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# Amelioration of cadmium-induced testes' damage in rats by the bone marrow mesenchymal stem cells

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ABSTRACT

Cadmium (Cd) and its compounds are highly toxic to virtually all organ systems of the mammals. Cd-induced testicular injuries have been reported in various animal species, using different protocols. The self-renewal capacity and the ability to generate different specialized cell types make the mesenchymal stem cells (MSCs) one of the ideal choices for restoring tissue damages of various etiologies. The use of bone marrow-derived MSCs (BM-MSCs) is among the most recent strategies to repair the Cd-induced testicular damage, but empirical studies in this regard are largely missing. Keeping in view the CD-induced testicular damage and the suggested restorative functions of BM-MSCs, the objectives of the current study were twofold: to induce testicular injury in Sprague-Dawley (SD) rats by a single intraperitoneal (i.p.) 2 mg/kg Cd injection; and to study the reparative potential of BM-MSCs in Cd-induced testicular damage in adult male rats. The SD rats were randomly divided into three groups (n = 10 each): control (untreated), Cd-group (i.p. 2 mg/kg Cd), and Cd + SC group (i.p. 2 mg/kg Cd plus two intravenous doses of 1 × 106 BM-MSCs via penile vein). After four weeks, Cd-group showed a significantly lower ($p < 0.05$) weight-gain, sperm count, and sperm viability, as well as led to testicular atrophy, necrosis, fibrosis, calcification, and marked perivascular lymphocytic infiltration, as compared to the untreated controls. As hypothesized, the rats exposed to Cd, but treated with BM-MSCs (Cd + SC group), showed a lesser degree of Cd-induced damage. In conclusion, the findings of current investigation indicate a reversal of Cd-induced testicular injury by BM-MSCs. The study supports the previously suggested notion that BM-MSCs can repair the Cd-induced testes' damage in rats.

## 1. Introduction

Cadmium (Cd) and its compounds are toxic. High Cd exposure has been reported among the workers in the construction and manufacturing industries (OSHA, n.d.). Apart from occupational exposure, humans are exposed to this heavy metal through diet and smoking (Yari et al., 2010). Cd causes cancer, and damages skeletal, respiratory, renal, gastrointestinal, cardiovascular, neurological, and reproductive systems (Agency for Toxic Substances and Disease Registry, 2012; OSHA, n.d.). As far as latter is concerned, Cd-induced testicular harm has been well documented in humans (Benoff et al., 2000; Marettová et al., 2015; Vigeh et al., 2011), and negative impact of Cd on testes has been reported in various animals (Marettová et al., 2015). Even the non-toxic dose of Cd has been reported to alter the normal gene expression in the testes (Zhou et al., 2004) and if the exposure is persistent, the testicular damage and its consequences can become overt (Marettová et al., 2015). In light of the unequivocal testicular damage by Cd and the high likelihood of Cd exposure, the development of innovative strategies to ameliorate Cd-induced testes' damage is warranted.

One such strategy has been the use of stem cells (SCs). Local injection of mesenchymal SCs (MSCs) has been reported to reverse testicular germ-cell injury in rats (Hsiao et al., 2015). The MSCs are self-renewable, multi-potent cells that exist in various tissues, ranging from adult mice to adult humans. The in vitro culture-expandability and genomic stability with fewer ethical concerns make MSCs a crucial agent in tissue repair, cell therapy, and regenerative medicine (Ullah et al., 2015). It is noteworthy that easy accessibility and the availability of advanced amplification technologies have made the bone marrow-derived MSCs (BM-MSCs) the ideal seed cells for repairing tissue injuries. In an azoospermic rat model, BM-MSCs were reported to potentially recover the spermatogenesis after localization to basal membrane of seminiferous tubules (Zhang et al., 2014).

Moreover, Wang et al. (2017) have shown that BM-MSCs inhibit the Cd-induced mitochondrial apoptosis and injury in the testes of Wistar

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rats. They administered up to 0.4 mg/kg intraperitoneal (i.p.) Cd-chloride saline solution, five times a week for five weeks for inducing testes injury. It would be intriguing to determine if the results of Wang et al. (2017) hold up in a different variety of rats, such as Sprague-Dawley (SD), with a slightly different mode of injury-induction. It should be noted that some investigators have used a relatively higher Cd dose. For example, in the Wistar rats, Kara et al. (2007) used 1 mg/kg subcutaneous Cd for 1 month, and Adamkovicova et al. (2014) used 30 mg/L Cd in drinking water for 90 days. Others have used a different variety of rats with a single intraperitoneal injection of Cd (Fouad and Jresat, 2015; Yiin et al., 1999). Single administration to establish an animal model is relatively easy, which makes the replication of studies involving Cd-induced testicular injury convenient for other researchers.

In order to validate the findings of Wang et al. (2017) and to investigate the scarcely researched topic of applying BM-MSCs to repair heavy metal-induced testicular injury, current study was undertaken. Specifically, the aims of the current study are: to investigate the induction of testicular injury in rats by Cd, based on sperm count, sperm viability, and histopathological findings; and to study the role of BM-MSCs in repairing Cd-induced testes' injury in these rats. The hypothesis was that BM-MSCs repair the Cd-induced injury in adult male rats' testes.

## 2. Materials and methods

### 2.1. Experimental animals

All protocols were in line with the official Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and were approved by institutional animal welfare regulatory committee. A total 30 Sprague Dawley SD, 12–14 weeks old adult male rats, weighing 150–200 g, were obtained from the Animal House of the Nile Center for Experimental Research, Mansoura, Egypt. Animals were kept at the recommended environmental and nutritional conditions, including access to fresh and clean water and food ad libitum. Rats were divided into 3 groups (n = 10 per group):

- **Group (1):** Control untreated healthy Sprague Dawley rats
- **Group (2):** Cd-group; each rat received 2 mg/kg i.p. cadmium chloride, dissolved in normal saline.
- **Group (3): Cd + SC group:** each rat received 2 mg/kg i.p. cadmium chloride dissolved in normal saline, followed by the first dose of intravenous (i.v.) $1 \times 10^6$ SCs in 0.2 ml Dulbecco's modified Eagle's medium DMEM via penile vein. The second dose of $1 \times 10^6$ SCs was given after one week.

Tissues samples from caudal part of testes and epididymis were taken at the end of the experiment for further analysis.

### 2.2. Isolation, culture, and expansion of BM-MSCs

Previously described protocol (Gabr et al., 2014) was followed. Briefly, the SD rats were anesthetized with halothane. The skin was sterilized with 70% ethyl alcohol before cutting the skin. The long bones, femurs and tibia were carefully dissected from adherent soft tissues. Bone marrow was obtained from the femurs and tibia. After dissection, these long bones were sterilized by immersion in 70% ethanol. The bone ends were cut, and BM was extruded by inserting a needle in one end through the bone shaft and injection of tissue culture media (DMEM, containing 10% fetal bovine serum (FBS)). The effluent was collected in sterile tubes. A single cell suspension was generated with gentle pipetting, and the cells were cultured in 75 $cm^2$ tissue culture flask containing 30 ml complete media, and incubated for 3 days at 37 °C in 5% humidified $CO_2$ incubator (Shel Lab, USA).

After 3 days, the non-adherent cells were discarded while, the adherent cells were considered as MSCs. The cultured cells were microscopically examined daily using inverted microscope (Olympus CKX41, USA) to follow up the cell growth. The cells were fed twice weekly with complete DMEM. After reaching confluence, the cells were detached with a solution of trypsin (Sigma) and ethylene diamine tetra-acetic acid (Sigma). Cells were re-suspended with complete DMEM and re-plated at 1:2 ratio.

### 2.3. Cell counting

Washed cells were suspended in 1 ml of appropriate media. As described previously (Gabr et al., 2014), 10 μL from washed cell suspension was removed for cell counting, using dilution factor between 2 and 10. Briefly, 10 μL of cells and 10 μL Trypan blue 0.4% (lonza, USA) were mixed, and 10 μL of the mixture was put in hemocytometer (Neubauer, Germany). Cells were counted under ordinary microscope (Olympus CX31, USA), using following equation in the process:

$$Number\ of\ cells/mL = average\ of\ counted\ cells \times dilution\ factor \times 10^4$$

### 2.4. Characterization of isolated stem cells

Previously described protocol (Gabr et al., 2014) was used. For flow cytometric analysis, the BM-MSCs at passage 3 were released by trypsinization. The cells were centrifuged at 300*g* for 8 min. After centrifugation, the cells were dissolved with phosphate-buffered saline (PBS) at l x l06 cells/ml. Fluorescent labeled direct antibodies (10 μL for each 100 μL sample) were used: phycoerythrin-labeled CD106 and CD90; and fluorescein isothiocyanate (FITC)-labeled CD45 and CD14 (Becton-Dickinson, USA). The labeled cells were analyzed after PBS-washing with argon ion laser (15mw) with 488 nm wavelength (FACSCalibur, Becton-Dickinson, USA). Cell Quest software (Becton-Dickinson, USA) was used to analyze 10,000 events.

### 2.5. MSC multi-lineage differentiation

As outlined before (Titmarsh et al., 2017), MSCs were plated in triplicate at $1.8 \times 10^4$ cells/$cm^2$ and when confluent, maintained at adipogenic induction medium for 14 days for adipogenic differentiation. For osteogenic differentiation, cells were plated at $3 \times 10^3$ cells/$cm^2$ and when confluent, maintained at osteogenic induction medium for 14 days. Likewise, the MSCs were plated in triplicate at $2.5 \times 10^4$ cells/$cm^2$ in chondrogenic induction medium for 14 days. The undifferentiated cells were used as controls.

#### 2.5.1. Adipogenic differentiation

Human MSCs were plated in triplicate at 1.8 3 $10^4$ cells/$cm^2$ in 12-well plates and cultured for 3 days. When confluent, the medium was replaced with Adipogenic Induction Medium (AIM) comprising maintenance medium supplemented with 1 mM dexamethasone, 10 mM insulin, 20 mM indomethacin, and 115 mg/ml 3-isobutyl-1-methylxanthine. Cells were maintained in AIM for 14 days, with AIM prepared fresh and replaced every 3–4 days. Undifferentiated cells used as controls (for each lineage) were kept in maintenance media for 14 days with replacement every 3–4 days.

#### 2.5.2. Osteogenic differentiation

Human MSCs were plated in triplicate at 3 3 103 cells/$cm^2$ in 12-well plates and allowed to attach overnight. Then, the medium was replaced with Osteogenic Induction Medium (OIM) comprising of maintenance medium supplemented with 10 nM dexamethasone, 25 mg/ml L-ascorbate-2-phosphate and 10 mM glycerol-2-phosphate. Cells were maintained in OIM for 14 days and medium was changed every 3–4 days.

#### 2.5.3. Chondrogenic differentiation

Human MSCs (2.5 3 $10^4$ cells) were pelleted in triplicate in