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Bio-Antioxidants Protect the Buffalo Bone Marrow Derived Mesenchymal Stem Cells against Oxidative Stress Induced During Freeze-Thaw Cycle

Navjot Kaur[1], M. Sharma[1*], M. K. Lonare[2], R. Udehiya[3] and D. Singh[1]

[1]Department of Veterinary Physiology and Biochemistry, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana – 141001, Punjab, India; mannsharma77@gmail.com
[2]Department of Veterinary Pharmacology and Toxicology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana – 141001, Punjab, India
[3]Department of Veterinary Surgery and Radiology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana – 141001, Punjab, India

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Abstract

The present investigation was planned to evaluate the effect of curcumin (CUR) and resveratrol (RES) on oxidative stress indicators (LPO, O2- radical, ROS and TPC), antioxidant status (GSH, GPx, SOD, CAT and GST) and cytotoxicity markers (ALP, LDH, GGT and CK-MB) in mesenchymal stem cells (MSCs) derived from buffalo bone marrow during the freeze-thaw cycle of one month. MSCs were isolated and cultured in high glucose DMEM supplemented with 15% FBS. MSCs were found to be positive for alkaline phosphatase (AP) activity and stem cell markers (CD73 and OCT4). Results revealed a significant reduction in ($p \leq 0.05$) in oxidative stress parameters and cytotoxicity markers, while, the levels of cellular antioxidants were found to be significantly increased ($p \leq 0.05$) in CUR/RES alone and in combination groups as compared to the control group. In conclusion, the addition of bio-antioxidants in the cryopreservation medium has improved the post-thaw cell recovery through suppression of oxidative stress-induced during the freeze-thaw cycle.

Keywords: Antioxidants, Buffalo, Curcumin, Mesenchymal Stem Cells, Oxidative Stress, Resveratrol

1. Introduction

Mesenchymal Stem Cells (MSCs) have created enormous enthusiasm amongst researchers because of their promising role in regenerative therapy and are well known for treating a wide range of degenerative diseases in human medicine[1]. The trend has also been initiated for the clinical use of these miracle cells in veterinary practice. Bone marrow-derived MSCs-based approaches are currently used for the treatment of tendon, ligament or cartilage/joint injuries in horses and dogs[2] and wound repair in cattle[3]. However, the risk of contamination increases, as well as characteristics of MSCs, may change when cell lines are maintained for a long period[4]. Therefore, the maintenance of characteristics of MSCs is crucial *in vitro* and it may be possible to preserve them by satisfactory cryopreservation technologies so that these cells can be used for clinical cases at any time. Furthermore, efficient cryopreservation may be more practical to establish stem cell banking for long term potential use and implementation of these cells into clinical applications.

DMSO has been the most widely used cryoprotective agent for stem cells. Reports from different studies indicate that the best cell recovery is obtained by controlled-rate freezing with 5–10% DMSO[5–10]. During cryopreservation, the change in temperature and osmolarity disturbs the membrane integrity and produces free oxygen radicals, which contribute to cell damage. Thus, an optimal protocol for the cryopreservation of stem cells that could overcome freezing-induced damage needs to be developed.

*Author for correspondence

Currently, the addition of certain antioxidants to the conventional freezing medium are found to improve post-thaw recovery of stem cells in human and mice[11–15], but no such published data is available in bovines. Moreover, previous work done in our laboratory resulted in 74.8% cell viability with 7% DMSO in combination with 3% glycerol using the slow freezing protocol for cryopreservation of buffalo bone marrow-derived MSCs[10] and still, there is a possibility to improve the cell recovery by adding certain antioxidants. In the present study, an effort has been made to evaluate the effect of the addition of bio-antioxidants curcumin (CUR) and resveratrol (RES) alone and in combination on oxidative stress in buffalo bone marrow-derived MSCs during the freeze-thaw cycle.

2. Material and Methods

2.1 Chemicals

Bio-antioxidants, Curcumin (CUR) and Resveratrol (RES) used in the present study were procured from Merck, Germany and Molekula Limited, UK, respectively. The chemicals and media used for the MSC culture were procured from the Sigma Aldrich, USA. Other chemicals of analytical grade, kits and enzymes used in the present study were obtained from established firms such as Bangalore-Genei, Himedia, Merck, Span Diagnostics Pvt. Ltd., Transasia Biomedicals Ltd., and Coral Clinical Systems.

2.2 Collection, Transportation, Isolation, Cul turing and Subculturing of Mesenchymal Stem Cells

The bone marrow samples were aspirated from iliac crest of the pelvis bone from buffalo calves of either sex aging between 6 months to 1 year old immediately after slaughtering of the animals under sterilized conditions from Dera Bassi slaughter house located in Chandigarh. After the collection, bone marrow samples were properly mixed with the anticoagulant to avoid coagulation and transported to the culture laboratory with minimum possible time at ambient temperature for further processing. The mononuclear stem cells were isolated from bone marrow samples with a density gradient method using histopaque-1077. Samples were loaded carefully on histopaque-1077 (5:3 ratio) in15 ml centrifuge tubes and centrifuged at 2500 rpm for 25 min at room temperature. The buffy coat was removed and washed twice with PBS at 800 rpm for 10 min to get the cell pellet. The cell pellet was then resuspended in red blood cell lysis buffer for 2 min. Cells were cleared of cell lysis buffer by washing with PBS twice followed by resuspension in stock Dulbecco's modified Eagle's medium (DMEM) and final washing at 800 rpm for 10 min. Finally, the isolated pellet cells were cultured in 25cm^2 culture flask as primary culture in high-glucose DMEM with L-glutamine, sodium pyruvate and sodium bicarbonate and supplemented with 15% FBS and gentamycin solution. The incubation of cells was done in a CO_2 incubator at 37°C in a humidified atmosphere with 5% CO_2. Subculturing was done when the cells reached to 70-80% confluency by trypsinization.

2.3 Cell Viability Assay (Trypan Blue Dye Assay)

Cell viability was determined after every passage by the trypan blue dye exclusion method. To perform the test, 10 µl of the cell suspension was added with an equal volume of 0.1% Trypan blue (1:1 ratio, dilution factor 2) and mixed well. The stained (Dead) and unstained (viable cells) cells were counted in the four corner primary squares of hemocytometer and viable cell number was calculated.

$$\text{Number of viable cells per ml} = \text{Total no. of cells}/4 \times \text{Dilution factor} \times 10^4$$

2.4 Characterization of Mesenchymal Stem Cells

2.4.1 Alkaline Phosphatase Staining (AP Staining)

Third passaged subcultured MSCs after reaching 70-80% confluency were subjected to alkaline phosphatase (AP) staining for characterization. After proper washing in PBS followed by fixation in 4% paraformaldehyde for 10 min, MSCs were incubated with 25 Mm Tris-HCl and 150 mM NaCl containing 8 mM $MgCl_2$, Naphthol AS-MX Phosphate (0.4 mg/ml) and FastRed TR salt (1 mg/ml) for 1 hr at 37°C as per the method of Gade and co-workers[16]. AP stain was removed after incubation and stained MSCs were then subjected to nuclear staining with Hoechst 33258 at a concentration of 1µg/ml for 5 min to make the nucleus visible. Dual stained cells were observed under a fluorescent microscope with the filters of respective wavelength.

2.4.2 Immunostaining

Immunostaining of CD73 and Oct 4 was performed in 3rd passaged cells as per the method described by Gade and co-workers[16] with minor modifications. Briefly, buffalo MSCs were washed thoroughly in PBS followed by fixation in 4% formalin solution for 2 min. Antigen retrieval was done by incubating the cells with 0.01 M Sodium citrate prepared in PBS for 20 min at 37°C. Non-specific bindings were blocked by incubating the cells with donkey serum (1:10 dilution in PBS) for 30 min. MSCs were then incubated with primary antibodies (1:50 dilution in PBS) for 1 hr at 37°C followed by incubation with secondary antibodies (1:200 dilution in PBS) conjugated with FITC/TR for 1 hr. Every step was followed by proper washing with PBS. The stained MSCs were observed under a fluorescent microscope with the filters of respective wavelengths.

2.5 Dose Standardization

Seven working concentrations of CUR and RES were prepared based upon previous literature concerned[17,18] in a

complete growth medium ranging from 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µM for CUR and 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.156 µM for RES. MSCs were exposed to these concentrations for the period of 48 hrs. Control cells were exposed to DMSO (1% for CUR and 0.2% for RES) which served as vehicle control, while, cells without any treatment served as normal control. After completion of the exposure period, cell proliferation assay was performed using Cell Counting Kit-8 (CCK-8 Kit) as per the manufacturer's instructions. Doses selected for CUR and RES were 3.125 µM and 0.156 µM, respectively, based upon the highest proliferation by comparing the final OD of different working concentrations of CUR (1.8278 vs 0.8336) and RES (1.7811 vs 0.8336) with the initial OD.

2.6 Cryopreservation of Mesenchymal Stem Cells

The healthy and growing cells in their exponential growth phase were cryopreserved for further study. Characterized MSCs were divided into four groups for cryopreservation. MSCs were cryopreserved with previous protocol followed in the laboratory[10] in group I which served as control. Group II MSCs were cryopreserved with CUR @ 3.125 µM, group III MSCs with RES @ 0.156 µM and group IV MSCs with CUR and RES in combination (3.125 µM+0.156 µM) in a standardized cryopreservative medium. All these media were prepared and stored at 4°C until use. Approx. 1 million viable cells were cryopreserved per ml of cryopreservative medium for one month using slow freezing protocol previously followed in the laboratory10. For the thawing of the cells, the frozen cryovials were removed from liquid nitrogen and placed immediately in a water bath maintained at 37°C. The cryovials were thawed rapidly within 1-2 min and the contents were then transferred to 15 ml centrifuge tubes. Cryopreservative medium was removed by centrifugation at 800 rpm for 10 min and stored in separate vials at -20°C for further enzymatic analysis. The cell pellet was suspended in PBS for washing at 800 rpm for 10 min followed by resuspending the cell pellet again in PBS. The viable cell number was determined by the trypan blue exclusion test as described earlier in section 2.3.

2.7 Preparation of Cell Lysate

After the determination of cell viability, PBS was removed to prepare cell lysate to estimate oxidative stress indicators and antioxidant enzymes using cell lysis buffer. Cell lysis buffer comprised of 30 ml of 150 mM NaCl, 10 ml of 5 mM of EDTA, 50 ml of 50 mM of Tris-HCl, 10 ml of 0.1% Triton-X100, 10 ml of 0.1% SDS and 10 ml of glycerol 0.1% and final volume was made to 1 litre with distilled water and pH was adjusted to 8.0 using 0.1N NaOH and 0.1N HCl solutions. 400 µl of ice-chilled cell lysis buffer was added to the cell pellet and placed on ice pack for 10 min. Gentle pipetting was done and then cell lysate was collected and immediately preserved at -20oC until analysis.

2.8 Assessment of Oxidative Stress Parameters

Lipid peroxidation in cell lysate was performed by the method of Stocks and Dormandy[19]. Malondialdehyde (MDA), an end-product of lipid peroxidation reacts with thiobarbituric acid (TBA) yields a pink-coloured trimethine complex exhibiting maximum absorption at 532 nm. O2- generation was estimated in terms of reduced nitroblue-tetrazolium (NBT) which form formazan as an index of superoxide anion generation as per the procedure described by Choi and co-workers[20]. 2,7-dichlorofluorescin diacetate (DCFHDA) assay was used to estimate total ROS (hydroxyl, peroxyl and other ROS) activity within the cells as per the method of Aranda and and co-workers[21] in suspension culture. ROS activity was measured in terms of percentage ROS positive cells. Total protein was determined by the method of Lowry and co-workers[22] using bovine serum albumin (BSA) as a standard.

2.9 Assessment of Cytotoxicity Markers

Cellular enzymes used as cytotoxicity markers such as lactate dehydrogenase (LDH), alkaline phosphatase (ALP), creatine kinase (CK-MB) and γ-glutamyl transferase (GGT) were measured in cryopreservative media collected after a freeze-thaw cycle of one month with the commercial kits as per instructions of manufacturers.

2.10 Assessment of Antioxidant Status

Antioxidant enzymes and non- enzyme were measured in cell lysates prepared from MSCs. SOD activity was estimated as per the method described by Madesh and Balsubramaniam[23]. It involves the generation of superoxide by pyrogallol auto-oxidation and inhibition of superoxide dependent reduction of tetrazolium dye MTT (3-(4-5 dimethyl thiozol 2-yl) 2,5- diphenyltetrazolium bromide) to its formazan which was measured at 570 nm. GST activity was estimated using the method of Habig and co-workers[24]. GST was determined spectrophotometrically as an increase in absorbance corresponding to an increase in conjugate formation between reduced glutathione (GSH) and 1-Chloro-2-4-dinitrobenzene (CDNB) at λ max 340 nm. GPx activity in cell lysate was measured by the method of Hafeman and co-workers[25]. The assay is based on the principle that GPx catalyzes the reaction between H2O2 and reduced glutathione (GSH) to form oxidized glutathione (GSSG) and water. The rate of oxidation of GSH by H2O2 is used as a measure of GPx activity. CAT activity was determined by the spectrophotometric method as described by Aebi[26] in the cell lysate. GSH was measured by the method of Beutler and Kelly[27]. This method depends on the reaction of reduced glutathione with 5,5-dithiobis-2-nitrobenoic acid which produces yellow-colored compound and is measured at a wave length of 412 nm against a blank reagent with a spectrophotometer.

2.11 Statistical Analysis

The data was subjected to statistical analysis using IBM SPSS Statistics software and Microsoft Excel. All the data were compared by employing One-Way Analysis of Variance (ANOVA) with Tukey's multiple comparisons as post hoc test. All the data were expressed as Mean ± SEM. A p value of <0.05 was considered as statistically significant.

3. Results

3.1 Establishment of MSCs Culture and Cell Morphology

Mononuclear cells were successfully isolated and cultured in 25cm^2 cell culture flasks and grew based on their plastic adherence property. In primary culture, MSCs showed round and non-fibroblastic morphology on day 0 after isolation (Figure 1 A). MSCs attached to cell culture flask after 72-96 hrs of seeding, consisting of round and spindle-shaped cells (Figure 1 B). Adherent cells further grew as fibroblast-like cells and proliferated slowly up to day 15 (Figure 1 C, D) after the initial seeding and took approx. one month to reach 80-90% confluency (Figure 1 E). The cells were sub-cultured after 80–90% confluency and were considered as 1st passaged cells. Subsequently, MSCs were sub-cultured to get a more homogenous and uniform population (Figure 1 F).

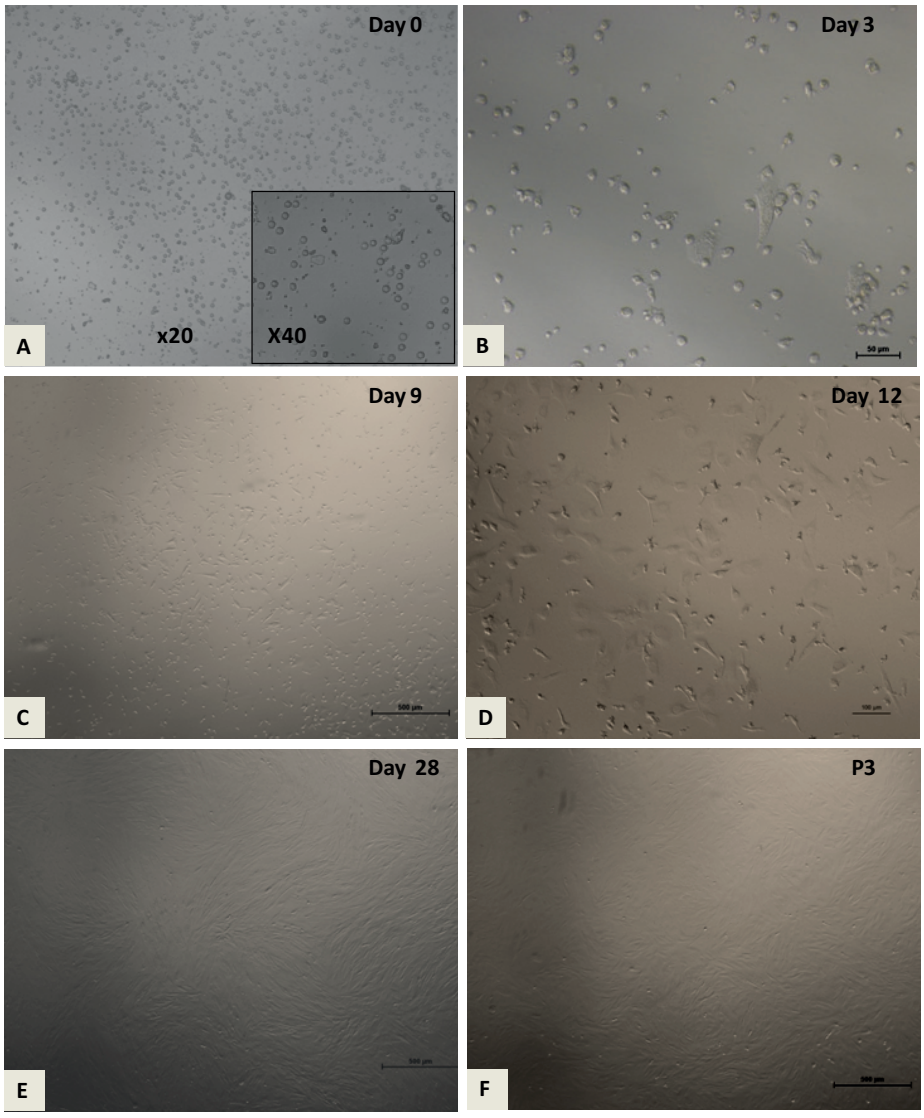


Figure 1. Development of buffalo bone marrow derived mesenchymal stem cells at different time. **A**. day 0; **B**. day3; **C**. day 9; **D**. day 12; **E**. day 28 (confluent monolayer); **F**. confluent monolayer at passage 3. (Original magnification: C, E and F: X4; D: X10, A: X20, Inset A and B: X40).

3.2 Characterization of MSCs

MSCs positive for alkaline phosphatase appeared red (Figure 2 A and B) after staining under a fluorescence microscope. Nuclear staining done with Hoechst is reflected as blue fluorescence. MSC-specific positive markers CD73 and OCT4 were used to characterize the cells by immunostaining. The bone marrow-derived MSCs showed positive expression as revealed by green fluorescence for CD73 (Figure 2 C and D) and red fluorescence for OCT4 (Figure 2 E and F).

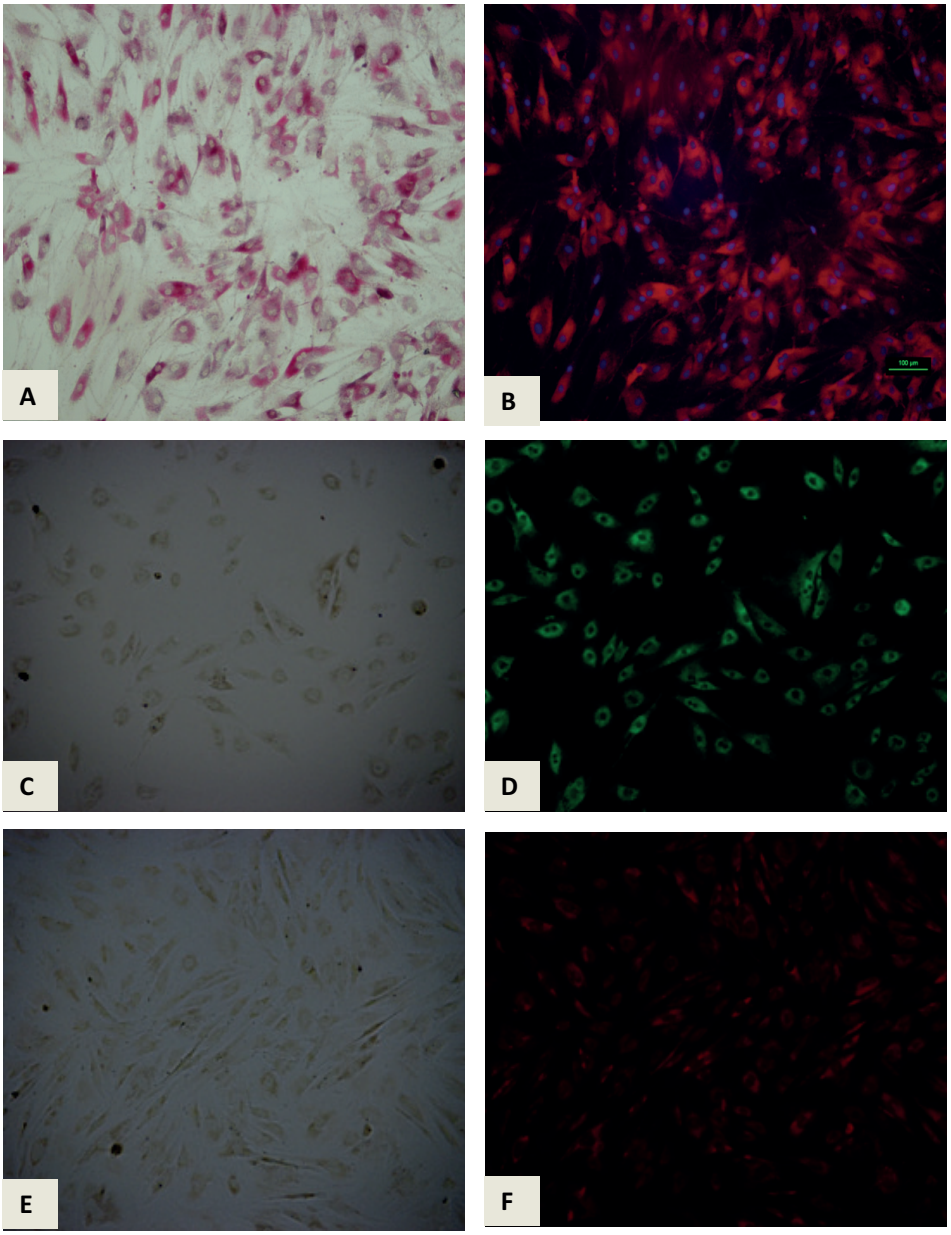


Figure 2. Representative photographs showing **(A and B)** Alkaline phoshphatase staining (nuclear staining was done with Hoechst reflected as blue fluorescence); **(C and D)** localization of CD73 with Fluorescein Isothiocyanate (FITC) conjugated secondary antibody; **(E and F)** localization of OCT4 with Texas Red (TR) conjugated secondary antibody in buffalo MSCs under bright **(A, C and E)** and fluorescent fields **(B, D and F)** of phase contrast inverted microscope (Original magnification : A-F: X10).

3.3 Effect on Oxidative Stress Indicators

A significant decrease ($p \leq 0.05$) in LPO was noticed in all treated groups when compared with the control group (Figure 3A). However, LPO values varied non-significantly among treatment groups. Total protein content (TPC) in MSCs exposed to CUR and RES alone and in combination showed non-significantly ($p > 0.05$) lower TPC impairment

as compared to the control group (Figure 3B), while, O^2- radical production significantly decreased ($p \leq 0.05$) when MSCs exposed to CUR and RES alone and in combination than the control group (Figure 3C). Among the treated groups, significantly ($p \leq 0.05$) lowest O^2- radical production was noticed in group IV than group II and III, whereas, O2- radical production varied non-significantly ($p > 0.05$) between group II and III.

Total ROS activity was measured in terms of dichlorofluorescin (DCF) fluorescence intensity with a fluorescent microplate reader corresponding to the percentage ROS positive cells. A significant decrease ($p \leq 0.05$) in ROS positive cells was noticed with the treatment of CUR and RES alone and in combination as compared to the control group, while, no significant difference ($p > 0.05$) was found among all the treated groups (Figure 3D).

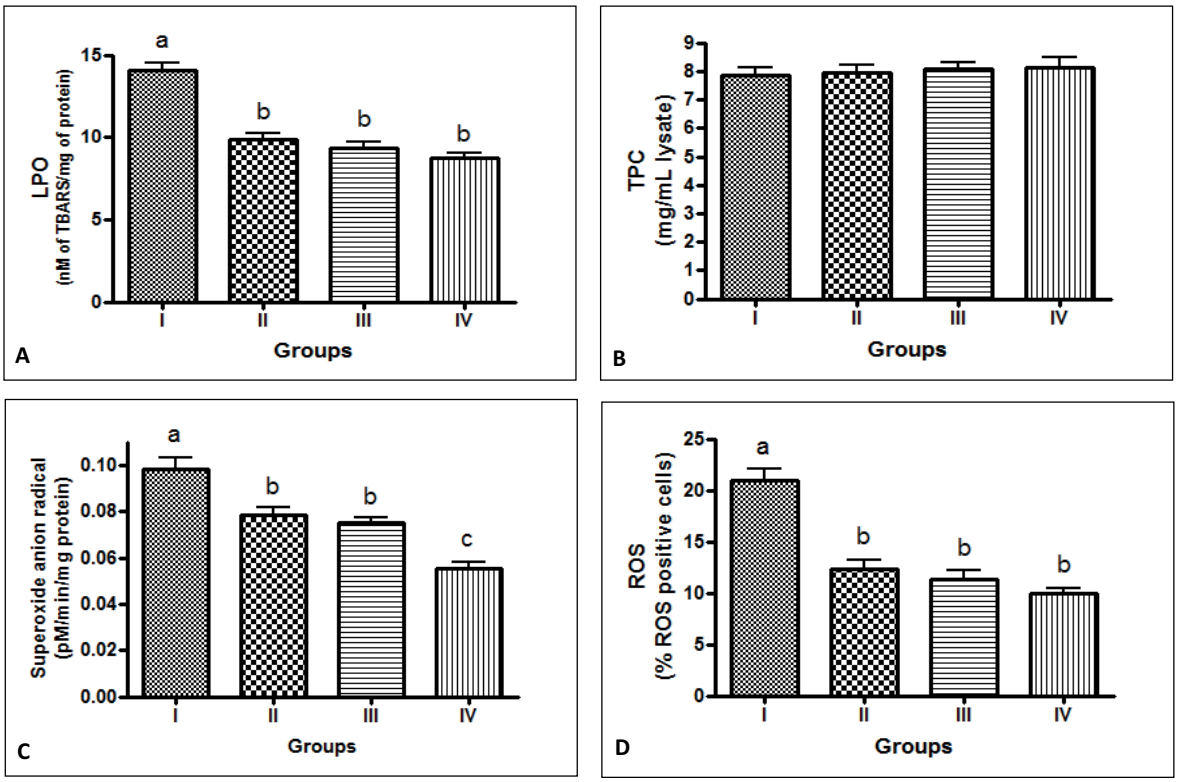


Figure 3. Effect of CUR and RES alone or in combination on oxidative stress markers after freeze-thaw cycle of one month on MSCs. Bar diagram showing mean values and SEM. Different superscripts represent significant difference ($p \leq 0.05$) among groups in One-way Analysis of Variance with Tukey's Multiple Comparison Test.

3.4 Effect on Cytotoxicity Markers

LDH and ALP activity was significantly lower ($p \leq 0.05$) in treated groups than the control group but, varied non-significantly among treated groups (Figure 4A, 4B). There was a significant decrease ($p \leq 0.05$) in the activity of CK-MB in bio-antioxidants treated groups either alone or combined when compared to the control group. Among the treated groups, CK-MB level was significantly ($p \leq 0.05$) low in the combined group than CUR treated group, whereas RES treated group showed non-significant ($p > 0.05$) changes with the other treated groups (Figure 4C). However, GGT activity was varied non-significantly ($p > 0.05$) among all the groups (Figure 4D).

3.5 Effect on Antioxidant Status

A significant increase ($p \leq 0.05$) in SOD and GPx activity was observed with the addition of CUR and RES alone and in combination than the control group (Figure 5A, 5B). GSH activity increased significantly ($p \leq 0.05$) in RES treated and combined group in comparison to the control group (Figure 5C), while the activity of CAT showed a significant increase ($p \leq 0.05$) in CUR treated and combined group (Figure 5D). No significant difference ($p > 0.05$) was noticed in GST activity in all the groups with the addition of antioxidants (Figure 5E).

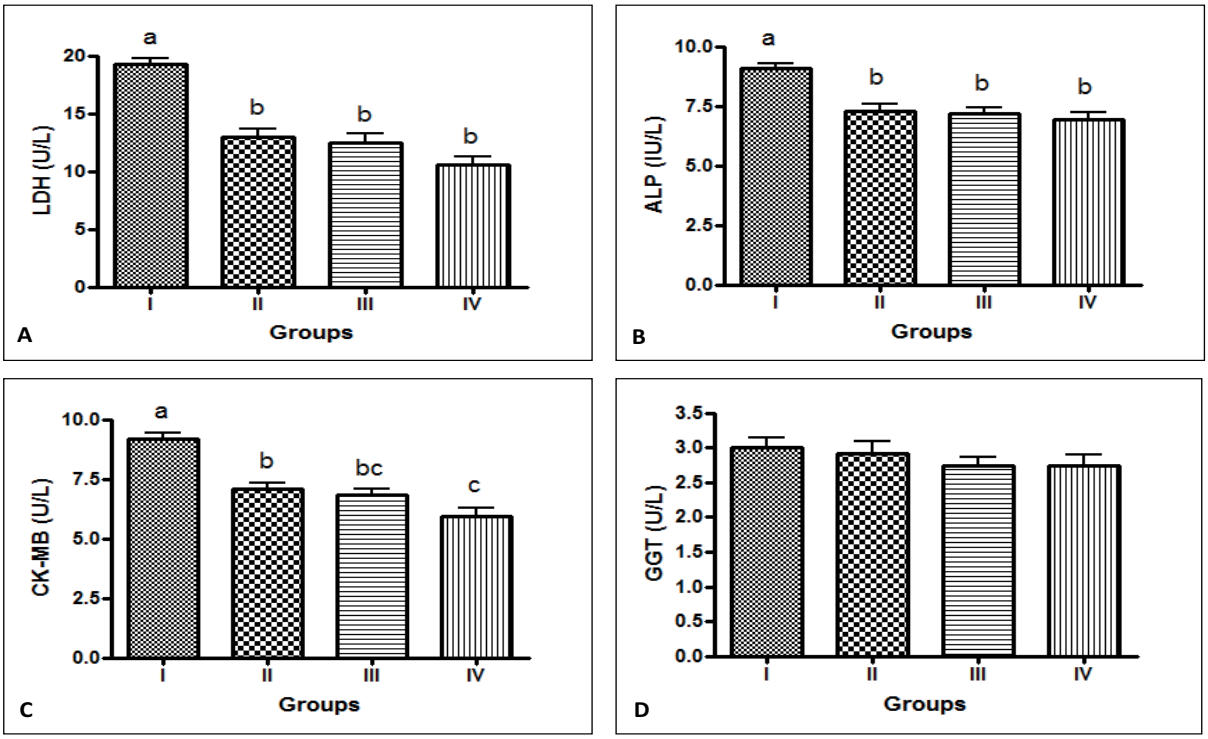


Figure 4. Effect of CUR and RES alone or in combination on cytotoxicity indicators after freeze-thaw cycle of one month on MSCs. Bar diagram showing mean values and SEM. Different superscripts represent significant difference ($p \leq 0.05$) among groups in One-way Analysis of Variance with Tukey's Multiple Comparison Test.

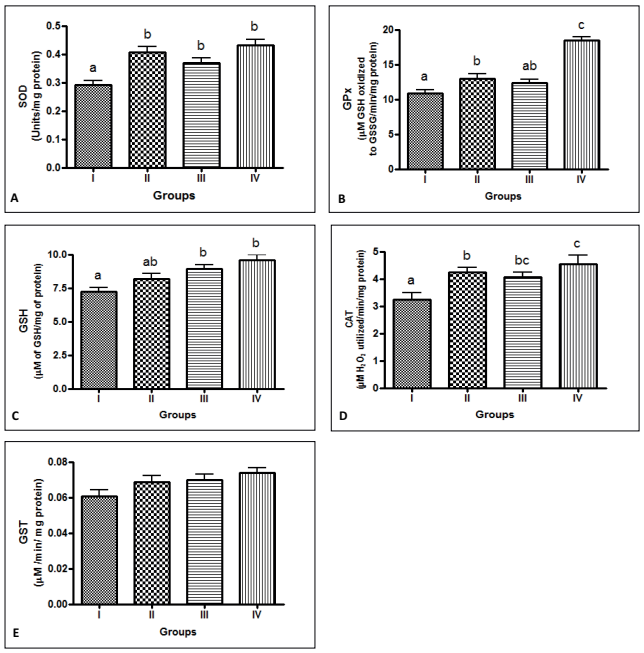


Figure 5. Effect of CUR and RES alone or in combination on antioxidants after freeze-thaw cycle of one month on MSCs. Bar diagram showing mean values and SEM. Different superscripts represent significant difference ($p \leq 0.05$) among groups in One-way Analysis of Variance with Tukey's Multiple Comparison Test.

4. Discussion

Damage due to freeze-thaw cycle to mammalian cells cannot be completely avoided even despite the optimization of cryopreservation protocols but still, endeavors has been made to reduce the freeze-thaw stress through this study with the addition of bio-antioxidants to the cryoprotective medium.

Peroxidation is a process that occurs mainly with polyunsaturated fatty acid residues of cells membrane[28]. Several studies showed that cryopreservation induces lipid peroxidation in sperms[29,30], hepatocytes[31], peripheral blood-derived mononuclear cells[32], and red blood cells[33]. Simultaneously, scientists also proved that the addition of antioxidants can reduce lipid peroxidation in cryopreserved cells[13,34–36]. In our study, CUR and RES either alone or in combination reduced the lipid peroxidation due to their antioxidant ability possibly by scavenging free radicals generated during the freeze-thaw cycle. Thus, prevented the cell membranes from oxidation. Several studies have shown that curcumin[37–42] and resveratrol[35,43,44] significantly decreased the levels of LPO. Curcumin can induce the activation of Nrf2 and induce the expression of antioxidant enzymes that inhibit free radicals from mediating peroxidation of membrane lipids or oxidative DNA damage[45,46].

Protein oxidation was detected in cryopreserved cells due to increased ROS accumulation[33,47–50]. As a result of protein oxidation, the biological activity of many proteins may be modified (increased/decreased) or completely lost. Oxidized proteins form aggregates and may have an inhibitory effect on the functional enzymatic systems even result in cell death[51]. But, the addition of antioxidants overcome the oxidative stress and protect the cellular proteins as reported by many researchers[13,33,34,36,52]. In our study also, CUR and RES alone as well as in combination prevented the proteins from oxidation and reflected as higher TPC in treated groups than the control group.

Superoxide anion radicals are generated biologically as monovalent reduction of molecular O_2 by NADPH oxidases (NOXs: a family of seven-membered enzymes) by catalyzing the transfer of one electron across the membrane from the electron-donating NADPH to the electron acceptor oxygen, thus reducing oxygen to form unstable O^2- radical[53]. Superoxide anion radicals were detected in the cells of various species undergoing cryopreservation and can be reduced with the addition of various antioxidants[13,33,36,54]. Bio-antioxidants, curcumin and resveratrol used in the present study effectively reduced the O2- radical production in MSCs. Our work is supported by the findings of Dudylina and co-workers[55] who investigated the antioxidant effect of quercetin, rutin, caffeic acid, curcumin and resveratrol to scavenge superoxide anion radicals generated by heart mitochondria in wistar rats. Similarly, Li and co-workers[52] reported that curcumin can inhibit oxidative stress and reduce the high expression of NADPH oxidase subunits in islet cells.

ROS are chemically reactive molecules containing oxygen that are needed for physiological activities such as intracellular cell signaling and homeostasis, cell death, immune defense against pathogens, and induction of mitogenic response at low/moderate concentrations[56–59]. An imbalance between the production of ROS and the ability of the antioxidant systems to detoxify these reactive intermediates results in oxidative stress that generates free radicals in excessive amounts cause damage to DNA, proteins and lipids, which can severely compromise cell health[60,61]. Such impairments of cellular functionality have been improved through the administration of antioxidants by scavenging reactive oxygen species. Various researchers worked on different antioxidants such as taurine[62], a combination of catalase with trehalose[63], trehalose[64-66], vitamin C[67], vitamin E[68], astragalosides[69], carboxylated poly-l-lysine[70], edaravone[71], hypotaurine[12], N-acetyl cysteine[9], catalase in combination with α-tocopherol[72], a combination of vitamin E and C[73], baicalein[14], iodixanol[74], and, quercetin[15] to the cell types such as mononuclear cells, pancreatic islets, germ cells, spermatozoa, dendritic cells, hepatocytes, hemopoietic cells and mesenchymal stem cells. In the present study, curcumin and resveratrol had been used to improve freeze-thaw stress-associated impairments and proved effective. Work done by Hirzel and co-workers[75] supported our findings and investigated a decrease in ROS production in human adipocytes when exposed to resveratrol and curcumin. A similar effect was noticed by Dudylina and co-workers[55] using antioxidants quercetin, rutin, caffeic acid, curcumin and resveratrol in the heart mitochondria of wistar rats.

Cellular enzymes like lactate dehydrogenase (LDH), alkaline phosphatase (ALP), creatine kinase (CK-MB) and gamma-glutamyl transferase (GGT) are the key partners and valuable indicators of cell damage under different disease conditions, stress, exposure to chemical agents etc. During cryopreservation, the change in temperature and osmolarity disturbs the membrane integrity and produces free oxygen radicals, which contribute to cell damage. In the present study, exposure of MSCs to cold stress and cryoprotectants during cryopreservation resulted in cell damage leading to leakage of cellular enzymes in cryopreservation medium and measured using commercial kits. Levels of LDH, ALP, CK-MB and GGT were found to be higher in the control group indicative of cell damage during cryopreservation which released these enzymes into the cryopreservative medium. But, the addition of bio-antioxidants either alone or in combination protected the cell from damage, thereby, decreasing the level of these enzymes in treated groups in the present study. Studies have shown that the level of cellular enzymes such as lactate dehydrogenase, adenosine triphosphate, caspase-3/7, aspartate aminotransferase, glutamate dehydrogenase, alanine aminotransferase, alkaline phosphatase, alpha-glutathione-S-transferase, creatine kinase and glucose-6-phosphate dehydrogenase increased due to cell injury under *in vitro*[76,77] and *in vivo*[78,79] conditions. The levels of these enzymes are direct indicators of the functional efficiency of the liver and hepatoxicity as studied by many workers[80–83]. Medicinal plants having antioxidant activity play a protective role to regain physiological changes due to hepatotoxicity[84]. Similar to our findings, curcumin supplementation resulted in a significant reduction in CK level in muscle damage in the rat model[85] and humans[86]. Gelen and co-workers[82] determined the protective effect of rutin and quercetin on 5-FU-induced cellular damage in the liver of Wistar rats and found increased levels of AST, ALT, LDH and ALP after supplementation.

Both enzymatic and non-enzymatic antioxidant systems are essential for cellular response against oxidative stress. The antioxidant molecules constitute the antioxidant defense grid in living system either by preventing free radical pro-

duction (first line of defense) or radical scavenging (second line of defense) or radical-induced damage repair (third line of defense). The antioxidant enzymes SOD, CAT and GPx can be categorized as first-line defense that suppress the formation of free radicals in cells by neutralizing any molecule with the potential of developing into a free radical or any free radical with the ability to induce the production of other radicals[87]. Transcriptomic studies using yeast DNA microarray have also shown that antioxidant genes such as SOD1, cytosolic catalase T (CTT1) and glutaredoxin-1 (GRX1) respond to freeze-thaw stress after cryopreservation[88]. Further, it was studied that all the antioxidant enzymes did not contribute equally in their ability to protect cells from freeze-thaw stress[89]. In this context, Park and co-workers already proved that yeast strains deleted for SOD1 and SOD2 were particularly sensitive to freeze-thaw stress, while single deletion of catalase and glutathione peroxidase were not as sensitive[90].

The levels of antioxidant enzymes were found to increase in the present investigation might be due to the prevention of production or scavenging of free radicals generated with the addition of bio-antioxidants, thereby, normalize the endogenous antioxidant balance of cells. The findings of our study can be supported with the findings of Iqbal and co-workers[91], who showed that dietary supplementation of curcumin enhances antioxidant and phase II metabolizing enzymes in the liver and kidney of mice. Similarly, researchers reported upregulation of endogenous antioxidants and phase II enzymes with the addition of resveratrol in cultured aortic smooth muscle cells resulted in cytoprotection against oxidative stress[92]. Eddine and co-workers evaluated the combined effect of quercetin and resveratrol and observed improved brain antioxidant status in male wistar rats[93]. Many other workers also demonstrated an elevation in levels of GSH, GPx, SOD and CAT with a decline in the level of LPO after being exposed to curcumin and resveratrol[94–98].

Change in oxidative stress, level of cytotoxicity enzymes and antioxidant status was noticed with exposure of mesenchymal stem cells to CUR and RES alone or in combination during cryopreservation in the present investigation which led to the conclusion that the addition of bio-antioxidants in cryopreservation medium suppressed the oxidative stress induced during the freeze-thaw cycle.

5. Acknowledgement

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