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RESEARCH PAPER

Artesunate-loaded Fe_3O_4 nanoparticles: A novel approach for combating 4T1 breast cancer cells *in vitro* and *in vivo*

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ABSTRACT

Objective(s): Breast cancer is one of the most common cancers among women, and current treatments are inadequate due to unwarranted side effects and lack of specificity resulting in off target consequences. Artesunate is a synthetic anti-malarial drug that exerts inhibitory effects on cancer cell lines via apoptosis and has been used treating some cancers. This study investigated the anticancer effects of Fe_3O_4 magnetic nanoparticles conjugated with chitosan, polyethylene glycol, folic acid, and artesunate *in vivo* and *in vitro*.

Materials and Methods: Nanoparticles were synthesized by co-precipitation; morphology and size were determined by scanning electron microscopy (SEM), and the presence of the components was verified by nanoscale Fourier transform infrared spectroscopy (FTIR). 4T1 murine mammary tumor cells were treated with nanoparticles, and cell viability was determined by MTT assay. 4T1 cells were also subcutaneously injected into BALB/c mice, and magnetic resonance imaging was carried out two weeks later to determine tumor size among the groups. Interferon gamma (IFN-γ) and IL-4 levels (in splenocytes culture supernatant) were measured by ELISA, and tumors, surrounding tissues, and mouse livers were histopathologically studied.

Results: The nanoparticle made in this article had good anticancer effects and caused apoptosis in cancer cells in breast cancer, and also strengthened the cellular immune system and further increased interferon gamma and increased the half-life of mice with cancer, while this nanoparticle It did not have the side effects of chemotherapy drugs.

Conclusion: Artesunate-containing nanoparticles decreased 4T1 cell viability and increased apoptosis to a greater extent than nanoparticles without the drug. *In vivo*, artesunate nanoparticles showed no toxicity and were more effective in decreasing tumor size than control. They were also associated with increased survival, increased IFN-γ, and decreased IL-4 levels in the spleen. The findings show the drug targets cancer cells effectively with minimal side effects due to its herbal nature and targeted nano delivery.

Keywords: *Breast cancer, Cancer therapy, Drug delivery, Nanoparticle*

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INTRODUCTION

Breast cancer is the most common cause of cancer-related deaths in women, comprising 18% of all cancers [1]. Artesunate, derived from *Artemisia Annua*, a Chinese plant with antimalarial properties, is a water-soluble compound that selectively induces apoptosis in cancer cells and inhibits angiogenesis *in vitro* and *in vivo*. Artesunate acts via the production of reactive oxygen species (ROS), catalyzed by iron in lysosomes and can activate the mitochondrial apoptosis pathway in breast cancer cells [2], including MCF-7 cells [2]. Artesunate also initiates BAK-mediated apoptosis in human lung adenocarcinoma [3] and exerts

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anticancer effects in chemotherapy-resistant neuroblastoma cell lines [4]. Furthermore, artesunate has been used to treat a case of human laryngeal cancer in India, with some success [5].

Artesunate exhibits low toxicity in normal cells [6], although it can cause toxicity in the bone and kidneys at high concentrations [7].

Cancer cells usually require folic acid, and its receptor is overexpressed on the surface of tumor cells, thus allowing folic acid to be used to target and internalize drugs in cancer treatment [8].

Polyethylene glycol, a compound with multiple applications in manufacturing and medicine, binds to various protein-based drugs to slow clearance from the blood, thus increasing circulation time and reducing toxicity [9].

Chitosan is a linear polysaccharide consisting of units of N-acetyl D glucosamine. This compound is used as a coating to protect metal-based nanoparticles against oxidation. Chitosan can also prevent the aggregation of small particles and maintain its structure in neutral and alkaline environments, but it dissolves in acidic environments of pH<6.5, such as those found in tumors. Chitosan can, therefore, trap the drug and subsequently release it into the tumor environment [10].

In a previous *in vivo* study, K506/AO2 leukemia cells were interperitoneally injected into nude mice. Administration of Fe_3O_4 nanoparticles containing daunorubicin and bromotetrandrine reduced drug resistance and decreased tumor mass [11]. Various studies have described different methods to produce superparamagnetic Fe_3O_4 nanoparticles. Although conventional methods use a similar approach to produce magnetic nanoparticles, they have limitations, including large particle size and instability in aqueous media [12].

To increase the biocompatibility of Fe_3O_4, carboxylated polyethylene glycol can be used, which increases the circulation time of nanoparticles in the blood [12]. To reduce the side effects of daunorubicin, a previous study described the coating of daunorubicin-loaded nanoparticles with oleic acid. The cytotoxic effects of the resulting nanoparticles in K562 cells increased with increasing drug concentration, with a calculated IC_{50} of 2.28 µg/ml after 24 hr of treatment [12].

Chertok B and his colleagues found that Bax expression was increased in cells treated with gambogic acid-loaded and Fe_3O_4 nanoparticles compared to gambogic acid treatment alone [13]. Other researchers have attempted to suppress tumor growth in animal models treated with magnetic anticancer drugs using an external magnetic field [14].

Artesunate-loaded nanoparticles for cancer treatment, formulated using chitosan and Fe_3O_4, increased drug effectiveness in terms of targeting (by using an external magnetic field) and delivery to cancer cells [6]. Fe_3O_4 nanoparticles also increase artesunate-related apoptosis in K562 cells by increasing bcl-rambo and decreasing survivin expression [7].

In this study, we used herbal artesunate drug for the treatment of breast cancer since this compound has fewer side effects than chemotherapy. Then, we have successfully targeted the drug with the help of iron and chitosan to reduce the off target side effects of drugs used in breast cancer.

MATHERIALS AND METHODS

Production of artesunate-loaded Fe3O4-FA-PEG-chitosan nanoparticles by co-precipitation method

First, 98 cc of distilled water was mixed with 2 cc glacial acetic acid, and 30 mg chitosan was added followed by 9 mg Fe_3O_4 nanoparticles in distilled water. The mixture was stirred at room temperature using a magnetic stirrer. The supernatant was discarded, 1 cc of the hydrophobic material and 30 cc of 96% ethanol were added, and the mixture was stirred at room temperature using a magnetic stirrer for 24 hr. The supernatant was removed, and the magnet with the remnant stayed at the bottom of the beaker (solution 1, containing chitosan, hydrophobic material, and iron oxide). Subsequently, 20 cc acetonitrile, 5 mg dcc, and 5 mg folic acid were added to a 50 ml Falcon tube (away from light) containing a magnet, and placed on a magnetic stirrer for 2 hr at room temperature and protected from light. Solution 1 was then added, and the mixture was stirred for a further 24 hr. Following sonication, 5 mg artesunate (Guilin Pharmaceutical Co., Ltd, China), 2 cc acetonitrile, and 0.5 mg PEG were adding and the mixture was stirred for 24 hr at room temperature, sonicated, and dried at 60°C for 24 hr. Finally, structural analysis was performed using Fourier transform infrared spectroscopy (FTIR), and images were captured by scanning electron microscopy (SEM).

Release profile of artesunate from Fe_3O_4-PEG-chitosan nanoparticles

Released artesunate nanoparticles were measured in 0.01 M phosphate buffer at pH 7.4

and 0.01 M citrate buffer at pH 4.5. Saluted nano drug (1 ml) was added to a dialysis sac with 100 ml citrate buffer and phosphate. Release studies were performed at 37 °C in a shaking water bath with sampling at specific intervals over 24 hr. For each sampling, 1 ml of the sample was removed and replaced with 1 ml of buffer. Samples were centrifuged at 15,000 rpm, and the supernatant was collected for optical density (OD) assessment.

Morphologic determination of nanoparticles by SEM

The morphology of the synthesized nanoparticles was studied by SEM (Hitachi S-4160 vace=15xv) after 20 mins of sonication.

Standard drug absorption curve

The wavelength at which the drug has a maximum absorption rate required to measure the amount of the released drug. A solution of artesunate of known concentration (0.2 mg/ml) in 70% alcohol (as solvent) was prepared, and the absorption spectrum in the 200–400 nm range was obtained.

Screening by nanodrop determined that artesunate had maximum absorption at 250 nm. Polymer solutions with different concentrations were prepared to avoid interactions between polymer and drug to ensure that Fe_3O_4 and PEG did not affect absorption at 250 nm. Drug accumulation was quantified using an absorption standard curve of absorption rate change to drug concentration.

Determination of the amount of loaded drug

After adding a solution containing artesunate and Fe_3O_4 to a polar solution, a portion of the drug becomes trapped in the formed nanoparticles, and it is necessary to quantify the extent of drug loading.

After mixing the two colloidal solutions, the resulting solution was centrifuged to precipitate the nanoparticles. The supernatant was removed, and the absorption rate was measured by nanodrop at 200 nm. The drug concentration was subsequently calculated using the standard absorption curve.

As the drug concentration in the initial solution added to Fe_3O_4 had already been determined, the concentration of the drug loaded onto the nanoparticles could be obtained by subtraction.

MTT assay

The 4T1 mouse breast adenocarcinoma cell line was used for *in vitro* studies (Pasteur Institute, Iran).

MTT assay was performed to determine the effect of artesunate-loaded Fe_3O_4-PEG-FA nanoparticles on 4T1 cells and natural WBC cells. The assay was performed on the following groups in triplicate:
1- Artesunate, 2- Fe_3O_4-chitosan-PEG-FA, 3- Hydroxyurea, 4- Untreated

Artesunate, nano drug, and nanocarriers were used at the following concentrations: 30, 60, 120, 240, and 300 µg/ ml.

Cells were cultured in 96-well plates in reduced-serum media (5% or 2.5% fetal bovine serum [FBS]). Cells were detached using trypsin and resuspended in 1 ml of culture medium containing 5% FBS, and cell Pyptazh was done. A 10 µL suspension volume was removed, 10 µL Trypan blue was added, and viable cells were counted. In continuation, the cell suspension was diluted in a way that each well had 200 ml of %5 FBS medium containing 10000 cells.

On day 2 of this experiment, 30, 60, 120, 240, and 300 µg/ml concentrations of artesunate and nanoparticles loaded with artesunate were added to cells. On the third day, special 24 hr culture dish was read.

Next, 100 µL of MTT solution was added to each well, and the plates were incubated for three h. The cellular environment was depleted, 100 µL isopropanol was added to dissolve the formazan crystals, and the plate was placed at room temperature for 30 min.

Absorption was read with a plate reader at 540 nm. These steps were repeated on days 4 and 5 of culture to determine the effects of treatment with artesunate and loaded nanoparticles for 48 and 72 hr. Cell viability was determined using equations (1) and (2).

(1) $\text{Cytotoxicity (\%)} = 1 - \frac{\text{mean absorbance of toxicant treated cells}}{\text{rbance of negative control}} \times 100$

(2) $\text{(\%) Viability} = 100 - \text{cytotoxicity}$

4T1 cell tumorigenesis in BALB/c mice

A total of 8 × 105 4T1 cells in 100 µl of saline were subcutaneously injected into the backs of 30 female BALB/c mice aged 6–8 weeks (5 groups of 6 mice). Tumor growth at the injection site was observed after approximately 10–14 days.

Treatment of tumor-bearing mice with FE3O4-chitosan-PEG-nanoparticles loaded with artesunate

Two weeks after the injection of 4T1 cells, tumor-bearing mice were treated as described in Table 1.

Table 1. Grouping of tested mice

No.	Groups	Treatment
I	Artesunate 0.5 mg/0.1 cc	Drug
II	Chitosan nanoparticles with FE FE_3O_4 PEG-FA 0.5 mg/0.1 cc	Nano system
III	Chitosan nano drug with FE_3O_4 PEG-FA Loaded with Artesunate 0.5 mg/0.1 cc	Nano drug
IV	Hydroxyurea 0.5 mg/0.1 cc	Hydroxyurea
V	Phosphate saline buffer 100 Lambda	Control

Tumor measurement

Tumor size was determined by MRI in mice anaesthetised by intraperitoneal administration of ketamine 50–70 mg/kg) and medetomidine (10 mg/kg).

lFN-γ And IL-4 measurement in splenic lymphocytes

On day 6 after treatment, mice were killed and splenectomised, and splenic lymphocytes were isolated with and without tumor lysate antigen and cultured for 3 days. The supernatant was collected for cytokine determination according to the kit protocol.

IL-4 measurement in splenic lymphocytes

IL-4 measurement was similar to that of lFN-γ, but the preparation of standards differed according to kit protocol.

Histopathological assessment

Tissues from tumors and surrounding tissues, from the liver of euthanised mice, and from liver biopsy samples of live mice on day 12 after treatment were collected. Haematoxylin and eosin (H&E) staining was performed.

Statistical analysis

Results are presented as the mean ± SEM. Data were analyzed using GRAPH PAD PRISM version 9 software and excel software (Microsoft Office 2013). Independent samples t-test performed the effect of the empty nano systems and nano drug and artesunate alone on cell viability at different concentrations for 24 and 48. When equal variances among groups could not be assumed, the Welch t Test statistic was used. Repeated measure ANOVA was used to analyze the change in tumor volume in the different treatment and control groups. Kaplan Meier survival rate was applied in mice treated with the nano drug compared with mice treated with the drug and nano system. A P-value < 0.05 was considered statistically significant.

RESULTS

Release profile of artesunate from Fe_3O_4-PEG-chitosan-FA nanoparticles

Rapid drug release was observed in the first 2 h, which subsequently showed a gradual release in PBS. Additionally, in the first hours, the rate of drug release in citrate buffer (tumor acidic environment) was more than in phosphate buffer (body physiological environment) (Fig. 1).

FTIR analysis

FTIR analysis confirmed the presence of Fe_3O_4 nanoparticles, chitosan, PEG, folic acid and artesunate in the synthesised nano drug (Fig. 2).

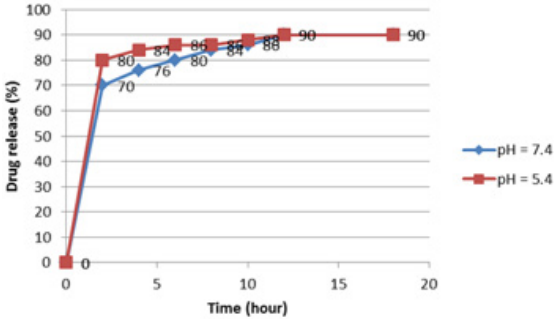

Fig. 1. Artesunate release from nano drug

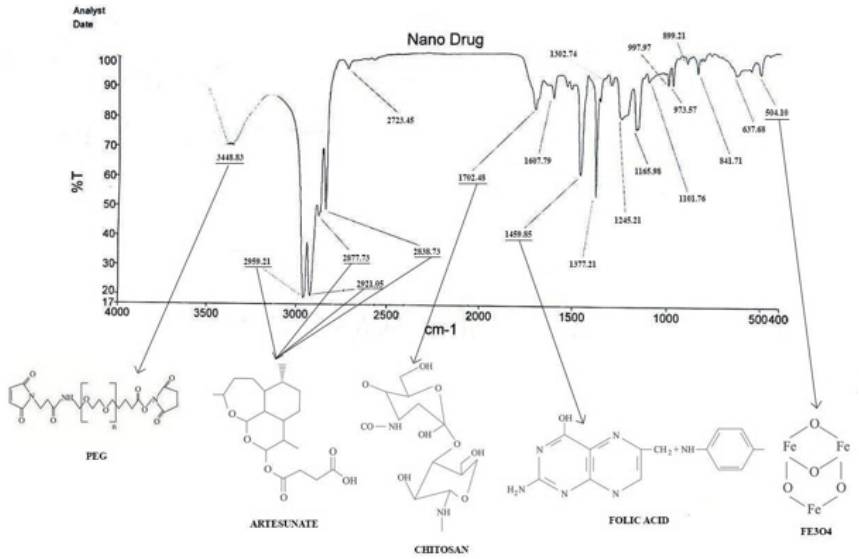

Fig. 2. FTIR diagram of the synthesised nano drug

MTT assay

The effect of the empty nanosystems and nano drug and artesunate alone on cell viability were compared at different concentrations for 24 and 48. Loaded artesunate in Fe_3O_4-Peg-chitosanFA nanoparticles significantly inhibited 4T1 cancer cell growth and decreased viability but had no significant effect on normal WBCs.

After 24 and 48 hr of MTT assay, the impact of the nano-drug on 4T1 cells increased with increasing concentration, but the effect was insignificant (P> 0.05).

Furthermore, the nano-drug at different concentrations had less effect than hydroxyurea in 4T1 cells, but the difference was not significant (P> 0.05) except at the concentration of 30 µg/ml at 24 h (P<0.05). The nano drug exerted a lesser effect than hydroxyurea in WBC, but the difference was not significant (P>0.05) (Fig. 3 A,B,C,D,E,F).

Effect of drug and nano drug on tumor growth

Mice-bearing breast cancer xenografts were treated with the nano-drug, drug, nanosystem, saline, and hydroxyurea by intraperitoneal injection (0.5 mg/ml, three times every other day). MRI

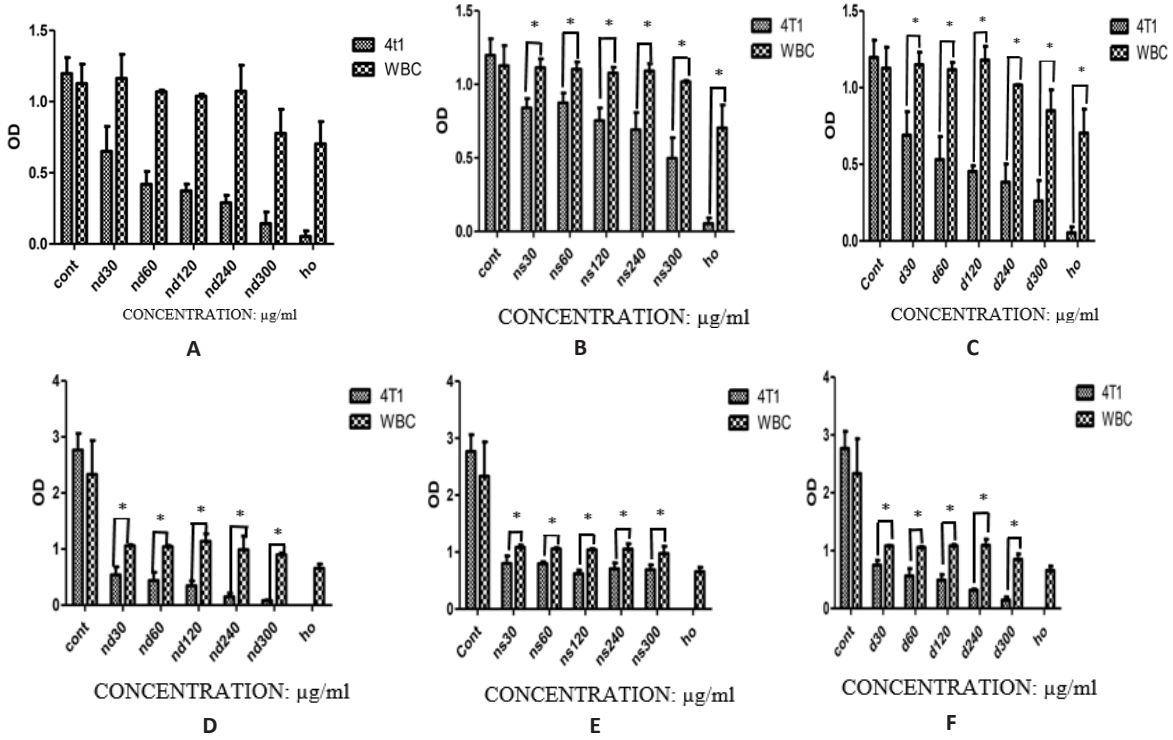

Fig. 3. **A.** 24 H MTT results of nano drug (nd) on cancer and normal cells: with increasing concentration of nano drug, its cytotoxicity effect increases significantly on 4T1, while its effect on WBC cells is less than hydroxyurea. Comparison of the effect of nano drug on 4T1 And WBC is significantly different (P< 0.05).cont:control,nd30:nano drug 30mg/ml,nd60:nano drug 60 mg/ml, nd120:nano drug 120 mg/ml, nd240:nano drug 240 mg/ml, nd300:nano drug 300 mg/ml,ho:hydroxy urea. B. 24 H MTT results of nano system (ns) on cancer and normal cells: the effect of increasing the concentration of nano system, its cytotoxicity increases significantly on 4T1 (except concentration 60), while its effect on WBC cells is less than hydroxyurea. The comparison of the effect of nano system on 4T1 and WBC is significantly different (P<0.05). cont:control,ns30:nano system 30mg/ml,ns60:nano system 60mg/ml, ns120:nano system 120mg/ml, ns240:nano system 240 mg/ml, ns300:nano system 300mg/ml,ho:hydroxy urea. **C**. 24 H MTT results of the drug (d) on cancer and normal cells: with increasing drug concentration, its cytotoxic effect increases significantly on 4T1, while its effect on WBC cells is less than that of hydroxyurea. Comparison of the effect of the drug on 4T1 and WBC is significantly different (P<0.05). cont:control, d30: drug 30 mg/ml, d60: drug 60mg/ml, d120: drug 120 mg/ml, d240: drug 240mg/ml, d300: drug 300 mg/ml,ho:hydroxy urea. **D.** MTT 48 H results of nano drug (nd) on cancer and normal cells: with increasing concentration of nano drug, its cytotoxicity effect increases significantly on 4T1, while its effect on WBC cells is less than hydroxyurea. Comparison of the effect of nano drug on 4T1 and WBC is significantly different (p < 0.05). cont:control,nd30:nano drug 30mg/ml,nd60:nano drug 60mg/ml, nd120:nano drug 120mg/ml, nd240:nano drug 240mg/ml, nd300:nano drug 300mg/ml,ho:hydroxy urea. **E.** The results of MTT 48 H nano system (ns) on cancer and normal cells: with increasing concentration of nano system, its cytotoxic effect does not increase significantly on 4T1, while its effect on WBC cells is less than hydroxyurea. Comparison of the effect of nano system on 4T1 And WBC is significantly different (P<0.05). cont:control,ns30:nano system 30 mg/ml,ns60:nano system 60mg/ml, ns120:nano system 120mg/ml, ns240:nano system 240 mg/ml, ns300:nano system 300 mg/ml,ho:hydroxy urea. **F.** 48 H MTT results of the drug (d) on cancer and normal cells: with increasing drug concentration, its cytotoxicity effect increases significantly on 4T1, while its effect on WBC cells is less than hydroxyurea. Comparing the effect of the drug on 4T1 and WBC significantly are different (P<0.05). cont:control, d30: drug 30 mg/ml, d60: drug 60 mg/ml, d120: drug 120 mg/ml, d240: drug 240 mg/ml, d300: drug 300 mg/ml,ho:hydroxy urea.

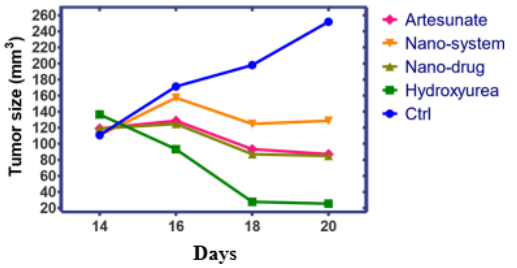

Fig. 4. Changes in tumor volume in the different treatment groups

measured tumor volume and was shown to differ among the groups, as shown in (Fig 4). Tumor volume in the groups treated with 1-artesunate, 2-Fe_3O_4/Peg/chitosan/FA loaded with artesunate,3-Fe_3O_4/Peg/chitosan/FA nanosystem, and 4-hydroxyurea (control) was decreased, although the differences were not significant (P=0.3021). In the group that received Fe_3O_4/Peg/chitosan/FA loaded with artesunate, tumor volume was reduced significantly compared to the group receiving the drug or nanosystem alone.

Effect of artesunate-loaded nanoparticles on splenic lymphocyte cytokine levels

The injection of the synthesized nano drug into tumor-bearing mice caused a significant increase in IFN-γ compared to the other groups.) P<0.05. (However, the reduction in IL-4 compared to that in other groups was not significant (P>0.05) (Fig. 5).

Histopathological analysis of tumors

In control mice, i.e., mice treated with physiological serum, tumor cells exhibiting mitosis and extensive blood vessels were observed (Fig. 6 A, B). Apoptosis was observed in mice treated with artesunate, with fewer blood vessels and less mitosis than the control (Fig. 6 C). Necrosis was observed in mice treated with nanosystem as a pale pink area with nucleolus pieces (Fig. 6 D). Furthermore, tumor necrosis, apoptosis, and reduced active mitotic nuclei and blood vessels were observed compared to the control group (Fig. 6 E). Extensive necrosis was observed in the tumors of mice treated with hydroxyurea (Fig. 6 F). Immune cell infiltration around the tumor was observed in mice treated with the nano-drug in addition to increased macrophages and neutrophils but decreased lymphocytes, which represents a disadvantage of this therapeutic approach (Fig. 6 H).

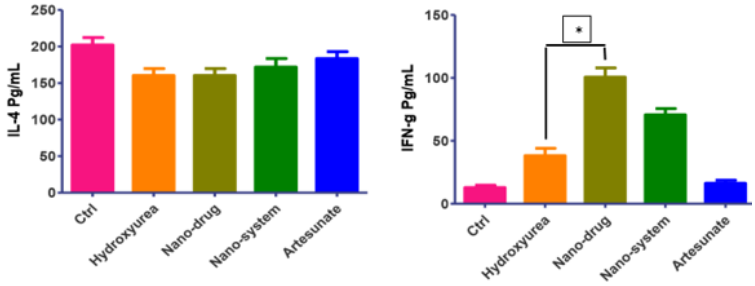

Fig. 5. IFN-γ and IL-4 levels in the different groups of tumor-bearing mice .The changes of IFN-γ in the groups were significant (P> 0.05). But the changes of IL-4 in the groups are not significant (P>0.05)

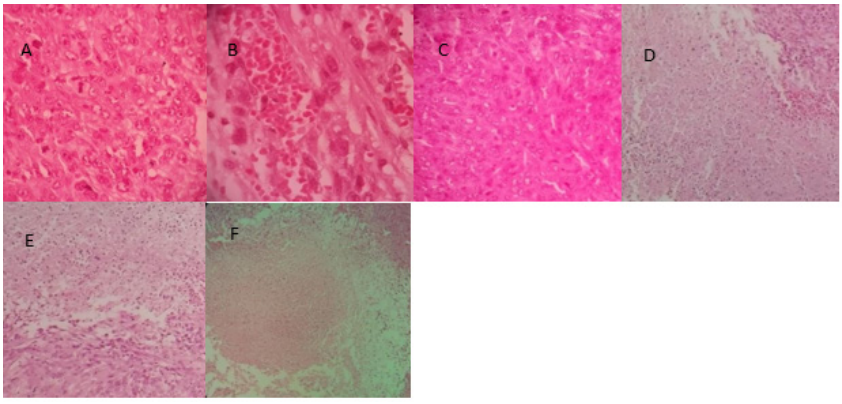

Fig. 6. A and B Mitosis and extensive blood vessels in tumors of control mice (×400 H&E). C. Apoptosis and decreased mitosis and blood vessels in tumors of mice treated with artesunate (×400, H&E). D. Necrosis in tumors of mice treated with nano drug (×400 H&E). E. Necrosis in tumors of mice treated with nano system (×400, H&E). F. Apoptosis and decreased mitosis and blood vessels in tumors of mice treated with nano drug (×400 H &E). F. Extensive necrosis in tumor and surrounding tissues in in mice treated with hydroxyurea (×100 H&E)

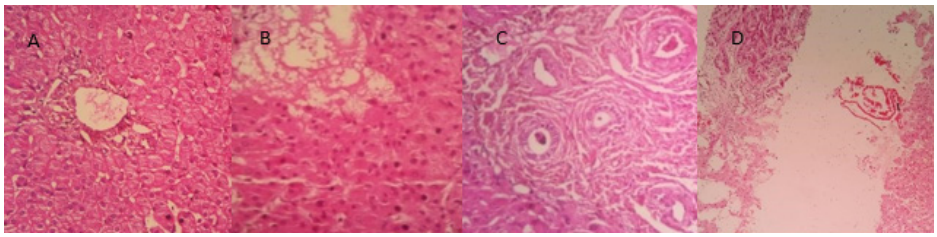

Fig. 7. A,B,C. Absence of metastases in the liver in mice treated with nano-drugs (x400 H & E).D. Metastasis in the liver autopsy of a control mouse (PBS recipient died on day 35). (x100 H & E)

Tumor metastasis in treated mice

No metastatic tumor cells were observed by autopsy and biopsy in mice treated with nanoparticles (Fig. 7 A,B,C). However, metastatic carcinoma was observed in the liver of a tumor-bearing mouse that had not received the nano-drug (Fig. 7 D).

Drug hepatotoxicity in treated mice

In the histopathological examination of the liver tissue in tumor mice receiving nano drug, there was no evidence of neutrophil infiltration and necrosis indicating hepatotoxicity. (Fig. 8).

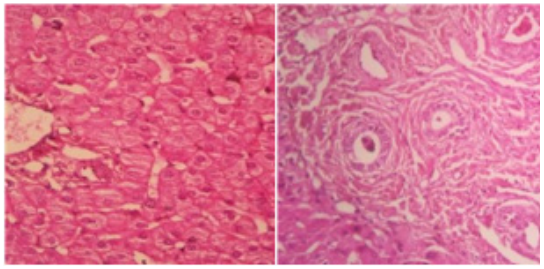

Fig. 8. Absence of neutrophils and necrosis in the liver of mice treated with synthesized nano drug (x400 H & E)

The survival rate of the treated mice

An increased survival rate was observed in mice treated with the nano-drug compared with mice treated with the drug and nanosystem, but the differences were not significant (P= 0.6269) (Fig. 9).

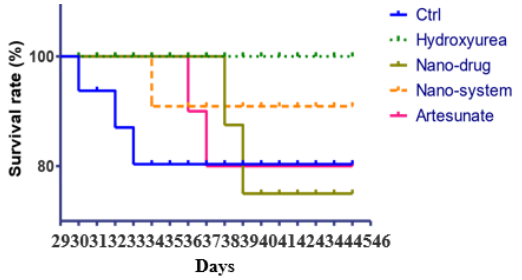

Fig. 9. The survival rate of mice in the various treatment groups. Nano drug: 40 days< -39-38, Artesunate: 40 days< -36-37, Nanosystem: 35-34-35 days, Hydroxyurea: 40<-40<-40<

DISCUSSION

Fe_3O_4 nano particle was previously shown to be non-toxic and completely biocompatible without causing mutation or chromosomal changes. Fe_3O_4 nanoparticles have also been shown to increase apoptosis in MCF-7 cells, with therapeutic effects, and to alter the cytokine profile in which macrophage acquired low responsive or inhibitory phenotype [15]. One advantage of magnetic drug delivery systems compared to other systems is reduced absorption by the reticuloendothelial system.

In summary, limitations exist to the successful use of iron-based nanoparticles *in vitro*, for example, changes in functional groups of drugs attached to nanoparticles, low loading nanomaterials disability for entering the tumor via blood arteries, drug transfer to endosomes and lysosomes instead of cytoplasm and nucleus, and decreased ability for targeted transfer because of weak ligand interaction and loss of magnetic properties. Furthermore, the heterogeneity of nanoparticles presents a problem [16]. However, 10–100 nm nanoparticles are effective for targeted drug delivery as these nanoparticles can escape from the reticuloendothelial system [17]. On the other hand, the size of synthesized nanoparticles in this study was 234 nm, which caused decreased uptake of the drug by the reticuloendothelial system and drug penetration to the tumor. In this study, we sought to synthesize a herbal-based remedy with low toxicity in normal cells but high anti-tumor effects and selected artesunate as a candidate because of its natural origin. According to the MTT results and evaluation of liver toxicity, artesunate toxicity was very low in normal cells, but it exerted a strong anti-cancer effect through the reduction in tumor size, mitosis, angiogenesis, metastasis, and apoptosis in tumors. We used a nanosystem to increase the anti-tumor effects of the drug, increase drug delivery, and strengthen the immune system against tumors.

MTT test results showed that the nanosystem exerted increased anti-tumor effects, whereas a comparison of tumor size after nano drug and

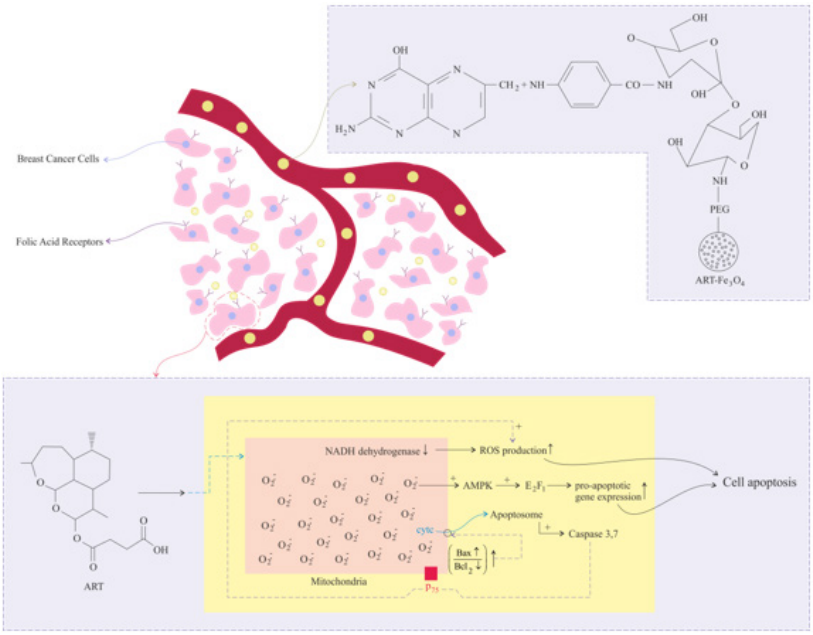

Fig. 10. Proposed mechanism for the proliferation inhibitory effects of Artesunate-loaded Fe_3O_4 nanoparticles in 4T1 breast cancer cells. (AMPK: 5' AMP-activated protein kinase, E2F1: transcription factor, P75: subunit of complex I of the mitochondrial electron transport chain)

drug showed that the nanosystem increased drug delivery and the anti-tumor effects of the drug.

Chitosan facilitated the release of drugs, which increased in the tumor environment. Of course, because of the presence of Fe_3O_4, external magnetic fields may be used to target nano drugs to the tumor, but this was not implemented here.

The significant increase following treatment with nanosystem suggests that the anti-tumor properties of the immune system may have been strengthened.

However, the significant reduction in IL-4 and low levels of infiltrated lymphocytes into the tumor indicated that an appropriate adjuvant should be used or the concentration of nanosystem components needs to be increased during synthesis.

In the previous studies, Artesunate has not been combined with a similar nanosystem, and its effects and limitations have yet to be fully studied and described.

Our study represents the first report on this nanosystem and the advantages of the synthesized nano-drug, including its safe profile and anti-cancer properties.

One reason for the lack of appropriate tumor response to treatment with artesunate may be drug resistance of breast cancer cells towards artesunate by the activation of NFκkB and AP-1 transcription factors, which regulate tumor progression and cause transcription, expression, and activation of MMP-1, and increased invasion, metastasis, and tumor resistance to the drug27. These factors may have contributed to the lack of a significant difference in tumor size and survival. These results indicate the need for further studies to elucidate the mechanisms involved (Fig. 10).

CONCLUSION

Fe_3O_4 nanoparticle is non-toxic and completely biocompatible and does not cause mutations or chromosomal changes.Fe_3O_4 nanoparticles increase apoptosis in MCF-7 cells and have therapeutic effects. Phagocytosis of nanoparticle containing Fe_3O_4 nanoparticle changes the cytokine profile of macrophage, which makes the macrophage less responsive or inhibitory phenotype. The advantage of magnetic drug delivery systems compared to other systems is the reduction of absorption by the reticuloendothelial system. Despite all these details, there are still many obstacles for the successful use of iron-based nanoparticles in the *in vivo* environment. Among them, one can change the functional groups of drugs to address issues such as to binding to nanoparticles, low loading rate, the inability of nanomaterials to enter the tumor via blood vessels, the transfer of drugs to endosome and lysosomes instead of cytoplasm and nucleus, reduced ability of targeted transfer due to weak connections with the ligand and the loss of

magnetic properties as pointed out.

In addition, the heterogeneity of nanoparticles is also a big problem. Also, the size of nanoparticles between 10 and 100 nanometers is very effective for targeted drug delivery. Because these nanoparticles can escape from the reticuloendothelial system, while the size of the nanoparticle synthesized in this study was 234 nm, which increases the uptake of the drug by the reticuloendothelial system and reduces the penetration of the drug into the tumor.In this study, we tried to synthesize a nano drug that has a herbal drug that has very few side effects and toxicity for normal cells and at the same time has high anti-tumor effects. According to MTT results and liver toxicity study, its toxicity for normal cells was very low, and at the same time, it had good anti-tumor effects, reducing tumor size, reducing mitosis, angiogenesis and metastasis, and causing apoptosis in the tumor.To increase the anti-tumor effects of the drug and increase drug delivery and increase and strengthen the immune system against the tumor, we used the nano system. This nano system contained Fe3O4, chitosan, polyethylene glycol and folic acid.According to the analysis of MTT test results in nano drug and drug, the nano system increases the antitumor effects of the drug. Based on the comparison of the results of tumor size reduction in nano drug and drug, we can infer that the nano system increases the drug delivery and anti tumor effects of the drug. Considering the significant increase of IFN-ɣ in nano drug compared to drug and other groups, it can be inferred that the said nano system strengthens the anti-tumor immune system. However, due to the lack of significant reduction of IL-4 and the low number of infiltrating lymphocytes in the tumor, it is suggested that either an appropriate adjuvant be used, or the concentration of the components of the nanosystem be increased in the synthesis, or otherwise the study time be increased. In the previous studies, artesonite was not combined with such a nanosystem, and its effects and side effects were not fully investigated, for example, the effect of Fe_3O_4 and artesonite on the apoptosis of K562 cell line and the anticancer effects of artesonite in combination with Fe_3O_4 and chitosan. Therefore, this research is completely new and unique in terms of nanosystem compounds and investigation methods, which adds to its significance .

In general, the synthesized nanomedicine has the following numerous advantages:

1- Artesonite drug is herbal and has very few side effects on normal cells compared to chemotherapy.

2. Low toxicity on human white blood cells in the MTT test compared to hydroxyurea

3- Being targeted towards cancer due to the presence of folic acid

4- Improved access in the acidic environment of the tumor due to the presence of chitosan

5-Having appropriate anti-cancer effects and reducing tumor size

6-Having more anti-cancer effects than artesonite alone or nanoparticle alone

7-Inducing apoptosis and necrosis and reducing mitosis in the tumor

8-Remostasis and reduction of angiogenesis

9. Not having liver complications

10. Increased survival rate in treated mice

11- Increasing IFN-γ and decreasing IL-4, which helps to strengthen cellular immunity and tumor removal

12- Biocompatibility and the ability to direct to the tumor with the help of external magnetic fields

But its disadvantages include its large size, which is easily removed by macrophages, and the low infiltration of lymphocytes towards the tumor in the treated mice. One of the important reasons for the lack of appropriate tumor response and the lack of acceptable treatment in artesonite treatment can be due to the development of drug resistance in breast cancer cells in AP-1 and NFKB to artesonite, which is through the activation of transcription factors and as a result of increased invasion and MMP-1, effective tumor progression is created, which causes transcription and expression and metastasis activity and more tumor resistance to drugs. These factors can collectively play a role in the non-significant differences observed in the study of tumor size reduction and survival study.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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