Possible Ameliorative Effects of Different Treatment Regimens Using Bone Marrow-mesenchymal Stem Cells of Doxorubicin-induced Toxicity in Female Wistar Rats

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Introduction

Doxorubicin (DOX) is an anthracycline antibiotic used for the treatment of solid tumors (e.g., breast, gastrointestinal, and ovary) and hematologic malignancies (e.g., leukemia and lymphoma). However, usage of DOX has been restricted due to common toxicities; nausea, vomiting, extravasation, myelosuppression, mucositis, diarrhea, alopecia, hyperpigmentation of nails, and the worst of all refers to cardiotoxicity which leads to irreversible cardiomyopathy and Congestive Heart Failure (CHF) besides nephrotoxicity and hepatotoxicity [1].

Mechanisms of doxorubicin-induced toxicity are multifactorial, including oxidative stress, disruption of iron metabolism (iron overload), calcium homeostasis dysregulation [2], and multiple forms of regulated cell death involving apoptosis, necroptosis, autophagy, pyroptosis, and ferroptosis [3].

Stem cell-based therapy is a promising technique for doxorubicin-induced toxicity. Although it remains in preclinical studies, many researchers have examined the role of regenerative medicine as an alternative therapy to heart transplantation [4] [5].

MSCs or stromal cells are adult, multipotent, self-renewing progenitor and immune evasive cells that exert therapeutic modality and have various properties that make them an adequate choice for the treatment of cardiac dysfunction including doxorubicin-induced toxicity; these properties include many items like; MSCs secrete multi-functional trophic (regenerative) factors

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that mediate cell communications, regulate cell differentiation and proliferation, and also have anti-inflammatory properties via producing hormones, extracellular vesicles (paracrine factors are secreted in encapsulated lipid bilayers), cytokines, interleukins, and growth factors that are participated in the process of cardiac remodeling through inhibiting fibrosis and cell death, stimulating vascularization, promoting tissue remodeling and repair and promoting wound healing [6] [7]. Besides, MSCs can improve cardiomyocyte survival because they can enhance C-kit+ cell differentiation and proliferation. Improving cardiac efficiency through antifibrotic effects and neovascularization features. In addition, MSCs inhibit oxidative stress by controlling the redox microenvironment and subsequently suppress apoptosis induced by reactive oxygen species (ROS) [8].

The therapeutic efficiency of MSCs is furthermore identified with a potential suppressive immunological effect, that can enhance the capability of pharmacological prophylaxis to prevent transplant rejection. Such an immunosuppressive effect nominales MSCs as a promising therapeutic candidate for graft-versus-host disease, multiple sclerosis, Crohn’s disease, and inflammatory kidney disease [9].

Several reports have demonstrated that MSCs can be isolated from the bone marrow (BM), adipose tissue, dental pulp, cervical tissue, placenta, muscle tissue, lung, synovial membranes, and umbilical cord blood. In this study, we have focused on bone marrow-derived mesenchymal stem cells (BM-MSCs), which are widely utilized in several cell-based therapies due to their great isolability, and survival capacity after transplantation [10].

The effectiveness of the treatment with BM-MSCs can be due to several factors, including the protocols used for the manipulation of the MSCs, which may influence the viability and therapeutic potential of the cells, the route of administration used to transplant them, besides the intrinsic differences in functional cardiac parameters and severity among participants [11].

Many studies proved that local or systemic injections with BM-MSCs enhanced cardiac functions, and ameliorated myocardial fibrosis, and the inflammatory response [12] [13].

This study investigates the role of BM-MSCs with different regimen strategies in recovering hepatic and renal functions and restoring the altered cardiac histopathology after the induction of toxicity by DOX in adult female Wistar rats.

Materials and Method

1. Chemicals

Doxorubicin hydrochloride (Adricin 50 mg/25 ml) was purchased from (Ebewe Pharma, Australia), thiopental sodium 500 mg was purchased from (Eipico) for induction of anesthesia in rats, neutral buffered formalin (10 %) was prepared in our lab, Dulbecco’s modified Eagles medium (DMEM, with Glucose and L-Glutamine) (Serana, Europe), fetal bovine serum (FBS) (Pan biotech, Germany), (Pen/strep) penicillin-streptomycin (10,000 U/ml penicillin-10 mg/ml streptomycin) (PAN biotech, Germany), Trypsin/ethylenediaminetetraacetic acid (EDTA) (0.05 % /0.02 in Phosphate buffer saline (PBS) (PAN biotech, Germany), Trypan blue dye 0.4 % (Lonza, Switzerland), Sodium chloride 0.9 % (Normal saline) (El-Nasr for Pharmaceutical Chemicals Co. Abu Zaabal, Egypt), absolute ethanol: used to prepare graduated ethanol (50, 70, 95 and 100 %), Paraffin wax: used for tissue blocking, 10 % formalin saline: used for tissue fixation, xylene as clearing agent, Hematoxylin and Eosin stains (H&E).

2. Experimental Animals

A total of 70 healthy adult female Wistar rats were included in the experiment, weighing 160 ±10 gm, purchased from (El-Nile Co. for Pharmaceuticals and Chemical Industries. Cairo. Egypt).

The animals were housed in metal steel cages and kept at a controlled temperature and 12 h light/12 h dark cycle throughout the experiment with a standard rat chow diet and water ad libitum. The study design and methods were approved by the ethics committee for animal experimentation at the Faculty of Pharmacy, (Boys), Al-Azhar University (Azhar-Pharmacy- 2023-010).

3. Preparation and injection of BM-MSCs

Sterile syringes are used to extract bone marrow from the femurs, which are excised from young female Wistar rats.

Bone marrow is flushed using isolation medium which is a mix of: Dulbecco’s modified Eagles medium (DMEM, with Glucose and L-Glutamine) supplemented with 20 % fetal bovine serum (FBS) and (Pen/strep) Penicillin-streptomycin (10,000 U/ml penicillin-10mg/ml
streptomycin) and this flushing is repeated until the marrow is removed and pipetted repeatedly to produce a single-cell suspension and the bone appears pale. Pipet this cell suspension through a nylon mesh filter (100 µm, Falcon®) to remove any muscle or bone spicules and remaining clumps. Resuspend the strained medium in fresh growth medium (89 % DMEM containing 10 % FBS and Pen/strep) and plate in a T50 flask [14].

Cells are preserved at 37°C in a 5 % CO₂ steril-cycle incubator. Non-adherent cells are removed carefully via replacing fresh medium every 2-3 days until tissue culture reaches 80-90 % confluence and is ready to be passaged (typically, confluency is achieved on the 7th day). When the primary culture becomes nearly confluent, the culture is treated with PBS (phosphate buffered saline) and cells are trypsinized with 0.25 % Trypsin/ethylenediaminetetraacetic acid (EDTA) (0.05 %/0.02) for 10 min at 37°C [15].

After centrifugation at (2400 rpm for 20 min) and pellet to culture, cells are suspended in DMEM and FBS and incubated in another culture flask, the resulting culture is considered the 1st passage. In our study, 2nd passage is used in which a purified population of BM-MSCs can be obtained 2 weeks after the commencement of culture [16].

After 2 cell culture passages, BM-MSCs were washed nearly 6 times with PBS and then treated with trypsin for 2 minutes. To generate a single-cell sheet, the cells were washed 3 times with DMEM then counted and prepared for injection.

Cell viability testing:
The viability of the MSCs was assessed by a trypan blue dye exclusion assay; which includes diluting the dye with PBS (0.4 % trypan blue/PBS) and then adding cell culture to an equal volume of diluted dye in a manner that depends on whether viable cells possess intact cell membranes and are excluded from the dye, while non-viable cells will have a blue cytoplasm [17].

Characterization of BM-MSCs:
MSCs are characterized by their adhesiveness to plastic and fusiform shape which is detected by an inverted microscope (Leica) and counted by hemocytometer then the cells are prepared for injection [18].

4. Experimental design protocol
After acclimatization, the rats were randomly divided into 7 groups (n=10) as follows:

Group C (control):
10 rats were injected with 0.1 ml of normal saline intra-peritoneal (IP) for two weeks and served as the normal control group.

Sixty rats were intravenously injected in the tail with a single dose of 16 mg/kg (IV) of DOX for the induction of acute cardiotoxicity. This schedule has been previously found to be consistent and reproducible for DIC (doxorubicin-induced cardiotoxicity) [19]. They were divided later into the following groups.

Group D (DOX):
10 rats that received DOX and were kept without treatment.

Treated groups (DOX-MSC groups): divided into subsequent 5 subgroups:

Group I: DOX (2 h-MSCs):
10 rats that received DOX as group D and after 2 h they were injected with BM-MSCs as a single dose of 1x10⁶ cells/rat.

Group II:  DOX (24 h-MSCs):
10 rats that received DOX and after 24 h, they were injected with BM-MSCs as a single dose of 1x10⁶ cells/rat.

Group III: DOX (48 h-MSCs):
10 rats that received DOX and after 48 h, they were injected with BM-MSCs as a single dose of 1x10⁶ cells/rat.

Group IV: DOX (72 h-MSCs):
10 rats that received DOX and after 72 h, they were injected with BM-MSCs as a single dose of 1x10⁶ cells/rat.

Group V: DOX (4 fractionated doses of MSCs):
10 rats that were DOX pretreated like before and were injected with BM-MSCs started after 2, 24, 48, and 72 h of DOX administration (Each rat was injected with 250,000 MSC/time) as accumulated 4 doses.

BM-MSCs are intravenously injected into the tail vein of rats by a single dose of (1x10⁶ cells/ml) for treated groups (I, II, III, and IV) and fractionated as 4 successive doses for group V; each dose equals 250,000 cells/ml. The time points were selected based on pharmacokinetic studies that exhibited that the average half-life of DOX lies between 12-48 h and due to acute cardiotoxicity, it emerges between 48-72 h [22]. Figure (1) demonstrates a schematic representation of the experimental groups of this research.

5. Collection of blood samples
All rats were anesthetized with a single intravenous injection of thiopental sodium (15 mg/kg b.w.) and the blood samples were withdrawn from the orbital plexus, using heparinized micro-capillaries [23]. Samples were allowed to clot for 30 minutes at room temperature before centrifugation. The serum was separated using a high-speed centrifuge at 4000 rpm for 10 min at 4°C, then the top yellow serum layer was pulled gently by a sterile pipette and prepared for analysis [24].

6. Assessment of serum biochemical markers
The separated serum samples were used to estimate levels of liver enzymes such as alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Besides kidney functions urea and creatinine levels were examined. These markers were measured using Epoch™ 2 Microplate reader Spectrophotometer, USA, according to the instruction of the experiment kits, and executed by the colorimetric method using a specific rat enzyme-linked immunosorbent assay ELISA. All reagents were purchased from a private Pharmaceutical Company (Bio Diagnostics Co, Egypt).

7. Histological examination of cardiac tissue
The cardiac tissue samples that were isolated from each group were fixed in 10% formalin, then embedded in paraffin wax blocks, sections cut (4-5 μm), and stained with hematoxylin and eosin [25]. The heart was cut into randomly transverse sections (basal, middle, and apical), the sections were examined under a light microscope, and then photomicrographs were taken.

8. Statistical analysis
The collected data were computerized and statistically analyzed using Graph Pad Prism®, version 8.0.2(Graph Pad Software, USA). Quantitative variables were demonstrated as mean ± SEM (Standard Error of Mean). To evaluate the differences between the studied groups, a one-way analysis of variance (ANOVA) was performed because the data were normally distributed, followed by Tukey-Kramer multiple comparison tests. Statistical significance was accepted at a level of p < 0.05.

Results

1. Characterization of BM-MSCs
Starting from small, round-shaped cells in culture and changing into larger, spindle-shaped cells with high differentiation and proliferation capacity. By the end of 2nd passage in culture, the cell population exhibited characteristic adhesiveness and displayed fibroblast-like-fusiform morphology, as shown in Figure (2).

2. Effect of BM-MSCs on Biochemical Assessments
Induction of acute toxicity with DOX has been noticed by the marked elevation in the serum ALT and AST levels as compared to the normal control group. On the other hand, there is a mild, but
not significant elevation in ALT and AST serum levels among 2, 24, 48, and 72 h of treatment with BM-MSCs and fractionated doses of BM-MSCs for 4 consecutive times as in 2, 24, 48 and 72 h when compared with the control group. There is a non-significant reduction in ALT and AST serum levels for BM-MSCs groups I, II, III, and IV when compared to DOX treated group, whereas BM-MSCs group V recorded a significant decrease \((p <0.05)\) in ALT and AST serum levels when compared with DOX treated group.

However, renal toxicity has been observed after DOX intravenous injection by significant elevation of serum levels of urea and creatinine, as compared to the normal control group. Administration of BM-MSCs at 2, 24, 48, and 72 h groups and fractionated group succeeded in restoring the serum levels of urea and creatinine while the fractionated group showed a significant amelioration among the all-different treated groups; followed by Group I and II (2 and 24 h respectively) and finally came groups III and IV (48 h and 72 h respectively) at the last line in alleviation the tissue damage as presented in Figure (3).

Fig. 2. Photomicrograph of BM-MSCs culture stages, A; on the 1st day of culture, B; on the 3rd day, C; at the end of 1st week (bar scale =200 µm).

Fig. 3. Effect of BM-MSCs on toxicity markers A; ALT, B; AST and renal function C; urea and D; creatinine parameters.
Effect of BM-MSCs on the histological features

The heart tissue of different groups of animals was histologically examined and the results were as shown in Figure (4).

Group C (control): revealed normal cardiomyocyte architecture, which shows a normal arrangement of cardiac muscle fibers, central nuclei, regular cell membrane, absence of vacuolation inside the cells, normal blood vessels, and no inflammation, Figure (4a).

Group D (DOX): showed marked interstitial edema, perinuclear vacuolation, inter-fibrillar hemorrhage, disarrangement, and degeneration of myocardium, Figure (4b).

Treated groups (DOX-MSC groups):

Group I: DOX (2 h-MSCs): showed improvement in the histopathological finding in the form of decreased edema between muscle fibers, absence of hemorrhage, no vacuolar changes, and normal arrangement of muscle fibers, Figure (4c).

Group II: DOX (24 h-MSCs): showed a slight improvement in histopathological findings in the form of moderate interstitial edema, few perinuclear vacuolations, no degeneration of muscle fibers, and no inter-fibrillar hemorrhage, Figure (4d).

Group III: DOX (48 h-MSCs): displayed muscle hypertrophy and muscle degeneration, Figure (4e).

Group IV: DOX (72 h-MSCs): showed marked interstitial edema, perinuclear vacuolation, inter-fibrillar hemorrhage, disarrangement, and degeneration of myocardium, Figure (4f).

Group V: DOX (fractionated): showed the same histological findings as in Group I (2 h-MSCs), Figure (4g).

These above results indicate that BM-MSCs showed cardio protection against DIC.

Discussion

DOX induces cardiotoxicity, limiting its beneficial use as chemotherapy. Acute cardiotoxicity caused by DOX is detected within three days of single-dose administration. Reducing doses would obstruct the efficacy of DOX and therefore bring about unsatisfactory therapy outcomes [26].

The heart is highly susceptible to DOX toxicity caused by oxidative stress and low regenerative capacity, as it possesses both; the highest content of mitochondria than any other

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**TABLE 1. Effect of BM-MSCs on serum liver, and renal function parameters in DOX-treated female rats.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Biomarker</th>
<th>ALT (U/ml)</th>
<th>AST (U/ml)</th>
<th>Urea (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
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<tr>
<td>Control</td>
<td>35.67±1.54</td>
<td>40.67±3.94</td>
<td>56.43±3.39</td>
<td>1.51±0.07</td>
<td></td>
</tr>
<tr>
<td>DOX</td>
<td>65.92±4.48</td>
<td>84.59±7.34</td>
<td>209.5±6.70</td>
<td>3.45±0.15</td>
<td></td>
</tr>
<tr>
<td>I: 2h</td>
<td>44.43±4.85</td>
<td>51.09±7.11</td>
<td>79.93±6.09</td>
<td>1.89±0.08</td>
<td></td>
</tr>
<tr>
<td>II: 24h</td>
<td>45.42±5.02</td>
<td>52.09±6.77</td>
<td>100.2±7.84</td>
<td>2.05±0.15</td>
<td></td>
</tr>
<tr>
<td>III: 48h</td>
<td>51.34±4.03</td>
<td>58.01±7.41</td>
<td>117.5±4.56</td>
<td>2.22±0.04</td>
<td></td>
</tr>
<tr>
<td>IV: 72h</td>
<td>48.22±4.46</td>
<td>63.89±7.50</td>
<td>156.0±3.06</td>
<td>2.59±0.03</td>
<td></td>
</tr>
<tr>
<td>V: fractionated</td>
<td>40.83±2.73</td>
<td>48.83±2.73</td>
<td>79.07±4.22</td>
<td>1.75±0.05</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM, n=10, a: significantly different from the control group, b: significantly different from the DOX group, c: significantly different from Group I, d: significantly different from Group II, e: significantly different from Group III, f: significantly different from Group IV.

Due to oxygen deprivation, mitochondria fail to supply the energy demand, and the myocardium is shifted to anaerobic glycolysis to generate limited energy. Oxygen deficiency results in a decrease in nutrient availability and insufficient elimination of metabolic waste products. This entire process contributes to ischemic heart failure [27]. In addition to cardiotoxicity, DOX can induce lethal toxicity in other organs, including the liver, kidney, and brain. DOX-induced liver impairment varies from non-specific alterations in liver structure to acute liver failure, cirrhosis, and an increase in serum liver enzyme ALT as well as AST [28].

In our study, DOX significantly elevated ALT, AST, urea, and creatinine serum levels, besides the histological deteriorations of the cardiac tissue, indicating hepatic, renal, and myocardial injury and confirming cardiotoxicity.

The proposed regimens influenced in restoration of almost the normal levels of biomarkers for treated groups that administered BM-MSCs at different durations.

The remarkable increase in serum ALT, AST, and cardiorenal biomarkers urea and creatinine, after DOX administration is correlated with the previous studies which proved that the disruption in cellular membranes of the renal and cardiac tissues causing leakage of these cytosolic components into the blood and subsequent increasing their serum concentrations [29].

Prevailing evidence from various studies has illustrated that DOX induces oxidative injury in the heart, kidney, liver, brain, and testes [30] which was confirmed in the current study through biochemical and histological investigations.

The suggested treatment with multiple timings of BM-MSCs significantly ameliorated the oxidative-induced damage [31].

Fig. 4. Transverse section photomicrographs of the heart for the seven groups; a: Group C (control group) showing normal appearance of cardiac muscle fibers (X 100), b: Group D (DOX) with marked edema, perinuclear vacuolation, hydropic degeneration (yellow arrow) inter-fibrillary hemorrhage (red arrow) and disarrangement with hypertrophy of muscle fiber (X 200), c: Group I shows slight edema between muscle fibers, central nucleus and normal appearance of muscle fibers. (X 100), d: Group II shows longitudinal with perinuclear vacuolation (yellow arrow) (X 100), e: Group III shows a zone of dark red normal myocytes retaining their nuclei interrupted by pale staining muscle showing cloudy degeneration (X 100), f: Group IV shows marked inter-fibrillar hemorrhage (yellow arrow), marked edema and disarrangement with hyper-eosinophilic of muscle fibers (X 200) and g: Fractionated group (V) shows longitudinal cardiac muscle which returns to normal cellular appearance (X 100).
The present work indicated the cardioprotective potential of BM-MSCs in female Wistar rats against DIC with its different regimens of BM-MSCs intravenous administration that succeeded in mitigating these acute toxicities through their direct cytotoxic radical scavenging activities and lipid peroxidation inhibition.

Our study also demonstrated DOX-induced liver toxicity based on the increment in levels of serum ALT and AST in the untreated DOX group versus the control one. The increase in these indices of liver markers may be due to an increase in the apoptotic processes in the hepatocytes which are the main target for reactive oxygen invasion [32].

An abnormal increase in these hepato-specific markers in serum indicates damage to hepatic cells, altering the permeability of the damaged cell membrane. Hence, enzymes are released from the cells into the bloodstream [33]. Both ALT and AST enzymes are abundant in the heart and liver; thus, their marked elevations have been correlated with both myocardial infarctions and liver injury.

Also, it was suggested that DOX-induced elevations of ALT and AST levels may contribute to hepatic cell death induced by the oxidative stress that causes leakage of hepatic and cardiac cytosolic enzymes. Conversely, different treated groups with BM-MSCs have significantly decreased serum ALT and AST compared to the DOX group [34].

These outstanding results may be due to the capacity of BM-MSCs as anti-inflammatory and antioxidant agents via catching and deactivating the free radicals that prevent hepatic damage, as previously mentioned.

In the current study, data indicated that DOX induced an increase in urea and creatinine levels. These results are compatible with previous research that proved DOX-induced nephrotoxicity in normal rats [35].

They demonstrated that leakage appeared in the injured kidney, and the levels of urea and creatinine serum in the BM-MSCs treated groups were found to be significantly lower than the DOX group, showing notable protection to the kidney tissues.

Besides, DOX-induced renal injury may be triggered by the production of mitochondrial reactive oxygen species (ROS) and membrane lipid peroxidation, then by inflammatory influences that cause increased capillary porousness and glomerular shrinkage [36].

The treatment with BM-MSCs with its different regimens after DOX administration caused significant amelioration in all previous biomarkers compared to the DOX group, indicating that BM-MSCs helped restrict the leakage of the enzymes and maintain membrane integrity. They also inhibit the iron-dependent production of free radicals, increasing their scavenging action and, therefore, norming lipids and preventing lipid oxidation.

When we compared the different treatment groups based on time trials, we discovered a significant resemblance between the healthy control group and the fractionated group, indicating that DOX nearly did not induce any side effects. This means that BM-MSCs transplantation has its effect through an indirect paracrine effect, while direct differentiation to the cardiomyocytes does not occur.

Hence, it can be hypothesized that this paracrine effect would be augmented if repeated doses of BM-MSCs were transplanted. This assumption is supported by previous studies that admitted that double doses of MSCs have greater effects than one single dose administration [37].

On the other side, 2 and 24-h groups exhibited marked significantly when compared to 48 and 72-h groups; this led us to another hypothesis that the earlier the BM-MSCs administration, the better the outcomes. As the BM-MSCs can perform their therapeutic and regenerative potential with much greater efficacy without interruption, the role of DOX as an anti-cancer drug is the main purpose of the study.

**Conclusions**

The current study highlights the importance of BM-MSCs in amelioration the effects of DIC and points out the potential protective roles of different regimens in treated groups via using different protocols of BM-MSCs through 2, 24, 48, 72-h and fractionated doses of BM-MSCs administration. Besides, this work showed that the effect of BM-MSCs work in a parallel mode with DOX without inducing any side effects or corrupting its role as an anticancer drug.

Results approved the efficacy of 3 groups of BM-MSCs administration (fractionated, 2, and
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24-h groups) after DOX treatment; thus, it is highly recommended for early treatment using BM-MSCs. Preclinical studies should be further investigated.

Data Availability Statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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