recorded great destruction of tumor tissue represented by the appearance of dead (arc) and necrotic cells (star) (Figure 4B).

Histopathological examination of the liver tissue in different animal’s groups:

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 5A). while treatment of female mice with 0.5mg/kg/day 5FUCNPs showed aggregation of inflammatory cells around the hepatic portal and hepatic veins with no appearance of tumor cells metastasis (Figure 5B).

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DISCUSSION

Conventional chemotherapeutic drugs are distributed nonspecifically in the body where they affect both cancerous and healthy cells, resulting in dose-related side effects and inadequate drug concentrations reaching the tumor. Recent progress in cancer nanotechnology raises exciting opportunities for specific drug delivery. Nanoparticles, particularly in the size range from 10 nm to 100 nm, are emerging as a class of therapeutics for cancer treatment (Wang et al., 2009). Chitosan is an interesting natural material occurring in abundance in the environment. Its excellent biocompatibility and several advantages due to its unique polymer cationic character render it highly useful for pharmaceutical applications (Thanoo et al., 1992). Chitosan nanoparticles (CNPs) have been previously synthesized as drug delivery for 5-FU. In the present study the cytotoxicity of 5FUCNPs on Ehrlich carcinoma cell line was carried out. The present study demonstrated that 5FUCNPs could exert a very high cytotoxicity against Ehrlich ascites carcinoma cell line. The median lethal concentration of 5FUCNPs was 20 μg/ml and at concentration of 30 μg / ml 5FUCNPs all Ehrlich carcinoma cells appeared to be burst. The cytotoxicity effect of nanoparticles is due to their adherence to the cell membrane, particle internalization and degradation of products in the cell culture medium or inside the cells (Abbasalipourkabir et al., 2011). Chitosan nanoparticles are positively charged due to the cationic characteristics of chitosan (Hu et al., 2002). 5FUCNPs could 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 (Qi et al., 2005). Mitra et al. (2001) studied the in vivo efficacy of using chitosan nanoparticles 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. In the present study when experimental animal’s gavage with 5FUCNPs, a delay in tumor size was recorded (El-Merzabani et al., 1979). The mechanism of nanoparticles in reducing the tumor size may be through the long-circulating nanoparticulate carriers. They can efficiently deliver the 5-FU to solid tumors by exploiting the enhanced permeability and retention (EPR) effect and thus can significantly enhance the therapeutic index of 5-FU or improve reducing undesirable side effects. Studies recorded that ultra-low size particles made of hydrogel polymer such as chitosan can efficiently be targeted to the tumor tissue through the combined effects of extravasation and long circulation in blood (Savita and Amarnath, 2009). On the other hand, context with the findings of many studies with the role of 5FUCNPs in tumor destruction, treatment of the experimental animals with 5FUCNPs great destruction and appearance of dead and necrotic cells represents many zones of sporadic underwent apoptotic cells in addition to the presence of apoptotic zone were detected in Ehrlich carcinoma (Zhang et al., 2008). There are many evidence showing that nanoparticles increase ROS production and in different types of cancer cells (Peters et al., 2007). In the present study treatment of experimental animals bearing Ehrlich carcinoma by 5FUCNPs recorded a significant increase in tumor MDA level compared to untreated tumor bearing group. The inverse linear relationship between the ROS level and the GSH level indicated that free radical species were generated by exposure to nanoparticles which reduced intracellular antioxidant levels (Fen et al., 2009). Treatment of experimental animals bearing Ehrlich carcinoma by 5FUCNPs developed a significant decrease in tumor GSH level compared to the level in tumor tissue of mice bearing Ehrlich carcinoma group. Furthermore, it has been well documented that GSH depletion cause mitochondrial dysfunction and changes in expression of distinct genes and pathways related to inflammatory responses and apoptosis including MAPK/ERK kinase, NFκB, MIP-2, caspase- 3, Bel-2 (Driscoll, 2000; Fubini and Hubbard, 2003).
It has been demonstrated in many in-vivo studies that angiogenesis is responsible for tumor growth and metastasis. VEGF, PDGF are angiogenic factors that play an important role in the process of tumor angiogenesis and in turn in tumor progression and metastasis (Dor et al., 2001). The present study shows that the treatment of the experimental mice-bearing EC with 5FUCNPs produced a significant decrease in the levels of TNF-α, PDGF and VEGF, against Ehrlich Carcinoma-bearing group due to the synergetic antiangiogenic effect of 5-FU together with CNPs which normalizes tumor vasculature, facilitates improved chemotherapy delivery, and prevents the recruitment of progenitor cells from the bone marrow (Kerbel, 2008). TNF-α was the major mediator of inflammatory response (Feghali and Wright, 1997). In EC bearing mice, the elevated cytokine (TNF-α) level may be attributed to oxidative stress. TNF-α act as tumor promoter because it stimulates cancer cells’ growth, proliferation, invasion and metastasis through activation of NF-κB signaling pathway (Ahmed et al., 2001). Our study showed reduced TNF-α level in the sera of 5FUCNPs treated mice. The decrease in TNF-α levels suggests an anti-inflammatory effect which might delay cancer progression (Agrawal et al., 2011).

Furthermore, the present study showed that the experimental mice bearing- EC recorded a significant increase in the liver LPO and a high significant decrease in GSH levels as a result of increased oxidative stress in liver tissue. The oxidative stress in the liver of mice bearing-EC induced hepatocyte cell death by either apoptosis or necrosis leading to liver injury and loss of liver function (Okuda et al., 2002). While treatment of experimental animals bearing Ehrlich carcinoma with 5FUCNPs recorded a significant decrease in tumor MDA and a high significant increase in GSH levels compared to untreated tumor bearing group These previous findings gave the fact that chitosan nanoparticles in combined with 5-FU diminished the damaged effect of free radicals, hence decreasing the level of lipid peroxidation resulting in decreasing liver oxidative stress and normalization of the liver tissue with histopathological disappearance of metastatic Ehrlich cells from liver tissue.

CONCLUSION

CNPs plays an important role in drug delivery of 5-FU through enhancement its cytotoxic, anti-tumor effect and diminishing its side effect. in addition, CNPs together with 5-FU inhibit Ehrlich Carcinoma growth by inhibiting the production of new tumor vacuolization

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