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Doxorubicin and liposomal doxorubicin induce senescence by enhancing nuclear factor kappa B and mitochondrial membrane potential

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ABSTRACT

Aims: Senescence is a state ensuing aging to eliminate age-associated damage with an irreversible cell-cycle arrest mechanism, which is historically believed to be one of the tumor responses to therapy. Doxorubicin as an anti-cancer drug has been used in cancer treatment for a long time. Liposomal doxorubicin (Ldox) is a liposomal formulation of doxorubicin, which increases the doxorubicin permanency. The aim of this study was to examine the toxicity of these two formulations by comparing them in terms of their ability to induce cellular senescence.
Main methods: The study groups included a control group, three DOX (0.75, 0.5, 0.1 mg/kg/BW) and three Ldox groups (0.1, 0.05, 0.025 mg/kg/BW). Heart tissues were studied regarding oxidative stress assessment, mitochondrial function, inflammatory markers and biochemical and histopathological evaluation. Real-Time PCR was used for P53 and SA β-gal expression.
Key findings: Based on the results, the highest doses of Dox and Ldox (0.75 and 0.1 mg/kg/BW respectively) significantly increased the level of inflammatory markers and according to other factors especially p53 and SA β-gal expression, both were able to induce senescence but the changes in Ldox were less tangible than the Dox.

1. Introduction

Aging is a multifactorial phenomenon, which leads to the deterioration of many functions and activities in living organisms [1]. As a result, the occurrence of age-associated conditions such as type 2 diabetes, Alzheimer's disease and cancer is rapidly soaring [2] and aging will soon turn into a major economic and social concern [3]. Cellular senescence is also known as an aging marker is a cellular response to restrict the proliferation of aged or defective cells, which via the compilation of effects within cells, contribute to the development of cancer [4]. In spite of the physiological role of senescence during normal development and its need for tissue homeostasis, senescent cells are assumed to resist apoptosis with altered patterns of gene expression [5]. Replicative senescence (RS) and stress-induced premature senescence (SIPS) constitute two major forms of senescence which share some features including telomere shortening and cell cycle arrest, as well as high activities of senescence-associated–β galactosidase and secretory phenotypes (SA-β-gal and SASP, respectively) [6]. Based on several studies, it is plain clear that the evaluation of β-galactosidase (β-gal) activity is most frequently done as a biomarker of senescent cells; a maker which shows lysosomal mass [7,8]. It is believed that main participants in aging include Reactive Oxygen Species (ROS), cellular senescence, and DNA damage as well as the proinflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) target genes, such as TNF- α and IL-1 [9].

Doxorubicin belongs to a nonselective class I anthracycline antibiotic, that is used for the treatment of a range of tumors, including hematological neoplasms [10], breast cancer, sarcomas, and leukemia [11]. DOX exerts its effects via its intercalation into DNA, DNA alkylation and DNA cross-linking inhibition of topoisomerase II, induction of apoptosis and free radical generation as a means of DNA damage and/or lipid peroxidation [12]. This agent is not tumor-specific and influences the growth of other cells as well. Consequently, the immune system becomes also affected and as a result the prevalence of infection

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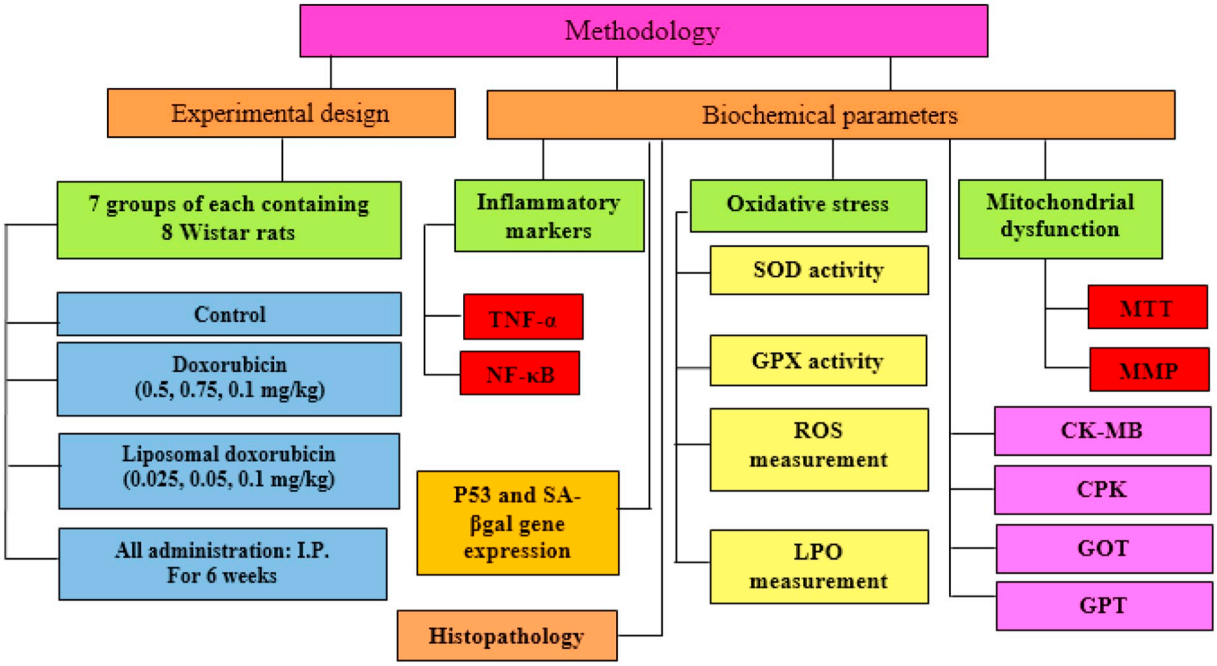

Fig. 1. The methodology of the experimental study is illustrated.

rises [13]. Senescence has been widely suggested as a probable tumor reaction to anticancer agents. According to some studies, DOX is suggested as a senescence inducer. For example, in one report due to the elevated activity of senescence-associated β-galactosidase, increased cdk-I expression and decreased telomerase activity, DOX was nominated as a senescence inducer. It was also found that acetylated p53 and promyelocytic leukemia protein as key factors of premature senescence were involved in the DOX-induced senescence in cardiomyocytes [14]. In another study, following the low dose exposure of HCT116 cells to DOX, the proliferation of the cells was mainly affected by the elevation of SA-β-galactosidase activity, p53, p21 and cyclin D_1 [15]. Based on a study by Irina Kozhukharova1 et al. high-dose exposure of DOX decreased the survival of human mesenchymal stem cells (MSCss). However low doses of this drug, caused early senescence in eMSCs (stem cell derived from menstrual blood cells), BMSCs (bone marrow stem cells) and AMSCs (adipose tissue stem cells) [16]. As stated above, DOX has a narrow therapeutic index. To achieve controlled release of this drug and to improve its selectivity and efficacy in the target organ, the use of carriers like liposomes were initially proposed by Gregoriadis in 1989 [17]. Liposomal doxorubicin (Ldox) was entitled as the first FDA-approved antineoplastic nano-drug [18]. As a result of encapsulation into liposomes, pharmacokinetic and pharmacodynamics of this drug changed, its circulation time was extended and the distribution of the drug was altered [19]. On such a basis, this study was designed to clarify the differences between DOX and Ldox in terms of cellular senescence in heart tissue.

2. Materials and methods

ELISA kits for the measurement of antioxidant enzymes activity and Rat NF-KB were purchased from Zellbio, Germany, Rat TNF-α kit was obtained from Diaclone, France. DOXO-cell® was obtained from Germany and liposomal doxorubicin (CAELYX®) was purchased from Belgium. All the remaining chemical material were purchased from Sigma-Aldrich (GmbH, Munich, Germany). Our experiment was performed using Qiagen RNAlater, RNA Isolation kit from Roche (Roche, Germany), and Revert Aid First Strand cDNA Synthesis Kit was purchased from Germany (Fermentas, Germany). and QuantiFast SYBR Green PCR Master Mix was from Qiagen (Hilden, Germany).

2.1. Animals

All the animal experiments were conducted under the general guidelines for the ethical laboratory animal use confirmed by MASUMS committee of ethics (IR.MAZUMS.REC.1397.3168). To perform the studies, male Wistar rats (weighing 200–250 g) were placed in a room for acclimatization at 20–25 °C, humidity of 50–55% and a 12 h light/dark cycle, during which all the rats has unlimited access to standard rat diet.

3. Experimental design

To determine the optimal doses, different doses of DOX (0.1, 0.5, 1) and Ldox (0.1, 0.05. 0.025) were used. Half of the treated animal at the highest dose of DOX died after 3 weeks; therefore, the sub-lethal doses were finally selected. In the previous studies, a dose of 0.06–2 mg/kg/BW of the liposomal formulation had been used [20]. For the liposomal formulation in our study, a dose of 0.05 mg/kg/BW and a higher and a lower dose was selected. All doses of DOX were based on its cumulative dose (16 mg/kg) [11,21]. Considering the length of injection and the cumulative dose of DOX, the dose of 0.5 mg/kg BW and a higher and a lower dose was also selected. Fifty-six male Wistar rats, were obtained from the animal center of Mazandaran university of medical sciences and were randomly allotted to seven groups of eight including: (1) control group (2, 3, 4) DOX (0.75, 0.5, 0.1 mg/kg/BW), and (5, 6, 7) Ldox (0.1, 0.05, 0.025 mg/kg/BW). The animals received daily peritoneal injections of each formulation for six weeks. The control group received neither DOX nor Ldox. At the end of this experiment, all animals were humanly euthanized under general anesthesia by an IP injecting of 60.6 mg/kg of Ketamine/Xylazine, and were immediately necropsied [11]. Blood samples were taken and prepared for biochemical analysis and the hearts were separated on ice and were homogenized in PBS. The homogenates were then centrifuged at 800 × *g* for 10 min at 4 °C to eliminate the debris and were appropriately stored for future analyses. A part of each tissue was used immediately for ROS detection. The flowchart of the experimental study design is shown in Fig. 1.

3.1. Activity assessment of glutathione peroxidase (GPX)

GPX activity was assessed in the prepared heart homogenates according to the protocol provided with the kit. A Zellbio kit (Berlin, Germany) was applied to check the activity of GPX, according to a glutathione reductase-coupled method. The principle of the test is the reduction of the hydroperoxide molecule by GPx which gives rise to the oxidized form of glutathione.

3.2. Checking the activity of superoxide dismutase (SOD)

SOD activity was measured by a kit (Zellbio, Germany). The principle of which was superoxide radical generation by xanthine and xanthine oxidase. These superoxide radicals then react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to create a red formazan dye. The extent to which this reaction is inhibited determines the activity of SOD. Data are shown as unit/mg of protein.

3.3. Lipid peroxidation (LPO) assessment

To perform this test, the method of Shokrzadeh et al. was followed [22]. In this method, the amounts of produced malondialdehyde (MDA) is an indicator of peroxidation.

3.4. Reactive oxygen species (ROS) assessment

Dichlorofluorescin-diacetate (DCFH-DA) was used to check the amounts of ROS. Shortly, DCFH was added to 2 ml of each heart homogenate supernatant (1 mg protein/ml) and was incubated for 15 min at 37 °C. at the amounts of the fluorescence produced was then checked at 480 nm (excitation) and at 520 nm (emission) by a fluorescence spectrophotometer [23].

3.5. Tumor necrosis factor (TNF-α) assessment

TNF-α (cachectin) is a major cytokine production of monocytes and macrophages. To perform this assay, the protocol included in Diaclone Rat TNF-α ELISA Kit was exactly followed. Briefly, 50 µl biotinylated anti-Rat TNF-α was added to 100 µl of samples and diluted standard. After 3 h incubation at room temperature and washing for three times, 100 µl Streptavidin-HRP was added, then after 30 min incubation and three times washing, 100 µl TMB and 100 µl H2SO4 were added and the absorbance was recorded at 450 nm.

3.6. Nuclear Factor-kappa B (NF-κB) assessment

To evaluate *NF-κB levels,* the protocol of Zellbio GmbH ELISA kit was precisely followed. On such a basis, 40 µl sample, 10 µl *NF- κB -Ab, 50* µl standards and 100 µl Streptavidin-HRP were mixed. After five times washing with washing buffer, 50 µl of each chromogen solution (A and B) was added and after 10 min *50* µl stop solution added. In the end OD was read at 450 nm.

3.7. Mitochondrial dysfunction

3.7.1. Mitochondrial preparation

The heart tissues were differentially centrifuged to purify the mitochondria. Two consecutive centrifugations were done. The homogenate was first centrifuged at 2000 ×*g* for 10 min at 4 °C to eliminate the pellet. Afterwards, another centrifugation at 10,000 ×*g* was done for 10 min to reach the pellet. After washing, the mitochondrial pellet was resuspended in the separation medium and was centrifuged once again at 10,000 ×*g* for 10 min. Tris buffer was used to resuspend the final pellets at 4 °C. Succinate dehydrogenase was measured to check if the isolation was successful. Freshly isolated mitochondria were used within 4 h of isolation and all experiments were strictly performed on ice to assure high-quality mitochondrial isolation [24].

3.7.2. Evaluation of mitochondrial function

To investigate the effects of Dox and Ldox on mitochondrial activity, an MTT test was used to measure the activity of succinate dehydrogenase as an indicator. The MTT assay was performed according to the method described in Shokrzadeh et al. method [25].

3.7.3. Mitochondrial membrane potential assessment

The uptake of cationic Rhodamine 123 which has fluorescent properties by the mitochondria was measured to assess mitochondrial membrane potential (MMP). After the addition the assay buffer to the mitochondrial solution, 10 mM of Rhodamine 123 was also added and the fluorescence intensity was recorded using a fluorescence spectrophotometer at 490 nm (excitation) and 535 nm (emission). Detailed steps were based on the protocol used in Mashayekhi V et al. method [26].

3.8. Histopathological assessment

The excised heart tissues were washed in ice cold PBS, fixed in paraformaldehyde (10%), and dehydrated in graded ethanol. The tissues were embedded in paraffin for 4 h. The blocks were then used to prepare 3 mm sections using a microtome and the sections were finally stained with hematoxylin and eosin for histopathological studies [27].

3.9. Gene expression

3.9.1. Total RNA extraction

All the specimens were kept in RNAlater solution at −20 °C prior to the isolation procedure. The exact total RNA isolation protocol described in the RNeasy® Plus Mini Handbook was followed. The RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) was used to perform the isolation. To eliminate any possible genomic DNA contamination, the samples were also treated with DNase (Qiagen, Hilden, Germany). RNase-free water was used to elude the extracted RNA of each sample and were then stored at −80 °C. Agarose gel electrophoresis was also used to check the quality of the extractions and the concentration of each RNA sample was finally measured at A260 using the Pico Drop 2000 (Thermo Fischer Scientific Inc.) [28].

3.9.2. cDNA synthesis

A cDNA synthesis kit (Fermentas, Germany) was used to prepare the required cDNA and the concentration and the purity of the product was finally checked using Pico Drop 2000 (Thermo Fischer Scientific Inc.).

3.9.3. Real-Time PCR

The RT-PCR for two target genes including the tumor-suppressor p53 and SA-βgal was done using a Roche Light CyclerTM system (Roche, Mannheim, Germany). The primers were designed using Allele ID 7.5. Primer sets and are shown in Table 1. The reaction mixtures were prepared at a final volume of 25 µl and constituted 12.5 µl of QuantiTect SYBR Green PCR master mix 1× (Qiagen, Hilden, Germany), forward and reverse primers (50 ng), and cDNA (50 ng). For SA-βgal, PCR conditions was denaturation at 95 °C for 10 min, followed by

Table 1
Sequence of the Oligonucleotide Primers used in qRT-PCR.

Target gene	Sequence (5′ - 3′)	Amplicon size (bps)
p53	Forward ATGGAGGATTCACAGTCGGA Reverse TTCCTCTGGGCCTTCTAACAA	183
SA-βgal	Forward AGCTATGACTATGACGCCCC Reverse CTTCCGTCACCGTCTTGAAC	175
Beta-actin	Forward GCCTTCCTTCCTGGGTAT Reverse GATCTTGATCTTCATGGTGCTA	199

40 cycles of 95 °C for 30 s, 60 °C for 40 s and 72 °C for 20 s, followed by melting curve analysis. For P53 amplification, all above condition was performed, but the annealing temperature was 62 °C. Gene expression analysis was carried out using the $2^{-\Delta\Delta CT}$ method [29].

3.10. Biochemical factors

Serum samples were used for measuring CK-MB, CPK, GOT and GPT using a biochemical analyzer (Olympus AU-660, Osaka, Japan).

4. Statistical analysis

The results were presented as mean ± standard deviation (SD). A One-way ANOVA test was used to statistically compare means. A Tukey post hoc test was also followed to show the difference among different groups. p values < 0.05 were deemed statistically significant.

5. Results

5.1. GPX and SOD activity

SOD and GPX activities in the DOX and Ldox groups had decreased significantly compared to the control group ($p < 0.05$) (Fig. 2, A and B). This significant reduction was more obvious in the DOX group compared to Ldox group.

5.2. Oxidative stress assessment

LPO levels in the DOX and Ldox groups was significantly higher than those of the control group ($p < 0.05$). Likewise, a significant rise in ROS production was observed in the treated groups compared to the control group ($p < 0.05$) (Fig. 2, C and D). Interestingly, ROS and LPO levels in the DOX group were higher than those of the Ldox group.

5.3. Effects of DOX and Ldox on mitochondrial function

After 1 h of incubation of mitochondria with these agents, a steep concentration-dependent fall in the mitochondrial conversion of MTT to formazan was observed (Fig. 3). This reduction in the DOX group was significantly higher than that of the Ldox group. It was also shown that the doses of Dox and Ldox especially the highest doses of each could increase MMP levels significantly which was higher in the DOX group compared to the Ldox group (Fig. 3).

5.4. Inflammatory markers

The results showed a significant increase in TNF-α in the DOX- and Ldox-treated groups in comparison with the control group. In addition, the level of NF-KB showed a significant increase in the DOX and Ldox groups which was higher in the DOX groups compared to Ldox groups (Fig. 4).

5.5. Biochemical factors

The results showed a significant increase in CK-MB and CPK levels in the DOX and Ldox groups. The lower doses of DOX and Ldox had no significant effects on these markers. It has been also shown that DOX and Ldox increase GOT and GPT compared to the control group which was related to the high dose of DOX and Ldox (Table 2).

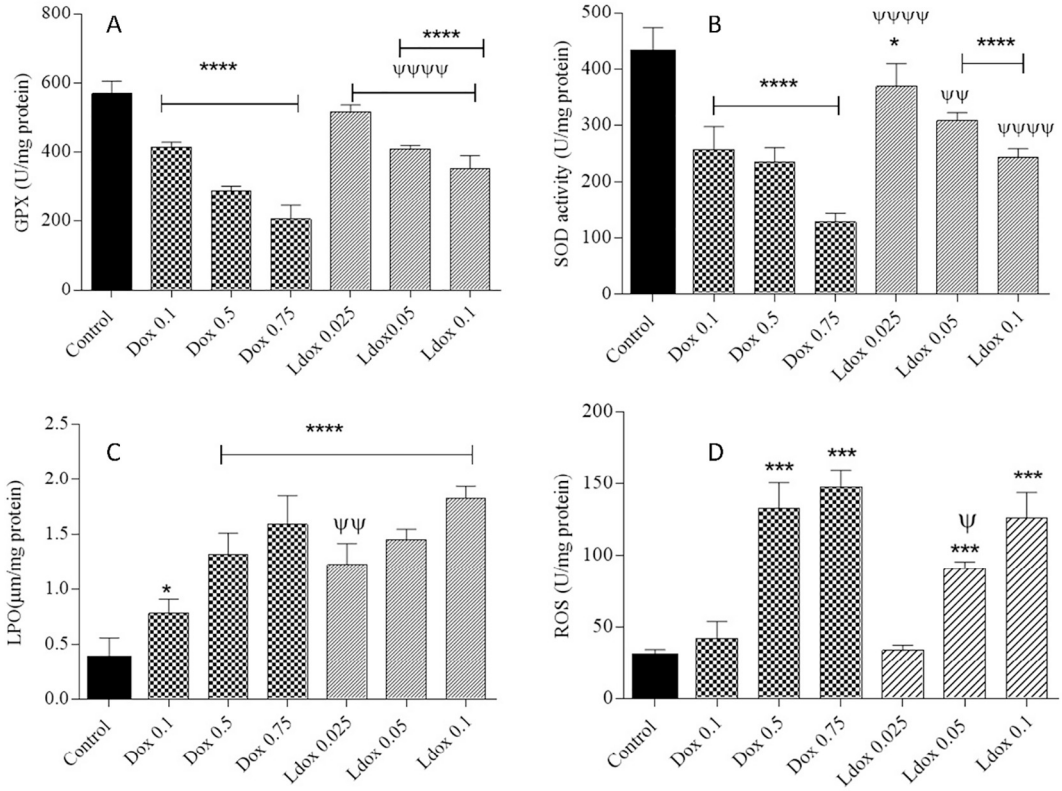

Fig. 2. Effects of treatments on oxidative stress parameters in rat heart tissue. Data are mean ± SD of eight animals in each group. The control group received normal saline; DOX groups received only doxorubicin at doses of 0.1, 0.5 and 0.75 mg/kg/BW; Ldox groups received only liposomal doxorubicin at doses of 0.025, 0.05 and 0.1 mg/kg/BW.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ Significantly different from control group. $^{\Psi}$ $p < 0.05$, $^{\Psi\Psi}$ $p < 0.01$, $^{\Psi\Psi\Psi}$ $p < 0.001$, $^{\Psi\Psi\Psi\Psi}$ $p < 0.0001$ Significantly different from the DOX group with the similar dose.

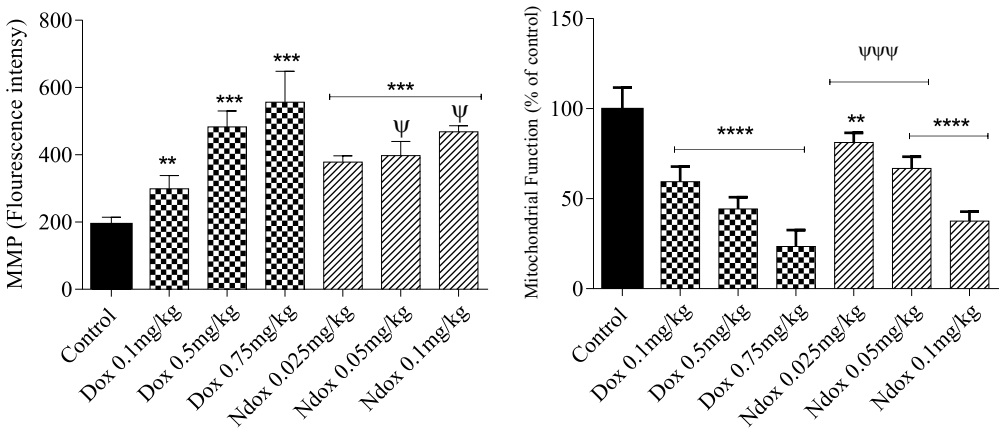

Fig. 3. Effects of treatments on mitochondrial parameters in rat heart tissue. Data are mean ± SD of eight animals in each group. The control group received normal saline; DOX groups received only doxorubicin at doses of 0.1, 0.5 and 0.75 mg/kg/BW; Ldox groups received only liposomal doxorubicin at doses of 0.025, 0.05 and 0.1 mg/kg/BW. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ Significantly different from control group. $^{\Psi}p < 0.05$, $^{\Psi\Psi}p < 0.01$, $^{\Psi\Psi\Psi}p < 0.001$, $^{\Psi\Psi\Psi\Psi}p < 0.0001$ Significantly different from the DOX group with the similar dose.

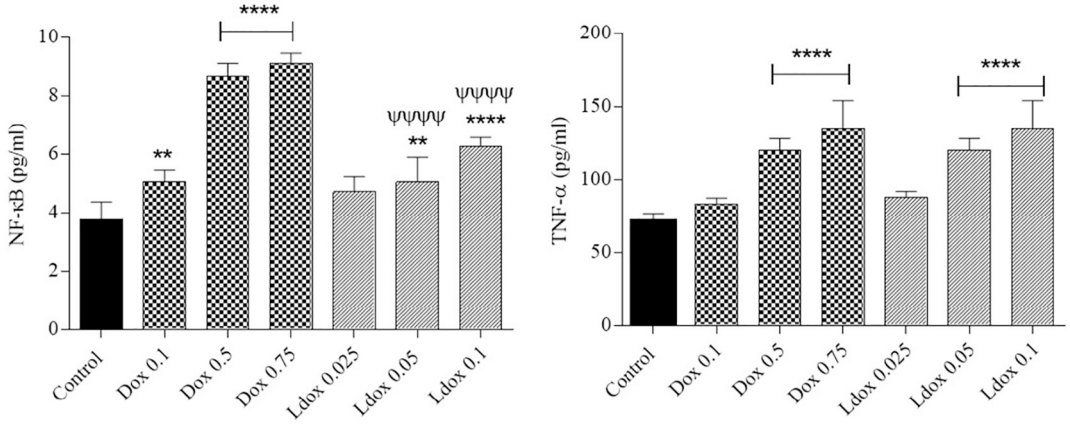

Fig. 4. Effects of treatments on inflammatory markers in rat heart tissue. Data are mean ± SD of eight animals in each group. The control group received normal saline; DOX groups received only doxorubicin at doses of 0.1, 0.5 and 0.75 mg/kg/BW; Ldox groups received only liposomal doxorubicin at doses of 0.025, 0.05 and 0.1 mg/kg/BW. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ Significantly different from control group. $^{\Psi}p < 0.05$, $^{\Psi\Psi}p < 0.01$, $^{\Psi\Psi\Psi}p < 0.001$, $^{\Psi\Psi\Psi\Psi}p < 0.0001$ Significantly different from the DOX group with the similar dose.

Table 2
Effects of various treatments on biochemical factors.

Markers	Control	DOX 0.1	DOX 0.5	DOX 0.75	Ldox 0.025	Ldox 0.05	Ldox 0.1
CK-MB	242.3 ± 58.71	443.7 ± 31.34	610.0 ± 32.23[a]	740.7 ± 131 [a]	325.3 ± 46.70	365.3 ± 110[b]	499.0 ± 68.55[a,b]
CPK	200.0 ± 27.87	352.7 ± 67.83[a]	425.0 ± 30.51 [a]	525.7 ± 30.99 [a]	248.0 ± 65.51	319.0 ± 25.87[a]	381.0 ± 34.18[a,b]
GOT	83.67 ± 13.43	130.7 ± 23.03	205.7 ± 19.43[a]	251.7 ± 12.50 [a]	99.00 ± 15.39	141.3 ± 24.54[a,b]	173.0 ± 16.82[a,b]
GPT	41.33 ± 3.51	54.00 ± 10.58	78.00 ± 5.00 [a]	85.00 ± 7.93[a]	55.67 ± 6.11	57.00 ± 10.15[b]	69.67 ± 3.05[a]

Data are mean ± SD of eight animals in each group. The control group received normal saline; DOX groups received only doxorubicin at doses of 0.1, 0.5 and 0.75 mg/kg/BW; Ldox groups received only liposomal doxorubicin at doses of 0.025, 0.05 and 0.1 mg/kg/BW. *a* Significantly different from control groups at $p < 0.05$. *b* Significantly different from DOX group at $p < 0.05$ with similar dose.

5.6. P53 and SA β-gal gene expression

A significant increase in P53 and SA β-gal gene expression was seen at the high doses of DOX and Ldox (Fig. 5). p53 gene expression enhancement was significantly higher at the highest dose of Ldox in comparison with the same dose of Dox.

5.7. Histopathological findings

Histological analysis of the heart tissues of the control group showed no abnormality. Whereas, the heart from the DOX- and Ldox-treated rats showed significant histological alterations in the cardiomyocytes such as congestion, focal myocytolysis and cytoplasmic

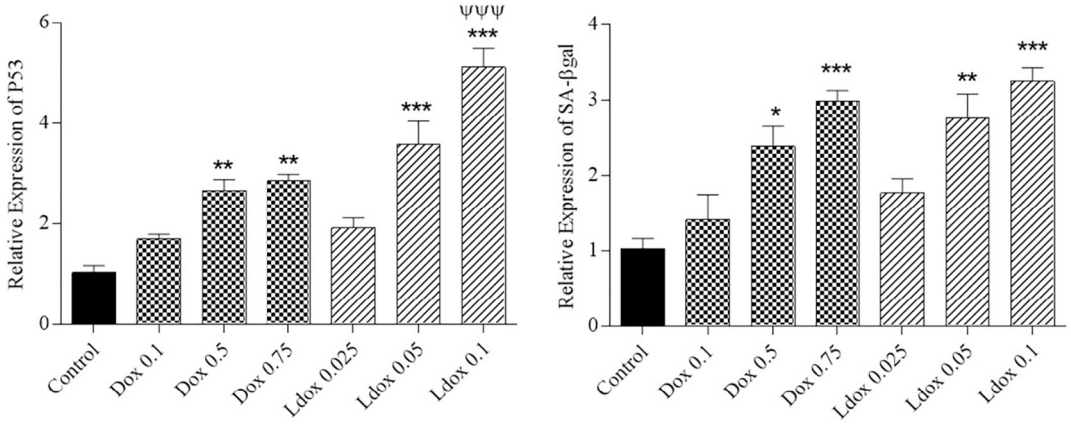

Fig. 5. Effects of treatments on P53 and SA β-gal gene expression in rat heart tissue. Data are mean ± SD of eight animals in each group. The control group received normal saline; DOX groups received only doxorubicin at doses of 0.1, 0.5 and 0.75 mg/kg/BW; Ldox groups received only liposomal doxorubicin at doses of 0.025, 0.05 and 0.1 mg/kg/BW. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ Significantly different from control group. $^{\Psi}$ $p < 0.05$, $^{\Psi\Psi}$ $p < 0.01$, $^{\Psi\Psi\Psi}$ $p < 0.001$, $^{\Psi\Psi\Psi\Psi}$ $p < 0.0001$ Significantly different from the DOX group with the similar dose.

vacuolization. These irregular changes were more obvious in all doses of DOX groups and at a high dose of Ldox (Fig. 6).

6. Discussion

DOX, belonging to a class of anthracyclines, is a widely used antineoplastic drug [30]. Over 40 years of DOX therapeutic use, different mechanisms of action have been proposed such as inhibition of topoisomerase II leading to DNA damage initiation [31], intercalation into DNA and the consequent macromolecule synthesis inhibition [32] and free radical generation followed by DNA damage or lipid peroxidation [33]. The most important dose-limiting complication of this compound is congestive heart failure [34]. One of the most critical and limiting factors in cancer chemotherapy is the inadequate uptake of chemotherapeutic drugs by target cells due to systemic toxicity, rapid clearance from circulation and the tumor cell membrane barrier. To overcome these limitations advanced drug delivery technologies have been proposed. Liposomes are used to entrap chemotherapeutic agents

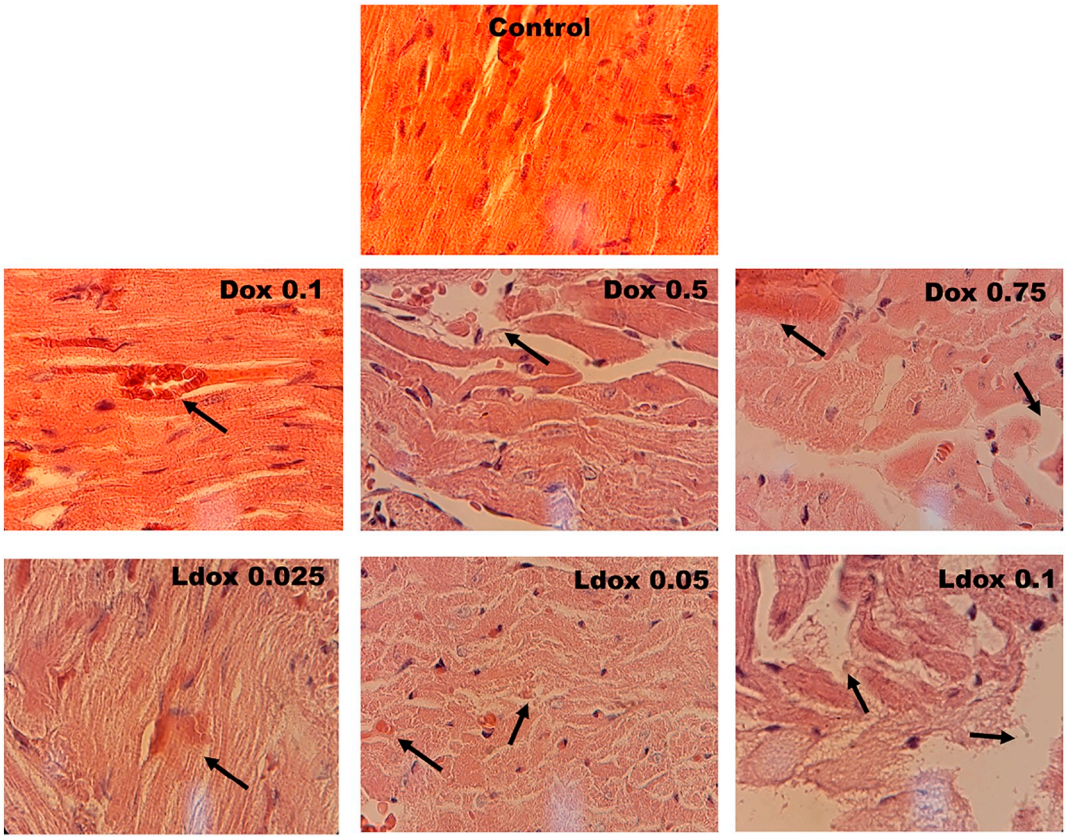

Fig. 6. Heart histological study in various groups. The control group received normal saline; DOX groups received only doxorubicin at doses of 0.1, 0.5 and 0.75 mg/kg/BW; Ldox groups received only liposomal doxorubicin at doses of 0.025, 0.05 and 0.1 mg/kg/BW. Normal structure in control group, Congestion, focal myocytolysis and cytoplasmic vacuolization in DOX and Ldox groups (H & E × 40).

by phospholipids, cholesterol and polyethylene glycol (PEG), to increase systemic drug circulation time and reduce toxicities [35]. Ldox (Doxil®) is the liposomal form of DOX which is able to increase drug accumulation in solid tumors because of its small size and high stability and lower toxicity such as cardiotoxicity [36,37].

Among the important mechanisms to hinder the progression of cancer cells is the induction of cellular senescence [38]. A study was conducted on the role of mTOR pathway in cellular senescence. The results showed that shTSC2 activation turned quiescence into senescence [39]. Likewise, it is also shown that activation of mTOR pathway in vascular smooth muscle cells as a senescence inducer, modified the level of proteins related to autophagy. Conversely, a drastic decrease of senescence-associated β-galactosidase (SA-β-gal) and increased levels of autophagy proteins have been shown after inhibition of mTOR pathway [40]. P53 has a complicated role in the senescence of organisms. Contrary to our research, it has been reported that p53 is able to suppress senescence which results in quiescence indicating a distinct function of p53 [41]. On the other hand, oxidative stress has a potential role in p53 induction [42], which is activated by phosphorylation at serine 15 as a marker of cellular senescence [43]. In this study, we determined the effects of DOX and Ldox on the induction of cellular senescence. It has been shown that DOX and Ldox increase the expression of P53. According to James J. Going's study, the role of SA-β-gal activity in senescence is vividly recognized in living organisms or cells but the data are sparse in in vivo state [44]. In current study, SA-β-gal expression has been enhanced at high doses of both DOX and Ldox, which may be a clue in senescence induction in in vivo state.

One of the complications of DOX administration is ROS induction both in normal and cancer cells [45,46]. ROS generation plays a crucial role in the initiation of premature senescence of cancer cells as a result of various stresses [47]. Our results showed that treatment with DOX and Ldox are related to increased ROS in cardiac cells. No significant increase was observed between two type of drugs regarding ROS generation. It is reported that treatment with cyclophosphamide, methotrexate and 5-fluorouracil (CMF), metabolites of anticancer agents induces lipid peroxidation through the inactivation of GPX, CAT (catalase), SOD, GR (glutathione reductase) and GST (glutathione *S*-transferase) in the breast cancer patients [48]. Production of H_2O_2 by human tumor cells held them under permanent oxidative stress [49]. Consequently, H_2O_2 and anthracyclines trigger NF-κB activation which leads to MnSOD expression and increases intracellular H_2O_2 production which skew the redox potential towards oxidative stress [50]. The results showed a significant increase of GPX levels in the DOX-treated group. Likewise, Ldox group also showed an increase in GPX levels which was higher than that of the DOX group. The data also demonstrated that SOD activity had been amplified in both DOX and Ldox treated groups. The effect of Ldox was more obvious indicating the potential safety and lower toxicity of the liposomal formulation.

The crucial role of mitochondria in energy supply has been proved in normal cell function. These vital organelles also interfere with apoptosis induction, cellular calcium and redox homeostasis [51,52]. On the other hand, mitochondria functions have been significantly influenced by chemotherapy in tumor cells [53]. It is recently demonstrated that energy metabolism impairment and mitochondrial alterations play an important role in anticancer therapy resistance and tumor progression [54,55]. It has been shown that remarkable modification of mitochondrial membrane potential (MMP) as well as mitochondria-mediated apoptosis which are also associated with mitochondrial functional alteration can lead to the impairment of respiratory chain complexes` activity [56,57]. In the present study, to examine whether DOX and Ldox affect mitochondria, we monitored the levels of MMP, as a reliable indicator of the mitochondrial function intactness. As shown in Fig. 3, an increase in MMP was observed in the DOX and Ldox groups. The increase in the DOX group was higher indicating that this form of drug has a more detrimental effect on mitochondria. Based on the results of the MTT test, the viability of cardiomyocytes was mitigated in both treated groups. ROS generation and MMP enhancement in addition to the observed P53 overexpression may explain the reason of such an increase in cellular senescence.

It has been shown that DOX enhances inflammatory cell infiltration, cardiac production of pro-inflammatory cytokines and necrosis in mouse heart [29,58,59]. Innate immune system activation which leads to the release of pro-inflammatory cytokines contributes to the pathogenesis of DOX-induced cardiotoxicity [60]. It is reported that DOX induces pro-inflammatory cytokines release such as tumor necrosis factor (TNF)-α, via the activation of nuclear factor kappa-B (NF-κB) [61]. In addition, increased TNF-α release leads to ROS generation [62]. Our results showed that Ldox treatment is associated with an increase in the level of TNF-α. This increase was higher in DOX treated groups compared to the Ldox-treated groups demonstrating that DOX has more detrimental effects on cardiomyocytes. NF-κB is one of the utmost regulators of cytokine synthesis which exists in the cytoplasm associated with regulatory proteins called inhibitors of κB (IκB). Phosphorylation of IκB is the most significant step to activate NF-κB which is mediated by IKK [62]. In the current study, significant increase was observed in the NF-κB level at all doses of both DOX and Ldox indicating an activation of transcription factors by anthracyclines. CK-MB, as an early indicator of myocardial damage, is a specific enzyme copiously found in the cardiomyocytes [23]. It is reported that DOX could impair cardiac contractile force, induce structural changes in the heart, and increase the levels of serum creatine kinase and CK-MB, two markers of cardiac injury [63]. CK is responsible for the conversion of creatine to phosphocreatine and acts as a modulator of the energy reservoir [64]. Dox has been shown to cause ferrous iron accumulation which in turn induces oxidative damage to CK, a modulator of energy storage. This way, Dox disrupts energy homeostasis by reducing both ATP and PCr (phosphocreatine) levels [65]. Based on our results, a significant increase in CK-MB and CK was observed in both Ldox- and DOX-treated groups.

7. Conclusion

The present study was carried out to evaluate the potential role of Dox and Ldox in cellular senescence. Based on the results, the doses of Dox (0.075, 0.5 and 0.1 mg/kg/BW) could induce senescence by increasing the activity of antioxidant enzymes and the expression of SA β-gal and p53 as well as mildly increasing inflammatory markers and mitochondrial instability. Ldox, especially at the dose of 0.1 mg/kg, contributed more to the senescence of cells and showed significant differences with the corresponding doses of Dox. Our results along with the safe history of Ldox on the intact cells invite for further assessment the safety of this therapeutic agent in clinical settings.

Authorship statements

MSh gave the idea, FSh, and HM were advisors; MF did the study and participated in the literature search and drafted the article; MM participated in drafting and editing the article. ZH, AM, MK, AD, AZ AM, and MM helped in performing the experimental part of the study. All authors were involved in data analysis and interpretation. MSh supervised whole study. All authors read and approved the final version.

Declaration of Competing Interest

The authors of this paper, declare that they have no conflicts of interests associated with this paper.

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