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IMPACT OF BONE MARROW MESENCHYMAL STEM CELLS AND THEIR DERIVED EXOSOMES ON REGENERATION OF CHEMOTHERAPEUTICALLY INDUCED DAMAGE IN RAT'S PAROTID SALIVARY GLAND

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ABSTRACT

Objective: To assess the impact of bone marrow mesenchymal stem cells (BMMSCs) and their derived exosomes on inducible nitric oxide synthase (iNOS) levels in cisplatin (CP) induced parotid salivary gland damage.

Methods: Cryopreserved BMMSCs line with cell density 10^6 was used in this study. BMMSCs derived exosomes were purchased from Nawah Scientific lab. Fifty-six male albino rats in good health weighting from 200-250 g were allocated into 4 groups (n=14); group I; rats received phosphate buffered saline (PBS), group II; rats were intraperitoneally injected with CP, group III; were injected with CP then after 3 days they were injected with BMMSCs via tail vein and group IV; received CP and after 3 days they were injected with BMMSCs-exosomes via tail vein. After 7 and 14 days, scarification of animals **was** done, and the parotid glands were collected and processed to be examined histologically and immunohistochemically using (iNOS) immune marker.

Results: Groups treated with BMMSCs, and exosomes showed marked decrease in the percentage of iNOS immunohistochemical positive reaction which was significantly lower in the group treated with exosomes in comparison to the group that received BMMSCs.

Conclusions: BMMSCs-exosomes have a potent effect in reducing the inflammatory effect of CP on rats' parotid salivary gland.

KEYWORDS: Parotid salivary gland, Cisplatin, Bone marrow, Mesenchymal stem cells, Exosomes, iNOS

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INTRODUCTION

Chemotherapy is a widely used treatment in some malignant tumors. Although it has the ability to damage cancer cells, it also causes damage in normal tissues. The severity of tissue damage is determined by the amount, the type and the length of time of the use of the chemotherapeutic drug [1].

Saliva plays an important role in innate immunity against infections in the oral cavity, so reduced salivary flow is one of the causes of the development of oral inflammation and increased dental caries [2]. Other oral side effects include mucositis, fungal infections, and neuropathies [3]. The reduction in salivary flow may arise from morphologic damage and alterations in salivary gland tissue [4].

CP is a powerful anti-cancer treatment which is extensively used in different types of human neoplasms[4]. CP always results in damage of deoxyriboneuclic acid (DNA), leading to formation of DNA adducts which is a crucial step in its cytotoxicity [5]. CP can also induce cytotoxicity by production of reactive oxygen species inside mitochondria which may induce apoptosis [6].

Stem-cell therapy is considered a well-known powerful modality that can be used in regenerative medicine to treat many diseases. One of the most reliable and functional tissues to obtain stem cells is the bone marrow as it has the power to self-renew, multiply and give other cell types. BMMSCs were confirmed to induce tissue regeneration after CP induced tissue damage in many organs [7, 8] as they can enhance cell proliferation in damaged tissues [9]. It is supposed that the paracrine release of many cytokines and growth factors mediates this effect. These factors include Vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), and caspase-3 which is an anti-apoptotic factor [10]. So, it is thought that the beneficial effect of BMMSCs in treatment of different diseases relies to a large extent on the paracrine effect of factors released from them like exosomes [11].

Exosomes are extracellular vesicles with a diameter ranges 30-100 nm. Different types of cells, including stem cells, secrete exosomes that are responsible for the paracrine effect of the cells releasing them [12]. They contain proteins, mRNAs and microRNAs that mediate exosomes action on target cells [13].

Nitric oxide (NO) is a gaseous free radical which has a small biological half-life. A group of the nitric oxide synthase (NOS) enzyme isoforms use L-arginine to build it up [14]. NO has a part in the formation of salivary amylase [15]. It is an important regulator of the functional processes in salivary gland either in physiological or pathological conditions [16]. It can interact with neurotransmitter receptors as a physiological messenger [17] or act as an inflammatory mediator in the development of many diseases [18].

Therefore, this study was designed to compare the impact of intravenously injecting BMMSCs and their exosomes on the level of iNOS, as regenerative agents against CP-induced cytotoxicity in the parotid glands of rats.

MATERIALS AND METHODS

This study was approved by the ethical committee of Faculty of Dentistry, Mansoura University; code no: MU-ACUC (DENT.MS.22.11.3) and according to ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments) for reporting animal research all procedures were performed.

Cryopreserved cell line from central lab of stem cells and biomaterials applied research, Faculty of Dentistry, Ain Shams University with cell density 10^6 was used to produce BMMSCs. BMMSCs derived exosomes were purchased from Nawah Scientific lab.

Study design

Fifty-six white albino male rats weighting 200-250 g (aged 16-18 weeks) were kept under

standard housing conditions in cages with normal accessibility to food and water in the animal house, faculty of medicine, Ain Shams Research Institute (MSRI). They were divided into 4 groups (n=14):

Group I: animals received 0.5 ml of PBS injected via the tail vein, so group I was the negative control group. **Group II:** animals were intraperitoneally injected with CP (single dose of 5 mg/kg) [19], it was considered a positive control group. **Group III:** the rats received the same intraperitoneal dose of CP as group II and after 3 days they received intravenous injection via the tail vein with BMMSCs (2×10^6 cells) suspended in 0.5 ml of PBS [20]. **Group IV:** the rats received single intra-peritoneal CP injection (5 mg/kg) and after 3 days they received intravenous injection via the tail vein with BMMSCs-exosomes (100 μg/kg/dose suspended in 0.2 ml PBS) [21].

Rats were scarified using overdose halothane anesthesia 7 and 14 days after injection of CP: 7 rats/group/time point. Bilateral parotid glands were excised, then immediately fixed in 10% formalin. The section was prepared for immunohistochemical staining of iNOS.

Statistical analysis

Data collected from iNOS expression were statistically counted in terms of mean ± standard deviation (± SD). The data distribution was checked to explore the normality of the numerical data, the mean and median values were calculated and Kolmogorov-Smirnov and Shapiro-Wilk tests were used. Parametric distribution of the data was found so; it was represented by mean and standard deviation *(SD)* values. The effect of different tested variables and their interaction were studied using the two-way ANOVA. Main and simple effects were compared by using pairwise t-tests with Bonferroni correction. The degree of significance was set at $p\leq0.05$ within all tests. IBM*® SPSS**® Statistics Version 26 for Windows was used to implement the statistical analysis was.

RESULTS

Immunohistochemical staining

The iNOS cytoplasmic immunoreactivity was noticed in all studied groups with different levels. Positive reaction is indicated by brownish staining. The negative control group which didn't receive any treatment showed the lowest level of iNOS expression in relation to other groups recorded (after 7 days; 0.97±0.09, and after 14 days; 1.04±0.11). The CP group displayed the highest levels of iNOS expression in comparison to either control or treated groups (after 7 days; 5.03±0.54, and after 14 days; 6.58±0.71). Regarding the treated groups, BMMSCs group revealed a significant decreased level of iNOS expression when compared to the CP group (after 7 days; 3.90±0.42, and after 14 days; 3.57±0.39) while, BMMSCs exosomes showed a significantly decreased level of iNOS expression than that of both cisplatin and BMMSCs groups (after 7 days; 2.88±0.31, and after 14 days; 2.24±0.24) **(Figure 1, 2)**.

Statistical analysis revealed significant difference between 7 days and 14 days in each group in the expression of iNOS, with $p<0.05$. However, the negative control group and BMMSCs group didn't show any significant difference between 7 and 14 days of treatment.

In relation to iNOS expression ANOVA test revealed a significant difference between the 4 groups with the highest mean value in the CP group then the BMMSCs group then the BMMSCs exosomes group and finally the negative control group with the lowest value after 7 days and also after 14 days with p-value ($P<0.001$). **(table. 1) (Figure. 2)**.

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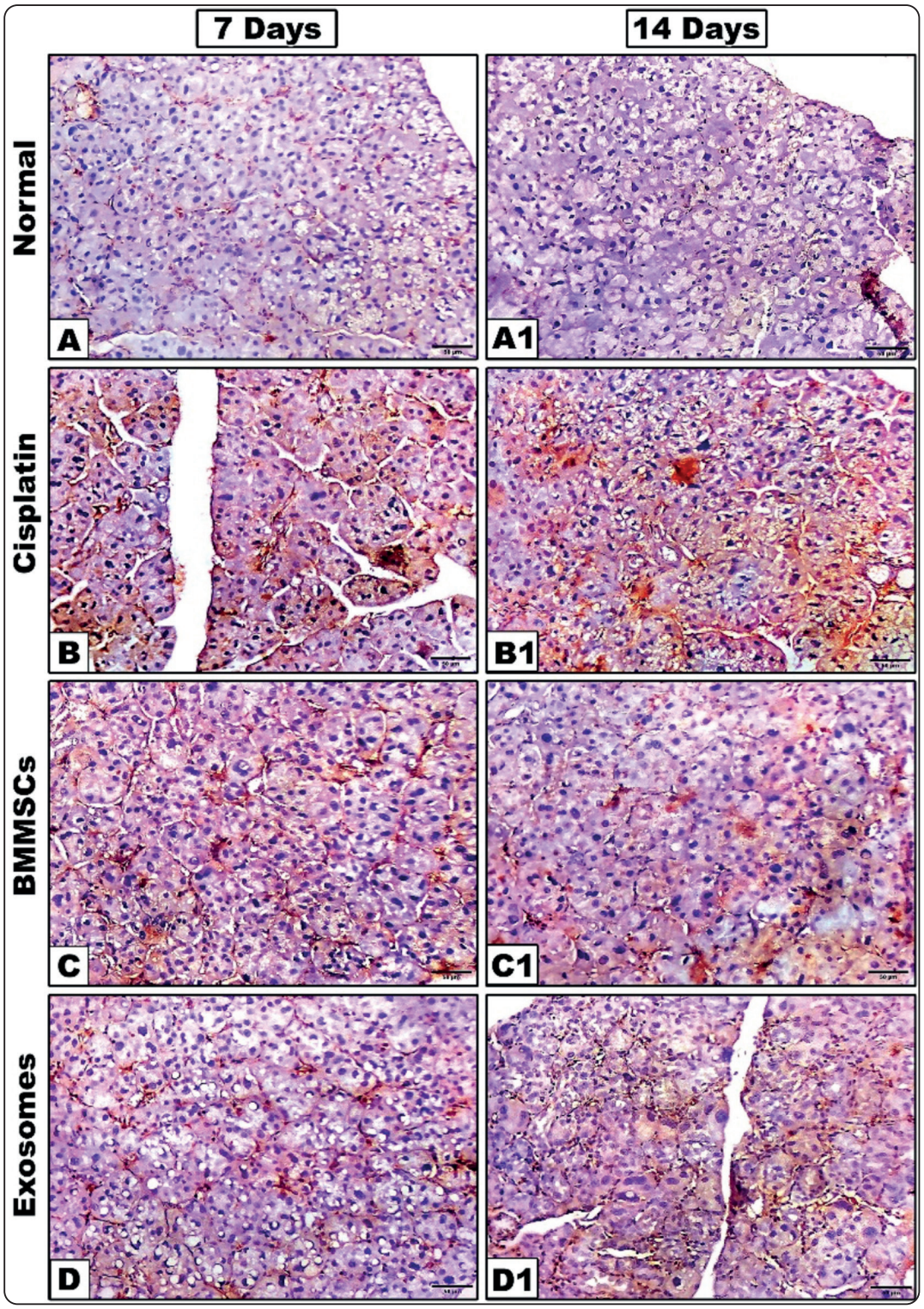

Fig. (1) Photomicrographs of the parotid gland sections stained with iNOS at 7 and 14 days' post-treatment. (A, A1) showing the negative control group, (B, B1) The CP group which showed the highest iNOS expression. (C, C1) The expression markedly decreased in BMMSCs and (D, D1) more decrease in the exosomes treated groups. (iNOS X 200)

TABLE (1) Descriptive statistics for iNOS Area% of different groups.

Groups	After 7 days	After 14 days	p-value(2)
Negative control	0.97±0.09	1.04±0.11	**0.243**
CP Group	5.03±0.54[a]	6.58±0.71[a]	**<0.001***
BMMSCs Group	3.90±0.42[a,b]	3.57±0.39[a,b]	0.152
Exosomes Group	2.88±0.31[a,b,c]	2.24±0.24[a,b,c]	**<0.001***
p-value(1)	**<0.001****	**<0.001****	

p-value 1 for one-way analysis of variance ***p-value 2 for Independent Sample t-test***

p-value > 0.05 is insignificant; ****p-value <0.05 is significant;*** *****p-value <0.001 is highly significant***

(a) Means there is a significant difference with the control group

(b) Means there is a significant difference with the CP group

(c) Means there is a significant difference with the BMMSCs group

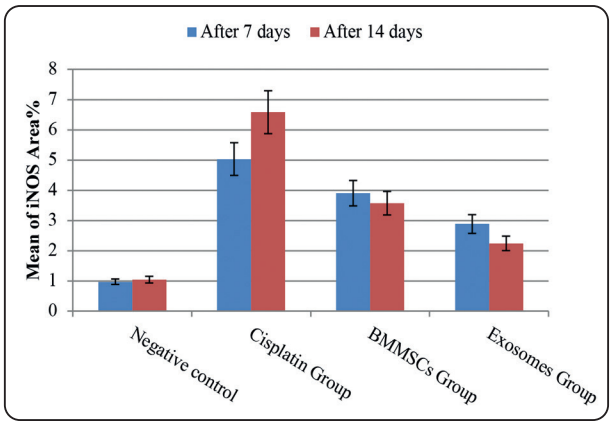

Fig. (2) Bar chart showing iNOS brown positive expression values for different groups.

DISCUSSION

Oral cancer is a common cause of death [22]. CP is an effective chemotherapy in treating cancers but it causes structural damage in the normal salivary glands tissue [4]. In the current study, BMMSCs were selected to evaluate their potential in counteracting this damaging effect of CP because of their regenerative abilities, particularly through paracrine action mediated by their secreted biologically active molecules including exosomes [23]. Thus, we further assessed the regenerative effects of exosomes that were reported to induce tissue regeneration with low immunogenicity and no vascular obstruction[24]. The used antigen in the current study to explore the regenerative potential was iNOS, which is an inflammatory mediator in many diseases [18].

In the present work, the immunohistochemical and statistical analysis of iNOS expression revealed that the CP group had significant difference in relation to the negative control group which agrees with ***He Y et al***. who found that iNOS increases 72 h after CP injection [25]. The mechanism of iNOS elevated expression was explained by ***Kaygusuzoglu E et al.*** who found that CP can activate NF-ϰB migration from the cytoplasm to enter the nucleus, acting together with ϰB elements to trigger iNOS transcription and expression of inflammatory factors like IL-6, tumor necrosis factor α (TNF-α) and IL-1β [26].

The result comes also in accordance with ***Leung EL et al***. who stated that CP upregulates iNOS among NOS isoforms and also suggested that iNOS may have a role in CP-induced apoptosis [27]. ***Fiscus RR et al.*** explained that iNOS induces apoptosis in mammalian cells by combining NO with superoxide anion, forming peroxynitrite, a powerful oxidizing substance that induces oxidative and nitrosative stress leading to toxicity and cell death [28].

Choi BM et al. mentioned that the NO produced from iNOS stimulates the release of cytochrome c from the mitochondria leading to activation of caspase and modulation of anti-apoptotic Bcl-2 proteins resulting in upregulation of p53 expression that induces cell death [29].

Regarding BMMSCs group a significant decreased levels of iNOS expression in relation to CP group was present. This was consistent with ***Sherif et al.*** who found that BMMSCs were able to ameliorate CP-induced tissue damage by decreasing inflammatory mediators as iNOS and TNF-α [30]. ***Xu C et al.*** stated also that MSCs can reduce iNOS by producing transforming growth factor β (TGF-β) [31].

Furthermore, several studies clarified that the anti-inflammatory effect of BMMSCs is achieved via their inhibitory action to the iNOS. ***Yang L et al.*** reported that BMMSCs mediate their powerful anti-inflammatory activity through the inhibition of iNOS synthesis and stimulation of synthesis of the anti-apoptotic VEGF [32]. Another study conducted by ***Li H et al.*** found that MSCs protective effect was mediated by reducing the number of M1 macrophages that are responsible for production of pro-inflammatory factors like iNOS and TNF-α and increasing M2 macrophages that are responsible for tissue repair [33]. Additionally, it was reported that BMMSCs can inhibit inflammation by blocking TNF-α pathway resulting in downregulation of iNOS, and on the other hand, BMMSCs stimulate IL-10 production which is an anti-inflammatory cytokine [34].

The BMMSCs exosomes group showed significant decrease in iNOS level in relation to CP group which comes in agreement with ***Mao F et al.*** who reported that MSCs exosomes reduces iNOS expression and macrophages recruitment to the inflamed tissues accompanied by decreasing expression of other proinflammatory cytokines such as IL-6 and TNF-α and increasing expression of the anti-inflammatory cytokine IL10 [35]. ***Ebrahim N et al.*** revealed that blocking the dysregulated Wnt signaling pathway is the mechanism by which BMMSCs-derived exosomes reduce iNOS expression and limit inflammatory responses [36]. ***Wen L et al.*** stated also that BMMSCs-derived exosomes are able to inhibit the pro-inflammatory factors including iNOS, IL-1β and TNF-α via upregulation of anti-inflammatory factors including IL-10 and TGF-β, accompanied by decreasing the ratio of M1/M2 macrophages [37].

In conclusion, it has been reported that BMMSCs and their exosomes can reduce the damage caused by chemotherapeutic agents in salivary glands. The results of this study may recommend the possible clinical application of BMMSCs or their exosomes to reduce salivary glands damage among patients on CP treatment.

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