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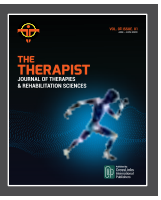

Original Article

Ascorbic Acid: A Potent Agent for Mitochondrial Damage Repair in H2O2 Treated Bone Marrow Mesenchymal Stromal Cells

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ABSTRACT

Bone Marrow Mesenchymal stromal cells (BMSCs) have shown an encouraging promise for cell-based treatments and regenerative medicine applications. Reactive oxygen species (ROS) can damage mitochondria and are detrimental to BMSC cell viability. Ascorbic acid, or vitamin C, is a crucial ingredient that is frequently added to culture media as an antioxidant. Its role in the proliferation of BMSCs has already been studied. However, no research has been done on its effects on the ability of BMSC to regenerate mitochondrial damage. **Objective:** To analyze the recovery of mitochondrial damage by H_2O_2-induced oxidative stress with Ascorbic Acid. **Methods:** BMSCs were cultured and treated with H_2O_2 in order to induce oxidative stress. The injured BMSCs were then treated with vitamin C and their regeneration and recovery from mitochondrial damage is investigated by cell viability assays, ELISA and gene expression profiling. Recovery from oxidative damage is checked through anti-oxidative enzymes. **Results:** Findings showed that supplementing with vitamin C greatly enhanced cell viability and proliferation. It significantly decreased the BMSC's generation of ROS brought on by H_2O_2. These results imply that Ascorbic Acid may enhance the rate of proliferation and reduces apoptosis by recovering the mitochondrial damage as evidenced by the down-regulation of BAX. **Conclusions:** H_2O_2 when given to BMSC could create oxidative stress which in turn damages these cells as evidenced by their decreased cell viability. Ascorbic acid was also observed to regenerate the cells from H_2O_2 injury with the help of increased cells' viability and proliferation and decreased apoptosis.

INTRODUCTION

Multi-potent bone marrow mesenchymal stromal cells are differentiated into multiple types of cells like bone, cartilage and fibroblast. BMSCs are considered to be the precursors of the body tissue. These cells were general but they have the ability to form specialized cells which performed specialized functions [1]. In the regenerative medicine field adult mesenchymal stem cell has a vital role. These cells can be isolated from any body tissue and minor differences and similar properties can be displayed because of their microenvironment origin; the display of characteristic occur after expansion. In vitro expansion can be obtained in serum-enriched media, fibroblast growth factor-enriched media, cytokine-free as well as in serum-free. In pathogenic conditions, oxidative stress played an important role. Cellular activities like cell attachment are negatively influenced by oxidative damage. On the other hand, antioxidants enhanced these activities. The basic cause of DNA damage and cellular senescence was thought to be oxidative damage. Due to defective mitochondrial functions, reactive oxygen species ROS was produced. Under hypoxic conditions when cells were treated with oxidants (H_2O_2) premature senescence was

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supported. Antioxidants enhanced the proliferation rate of bone marrow and adipose-derived MSCs while the effect of ROS remain un-cleared [2]. Cell viability of bone marrow stromal cells reduced by oxidants and antioxidant plays a vital role in the protection of cells from oxidant cytotoxicity was reported. Elimination of organelle and injured cell take place through a complex process referred to as apoptosis [3]. Antioxidants like Vitamin-C were water-soluble and necessary for the functioning of immune systems. A key role was played by Vit-C in other ECM components and the biosynthesis of collagen. Throughout the human body, it acted like co-factoring many biological reactions. When Vit-C is supplied to the culture medium, it increased the DNA synthesis and proliferation rate of BMSCs [4]. It acts as a scavenger of nitrogen species and reactive oxygen species. To get entry into the cells required a channel to pass the hydrophobic lipid bilayer of the plasma membrane. So, sodium-dependent Vit-C transporter 2 has been shown that transported Vit-C into BMSC. On the basis of this background, we developed an experiment in which we investigated the effect of Ascorbic acid on the injury regeneration of MSCs in bone marrow via an enhanced rate of proliferation and decreased rate of apoptosis.

METHODS

At The University of Lahore from the animal house after submitting the signed letter to the lab attendant a 2-month-old albino rat was purchased. After dissecting the rat, BMSC (bone marrow stem cells) were collected. For the purpose of isolating BMSCs, the bone marrow of C57BL/6 GFP-positive mice was collected by flushing their tibias and femurs [5]. Cells were centrifuged and resuspended in culture media in 25 cm2 culture flasks. The culture medium used was HG-DMEM supplemented with 100 g/ml Penicillin G, 100 U/ml Streptomycin and 20% FBS. When cells were 80-90 % confluent, 3-4ml trypsin was added to the flask. Incubate it at 37ºC, 5% CO_2 for 10-15 minutes. When cells became detached, 1-2 ml PBS was added to stop the activity of trypsin. The detached cells were then transferred to a sterilized 15ml falcon. After centrifugation, the pellet was re-suspended with 10% DMEM. And these cells transferred to a new T-75 flask. To check the cytotoxicity of Bone marrow mesenchymal stromal cells H_2O_2 injury was given with concentrations of 0.5mM, 1mM, 2mM, and 5mM for 2 hours and then gave the treatment of Ascorbic Acid with concentrations 30ug/ml, 60ug/ml, and 90ug/ml were used. A 96-well plate was used to conduct the 3-(4, 5-dimethylthiazol-2yl) -2, 5-diphenyltetrazolium bromide (MTT) assay (Invitrogen Inc., USA) for the evaluation of the proliferative capacity of BMSC. Phosphate buffer saline (PBS) was used to wash a monolayer of cells before they were cultured for two hours in 100 l of serum-free DMEM medium and 25 l of MTT solution (5 mg/ml). After being solubilized by 10% sodium dodecyl sulphate (SDS), the purple-colored formazan crystals emerged and the 570 nm absorbance was measured. The previous technique was used to calculate the percentage viability [6]. By using Trizol-method total RNA was extracted from all pre- and post-treated BMSC with Ascorbic acid. In different experimental groups, 1.5ml Trizol-reagent was added and saved in Eppendorf. These Eppendorf were further mixed properly and centrifuged again at 1200rpm for 10 min at 4ºC. After centrifugation, a pellet appeared which contained RNA and the supernatant was discarded. Afterwards, the pellet was cleaned twice with 70% ethanol by centrifuging them at 12000rpm [7]. After discarding ethanol, the pellet was dried and then added the 50ul DEPC water. By using 7ul of RNA, 1ul dNTPs (10mM) and 2ul of poly T were used to synthesize complementary DNA. This reaction mixture was shaken properly and incubated at 65ºC for 5 minutes and then immediately kept on ice. After that, 2ul of 5x reaction buffer, 1ul M-MLV reverse transcriptase and 7ul water were added and incubated for three steps: at 42ºC for 60 minutes, 70ºC for 5 minutes and 4ºC for minutes. The total reaction mixture was 20ul of c.DNA. Different groups of primers such as GAPDH for optimization of c.DNA, proliferation markers like TOP2A, PCNA, and KI67 and apoptotic markers such as BAX, CASPASE, and p53 were used for PCR. To make a PCR product, the reaction mixture is first prepared. After running PCR, these products were run at 1% agarose gel. In a 96-well plate, ELISA was performed for VEGF, P53 and ANNEXIN to check the apoptosis and angiogenesis of BMSC. 100ul of capture antibody in coating buffer was transferred to each well. After adding these the Plates were incubated at 4ºC overnight. The next day, captured antibody was carefully removed, and saved and washing was done with TBST solution three times. After washing, 100ul secondary antibody HRP (Horse reddish peroxidase) was added to each well and left it over overnight. Then, the secondary antibody was removed, and saved and washing was done with TBST. Afterwards, 100ul of Tetramethyl benzidine (TMB) was added for the detection of HRP as substrate solution for 20 minutes. After sufficient color development adds 100ul of stop solution 0.18M H_2SO_4 in each well. Absorbance was taken out at 450nm. Dead cell assay was Performed for live and dead cells in which Trypan blue was used as a prohibiting agent. [8]. The cells from the various study groups were washed with PBS three times before being incubated for five minutes with 50ul of Trypan blue. Cells were then three times rinsed in PBS and examined under a microscope. Trypan blue-stained cells were thought to be deceased. The crystal violet staining method

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was used to identify live cells. For this measurement, a 96-well plate was employed. After removing the medium from the dish, three PBS washes were performed. After washing, 2% ethanol mixed with 0.1 % crystal violet dye was poured into each well. It was kept at room temperature for 15 minutes. Cells were washed once more after the dye was withdrawn. In each well, 100ul of 1% SDS was then added and allowed to sit for 5–10 minutes. On the microtiter plate, absorbance was calculated at 595 nm. Antioxidant enzymes were checked with Glutathione Reductase, Catalase, Superoxide Dismutase and Ascorbate Peroxidase assay. Experiments were done in triplicates[9]. Standard Error Mean was checked by GraphPad.

RESULTS

The cell protective effect of Ascorbic acid on H_2O_2- induced cytotoxicity in the BMSC was observed. MTT assay for all groups shows a significant difference between the viability of treated groups and control. Significantly increased proliferation of BMSC is observed at 1Mm concentration of H_2O_2.

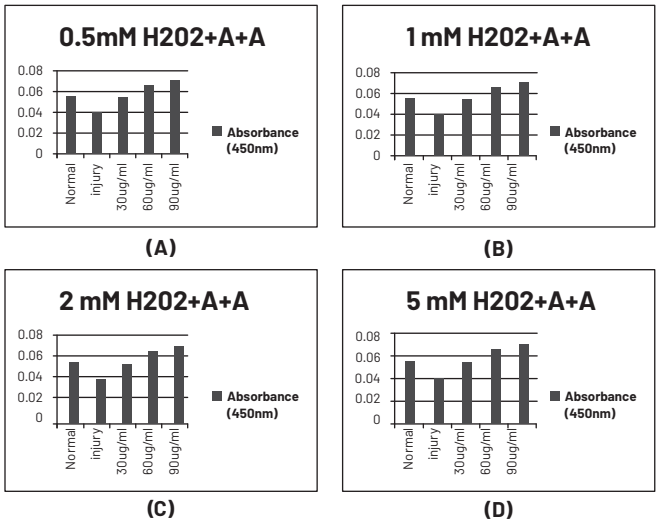

Figure 1: (A) In order to check the cytotoxicity levels in H_2O_2 post-treated BMSCs MTT assay was performed.

BMSC proliferation is dose- and time-dependently regulated by ascorbic acid. Ascorbic acid's effects on BMSCs were verified by the results of a cell proliferation assay (B) By calculating IC_{50}, we observed that at 1.19mM 50% of the viability of BMSCs occurred (C) 2mM H_2O_2 when Co-incubated with various concentrations of Ascorbic acid, decreased the 40% viability rate of BMSCs (D) at 5mM 30% viability of BMSC decreased as compared to other concentrations of H_2O_2. The value was shown as mean ± SEM where p <0.0001* showed significant values

Cell viability detection was done through crystal violet, trypan blue and ELISA VEGF (Figure 2).

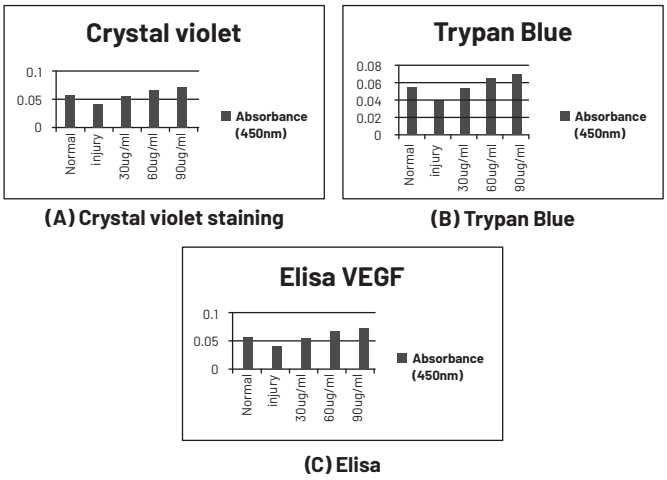

Figure 2: (A) Graphical representation of crystal violet staining showed increased viability of BMSC on the increasing dose of H_2O_2 as compared to control the ratio of dead cells become decreased as compared to the untreated cells (B) Percentage analysis of non-viable cells by trypan blue assay. 1 mM concentration of H_2O_2 Showed significantly low viability of BMSC in comparison with treated groups of BMSC. (C)Growth factor VEGF showed increased proliferation and decreased apoptosis analyzed by ELISA The absorbance was taken at 450nm

Gene profiling through PCR showed increased proliferation of treated BMSC at PCNA and TOP2A as compared to KI-67. Ascorbic acid increases BMSC survival against H_2O_2-induced apoptosis. Live cells increased as we increased the concentration of ascorbic acid. The value was shown as mean ± SEM where p <0.0001* showed significant values (Figure 3).

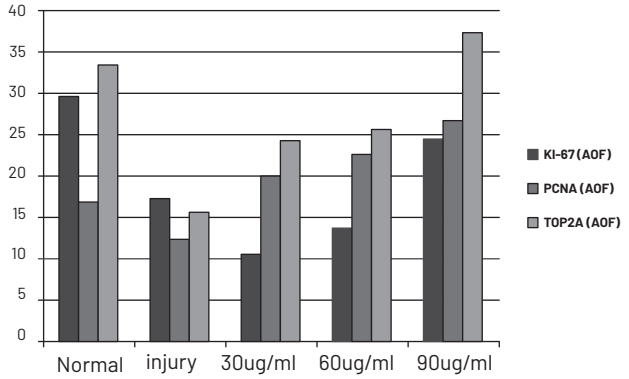

Figure 3: Representation of the increased angiogenesis through VEGF in post-treated groups. The value was shown as mean ± SEM where p<0.0001* showed significant values

Comparative analysis showed decreased apoptosis in figure 4.

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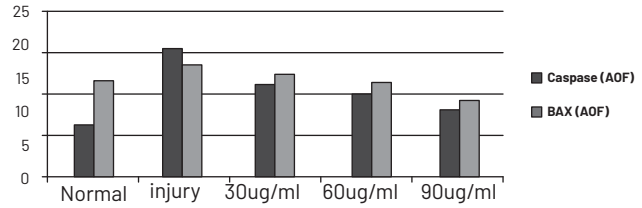

Figure 4: Expression analysis of apoptosis in treated groups of BMSCs through Annexin-V and p53 is decreased as compared to the untreated group

Post-treated BMSC treated with these enzymes showed increased activity versus control with standard error. More proliferation and less apoptosis were observed in treated BMSCs as compared to the untreated group.

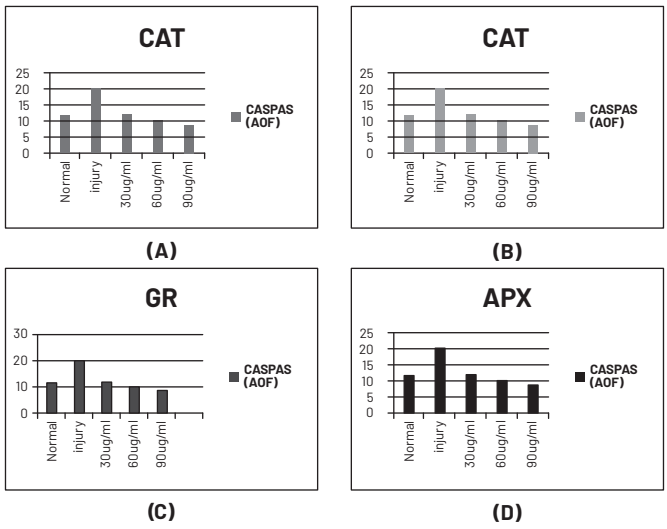

Figure 5: Evaluation of antioxidants graphs showed the antioxidant activity of SOD (A), CAT (B), GR (C) and APX (D) These figures show the levels of antioxidants in treated BMSC versus control the absorbance was taken at 560nm, 240nm, 340nm and 240nm respectively

DISCUSSION

Many studies have observed that ascorbic acid-induced various cellular responses. Previous study by Choi *et al.*, showed that low concentrations of ascorbic acid enhanced the proliferation rate of mesenchymal stromal cells [10]. A recent study by Vicencio *et al.*, revealed that during culture ascorbic acid modulated BMSC proliferation and served as a positive regulator of BMSC proliferation. Different concentrations of ascorbic acid in culture 30ug/ml, 60ug/ml, and 90ug/ml modulated the proliferation rate of mesenchymal stromal cells without loss of the cell's differentiation capacity. Crystal violet staining in our experiment showed increased viable cells (Figure 2). Our results demonstrated when trypan blue was performed it showed a decreased death rate of BMSCs as compared to the crystal violet. We examined the assumption that the ascorbic acid could direct and modulate BMSC growth. Angiogenesis inducer VEGF was an important regulator of neovascularization and it could easily proliferate the proliferation. In our experiment when BMSCs were treated with ascorbic acid, VEGF was up-regulated and enhanced the proliferation of BMSCs. Although expression p53 was associated with regulation of both senescence and apoptosis [11]. In a previous study by Xu *et al.*, showed that when BMSCs undergo cellular senescence, which was followed by the increased expression of p53, the level of apoptosis was up-regulated [12]. Our experiment indicated that BMSCs when treated with ascorbic acid, the expression level of p53 and annexin was downregulated as compared to the increased expression of VEGF (Figure 3). PCNA was the accessory protein for the functioning of DNA polymerase, the molecule essential for cell proliferation and intracellular DNA replication, Ki-67 was a molecule related to PCNA activity and chromatin closely, and Ki-67 is used as a cell proliferation indicator. Bax was the main molecule regulating apoptosis in the mitochondria [13]. The Bcl-2 family member BAX played a crucial part in the apoptosis process. Once the apoptosis process has been started CASPASE-3 cleaved into more apoptotic factors such as CASPASE-6 and CASPASE-9 and these factors become activated which further enhanced the effect of apoptosis on BMSC [14]. In our comparative apoptotic study, we concluded that BAX and CASPASE showed lower levels of apoptosis in BMSC while proliferative markers PCNA and TOP2A showed increased expression as compared to the KI67. The reduction in cellular proliferation and apoptosis induction in cancer cells is linked to the effect of cytotoxicity. Similar to this, our findings demonstrated that Kalonji extracts have the ability to induce apoptosis by up-regulating apoptotic markers like BAX, Caspase-3 and p53 reduce proliferation by down-regulating proliferative genes (PCNA, TOP2A, Ki67) in HeLa and HepG2 cells. Cell viability of bone marrow stromal cells reduced by oxidants and antioxidant plays a vital role in the protection of cells from oxidant cytotoxicity was reported [15]. In a recent study, we analyzed that ascorbic acid has a positive effect on the survival of BMSCs towards H_2O_2 that induced cell death. We found that the proliferation rate was enhanced with increased expression of SOD, and CAT. GR and the apoptosis rate were decreased [16]. The imbalance between the antioxidants and oxidants is referred to as oxidative stress which further leads to cellular death [17]. ROS basically formed by the reduction of an O_2 molecule that contained oxygen with unpaired electrons. Precursor of ROS which was a superoxide anion, formed by the transfer of one electron to an O_2 molecule [18]. SOD catalyzed this superoxide anion and further produced hydrogen peroxide. This H_2O_2 easily crossed the cell membranes and caused damage in the cells because it's a

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stable molecule as compared to superoxide anion. For the breakdown of H_2O_2, antioxidant enzymes SOD, and CAT will come and catalyze this molecule into water and oxygen [19]. Inactivation of proteins and DNA fragmentation is caused by the production of ROS which ultimately leads to apoptosis. In order to exert anti-oxidative function, CAT, GSH and SOD have the ability to scavenge the free radicals. Treatment with ascorbic acid was found to significantly decrease ROS- production and increase the anti-oxidative effect [20].

CONCLUSIONS

From the study, it was observed that H_2O_2 when given to BMSC could create oxidative stress which in turn damages these cells as evidenced by their decreased cell viability. Ascorbic acid was also observed to regenerate the cells from H_2O_2 injury with the help of increased cells' viability and proliferation and decreased apoptosis.

Authors Contribution

Conceptualization: RM
Methodology: SJA, SM
Formal Analysis: LM
Writing-review and editing: LK, NN, AM

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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