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Research Article

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Attenuation of Doxorubicin Induced Genotoxicity in HepG2 Cells: Effect of Melatonin Loading Chitosan-Tripolyphosphate Nanoparticles on Oxidative stress

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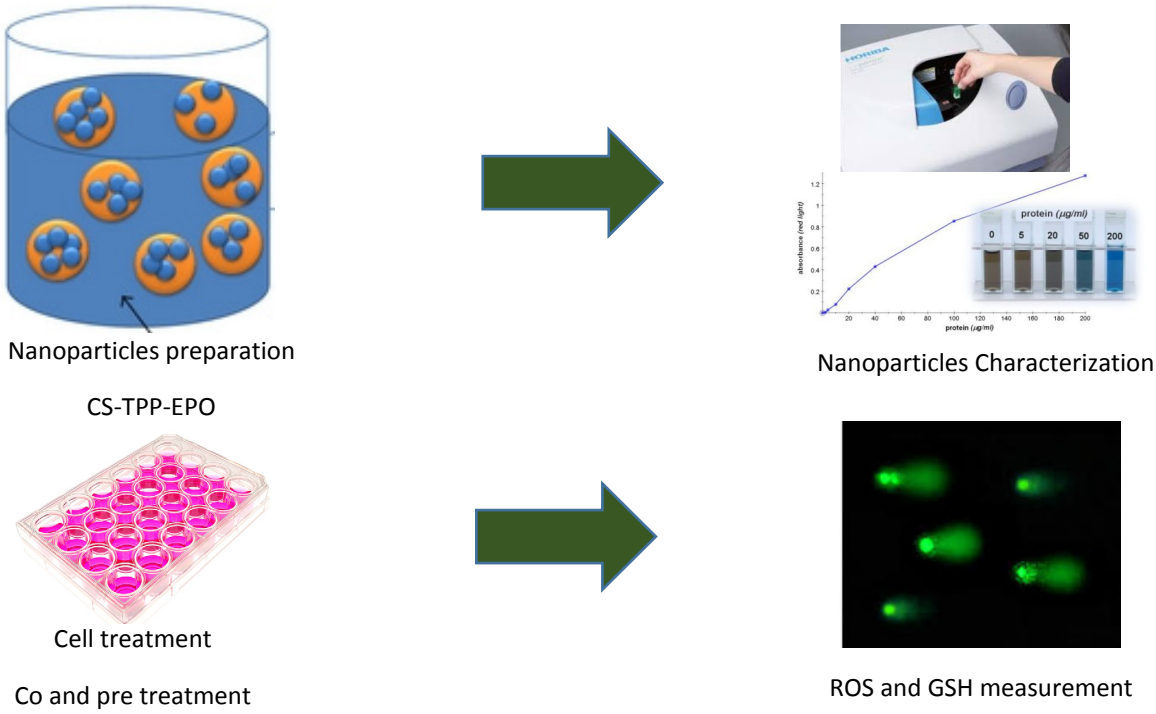
Abstract

Doxorubicin is a chemotherapy medication applied in the treatment of numerous cancers. Like other topoisomerase II inhibitors, doxorubicin has been shown genotoxic effects in both in vitro and in vivo models. Melatonin acts as a potent antioxidant. In additional, anti-inflammatory, anti-apoptotic, cytoprotective and genoprotective effects of melatonin have been reported in previous studies. The aim of present study was to determine protective role of melatonin nanoparticles against doxorubicin induced-genotoxicity. HepG2 cells were treated with various concentrations of doxorubicin, melatonin and nano melatonin in both pre- and cotreatment conditions and then analyzed via comet assay. Besides the intracellular reactive oxygen species and glutathione levels have been assessed. The results of current study show that doxorubicin induced a clear genotoxic effect in HepG2 cells. Melatonin and its nanoparticles decreased the genotoxic effects of doxorubicin significantly in both types of experiment states that exhibited by comet assay. Furthermore, both forms of melatonin decreased the intracellular reactive oxygen species generation and increased the intracellular glutathione contents in HepG2 cells. However, nano melatonin was more effective in attenuating of oxidative stress and DNA damage induced by doxorubicin.

Keywords: Doxorubicin, Melatonin, Genotoxicity, Comet Reactive Oxygen Species, Glutathione



Introduction

Doxorubicin, one of the highly active chemotherapeutic agents, is widely applied in clinical for treatment of various cancers such as acute lymphoblastic leukemia, hepatocellular carcinoma, ovarian carcinoma and breast carcinoma [1-3]. This drug acts as a topoisomerase II inhibitor and exerts its anti-cancer effects via causing DNA damage and inducing cell cycle arrest in cancer cells [4,5]. However, the use of doxorubicin is limited by its deleterious effects including myelosuppression, cardiotoxicity and hepatotoxicity [1,6,7]. Literature so far reports that doxorubicin induced apoptosis following DNA damage is an important mechanism for its toxic effects in various cell lines [8-9]. In addition, numerous other mechanisms by which doxorubicin would exert cytotoxicity have been demonstrated. Some of these mechanisms are generation of reactive oxygen species (ROS) and subsequently oxidative stress, formation of DNA double-strand breaks and prevention of DNA replication [10]. A prominent feature of doxorubicin is DNA damage [9]. The genotoxic effects of this drug have been reported in several in vitro and in vivo studies [11].

Melatonin (N-acetyl-5-methoxytryptamine), (MT), an endogenous hormone, exhibits a potent antioxidant effects directly by scavenges •OH and peroxynitrite anion ($ONOO^-$) and indirectly by supporting superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities [12,13]. Melatonin promotes the activity of glutathione reductase in organism cells and also stimulates glutathione production in cells via motivates γ-glutamylcysteine synthase [14]. This protective agent can reduce oxidative damage in both the lipid and aqueous milieus of cells because of its amphiphilic feature [15]. Melatonin has also a number of other important functions, such as anti-inflammatory, anti-apoptotic and cytoprotective effects [16,17]. Previous studies demonstrated that melatonin has protective effects against chemotherapeutic agents induced genotoxicity in both in vitro and in vivo models [18,19]. Although, melatonin acts as a highly potent ROS scavenging antioxidant, but this molecule have not enough efficiency to reduce genotoxic damage because of its weak bioavailability and its short half-life [20].

Nowadays, Nanoparticles have been applied in medicine because of their ability to deliver drugs in the ideal dosage range that lead to enhance therapeutic efficiency of the drugs and attenuate side effects of them [21,22]. On the other hand, numerous polymers have been used in drug delivery investigation as they can successfully deliver the drugs to the target location [23]. Chitosan (CS), a type of natural biopolymer, is one of these polymers that extracted from the shells of crustaceans [24]. This component has been used widely for medical and pharmaceutical applications such as targeted drug delivery and drug transport [25]. In the current study, CS and tripolyphosphate (TPP) nanoparticles comprising melatonin by an ionotropic gelation method were made.

The Single-cell gel electrophoresis assay (comet assay) is a rapid and sensitive method for detecting genotoxic and geno-protective effects of both chemical and nonchemical compounds and is accepted as a valid technique for determining DNA damage in individual cells [26-28]. The present study was designed to evaluate the potential protective effect of melatonin nanoparticles against doxorubicin-induced genotoxicity in HepG2 cell lines. To achieve this, we assessed the DNA damage level with comet assay in HepG2 cell lines treated with doxorubicin and melatonin nanoparticles in co- and pretreatment conditions. Furthermore, ROS generation and intracellular glutathione (GSH) levels as parameters of oxidative stress were also investigated to reveal probable mechanisms of doxorubicin induced genotoxicity.

Materials and Methods

Chemicals

Doxorubicin, melatonin, CS (Mw, 550,000; deacetylation degree, 90%) and TPP were purchased from Sigma-Aldrich, France. H2O2, NaCl, NaOH, EDTA, NaH2PO4, Na2CO3, Triton X-100, Tris and Coomassie Brilliant Blue were acquired from Merck Co. (Germany). Low melting point agarose (LMA), Na2HPO4, KCl, and ethidium bromide were supplied by Sigma Co. (USA). DCFH-DA probe and mBCl were purchased from sigma Aldrich (USA). The RPMI 1640 medium, antibiotics and fetal bovine serum (FBS) were obtained from Biosera (France). Normal melting point agarose (NMA) was from Cinnagen Co (Iran), and HepG2 cells were products of Pasture Institute (Iran). All other chemicals and reagents used in present study were of analytical grade.

Preparation and Characterization of the CS-TPP Nanoparticles

CS-TPP nanoparticles were prepared using ionic gelification technique as previously described (Shokrzadeh and Ghassemi-Barghi 2018). Briefly, a 1% CS in 1% acetic acid was made at ambient temperature, using magnetic shaking. TPP aqueous solution was added drop by drop into the CS solution, then the pH was adjusted to 5.7. Amount of TPP solution used depends on the concentration of the CS, that yielded a final CS to TPP mass ratio of 4: 1. The CS/TPP nanoparticles were scattered in ethanol and after that, the size of particle, size distribution of the nanoparticles (PDI) and zeta potential were assessed using a particle size analyzer (zeta analyzer, Malvern, UK).

Determination of the Encapsulation Efficacy in the CS Nanoparticles

For encapsulation of melatonin on CS/TPP nanoparticles, melatonin was dissolved in the CS solution at the end concentration of 100 μmol/L prior to the addition of TPP. Loaded NPs were collected by centrifugation at 10,000 rpm for 30 min. The encapsulation efficiency (EE) of melatonin was determined by the Bradford protein assay spectrophotometric method at 592 nm. The CS/TPP encapsulating rate was calculated as follows:

EE% = (T Melatonin- S Melatonin)/T Melatonin × 100%

Where EE is the encapsulation efficiency, T Melatonin is the total content, and S Melatonin is the melatonin content in the supernatant.

In Vitro Release Assay

The in vitro release procedure of nanoparticles was as follows. In brief, 2 mL of the total of encapsulated melatonin in CS-TPP nanoparticles was added in a dialysis bag (cutoff 12,000 Da) and placed in 100 mL of deionized water to segregate the free drug. Following this step, 20 ml of phosphate-buffered saline (pH 7.2) was added to the dialyzing bag and then stirred at 120× g at 37 ° C. A total of 0.5 mL supernatant elicited 5-min intervals and the melatonin contents were evaluated by the Bradford protein assay [29].

Cell Culture

Human hepatoma (HepG2) cell line was purchased from Pasture Institute of Iran. Cells were cultured in RPMI 1640 medium which contained 5% fetal bovine serum and 1% penicillin/ streptomycin solution in an atmosphere of 5% CO2 at 37°C [30].

Experimental design

HepG2 cells were applied to evaluate the genotoxicity and some of oxidative stress parameters. Therefore, Cells were seeded on 24-well culture plates at 25× 10^4 cells/well. After overnight growth, the cells were exposed to melatonin and melatonin nanoparticles at fallowing concentration: 100, 200, and 400 µmol/L, simultaneously and 24 hours prior doxorubicin treatment (1 µmol/L). Untreated cells have been chosen as control group. The concentrations were selected by previous study [31-33].

Comet Assay

DNA damage was measured by alkaline single cell gel electrophoresis (SCGE) method according to Singh et al. study with slightly modified. HepG2 cells were seeded as above. The cells were exposed with doxorubicin for 1 h to analyze the genotoxic effect and the cells were exposed with melatonin, its nanoparticles, and doxorubicin in co- and pretreatment conditions to analyze the anti-genotoxic effect. Untreated cells were considered as a negative control. In brief, 10 µL of cell suspensions were mixed with 100 µL of low-melting-point agarose (LMA). The mixture was expanded onto microscope slides which were pre-coated with 1.0% normal-melting point agarose (NMA). After solidification of the agarose (4 ° C, 10 min), slides were lysed in cold lysis buffer including 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH 10, containing 10% DMSO and 1% TritonX-100 (4° C,1 h). Then, slides were submerged in electrophoretic buffer including 1 mM Na2-EDTA, 300 mM NaOH, pH>13 for 40 min to open DNA prior to electrophoresis. The electrophoresis was performed at 25 V and a current of 300 mA for 20 min. Then, the slides were washed with cold neutralization buffer solution three times for 15 min. Subsequently, the slides were stained with 30 µL ethidium bromide and observed with a fluorescence microscope. The most reliable factors, tail moment, tail length and tail DNA percentage were measured using Comet Score software project (CASP) to assess DNA damage [34].

Measurement of reactive oxygen species (ROS)

Total intracellular ROS were measured using 2', 7'-dichlorofluorescin diacetate (DCFH-DA) as a probe. The principle of the technique is that ROS can oxidize DCFH to a highly fluorescent 2′,7′-dichlorofluorescein (DCF). After 24 h incubation, cells were exposed with doxorubicin alone and with both regular melatonin and its nanoparticles 24 h before doxorubicin exposure. After that, the culture medium was removed and the cells were washed twice with HBSS. Then, the cells were treated with doxorubicin (1 µmol/L) for 1 h and were washed twice with HBSS and incubated in 2ml of culture medium without FBS. DCFH-DA (10 µmol/L) was added to cells and incubated for 30 min. finally, the cells were washed twice with PBS and upheld in 1 ml of culture medium. The ROS levels were measured by analyzing cells by fluorescence plate reader with excitation wavelength at 488 nm and emission wavelength at 535 nm [22].

Measurement of Intracellular GSH Levels

The content of GSH was evaluated by using a fluorescent indicator of GSH, monochlorobimane (mBCl). Cultured cells were treated with doxorubicin (1 µmol/L) for 24 h in the presence or absence of different concentrations of melatonin and its nanoparticles pretreatment for 3 h. Treated cells were then incubated with 40 µmol/L mBCl for 1 h in a staining solution including 5 mmol/L glucose, 1 mmol/L CaCl2, 0.5 mmol/L MgSO4, 5 mg/mL BSA for 30 min at 37 ° C in the dark condition. Intracellular GSH level was determined using a spectra fluorescent plate reader (λex = 380 nm and λem = 460 nm) [34].

Statistical Analysis

We used the most frequently factors of DNA damages in the comet assay including tail moment, tail length and percent of DNA in tail for statistical analysis in this study. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was applied. Statistical significance was considered as $p < 0.05$.

Results

Characterization of CS-TPP-MT nanoparticles

As shown in table 1 the particle size, size distribution (polydispersity index (PDI)), and zeta potential of particles were measured by Zetasizer (Malvern Instruments, Worcestershire, UK), based on the dynamic light scattering (DLS) technique. The release profile of CS TPP MT nanoparticles demonstrated a biphasic release: an early burst release phase and a slow release phase, (figure 1).

Table 1. The characterization of CS-TPP-MT nano particles

size	PDI	Zeta potential	Encapsulation efficacy
110 nm	0.28	45mV	75%

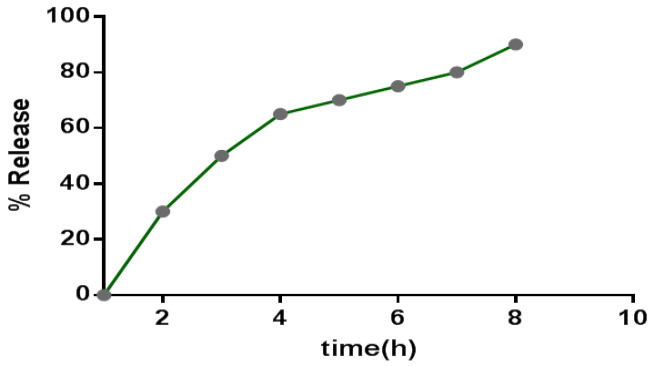


Figure 1: Release curve of MT from chitosan-tripolyphosphate-MT nanoparticles

Study of the Effect of regular MT on the Doxorubicin-induced DNA damage

The potential genoprotective effect of regular MT was evaluated via the alkaline comet assay. Results of the software scoring and

percentage of total DNA damage induced by doxorubicin and prevention by MT were shown in Fig.2. We used 1 µM of doxorubicin as optimum genotoxic concentration and MT in the co and pre-treatment conditions decreased significantly ($p < 0.0001$) the level of DNA fragmentation as compared to the doxorubicin group.

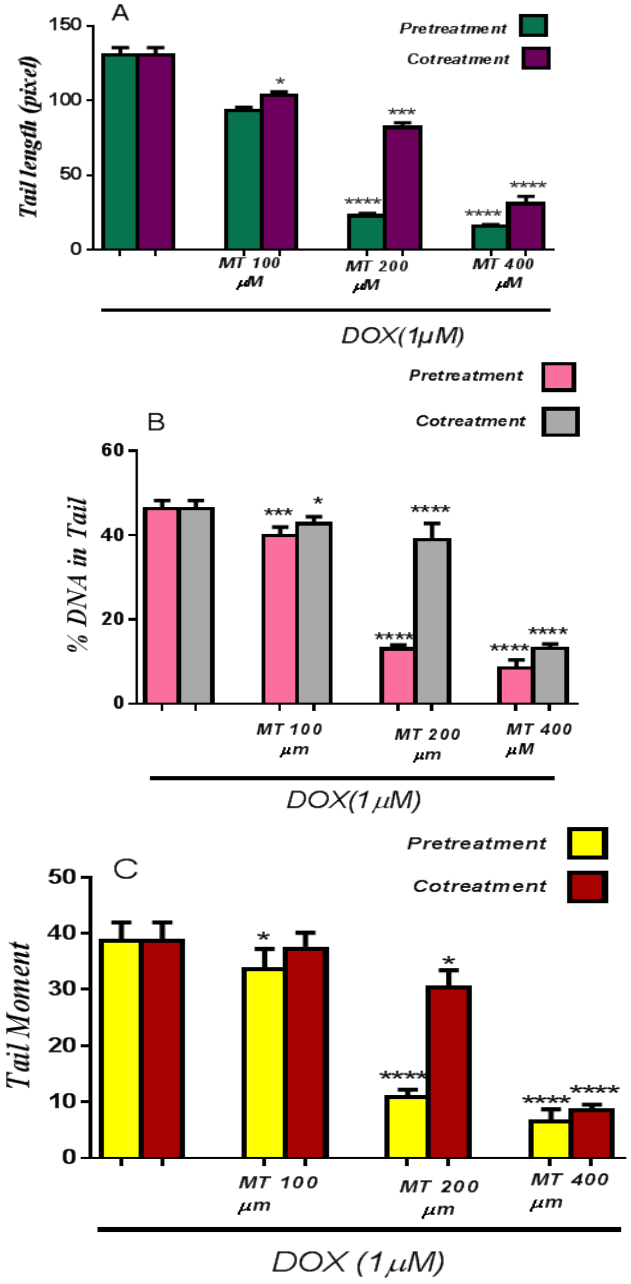


Figure 2. Comparison of three studied factors in MT plus doxorubicin treated groups A; Tail length, B; % DNA in tail and C; Tail moment. Each graph has been represented as Mean ± SEM. The sign (****), (***) and (*) show significantly decreased results (respectively p<0.0001, p<0.001 and P<0.05) in compare with the doxorubicin group.

Study the effect of CS-TPP-MT nanoparticles on the doxorubicin induced DNA damage

The genoprotective effect of CS-TPP-MT nanoparticles was explored via the alkaline comet assay. Results of the data analyzing were shown in Table 2. We observed that doxorubicin treatment at 1 µM induced a significant ($p < 0.001$) increase in DNA damage as compared to the control group. CS-TPP-MT nanoparticles decreased significantly ($p < 0.0001$) the level of DNA fragmentation as compared to the doxorubicin group. Also CS-TPP-MT nanoparticles are more effective than regular MT (P<0.0001).

Table 2. Genoprotective effects of CS-TPP-MT Nanoparticles on HepG2 cells using the comet assay.

Treatment (Mean±SEM)	Tail length (Mean±SEM)	%DNA in Tail (Mean±SEM)	Tail moment (Mean±SEM)
Control (DOX 1µM)	130.7. ±4.5	46.26±1.9	37.6±1.2
Regular MT (400 µM)	30.9 ±.4.9 *	8.4±.1.9	5.4 ±.2*
CS-TPP-MT NPs (100µM)	14.5±0.8*#	1.5±.09*#	0.9±.02*#

Table 2. The genoprotective effect of regular MT and CS-PP-MT nanoparticles compared with control groups on tail length (pixels), percentage of DNA in tail, and tail moment (pixels) ,that are represented as mean± SEM. * and # mean value was significantly different from control and regular MT group ($p < 0.0001$),(one-way ANOVA followed by turkey's post hoc test).

Study the ROS generation

To investigate the role of oxidative stress in doxorubicin -induced genotoxicity, we used DCFH-DA, a cell-permeable fluorescent dye, to examine the ROS generation in HepG2 cells in response to doxorubicin stimulation. Incubation with doxorubicin (1µM) for 1 h showed a considerable increase in oxidant-induced 2_, 7_-dichlorofluorescein fluorescence in HepG2 cells (Fig. 3). H2O2-mediated DCF fluorescence occurred after 1h incubation with doxorubicin in HepG2 cells (p<0.0001). This suggests that doxorubicin, induce intracellular oxidative stress, involved in its genotoxicity. After that cells were treated with MT in pre-treatment condition and subsequently examined. Regular MT and CS-TPP-MT nanoparticles were significantly (p<0.0001) reduced ROS generation as compared to the doxorubicin group. Untreated cells served as control.

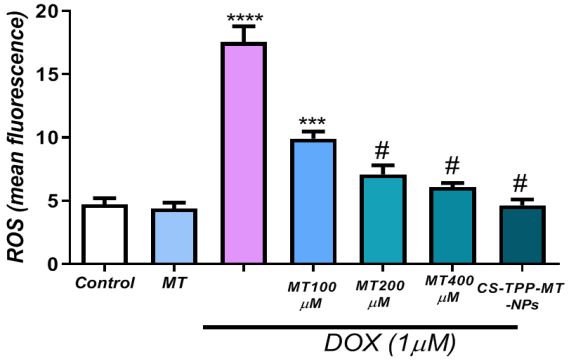


Figure 3. Study the effect of melatonin on doxorubicin -induced ROS generation. (****) and (***) show significantly increased results (respectively p<0.0001, p<0.001) as compared to the control

group. The sign (#) show significantly ($p<0.0001$) decreased compared to the doxorubicin group.

Study the effect of doxorubicin on intracellular levels of GSH

We first examined the effect of doxorubicin on the intracellular levels of GSH using mbci which readily enters cells to form a fluorescent GSH-bimane adduct that can be measured fluorometrically. As shown in fig.4 A, within 1h after treatment, the intracellular levels of GSH were reduced ($p<0.0001$). This finding was subsequently confirmed by an enzymatic assay using glutathione reductase and 2-vinylpyridine. Next, we measured the intracellular levels of GSH in cells after treatment with MT and doxorubicin in pre- treatment condition. As shown in fig.4, MT (400µM) and CS-TPP-MT nanoparticles were significantly ($p<0.0001$) increased GSH levels a4s compared to the doxorubicin group.

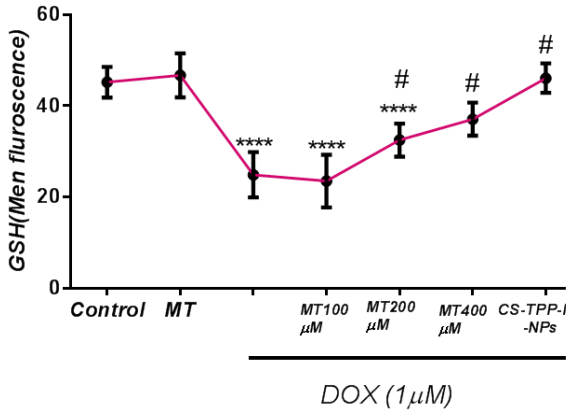


Figure 4. The effect of MT on the levels of intracellular GSH were determined. ANOVA analysis revealed that MT, significantly inhibited the effects of doxorubicin on the levels of GSH. Sign (***) show significantly decreased results (respectively $p<0.0001$ as compared to the control group. Sign # show significantly ($p<0.0001$) increased as compared to the doxorubicin group.

Discussion

Doxorubicin, one of the anthracycline anti-tumor antibiotics, has been shown immense efficacy in treatment of solid and liquid tumors [35,36]. In spite of the widespread usage in cancer therapy, the exact mechanisms of action of this drug is not completely understood. However, topoisomerase II poisoning, DNA adduct formation and oxidative stress have been suggested as involved mechanisms in doxorubicin- induced cancer cell death [37].

Numerous investigations showed that DNA is the main cellular target of doxorubicin [38,39]. Actually, doxorubicin exhibits its antitumor activity via interact with cellular DNA [40]. This drug is well known to induce genotoxic effects such as single- and double-strand DNA breaks, chromosomal rearrangements and mutation [41,42]. The risk for the development of second malignancies enhance after exposure to drugs targeting topoisomerase II such as doxorubicin [43]. That may be related to genotoxicity induced by this drug [42,43].

Melatonin is not a simple antioxidant and anti-inflammatory agent. Experimental investigations reported that melatonin, via different mechanisms reduces the genotoxicity that induce by different chemotherapy and non-chemotherapy agents [44-46]. The results of Karamian et al. study showed that melatonin had a potent antigenotoxic effect against diazinon induced DNA damage [47]. Kim et al. demonstrated that melatonin decreased doxorubicin induced genotoxicity [48]. Kangsadarn Bennukul et al. subsequently showed that MT showed protective effect against the mutagenicity of cisplatin, in HepG2 cells [49]. In another study MT protects normal tissues from doxorubicin toxicity and senescence while cytotoxicity against tumor cells is maintained [50]. Besides results obtained from previous studies have been proven that MT protects against genotoxicity induced by diazinon in peripheral blood lymphocytes. Other reports documented that melatonin protects normal tissue against lead acetate, parquet and some other toxicants [51,52].

Despite melatonin is widely used in clinical, its efficacy may be decrease because of its low oral bioavailability (3%) and its short biological half-life (54min) [53]. To overwhelm these problems and enhance its efficiency, suitable delivery system (nanoparticulated system) for melatonin has been investigated that indicated to be more effective in comparison to immediate release formulations [54].

Schaffazick and colleagues demonstrated that nanoparticles containing melatonin induced a significant enhance in the antioxidative effect of melatonin against lipid peroxidation [55]. Mariele et al. study showed that melatonin nanocapsules were better than melatonin to protect against paraquat induced cytotoxicity and genotoxicity in A549 cells [19]. The finding of our previous work indicated that melatonin nanoparticles were more effective than regular melatonin against doxorubicin induced genotoxicity [31]. In accordance with previous studies, we also determined that melatonin loading chitosan-tripolyphosphate nanoparticles were potent to reduce DNA damage induced by doxorubicin.

In the present study, the size of the nanoparticles was ~110 nm and the melatonin EE was 75%. The in vitro release profile of melatonin nanoparticles showed biphasic distribution and the release after 7 h was ~87%. This indicates that CS-TPP nanoparticles exhibit good release properties.

In this study, DNA strand breaks were observed in HepG2 cell lines after exposure to doxorubicin. Although both melatonin and its nanoparticles decreased doxorubicin induced genotoxicity, but melatonin nanoparticles were more effective than melatonin in reduce DNA damage in both pre- and treatment conditions.

Doxorubicin induced ROS generation and subsequently oxidative stress can lead to genotoxicity [34,53]. ROS are known to cause DNA damage such as single- and double-strand breaks after exposure to doxorubicin [45]. The results of present study revealed the significant increase in ROS production in HepG2 cell lines after doxorubicin treatment. GSH, the main non-enzymatic antioxidant, reacts with free radicals and inhibits the deleterious effects of them. Decreasing GSH contents was observed in various studies after exposure to genotoxic agents [54-56]. We also showed the significant reduce in GSH levels after exposure to doxorubicin.

In this study, to evaluate the ability of melatonin nanoparticles to reduce oxidative stress as an involved mechanism in doxorubicin induced genotoxicity, HepG2 cell lines were treated with both melatonin and its nanoparticles and then ROS and GSH levels were

measured. Our results indicate that treatment of HepG2 cell lines with melatonin nanoparticles and regular melatonin significantly decrease the ROS and increase the GSH levels, however, melatonin nanoparticles were highly effective in regulation of these parameters. Although, we showed that melatonin nanoparticles were highly effective in reducing DNA damage but more studies are needed to fully understand involved genoprotective mechanisms of melatonin nanoparticles.

Conclusion

We found that melatonin loading chitosan-tripolyphosphate nanoparticles can protect HepG2 cell lines from the genotoxic effects of doxorubicin. Melatonin nanoparticles were the most potent at reducing oxidative damage in comparison to regular melatonin. Actually, nanotechnology ameliorated the melatonin protective effects.

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