ORIGINAL ARTICLE

Histochemical studies of Mesenchymal Stem Cells Effect on Possible Adverse Effects of Rapamycin Drug; on Lymph Node and Peyer's Patches in Adult Male Albino Rats

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Abstract

Background: ***The possible effects of Bone marrow-derived mesenchymal stem cells (BM-MSCs) on the possible adverse effects of immunosuppressive drugs, which are commonly used nowadays. In this study, we assess the therapeutic potential effect of BM-MSCs on adverse effects induced by rapamycin overdose treatment by evaluating the histology and functional integrity of lymph nodes and Peyer's patches under amplified BM-MSCs /or rapamycin treatments compared with the control group. The present study was carried out on 40 healthy albino rats of different age groups. The rats were divided into four groups. Analytical methods include flow cytometry, ELISA for cytokine detection, and histological staining protocol assessments.***

Results: ***Findings indicated that as rapamycin concentrations increased, BM-MSC viability decreased. Histological evaluations revealed improved structures in lymph nodes and Peyer's patches when BM-MSCs were co-administered with rapamycin, suggesting that BM-MSCs can counteract rapamycin's immunosuppressive effects.***

Conclusion: ***Integrating BM-MSC therapy could enhance its therapeutic safety profile, making it a viable approach for reducing the adverse effects of immunosuppressive regimens in clinical practice.***

Keywords: Rapamycin; BM-MSCs; lymph node; Adverse effects; Peyer's Patches

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells found in adipose tissue, bone marrow, or umbilical cord. They have the ability to expand, differentiate in vitro and vivo, and have immunoregulatory properties.[1] MSCs have been proposed as regenerative therapeutics for diseases like ischemia, chronic wound healing, and osteoarthritis. BM-derived MSCs contribute to healing by producing cytokines, growth factors, and cell lineages.[2,3] They must differentiate into osteocytes, chondrocytes, and adipocytes in vitro and adhere to plastic surfaces.[4]

Rapamycin, also known as Sirolimus or Rapamune, is an immunosuppressive and anti-proliferative drug used to prevent transplant rejection.[5] It inhibits the mammalian target of rapamycin (mTOR) pathway, impairing protein synthesis and improving autophagic flux.[6] This property has been applied to senescent cells, revealing rapamycin as an effective senotherapeutic drug in preclinical studies.[7] Research is focused on improving optimal mTOR inhibitors to protect MSCs from early senescence and enhance their immunomodulatory potential.[8] Rapamycin has been found to reverse replicative senescence and induce therapeutic efficacy in treating ischemic illness in mice.[9]

Accepted 15 March 2025.
Available online 31 May 2025

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https://doi.org/10.21608/aimj.2025.446588

mTOR, a key nutrient metabolic pathway, is essential for controlling cell cycle progression and survival in human cancer.[10] It is often activated by oncogenic alterations, leading to interest in targeting mTOR as an anticancer therapeutic strategy.[10] Rapamycin targets mTOR in adipogenesis, a key developmental process associated with metabolic homeostasis and nutritional signal transduction.[11,12]

In this study, we aimed to evaluate the therapeutic potential of BM-MSCs amplified in rapamycin overdose treatment by assessing the histology and functional integrity of lymph nodes and Peyer's patches, immune cell population and function, and molecular analysis of soluble inflammatory cytokines, tissue damage, and tissue repair markers

2. Patients and methods

Experimental animals

The study involved 40 adult male albino rats, purchased from SLAC Laboratory Animal Co., Ltd., to evaluate the therapeutic potential of BM-MSCs on the adverse effects of the rapamycin immunosuppressor drug. The animals were divided into four groups:

Control group (Ck): Ten rats will receive intraperitoneal injection with PBS solution at (1.0ml/kg/day intraperitoneal (i.p.)).

Rapamycin group (Rapa): Ten rats in which immunosuppression will be induced by rapamycin drug (Sigma-Aldrich; Merck KGaA) dissolved in PBS at 1.0 mg/mL for intraperitoneal injection at doses of 1mg/k/day i.p. from days 0 to 10.

BM-MSCs group (BM-MSCs): Ten rats will be injected once with BM-MSCs labeled by PKH26 dye (1 × 10^6 cells/mL, i.v.) suspended in complete medium at day 0, and dissolved in PBS at 1.0 mg/mL for intraperitoneal injection at doses of 1mg/k/day i.p. from days 0 to 10.

Rap+ BM-MSCs group: Ten rats will be injected once with BM-MSCs labeled by PKH26 dye (1 x 106 cells/ml, i.v.) suspended in complete medium at day 0, in addition to being injected with rapamycin at doses of 1mg/k/day i.p. from days 0 to 10 days.

All rats were sacrificed at the end of the experiment and sacrificed by cervical dislocation. Dislocation and lymph nodes and Peyer's patches were removed and fixed in 10% neutrally buffered formalin for 24 hours. A microscopic investigation was conducted using techniques approved by the research ethics committees and the protocols of Al-Azhar University.

Isolation of BM-MSCs

The study focuses on the preparation of Bone marrow-derived mesenchymal stem cells from rats. The process involves flushing the tibiae and femurs of 6-week-old male white albino rats with Dulbecco's modified Eagle's medium, centrifuging, and cultured in a 25 cm2 culture flask. The cells are then incubated at 37°C for 12-14 days, then washed twice and trypsinized. The resulting first-passage cultures are identified by their morphology, adherence, and differentiation ability.

Phenotypic analysis of BM-MSCs by Flow cytometry:

The isolated cultured BM-MSCs cells (3rd,4th passages) were morphologically characterized using flow cytometry. Analyses of the expression of cell surface markers were performed on a population of live single cells. BM-MSCs were washed in phosphate-buffered saline and incubated for 30 min (4 0C) with the monoclonal antibodies labeled with anti-CD3, anti-CD4, anti-CD8, and anti-CD21. Dead cells were stained using fluorescent dye (Invitrogen, USA) and added to the samples 10 min prior to flow cytometry. Data (100,000 events per sample) were obtained using an LSRII flow cytometer (Biosciences, USA) and analyzed using Flow software (Tree Star, USA).

Labeling MSCs with lipophilic fluorescent dyes:

Prior to the experiments, BM-MSCs grew to 70% confluence and were washed with PBS. For staining with PKH26, BM-MSCs were incubated in a staining cocktail consisting of DMEM medium without serum (83.3% v/v) complemented with 1.1% (=1 vol) of PKH26 solution in diluent at 100% and incubated for one h in a humidified Materials and Methods 35 incubator (37°C, 5% CO2). Then the staining was stopped as described by the supplier (Sigma-Aldrich). The PKH26-labeled BM-MSCs were maintained in MSC growth medium for the following experiment.

In vitro pretreatment of BM-MSCs with rapamycin

BM-MSCs were divided into two groups: a. The untreated BM-MSC group was used as a negative control. b. The second group involved BM-MSCs treated with 100 nmol/L (9.14 g/L) of rapamycin in the culture medium, and the cells were subsequently incubated for two hours.

Histological staining techniques

The study evaluated the impact of Rapa-Treated BM-MSCs on lymph nodes and Peyer's patches tissues by staining them with Hematoxylin & Eosin, immunohistochemical staining, and fluorescent cell homing detection, and Masson's trichrome staining protocol, then examination of the stained slides using light microscopic techniques.

Chemical and molecular study

Oxidative Stress Status:

The study aimed to assess the antioxidant role of BM-MSCs in mitigating the immunotoxicity impact of overdose rapamycin on lymph nodes and Peyer's patches. Oxidative stress markers (MDA, TAC, and NO) in lymph nodes and Peyer's patches

tissues were assessed. Rapa-treated BM-MSCs showed potential in mitigating these markers. The study also measured the reaction of lipid peroxidation, malondialdehyde, with thiobarbituric acid (TBA), and total antioxidant capacity (TAC).

3. Results

Morphological characterization of isolated BM - derived mesenchymal stem cells

BM-derived mesenchymal stem cells were collected from rat bone marrow and harvested from male white albino rats. They were cultured in falcon tubes and incubated for 14 days. The morphology of the cells was identified using a suspension, labeled with markers, and incubated for morphological characterization. The cells were expressed on their surface, with some having irregular shapes. The morphological characters and growth curve of the collected BM-MSCs is shown in figures 1,2.

In Vitro Pretreatment of BM-MSCs with Rapamycin

The Effects of rapamycin Drugs on cell viability:

To evaluate the cytotoxicity effect of rapamycin on metabolic activity of B-MSCs, the cell viability of untreated and rapamycin treatment BM-MSCs cells in the presence of optimum Rapa concentration (50, 100, 200, 400, 600, 800, and 1000 µg/ml) in the culture medium for 48 h, and the cell viability of these cells was assessed according to WST-1 assay protocol as compared with chemical standard (Tamoxifen).

Results were shown in table 1 and fig. 3

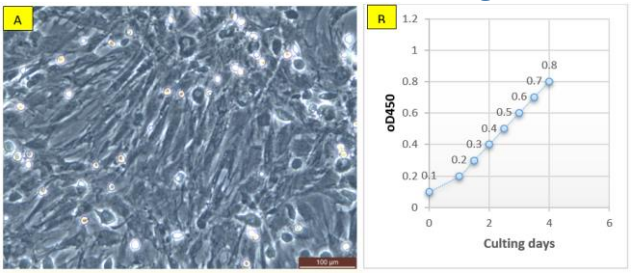

Figure 1: Morphology and proliferation of BM-MSCs harvested by flushing the tibiae and femurs approaches of 6- week-old male white albino rats obtained using isolation approaches, (A) morphology of non-uniform population with BM-MSCs cells in suspension soon after the earliest passage , the BM-MSCs was viewed using an inverted microscope. (B) Growth curves were measured by a CCK-8 assay.

Non-uniform population with BM-MSCs cells in suspension soon after the earliest passage

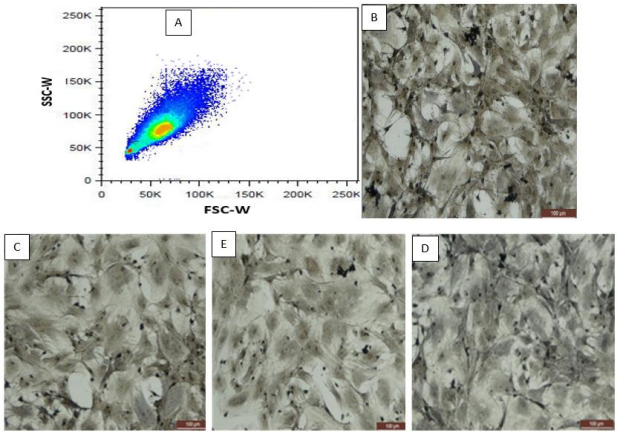

Figure 2. Characterization of BM-MSCs.The cell suspension population cultured for 16 days analyzed by flow cytometry (A). BM-MSCs express on their surface the classical markers of mesenchymal stem cells, e.g., CD19 (B), CD73 (C), CD90 (D) and CD105 (E).

Table 1. Cytotoxic effect of different concentrations of rapamycin on BM-MSCs cell viability as compared with positive cytotoxic chemical (Tamoxifen) after 48h incubation. Values are represented as mean + SD measured in triplicate.

DOSE (µG/ML)	RAPAMYCIN	TAMOXIFEN
CONTROL	100±0.0	100±0.0
50	87.11±3.45	83.41±4.09
100	81.03±2.60	73.9±4.60
200	73.4±3.04	57.4±3.28
400	54.8±2.72	45.2±3.46
600	42.6±2.06	26.6±1.94
800	26.1±1.64	18.97±1.04
1000	16.59±0.83	12.89±0.89
IC50 VALUE	521.47±39.25	501.14±34.58

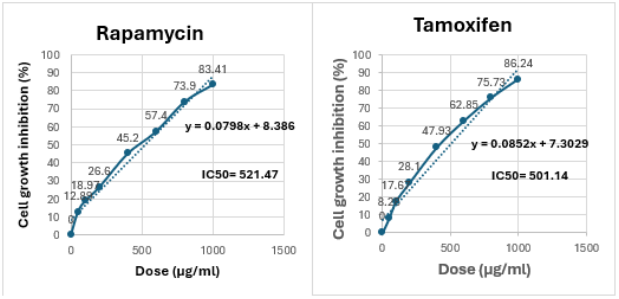

Figure 3. Cell growth inhibition (%) and inhibitory concentration (IC50) of different concentrations of rapamycin (on BM-MSCs culture as compared with positive cytotoxic chemical standard (Tamoxifen) after 48h incubation. Values are represented as Mean± SE measured in triplicate.

Histological staining of rat lymph nodes and Peyer's patches:

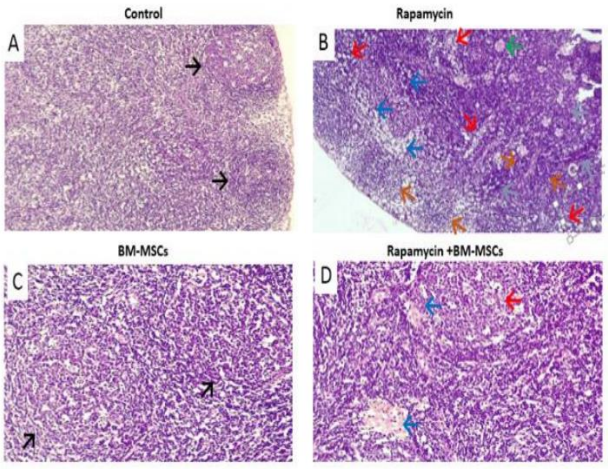

Figure 4. Photomicrographs of H&E staining of rat lymph nodes sections of rapamycin immunosuppressive drug, BM-MSCs cell and combination of both treatment rats compared with untreated group demonstrating morphological structure changes. A: Lymph node of control group with normal structure, LN lobule (black arrow). B: Lymph node of rapamycin group showed, Atrophy (gray arrow), LN inflammation (blue arrow), Lymphocytes proliferation (B-cell) (red arrow), follicular lymphoma (brown arrow) and macrophage (green arrow). C. Lymph node of BM-MSCs group showed regular structure of LN and LN lobule (black arrow). D: Lymph node of rapamycin +BM-MSCs group showed improvement of the disturbed structure, remain LN inflammation (blue arrow)

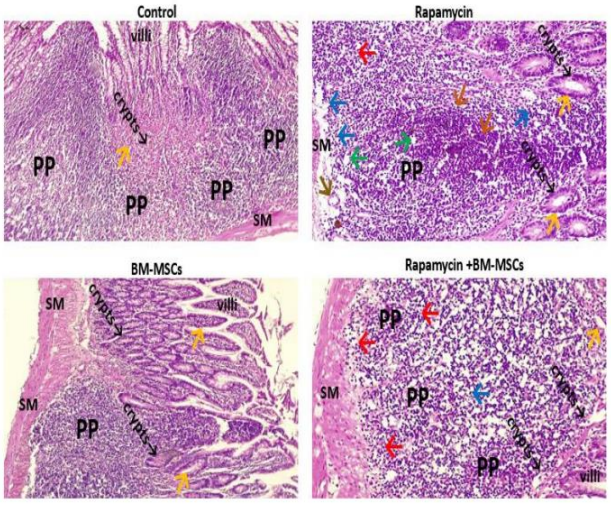

Figure 5. Photomicrographs of H&E staining of rat Peyer's patches sections of rapamycin immunosuppressive drug, BM-MSCs cell and combination of both treatment rats compared with untreated group demonstrating morphological structure changes. A: Peyer's patches of control group with normal structure of Peyer's patches (PP), smooth muscle (SM), villi, and crypts. B: Peyer's patches of rapamycin group showed necrosis (dark brown arrow), crypts (orange arrow), LN inflammation (blue arrow), Lymphocytes proliferation (T-lymphocytes) (red arrow) and Peyer's patches lymphoma (brown arrow). C. Peyer's patches of BM-MSCs group showed regular structure of PP. (red arrow). D: Peyer's patches of rapamycin +BM-MSCs showed improvement of the disturbed structure, remaining PP lymphocytes (red arrow) and tissue inflammation (blue arrow).

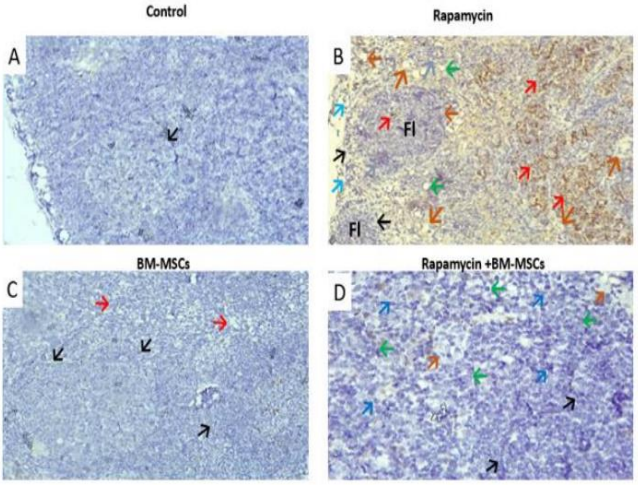

Figure 6. photomicrographs of IHC staining using CD3, CD8, CD19 and CD25 immunological markers. of rat lymph nodes sections of rapamycin immunosuppressive drug, BM-MSCs cell and combination of both treatment rats compared with untreated group demonstrating morphological structure changes. A: Lymph node of control group with normal structure, LN lobule (black arrow). B: Lymph node of rapamycin group showed, Atrophy (gray arrow), LN inflammation (blue arrow), Lymphocytes proliferation (B-cell) (red arrow), follicular lymphoma (Fl), necrosis (dark brown arrow) and macrophage (green arrow). C. Lymph node of BM-MSCs group showed regular structure of LN and LN lobule (black arrow). D: Lymph node of rapamycin +BM-MSCs group showed improvement of the disturbed structure, remain LN inflammation (blue arrow) and necrosis (dark brown arrow)

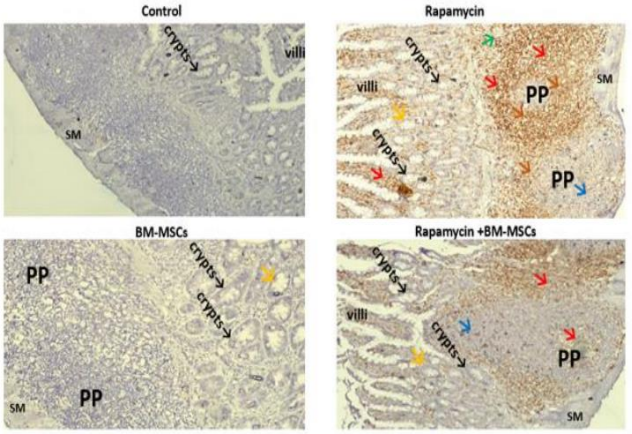

Figure 7: photomicrograph of IHC staining using CD3, CD8, CD19 and CD25 immunological

markers of rat Peyer's patches sections of rapamycin immunosuppressive drug, BM-MSCs cell and combination of both treatment rats compared with untreated group demonstrating morphological structure changes. A: Peyer's patches of control group with normal structure of Peyer's patches (PP), smooth muscle (SM), villi, and crypts. B: Peyer's patches of rapamycin group showed crypts (orange arrow), LN inflammation (blue arrow), Lymphocytes proliferation (T-lymphocytes) (red arrow) and Peyer's patches lymphoma (brown arrow). C: Peyer's patches of BM-MSCs group showed regular structure of PP. (red arrow). D: Peyer's patches of rapamycin +BM-MSCs group showed improvement of the disturbed structure, remaining PP lymphocytes (red arrow) and tissue inflammation (blue arrow)

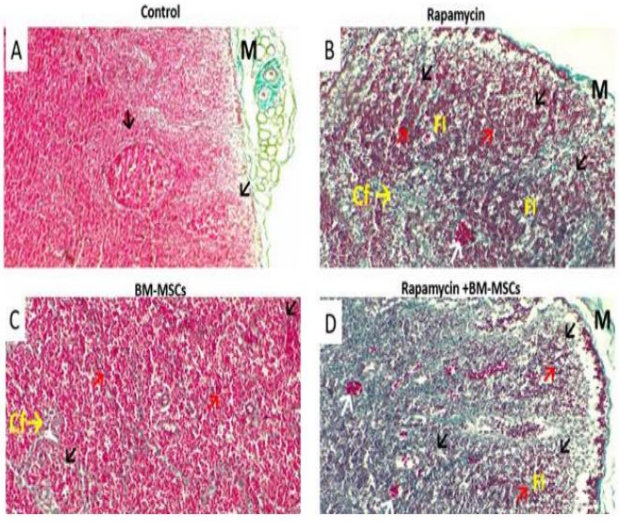

Figure 8. Photomicrographs of Masson's trichrome staining of rat lymph nodes sections of rapamycin immunosuppressive drug, BM-MSCs cell and combination of both treatment rats compared with untreated group demonstrating morphological structure changes. A: Lymph node of control group with normal structure, Muscle (M0, and LN lobule (black arrow). B: Lymph node of rapamycin group showed, LN Atrophy (white arrow), Lymphocytes proliferation (T-cell) (red arrow), blue stained collagen fibers (Cf) (yellow arrow) and follicular lymphoma (Fl). C. Lymph node of BM-MSCs group showed regular structure of LN and LN lobule (black arrow). D: Lymph node of rapamycin +BM-MSCs group showed improvement of the disturbed structure, remain LN Atrophy (white arrow)

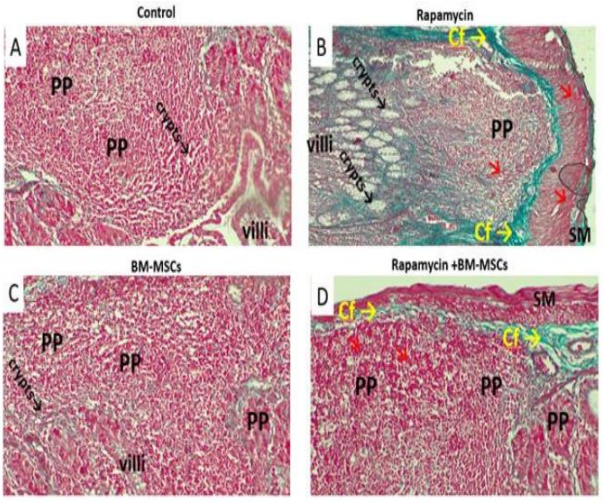

Figure 9. Photomicrograph of Masson's trichrome staining of rat Peyer's patches sections of rapamycin immunosuppressive drug, BM-MSCs cell and combination of both treatment rats compared with untreated group demonstrating morphological structure changes. A: Peyer's patches of control group with normal structure of Peyer's patches (PP), smooth muscle (SM), villi, and crypts. B: Peyer's patches of rapamycin group showed, crypts (orange arrow), blue stained collagen fibers (Cf) (yellow arrow), Lymphocytes proliferation (T-lymphocytes) (red arrow) and Peyer's patches lymphoma (brown arrow). C. Peyer's patches of BM-MSCs group showed regular structure of PP. (red arrow). D: Peyer's patches of rapamycin +BM-MSCs showed improvement of the disturbed structure, remain PP lymphocytes (red arrow) and blue stained collagen fibers (Cf) (yellow arrow)

Evaluation of the Oxidative Stress State in LN, PP tissues:

The study examined the biochemical, oxidative stress state, and relative expression of immunomodulatory genes in lymph nodes and Peyer's patch tissues from different experimental rat groups. The rats were treated with rapamycin, BM-MSCs, rapamycin, and BM-MSCs cells, as well as untreated control groups. The rapamycin treatment significantly increased oxidative stress and lipid peroxidation levels in lymph node tissue, while BM-MSCs cell treatment decreased MDA levels. Total antioxidant capacity increased in lymph node tissue, and total antioxidant capacity (TAC) levels increased in Peyer's patches tissue. SOD antioxidant enzyme activity increased in lymph node tissue, and catalase activities increased in lymph node tissue. The results suggest that rapamycin treatment can improve immune function and reduce oxidative stress in lymph nodes and Peyer's patch tissues.

Table 2. Effects of MSCs pretreated with rapamycin immunosuppressive drug on oxidative stress/antioxidant levels in lymph node and Peyer's patches tissues. Data are represented as mean + SD measured in triplicate.

TREATMENTS	DOSE	MDA (NM/MG PROTEIN)		TAC (UMG-1MIN-1 PROTEIN)		SOD (UMG-1MIN-1 PROTEIN)		CAT (UMG-1MIN-1 PROTEIN)	
		LN	PP	LN	PP	LN	PP	LN	PP
CONTROL	0	2.50±0.1 g	4.617±0.04 d	11.466±0.13 c	3.47±0.075 h	46.260.041 d	38.39±0.04 d	20.31±0.03g	18.8467±0.055h
RAPAMYCIN	1mg/k/day	5.6467±0.05c	7.08±0.03 a	22.02±0.116 b	6.91±0.055f	53.737±0.07 c	55.85±0.05 c	23.6067±0.06f	26.25±0.0e
BM-MSCS	*1 x 10⁶ cells/ml*	1.96±0.06 d	3.85±0.05 f	9.27±0.055 d	4.37±0.03g	62.17±0.046 b	63.47±0.044 b	33.9167±0.055 c	31.53±0.0550.06 d
RAPA. + BM-MSCS	both	4.42±0.08e	6.71±0.1 b	28.11±0.035 a	8.11±0.035e	71.5±0.06 a	68.217±0.04 a	38.1067±0.055 a	37.7667±0.06 b
LSD		4.58±0.041		24.64±0.058		33.11±0.03		19.26±0.055	

*Lymph node *LN); Peyer's patches (PP)

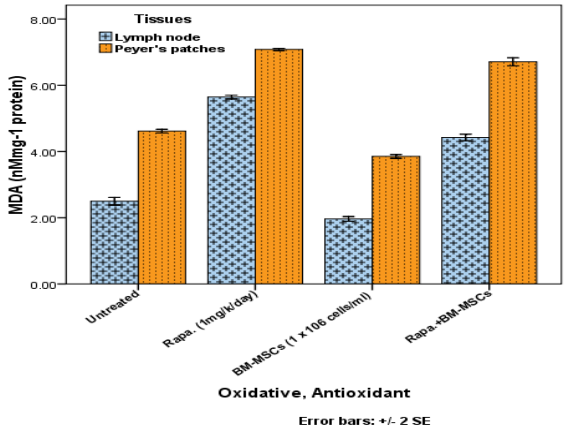

Figure 10. Effects of MSCs pretreated with rapamycin immunosuppressive drug on MDA levels in lymph node and Peyer's patches tissues. Data are represented as a mean + SE measured in triplicate.

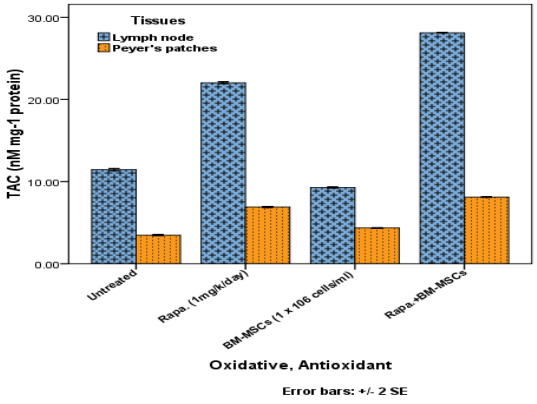

Figure 11. Effects of MSCs pretreated with rapamycin immunosuppressive drug on TAC levels in lymph node and Peyer's patches tissues. Data are represented as a mean + SE measured in triplicate.

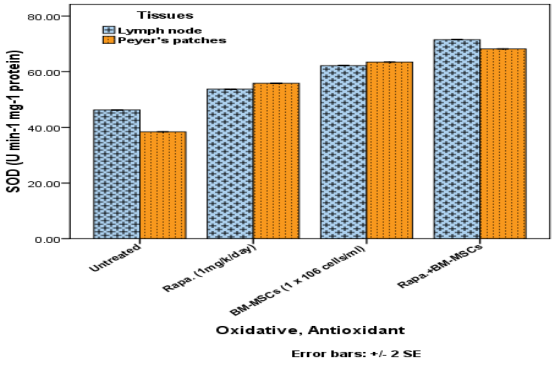

Figure 12. Effects of MSCs pretreated with rapamycin immunosuppressive drug on SOD antioxidant enzyme activity in lymph node and Peyer's patches tissues. Data are represented as a mean + SE measured in triplicate.

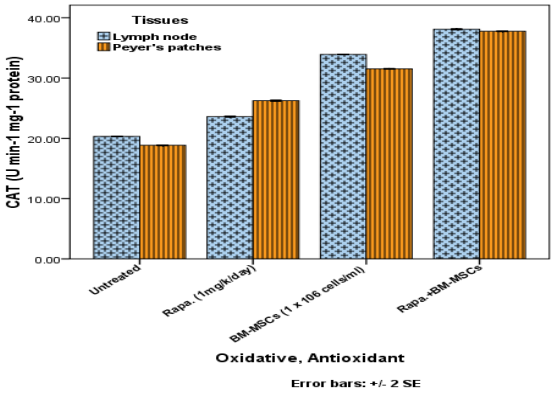

Figure 13. Effects of MSCs pretreated with rapamycin immunosuppressive drug on CAT antioxidant enzyme activity in lymph node and Peyer's patches tissues. Data are represented as a mean + SE measured in triplicate.

4. Discussion

Rapamycin is a widely used immunosuppressive drug to prevent organ transplant rejection, but it has various side effects, particularly on precursor cells and tissue healing. It negatively impacts mesenchymal stem cell (MSC) function, inhibiting their activity and inducing apoptosis, as noted in previous studies.[13,14] This research aimed to isolate and culture BM-MSCs from adult albino rats to assess their potential in mitigating the adverse

effects of rapamycin. Over the culture period, BM-MSCs exhibited varied morphologies, with significant changes noted by day nine, where a dense population of spindle-shaped cells formed, consistent with findings from Mok et al.[15] and Sasaki et al.[16]. Cytotoxicity assays revealed a dose-dependent toxic effect of rapamycin on BM-MSCs, leading to decreased cell viability and affected metabolic activity, with a maximum viability of 87.11% observed at lower concentrations (50 µg/ml) and a minimum of 16.59% at 1000 µg/ml. The pretreatment of BM-MSCs with rapamycin resulted in a significant decrease in the production of several key cytokines, including HGF, VEGF, CCL2, IL-6, and IL-8, indicating an overall reduction in inflammatory responses, consistent with the results of Amira Awadalla et al. [17]. Furthermore, flow cytometry analysis highlighted morphological apoptosis in these cells after rapamycin treatment. Histological analyses showed atrophy and inflammation in lymph nodes and Peyer's patches within the rapamycin-treated group, while BM-MSCs exhibited some restorative effects on the tissue structure, despite persistent inflammation. Immunological evaluations indicated a disturbed lymphocytic distribution in the rapamycin group, which improved in the BM-MSC-treated group. Overall, the study suggests that rapamycin's immunotoxicity significantly impacts BM-MSCs, hindering their therapeutic potential in tissue healing. The findings underscore the importance of further exploring the interactions between immunosuppressive therapies and stem cell treatments to enhance healing post-transplantation .[17, 18]

4. Conclusion

While rapamycin remains a critical immunosuppressive agent, integrating BM-MSC therapy could enhance its therapeutic safety profile, making it a viable approach for reducing the adverse effects of immunosuppressive regimens in clinical practice.

Disclosure

The authors have no financial interest to declare in relation to the content of this article.

Authorship

All authors have a substantial contribution to the article

Funding

No Funds : Yes

Conflicts of interest

There are no conflicts of interest.

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