Curcumin coated gold nanoparticles attenuates doxorubicin-induced cardiotoxicity via regulating apoptosis on mice model

Zeynab Sharifiaghdam[1], Fereshteh Dalouchi[1], Maryam Sharifiaghdam[2], Elnaz Shaabani[2], Fatemeh Ramezani[1], Farnaz Nikbakht[1], and Yaser Azizi[1]

[1]Iran University of Medical Sciences
[2]Ghent University Department of Pharmaceutics

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Abstract

Doxorubicin (DOX) is one of the most widely used chemotherapy agents which is associated with several adverse effects on heart tissue including cardiomyopathy. Curcumin (Cur), a well-known dietary polyphenol could exert important cardioprotective effect but its biological application is limited by chemical insolubility. In order to overcome this problem in this study, we synthesized gold nanoparticles completely based on curcumin (Cur-AuNPs). This nanoparticle, on the one hand, could bring out a stable delivery way for curcumin that cause more solubility and on the other hand, will cause more efficacy. UV-Visible, size, surface charge, TEM and FTIR characterization also in-vitro cytotoxicity effect on H9C2 cells were performed for Cur-AuNPs. Biological efficacy of Cur-AuNPs was evaluated after acute cardiotoxicity induction in Balb/c with DOX injection in comparison to carrier-free curcumin. Heart protective effect of Cur-AuNPs was evaluated 24 h after toxicity induction by quantifying serum biomarkers, myocardial histological changes and cardiomyocyte apoptosis. Long term Cur-AuNPs protective effect was evaluated for heart/body weight changes and myocardial fibrosis after 14 days of toxicity induction. The results revealed that Cur-AuNPs delivery system was capable to apply heart protection in a much more effective way than curcumin. Cur-AuNPs efficacy is evident both in the short-term results, 24 h after toxicity induction, by reduction of serum biomarkers and apoptotic proteins (Bax and Caspase-3) and no sign of interfibrillar haemorrhage, and intercellular spaces in microscopic images also in the long-term study,14 days later, that indicate Cur-AuNPs could successfully prevent body and heart weight loss.

Introduction

Doxorubicin (DOX), as a member of anthracyclines class, is a potent and one of the most widely used effective chemotherapeutic agents for the cancer treatment. It is commonly used for treatment of some hematological and solid tumor's malignancies in both adults and children. However, its clinical application causes dose-dependent cardiotoxicity, which often results in severe cardiomyopathy, cardiac heart failure, and even transplantation or death in cancerous patients[1-3]. Many studies indicate that reactive oxygen species (ROS) production and apoptosis induction in cardiomyocytes are the principal mechanisms of cardiomyopathy in DOX administration[4-6]. Other proposed mechanisms in DOX-cardiotoxicity include intracellular calcium disturbances, nucleic damages, impaired cardiac energy homeostasis, and autophagy [7,8]. According to some reports, DOX could activate both extrinsic and intrinsic apoptotic pathways [9], also could evoke the fibrotic signaling pathways such as activation of matrix metalloproteinases (MMPs) [10]. Therefore, any strategy for reducing of apoptosis and fibrosis in patients treated with DOX can prevent DOX-induced cardiotoxicity.

Curcumin (Cur), a natural yellow pigment derived from Curcuma longa, has been reported to exhibit anti-inflammatory, antioxidant, and antimicrobial properties [11,12]. In addition, studies have confirmed that Cur

has unique protection properties against cancer, apoptosis, and oxidative stress[13-16]. Cur is not only able to inhibit superoxide, hydrogen peroxide and nitric oxide radicals, but also can increase the activity of antioxidant enzymes [17,18]. Beneficial antioxidant action of Cur has been reported in diabetes, allergies and Alzheimer disease, also it could exert important cardio protective effect through reducing of oxidative stress and mitochondrial damage[18,19]. Previous studies explored the use of combining traditional chemotherapeutic agents with natural compounds, with encouraging results, in which Cur is reported to have increase effect on efficacy of chemotherapeutic agents [20]. Studies indicated that Cur can have reversal effect in doxorubicin-resistant breast cancer cell lines and have confirmed that Cur inhibits tumor growth and can play a synergistic effect with a variety of chemotherapy drugs [21]. DOX combined with Cur is not a new concept for the treatment of malignant tumors. It has been reported in the previous literatures with the ability to reduce the cardiotoxicity of the DOX, but biological application of Cur is limited by its own chemical insolubility in water that cause drug inefficacy[22]. In order to overcome this problem, a large number of nanocarriers have been developed to improve the bioavailability of Cur and its biological efficacy, such as curcumin attachment to the gold nanoparticles[23].

In recent years, gold nanoparticles have attracted more attention in biomedical application [24-26]. This interest is due to the unique properties of gold nanoparticles including tunable Surface Plasmon Resonance (SPR), biocompatibility, high surface reactivity and oxidation resistance. This properties make them suitable for multifunctional applications such as diagnostic application and drug carrier [27-29]. There are different methods for preparation of gold nanoparticles such as physical, chemical and biological synthesis [30,31]. Many studies have revealed toxic effect of chemical methods in biomedical applications[32]. Application of plant extract are of more interest than previous ones because this green synthesis is affordable, environment friendly and safe for clinical applications. Plant extract such as lemongrass, resveratrol, curcumin, etc. have all been used in previous studies as a capping agent that cause to have more stable nanoparticles that at the same time, the benefits of their surface capping can be used in clinical applications [33-35].

Considering the above-mentioned Cur potential in cardiotoxicity prevention from doxorubicin treatment, in this study, we first focused on carrier system to improve the bioavailability of Cur. So, we synthesized a gold nanoparticle reduced with curcumin, generating in this way Cur-AuNPs that are easily soluble in water for further using. Then, we investigated Cur-AuNPs biological effect in DOX-induced cardiotoxicity with emphasizing on its anti-apoptotic effects.

We first synthesised and investigated of physical characterization of Cur-AuNPs including; UV-Visible, size, surface charge, TEM and FTIR then in-vitro cytotoxicity effect of Cur-AuNPs were carried on H9c2 cells. In addition, in-vivo studies were performed in Balb/c with DOX-induced cardiotoxicity for tracking Cur-AuNPs effects in serum marker changes, and evaluation of myocardial histological changes, cardiomyocyte apoptosis, and myocardial fibrosis

The results revealed that, Cur-AuNPs delivery system was capable to apply heart protection effect and could reduce risk of cardiotoxicity in the case of doxorubicin treated mice when it was injected after doxorubicin.

Experimental Section

Materials Curcumin, Doxorubicin, Ketamine, and Dimethyl sulfoxide were purchased from Sigma-Aldrich. Penicillin/Streptomycin solution (5000 IU/mL penicillin and 5000 µg/mL streptomycin) (P/S), Fetal Bovine Serum (FBS), Trypan Blue, 0.25% Trypsin-EDTA, and Dulbecco's Modified Eagle's medium (DMEM) were supplied by Gibc, UK. Recombinant anti-Bax antibody (ab32503), anti-Bcl-2 antibody (ab59348), and anti-Caspase-3 antibody (ab44976), were purchased from abcam, UK. Aspartate Aminotransferase Activity Assay Kit (ab105135) abcam, UK. cTnI AccuBind ELISA Kits (Lake Forest, California, USA). Alanine Transaminase Activity Assay Kit (Colorimetric/Fluorometric) (ab105134). LDH Assay Kit / Lactate Dehydrogenase Assay Kit (Colorimetric) (ab102526). CK - MB Hitachi_serise (Pars Azmoon, Tehran, Iran).

Preparation of gold nanoparticles coated with curcumin (Cur-AuNPs)

Cur-AuNPs were synthesized by using curcumin as a reducing and capping agent. Briefly 120 µL of curcumin

(20 mM solution) dissolved in dimethyl sulfoxide (DMSO) added to 7 mL of deionized (DI) water with adjustment of pH on 9-10 under constant rotating at room temperature for 5 minutes. After releasing enough hydroxyl groups from curcumin solution, for reduction of Au ions, 2.5 ml of HAuCl4 salt was added drop-wisely to the curcumin solution. After that, volume of the solutions was increased to 10 ml by addition of DI water. Then, we kept stirring solution for further 4 h that helped to complete the reaction. Turning the yellow color of HAuCl4 solution into ruby red is the visible sign of nanoparticle synthesis. Finally, the solution was centrifuged at 4000 g/min for 5 min using filtering tubes to wash off all un-reacted materials.

Characterization of Cur-AuNPs

UV-Visible characterization

UV-visible absorption spectra of Cur-AuNPs solution were taken over the wavelength range 190 to 900 nm using UV-Visible spectrophotometer (model Bio Aquarius CE 7250, United Kingdom) to estimation nanoparticles average size and stability in different working medium including, water, normal salin 9% (serum) and Dulbecco's Modified Eagle Medium (DMEM) based on Surface Plasmon resonance spectra (SPR).

Size and surface charge determination of Cur-AuNPs

Hydrodynamic diameter and zeta potential of the Cur-AuNPs were analyzed using Zetasizer Nano (Malvern, Worcestershire, UK) at 25 °C. The measurements for the size and zeta potential were done in triplicate (n=3) and the results were reported as mean ±SD.

- Fourier transform infrared (FTIR) Spectroscopy Fourier transform infrared (FTIR) analysis was used in order to determine functional groups on Cur-AuNPs and interactions between Au^{3+} and curcumin. The nanoparticles were washed and lyophilized to make them powder with Telstar freeze dryers Lyo Quest -85 (Spain). Then sample was prepared by mixing NPs with KBr (1:50, w/w) and pressing into uniform pellets to do scanning between wavenumber range 4000–400 cm^{-1} using Nicolet iS10 spectrometer (USA).
- Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to measure size, shape and morphology of nanoparticles using transmission electron microscope (Zeiss-EM10C-100 KV (Germany)). For this purpose, 50 µL of Cur-AuNPs were loaded on the carbon coated hexagonal copper grids for around 20 minutes and then followed by several times washing steps in double distilled water and air-dried for imaging.

In vitro Cytotoxicity Evaluation of Cur-AuNPs

The cytotoxicity of Cur-AuNPs on H9c2 heart cells was assessed by MTT assay, which measures the reduction of the yellow dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple formazan crystal, mainly by activity of the mitochondrial enzymes cytochrome oxidase and succinate dehydrogenase in living cells. In this regard, cells were grown in DMEM medium supplemented with 10% FBS, and 100 µg/mL P/S in a humidified incubator at 37°C with 5% CO_2 atmosphere. After sufficient growth, cells were removed from the plate by trypsin and 3×10^4 cells/well was seeded in three 96-well cell culture plates and were allowed to attach overnight for the experiments. Next day, cells were treated with prepared Cur-AuNPs at different concentrations concentration (1/5, 3, 6/25, 12, 25, 35, 50 ppm) for 4 h at 37°C in cell culture medium. Next, cells were washed with PBS 1x and incubated in fresh cell medium for an additional 24, 48 and 72h before they were prepared for MTT assay. In mentioned time points, 20 µl of MTT solution in PBS (5mg/ml) was added into each well and the cells incubated for 4 h after that, supernatants were removed and crystals of formasan were dissolved in DMSO (100 µl/well). As negative control, a blank sample with medium was used, while as a positive control untreated cells were used. UV absorbance was measured on a plate reader (Biotek, United States), and at 570 nm (metabolic activity) and 690 nm (reference wavelength).

Animal study

Sixty male Balb/c mice (8-week-old), were used in this study. Animals were obtained from the Experimental Animal Center of Iran University of Medical Sciences. All animals had freely access to food and water. Animals were fed with standard laboratory rodent diet pellets and maintained on a 12-h dark/light cycle in a room with humidity and temperature 22 ± 2°C. This study was performed accordance with the U.S National Institutes of Health for the care and use of laboratory animals' guidelines (NIH Publication No. 85-23, revised 1996). Also All assays were approved by the ethics committee of Iran University of Medical Sciences (Tehran, Iran) (IR.IUMS.FMD.REC.1397.215).

Before animal study, concentrated stock solutions of Cur-AuNPs were sonicated to dissipate any aggregation then filtered with Whatman® Puradisc30 syringe filters and concentration of that was determined with ICP-MS (inductively coupled plasma- mass- spectrometry). Cur-AuNPs solution at 100, 200 and 400 µg/kg was made by diluting in normal saline to prepare a physiological solution for tail vain injection. Amount of injected curcumin is equally same as its amount in Cur-AuNPs synthesis. In the present study, there were two experimental protocols for animal treatment. In the first protocol; sixty male mice were divided into eight groups as shown in Table 1. According to this protocol-1, 24 hours after treatment, animals were weighed. After anesthesia with injection of ketamine hydrochloride (80 mg/kg) and xylazine (8 mg/kg), intraperitoneally, blood samples were collected from heart and centrifuged at 4@C (800 g) for 10 min. Supernatant used for measurement of cardiac injury markers: Lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB) and cardiac troponin I (cTnI) and also liver injury markers: Alanine aminotransferase (ALT) and aspartate aminotransferase (AST). For histopathological studies, samples were fixed in a 10% formalin solution for 48 hours, embedded in paraffin blocks, and finally cut into 6 µm in thickness by microtome. At the end, prepared samples were stained with hematoxylin and eosin (H&E) for histological analysis and immunohistochemical staining to evaluate apoptosis in cardiomyocytes.

In the second protocol; sixty mice were randomly divided into four groups: 1) Control group, 2) DOX acute cardiotoxicity 3) DOX+Cur-147, and 4) DOX+Cur-AuNPs-400; Animals were kept for 14 days then under deep anesthesia with injection of ketamine hydrochloride (80 mg/kg) and xylazine (8 mg/kg) intraperitoneally, animals were scarified and the heart tissue was extracted by thoracotomy and weighed to calculate heart weight (HW) to body weight (BW) ratio (HW/BW), Masson's trichrome staining was used to evaluate cardiac fibrosis.

Measurement of Serum parameters

Lactate Dehydrogenase (LDH): 24 hours after the first protocol, to evaluation of heart damage we quantified LDH activity in serum. LDH converts pyruvate, the final product of glycolysis, to lactate in hypoxic conditions and during injury or toxic damages, the cells will release LDH into the bloodstream so its quantification in serum will give the singe of damage and toxicity of tissue and cells. Based on protocol we first prepared 1.25 mM NADH Standard Solution. All reaction wells were prepared including: Standard wells = 50 µL standard dilutions, and serum samples can be tested directly by adding to wells = 2 – 50 µL samples (adjust volume to 50 µL/well with LDH Assay Buffer). Then, the Reaction Mix was prepared by mixing 48 µL LDH Assay Buffer with 2 µL LDH Substrate Mix and 50 µL of that was added into each standard, and samples. Measurement carried on immediately at OD 450 nm at 2 time points. Absorbance values for each standard is a function of LDH activity. Activity of LDH in the test samples was calculated as: ΔA450nm = (A2 – A1) Where: A1 is the sample reading at time T1 and A2 is the sample reading at time T2. Then ΔA450 nm was used to obtain B nmol of NADH generated by LDH during the reaction time (ΔT = T2 – T1). Based on protocol activity of LDH in the test samples is calculated as:

$$LDH\ Activity\ = \frac{B}{(T) \times V} \times D\ = nmol/min = mU/ml$$

B: Amount of NADH in sample well calculated from standard curve (nmol). ΔT: Reaction time (minutes). V: Original sample volume added into the reaction well (mL). D: Sample dilution factor. Unit Definition: 1

Unit LDH = amount of enzyme that catalyzes the conversion of lactate to pyruvate to generate 1.0 µmol of NADH per minute at pH8.8 at 37°C.

Creatine Kinase MB (CK-MB): CK-MB, is a ready to use chromatography based kit to fast detection of myocardial infarction (MI) that will be in the highest level 24 h after injury. To perform the test, simply we dropped the serum samples on a cassette of kit and then wait to samples migration along the capillary chromatographic membrane. In positive samples, the result will be determined by the appearance of a colored bar relative to the control group.

Cardiac-specific Troponin I (cTnI): In addition, Cardiac-specific Troponin I (cTnI) concentrations (ng/ml) were measured by cTnI AccuBind ELISA Kits (Lake Forest, California, USA). cTnI is a protein found in the heart and has been known as a marker of heart attacks and myocardial cell death. As the concentration of it's subunits will increase in circulation after degradation of cardiac myofibrils it has been considered as a specific biomarker. Principle of this method is based on dose response curve that briefly in this method first, Troponin-I calibrator manufactured at different concentration (ng/ml) and samples from serum are add to the streptavidin coated well, then, biotinylated monoclonal and enzyme labeled antibodies were mixed to make a sandwich form on the well. After the completion of required incubation time, un-bound materials were separated with wash buffer and finally enzyme activity were quantified by addition of working signal reagent and incubation for 5 min in dark. Based on the relative light unit (RLUs), dose response curve were drew to calculate cTnI concentrations in samples.

Aspartate aminotransferase (AST): AST, also known as Aspartate transaminase and Glutamate-oxaloacetate transaminase (GOT) assay that was performed for myocardial infarction detection. This protocol is based on glutamate detection and for that converts from a nearly colorless probe to color (λmax = 450 nm) is detectable. In this method we followed kit protocol (ab105135). Briefly, we first prepared Glutamate Standard with dilution of 10 µl of the 0.1M Glutamate Standard with 990 µl Assay Buffer to generate 1 mM glutamate. For next step, we add 0, 2, 4, 6, 8, 10 µl of that into each well individually and adjust the final volume to 50 µl/well with Assay Buffer to generate 0, 2, 4, 6, 8, 10 nmol/well. Then 100 µl of the Reaction Mix containing Assay Buffer (80 µl), Enzyme Mix (2 µl), Developer (8 µl), and Substrate (10 µl) was added to each well containing the samples. In the next step, glutamate standard curve was plotted after reading OD450nm with microplate reader at (A1) at T1 (T1 > 10 min) then again (A2) at T2 after incubating the reaction at 37°C for 60 min. Based on reading formula of ΔA450nm= A2 − A1 ΔA450nm was used to obtain B nmol of glutmate. At the end, aspartate aminotransferase (AST) activity in the test samples calculated by following equation:

$$AST\ Activity\ = \frac{B}{(T2 - T1) \times V} \times D\ = nmol/min = mU/ml$$

B: The glutamate amount (nmol) calculated from the Standard Curve.T1: The time of the first reading (A1) (in min). T2: The time of the second reading (A2) (in min). V: The original sample volume added into the reaction well (in ml).D: The sample dilution factor.

Unit Definition: One unit of AST is defined as the amount of AST which generates 1.0 µmol of glutamate per minute at 37 °C

Alanine Transaminase Activity (ALT): ALT enzyme is also called serum glutamic pyruvic transaminase (SGPT) or alanine aminotransferase. (ALAT) is found in serum and in various bodily tissues, but is usually associated with the liver. In the ALT assay protocol, ALT transfers an amino group from alanine to α-ketoglutarate; producing pyruvate and glutamate. The pyruvate is detected in a reaction that converts a nearly colorless probe to a form that is colored (OD_{max}= 570 nm) and fluorescent (Ex/Em = 535/587 nm). Briefly, first all reaction wells were prepared including: Standard wells = 20 µL Standard dilutions, and sample wells = 2 − 20 µL samples (adjust volume to 20 µL/well with ALT Assay Buffer). Then, the Reaction Mix was prepared from ALT Assay Buffer, OxiRed Probe, ALT Enzyme Mix, ALT Substrate based on

formula in protocol and 100μL of that was added to each well. Measurement was carried on a microplate reader, OD=570 nm, 2 time point T1 and T2. Activity of ALT is calculated as:

ΔA570 nm= A2 – A1, where: A1 is the sample reading at time T1 A2 is the sample reading at time T2.

Same as previous protocol we used the ΔA570nm to obtain B nmol of Pyruvate generated by ALT during the reaction time (ΔT = T2 – T1).

Concentration of pyruvate in the test samples is calculated as:

$$ALT\ Activity\ = \frac{B}{(T2-T1)\times V} \times D\ = nmol/min = mU/ml$$

B: Amount of pyruvate from Pyruvate Standard Curve. T1: The time of the first reading (A1) (in min). T2: The time of the second reading (A2) (in min. V: original sample volume added into the reaction well (in mL). D = sample dilution factor.

Unit Definition: 1 Unit ALT = amount of ALT which generates 1.0 μmol of Pyruvate per min at 37°C.

Detection of apoptosis in heart sections

24 hours after the first protocol, cardiomyocyte apoptosis was evaluated via the immunohistochemical staining with Bax, Bcl-2 and Caspase-3 primary antibodies. The slides were then analyzed with a light microscope under a high power field (×400). The digital photomicrographs were taken from 6 random fields within each section. The number of apoptotic myocardial cells in each field were determined as the number of apoptotic cells per field.

Measurement of myocardial fibrosis

14 days after treatment according to the second protocol, 6 μm sections were stained with Masson's trichrome (Sigma-Aldrich Co., MO, USA). Cardiac fibrosis was also calculated by Photoshop software in the heart sections (Ver. 7.0, Adobe System, San Jose, CA, USA). Myocardial fibrotic area was calculated as the ratio of the interstitial fibrotic area relative to the total specimen area.

Result

Cur-AuNPs synthesis and Characterization Light exposed metallic nanoparticle like gold nanoparticles can do collective coherent oscillation with electromagnetic field. The amplitude of the oscillation reaches maximum at a specific frequency, called surface plasmon resonance (SPR), that it's intensity and wavelength depends on the factors affecting the electron charge density on the particle surface such as particle size. Particle size can be estimated based on Mie theory according to the following equation [36]:

$$C_{\text{ext}} = +\frac{24\pi^2 R^3 {\varepsilon_m}^{\frac{3}{2}}}{y} \times \frac{\varepsilon_i}{{(\varepsilon_r + 2\varepsilon_m)}^2 + {\varepsilon_i}^2}$$

Where Cext is the extinction, λ is the wavelength of the incident light, ε_r is the real part of complex dielectric constant, ε_i is the imagery part of the dielectric function of the metal, ε_m is the dielectric constant.

Cur-AuNPs scheme is shown in Figure 1-A. As can be seen in Figure 1-B UV-Visible spectra of Cur-AuNPs is showing slightly decrease in the SPR intensity after washing that can be due to removing of extra curcumin around the nanoparticles. Estimated average particle size for Cur-AuNPs based on UV-Vis absorbance peak in 528 nm was about 7 nm. The SPR spectra of Cur-AuNPs also were checked after dispersion in DI water, serum, and DMEM to determine the stability of nanoparticles with changing working medium. The absorbance spectra of Cur-AuNPs in three different mediums were shown in Figure 1-C, without any considerable change. Estimated size was almost similar for all samples which is a good sign indicating nanoparticles stability in different mediums.

With changing working medium from water to normal saline or DMEM, DLS data showed increasing in hydrodynamic diameter from 32.0±3.6 in water to 180.3±3.2 and 287.6±6.5 nm in DMEM and serum, respectively. This size alteration happened by changing surrounded ions around Cur-AuNPs Figure 1-(D-F). In addition, dynamic light scattering (DLS) was applied to measure the hydrodynamic diameter of the nanoparticles and results showed that the hydrodynamic size of Cu-AuNPs was about 32.0±3.67 nm, with narrow size distribution (PDI ˜0.3) and negative zeta potential -42.0±0.2.

Moreover, TEM micrographs confirmed formation of nanoparticles (Figure 1-H) and almost their spherical shape with average particle size 9.6 ± 0.4 nm (by measuring the size of about 500 particles). This size difference comes from the difference in the measurement method, which in DLS we have hydrodynamic diameter but TEM gives a real diameter.

To investigate the presence of curcumin on the surface of AuNPs we performed FTIR analyses as shown in Figure 1-G to find the C=C and C=O stretching band, and CH3 and C-CO-C bending bands as the signs of curcumin presence in the sample. In addition advent of metal-curcumin combination band was seen at ˜ 450 cm^{-1} which was absent in the spectrum of curcumin[37,38]. All of these results are a sign of presence and strong curcumin molecules capping on the surface of AuNPs that cause to have the stable nanoparticles.

Cell viability assay

MTT assay of Cur-AuNPs on H9c2 heart cells was carried on 24, 48 and 72 h after cells treatment for seven NPs concentrations from 1.5-50 ppm. As shown in Figure-2, the viability of H9c2 cells exposed to Cur-AuNPs remained at more than 80%, up to 50 ppm after 24 h, suggesting no toxicity from such a green synthesized Cur-AuNPs. But cultured cell for 48 and 72 h were represented some amount of toxicity but still more than 60% that is not reached to IC50.

Effect of Cur-AuNPs on serum markers

Collected results from cardiac (LDH, CK-MB, cTnI) and liver injury markers (AST, ALT) were analyzed and shown in Figure-3(A-E). Treatment with DOX significantly increased LDH, CK-MB, cTnI markers ($p<0.001$, $p<0.003$ and $p<0.001$, respectively) and liver injury markers ($p<0.0001$) in comparison with control group. Based on obtained results from quantification of LDH serum level (Figure-3A), animals treated with DOX+Cur-AuNPs200 and DOX+Cur-AuNPs400 have shown the most effective results that the amount of LDH was significantly lower than all other treatment groups ($p< 0.0001$). In the group treated with DOX high level of LDH is release to serum compare to control ($p< 0.0001$) but results are indicating that curcumin injection alone can prevent heart damage from DOX treatment but its efficacy even in high concentration DOX+Cur147 group is significantly lower than when we have DOX+Cur-AuNPs groups ($p<0.0001$).

Results of CK-MB analysis is shown in Figure-3B indicating that CK-MB biomarker level is increased significantly in DOX, DOX+Cur40, DOX+Cur73 and DOX+Cur-AuNPs100 groups ($p<0.0001$, $p<0.003$, $p< 0.04$ and $p<0.03$ respectively) compared to control group. Also decreased in all DOX+Cur and DOX+Cur-AuNPs groups in comparison with DOX group ($P<0.0001$). But effective results to prevent heart damage of DOX induction is obtained in the group of DOX + Cur-AuNPs400 that is almost same as control group (P˜ 1).

Results of cTnI quantification is shown in Figure-3C. As can be seen, statistical analysis were showed that serum level of cTnI was increased in all groups except the Cur-AuNPs400 group compared with control ($P<0.0001$). In addition, treatment with different doses of curcumin (40, 73 and 147 μg/kg) had significant effect in decrease of cTnI serum level ($p< 0.02$, $p< 0.007$ and $p<0.0001$ respectively) and also Cur-AuNPs (100, 200 and 400 μg/kg) ($p<0.0001$) reduced serum level of cTnI in comparison to DOX group. When we compared serum levels of cTnI between 2 effective dose of DOX+Cur147 and DOX+Cur-AuNPs400, results indicated that, attached curcumin on AuNPs surface was presented more effective that significantly decreased cTnI biomarker level in serum ($p<0.0001$). DOX+Cur-AuNPs400 effect was 1.6 times better than DOX+Cur147, comparable with control group, this is while they have same amount of curcumin.

Analyzed data from AST and ALT quantification of animal groups is depicted in Figure-3(D,E). Our results

showed that as we expected AST and ALT levels were significantly increased in DOX- treated group same as all previous DOX treated groups in comparison with control group ($P<0.0001$). Furthermore, the level of AST in animal treated with different doses of curcumin (40, 73 and 147 μg/kg) and Cur-AuNPs groups ($p<0.03$, $p<0.02$ and $p<0.002$ respectively) and ($P<0.0001$) decreased in comparison with DOX group. But again the most effective group to prevent heart damage and following that decrease serum biomarker level is group treated with Cur-AuNPs400 comparable with DOX+Cur147 group ($P< 0.002$). In addition, serum level of ALT was decreased in DOX+Cur-AuNPs200 and DOX+Cur-AuNPs400 groups in comparison with DOX group ($p<0.043$, $p<0.0001$ respectively). Our results also showed that treatment with Cur-AuNPs400 was more effective than different doses of curcumin (40, 73 and 147 μg/kg) ($p<0.0001$, $p<0.0001$ and $p<0.001$ respectively) and also comparable with Cur-AuNPs100 and Cur-AuNPs200 groups ($p< 0.001$ and $p<0.012$).

Quantification of serum biomarkers after DOX induction toxicity showed that for 2 groups with curcumin; DOX+Cur147 and Cur-AuNPs400 had the best efficacy. In this way they were selected as an efficient concentration for further experiment.

Effect of Cur-AuNPs on myocardial histological change

Acute DOX-induced toxicity was assessed by H&E staining 24 hours after injection. Results are shown in Figure-4. The heart sections from control group showed regular cell distribution and normal myocardium morphology. However, the cardiac myofibrils in DOX group showed increasing of intercellular spaces, loss of myofibrils, interfibrillar hemorrhage, vacuolization of the cytoplasm and cellular necrosis. Myocardial histology from DOX+Cur-AuNPs400 treated animals showed that there were no interfibrillar hemorrhage, and intercellular spaces were reduced. Our results also showed that in DOX+Cur treated groups, curcumin could not prevent the toxic effect of DOX.

Effects of Cur-AuNPs on cardiomyocytes apoptosis

In this study, we performed immunohistochemical staining to investigate DOX-induced apoptosis and the effects of Cur-AuNPs400, 24 hours after treatment. The arrows in Figure-5A represent Bcl-2, Bax and Caspse-3 positive cells (brown color). As can be seen in Figure-5B, the number of Bcl-2 positive cells are decreased in DOX and DOX+Cur147 groups as compared with control group ($P<0.0001$ and $P<0.001$ respectively). Whereas, in the Cur-AuNPs400 treated group, number of Bcl-2 positive cells are not deceased that is comparable with control group without significant difference ($P>0.670$). But there was significantly deferent in comparison with DOX+Cur147 group ($P<0.03$). On the other hand, expression level of Bax is increased in DOX and DOX+Cur147 groups in comparison with control group (Figure-5,C $P<0.0001$). This is while Cur-AuNPs400 treatment significantly reduced number of Bax-positive cells in comparison with DOX and DOX+Cur groups ($P<0.004$ and $p<0.06$, respectively). Expression level of caspase-3 is increased in DOX and DOX+Cur147 and Cur-AuNPs400 groups in comparison with control group ($P<0.0001$, $p<0.001$ and $p<0.032$, respectively). Moreover, the number of caspase-3 positive cells are decreased significantly compared with DOX group after treatment with Cur-AuNPs400, (Figure-5D, $P<0.001$). All results are indicating that Cur-AuNPs400 have the best anti-apoptotic effect, and it can inhibit toxic effect of DOX in cardiac cells.

According to the second protocol, after 14 days we studied HW/BW ratio. The body and heart weight in DOX group and DOX+Cur147 were significantly decreased compared to control group ($P< 0.0001$ and $P< 0.0001$ respectively). Our results also showed that treatment with Cur-AuNPs400 in DOX-intoxicated mice prevented body and heart weight loss compared with DOX group ($P<0.0001$ and $p<0.003$ respectively). The ratio of heart weight to body weight was not significantly changed between all groups ($P>0.05$). At the end of study, there was not mortality in groups except four mice in DOX group and two in DOX+Cur147 (Table 2).

Effect of Cur-AuNPs on myocardial fibrosis

Our results showed that, there was no significant difference in the amount of fibrosis between control, DOX+Cur147, and Cur-AuNPs400 treated groups ($P>0.05$). In the other hand, staining with Masson's

trichrome after 14 days showed that there was not collagen deposition and fibrosis in DOX, DOX+Cur147, and Cur-AuNPs400 compared with control group ($P>0.05$) (Figure-6).

Discussion

In the present study, we have studied the effects of curcumin coated gold nanoparticles (Cur-AuNPs) on DOX-induced acute cardiotoxicity in male mice. DOX, as a member of anthracyclines, is using for cancer treatment. However, it's clinical application is associated with cardiotoxicity [39]. In this study, acute cardiotoxicity in animals were induced by single dose injection of DOX (20 mg/kg). In previous studies this dose of DOX lead to cardiotoxicity[40]. Also our results showed that DOX treatment caused decrease in HW, BW and ratio of HW/BW, and increase in serum level of heart damage biomarkers including LDH, CK-MB, AST, ALT, and cTnI. In addition we could induce cardiomyocytes apoptosis and histopathological changes in heart tissue with DOX injection. The present results are similar to previous reports and Indicates success in cardiomyocytes inducing by DOX injection [41].

In addition, we reported that Cur-AuNPs treatment significantly reduced myocardial apoptosis in DOX+Cur-AuNPs animals, also could improve histopathological change and return serum levels of heart and liver injury markers to normal level. Our result indicated that Cur-AuNPs has a protective effect against cardiotoxicity induced by DOX.

Previous studies indicated that Cur is a natural compound well known as antioxidant and cardioprotective agent [16]. But because of low solubility of curcumin in the body fluids, the protection effects are limited [23]. In this study, curcumin coated gold nanoparticles, Cur-AuNPs, were synthesized completely base on curcumin that had dual role of reducing and capping agents to prepare 1) a stable delivery way for curcumin; cause more solubility of that 2) curcumin protection to have more efficacy; soluble and stable Cur-AuNPs are likely to have more penetration into the tissue and have effect. Our FTIR results confirmed present of curcumin in the structure.

NPs characterization indicated that synthesized Cur-AuNPs have a small size that is around 32.0 nm, and 9.6nm with DLS and TEM, respectively. With minus zeta potential 42. Since the basis of our synthesis is based on curcumin, and Au here acts as a central nucleus that helps curcumin to deposit on it, so any changes in NPs will indicate the collapse of the structure. In this way we tracked SPR spectra of Cur-AuNPs and results showed that particles were remained stable in DI water, normal saline and DMEM without any SPR shift. All studied concentration of Cur-AuNPs on H9c2 cell line showed cell viability above IC50 so we calculated injection dose base on curcumin in synthesis and above IC50.

In the first protocol of animal study after 24 h of DOX injection; acute dose of DOX induced cardiotoxicity and led to increase serum level of LDH, CK-MB, AST, ALT, and cTnI. In addition cardiomyocytes apoptosis and histopathological changes. Also in the second protocol after 14 days of DOX injection, we observed that acute dose of DOX caused decrease in heart weight and body weight. Masson 's trichrome staining after 14 days showed that although there was slight tissue change, but it did not reach the stage of cardiac fibrosis. Whereas, single dose of Cur-AuNPs400 in DOX intoxicated mice prevented this increase. It seems that Cur-AuNPs by preserving structural of the heart could prevents the increase of markers.

According to previous studies treatment with DOX caused cardiac fiber loss, interfibrillar hemorrhage, vacuolization and myocardial necrosis[42,43]. In line with previous study, our microscopic observations confirmed these changes in heart tissue in DOX treated group after 24 hours. In contrast, our histological results showed that treatment with Cur-AuNPs400 prevented myocardial changes in compared to the DOX group. It seems that Cur-AuNPs400 by preventing cardiac cell apoptosis could prevent heart tissue changes. Cardiomyocytes apoptosis is the critical feature of DOX cardiotoxicity [44,45]. Many studies have reported that DOX stimulates free radicals formation which is necessary for cardiac apoptosis [46-48]. Although, the exact mechanisms of DOX-induced cardiotoxicity are not yet completely clear. Many study supports the significant role of apoptosis after a single dose of DOX in cardiomyocyte [49,50]. The Bcl-2 protein family play a key role in cell apoptosis. In this family, there are both pro-apoptotic proteins such as Bax and Casp-3 and anti-apoptotic proteins such as Bcl-2. It is proven that during the apoptosis process, cardiac Bax and Casp-3

expression levels raise and Bcl-2 protein expression reduces in DOX induced cardiotoxicity[41]. Our results showed that DOX caused to decrease expression levels of Bcl-2 as one of the most important regulators of cell death and increased Bax and Casp-3. In contrast, single intravenous injection of Cur-AuNPs400 significantly increased Bcl-2 and decreased Bax and Casp-3 releasing to blood stream. These data showed that Cur-AuNPs400 had a beneficial effect on myocardial apoptosis prevention. In long term study, DOX led to decrease the BW and HW after 14 days, but there was no change in HW/BW after 2 weeks. These results are in agreement with previous studies [51]. It may be due to the myofibrils loss, myocardial necrosis and food intake reduction[52]. Our results also showed that heart and body weight loss were found to be inhibited successfully in the Cur-AuNPs400 treated groups. This data is proving the beneficial effects of Cur-AuNPs on myocardial structure and shown that Cur-AuNPs could prevent cardiac fiber loss, myocardial necrosis and body weight loss.

There is evidence that acute and chronic cardiac toxicity is associated with collagen accumulation in tissue [53]. Induction of cardiac toxicity by DOX and evaluation of cardiac fibrosis after 14 days showed that, although tissue changes were evident, but these changes did not reach the collagen deposition stage and cardiac fibrosis. We agree with Tiam Feridooni et al., also found that single dose of DOX injection caused to cardiac fibrosis after 3 days, but the rate of this fibrosis decreased after 7 days [54]. Another study showed that cardiac fibrosis had disappeared 5 days after DOX injection[55]. In this study, we assume that the cause of non-fibrosis after 14 days is because to acute injection of DOX has inactivated the MMPs, also the secondary pathways that cause fibrosis, such as inflammation and neurohormonal pathways, were not activated. It which was attributed to the lack of and not activated of secondary pathways of fibrosis [54].

Conclusion

Based on this study we can suggest that Cur-AuNPs400 has protective effects on doxorubicin-induced cardiomyopathy. Cur-AuNPs400 attenuate myocardial damages and apoptosis via increasing Bcl-2 and decreasing Bax and Casp-3 expression. However, more detailed studies are essential to confirm the findings.

Statistical analysis

All data were shown as mean ± SEM. GraphPad Prism software (Version 5.00) was used for statistical analysis. For comparisons among different groups, normally distributed data were analyzed by one-way ANOVA and Tukey test as a post-hoc analysis. P-value <0.05 was considered to be statistically significant.

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Conflict of interest statement

The authors report that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Z.S.A performed experimental tests, collected the data and wrote the manuscript draft F.D. contributed in performing the experiments and preparation of Figure; M.S.A and E.S. participated in the preparation of Nanoparticles, and revised the initial draft; F.R. participated in cellular techniques; F.N. analyzed the data and revised the initial draft; Y.A. processed the data and supervised the whole project and manuscript preparation; All authors reviewed the manuscript.

DATA AVAILABILITY STATEMENT

All data supporting the findings are available from the corresponding author upon reasonable request.

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	Group name	**Group information**
Protocol 1	**Control (Ctrl)**	Animals received intraperitoneal (i.p)
	Doxorubicin (DOX)	Animals received a single dose of DO
	Doxorubicin and Curcumin-40 (DOX + Cur40)	Animals received single intravenous (
	Doxorubicin and Curcumin-73 (DOX + Cur73)	Animals received single i.v injection o
	Doxorubicin and Curcumin-147 (DOX + Cur147)	Animals received single i.v injection o
	Doxorubicin and Cur-AuNPS-100 (DOX + Cur-AuNPs100)	Animals received single i.v injection o
	Doxorubicin and Cur-AuNPS-200 (DOX + Cur-AuNPs200)	Animals received single i.v injection o
	Doxorubicin and Cur-AuNPS-400 (DOX + Cur-AuNPs400)	Animals received single i.v injection o
Protocol 2	**Control (Ctrl)**	Animals received (i.p) injection of ap
	Doxorubicin (DOX)	Acute cardiotoxicity induced by DOX
	DOX+Cur147	After acute cardiotoxicity induction,
	DOX+Cur-AuNPs400	After cardiotoxicity induction, they r

Table 2. Mortality rate and effect of Cur-AuNPs on body weight, heart weight and heart weight/body weight after 14 days of treatment (n=5).

Treatment	Control	DOX	DOX +Cur $_{147}$	DOX + Cur-AuNPs$_{400}$
Mortality	0	4	2	0
HW (mg)	0.52 ± 0.04	0.32 ± 0.02***	0.36 ± 0.04**	0.45 ± 0.06++++
BW (g)	34 ± 1.15	23.6 ± 1.67***	26.2 ± 1.64***	32.57 ± 1.39++++++++++
HW/BW (mg/g)	15.45 ± 1.42	13.79 ± 2.11	14.04 ± 1.97	14.84 ± 2.22

BW; Body weight (g), HW; Heart weight (mg), HW/BW; Heart weight/body weight, DOX; Doxorubicin, Cur; Curcumin, Cur-AuNPs; Curcumin coated gold nanoparticles. Data are presented as mean ± SEM. * P< 0.05, ** P< 0.01 and *** P < 0.001 vs. control group. + P< 0.05, ++ P < 0.01 and +++ P < 0/001 vs. DOX group and. ++ P < 0.05, ++?++ P < 0.01 and ++?++?++ P < 0/001 vs. Cur147 group. one-way Variant analysis or # the Kruskal-Wallis test.

Figure 1: (A) Schematic image of Cur-AuNPs. Cur-AuNPs; curcumin coated gold nanoparticles. (B) UV-Vis absorbance spectra of Cur-AuNPs supernatant after and before washing. Cur-AuNPs; curcumin coated gold nanoparticles. (C) Stability of Cur-AuNPs. UV-Vis absorbance spectra of Cur-AuNPs in different mediums. Cur-AuNPs; curcumin coated gold nanoparticles. DLS data hydrodynamic diameter of

Cur-AuNPs in (D) water, (E) serum and (F) DMEM. Cur-AuNPs; curcumin coated gold nanoparticles. (G) FTIR spectrum of Cur-AuNPs. (H) TEM micrographs of Cur-AuNPs, (inset) Corresponding size distribution histogram. Cur-AuNPs; curcumin coated gold nanoparticles.

Figure 2: Cell viability of H9c2 cells after exposure to curcumin coated gold nanoparticles (Cur-AuNPs). Cells were treated with different concentrations of Cur-AuNPs (1.5-50 ppm) for 24h, 48h and 72h. The cell viability was determined by MTT assay.

Figure 3: Effect of Cur-AuNPs on LDH, CK-MB, cTnI, AST and ALT after 24 h in all experimental groups(n=5). LDH; Lactate dehydrogenase (IU/L), CK-MB; Creatine kinase-MB (IU/L), cTnI; Troponin I (ng/ml), AST; Aspartate aminotransferase (IU/L), ALT; Alanine aminotransferase (IU/L), DOX; Doxorubicin, Cur; Curcumin, Cur-AuNPs; Curcumin coated gold nanoparticles. Data are presented as mean +- SEM. * P< 0.05, ** P< 0.01 and *** P < 0.001 vs. control group. + P< 0.05, ++ P < 0.01 and +++ P < 0/001 vs. DOX group and. ++ P < 0.05, ++?++ P < 0.01 and ++?++?++ P < 0/001 vs. Cur147 group.

Figure 4: Histological changes of myocardial tissue after 24 h of treatment (n=5). Transverse sections of the hearts from apex to base stained with H&E to detect myocardial changes. 400 X magnifications. DOX; doxorubicin, Cur; Curcumin, Cur-AuNPs; Curcumin coated gold nanoparticles.

Figure 5: (A) Immunohistochemical staining 24 h after treatment (n=5). (B) Quantification of positive cell numbers of Bcl-2, (C) Bax and (D) Caspase-3. The arrows represent Bcl-2, Bax and Caspse-3 positive cells (brown color) (100 x magnification). Blue points represent nuclei of cells. DOX; Doxorubicin, Cur; Curcumin, Cur-AuNPs; Curcumin coated gold nanoparticles. Data are presented as mean +- SEM. * P< 0.05, ** P< 0.01 and *** P < 0.001 vs. control group. + P< 0.05, ++ P < 0.01 and +++ P < 0/001 vs. DOX group and. ++ P < 0.05, ++?++ P < 0.01 and ++?++?++ P < 0/001 vs. Cur147 group.

Figure 6: Histological analysis of fibrosis at day 14 after treatment (n=5). Transverse sections of the hearts from apex to base stained with Masson's trichrome to detect interstitial fibrosis. 100 X magnification DOX; Doxorubicin, Cur; Curcumin, Cur-AuNPs; Curcumin coated gold nanoparticles.

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