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N-ACETYLCYSTEINE PREVENTS CORD DERIVED STEM CELLS FROM H_2O_2 INDUCED INJURY IN VITRO

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ABSTRACT

Stem cell therapy is a promising approach for repair of tissue injury. However, the efficiency of transplanted stem cells is greatly hampered by the injury conditions in the body. The purpose of present study was to investigate the potential role of an antioxidant N-acetylcysteine (NAC) in in-vitro injury conditions on Wharton's Jelly (WJ) derived Mesenchymal stem cells (WJ-MSCs). Injury was inflicted in WJ-MSCs by treating with 100 µM H_2O_2 and 1 mM NAC was employed to WJ-MSCs during this injury condition. Cell viability, death and proliferation were assessed by trypan blue exclusion assay, MTT assay and LDH release respectively. Oxidative stress was evaluated by levels of glutathione (GSH), malodialdehyde (MDA) as well as by activity of superoxide dismutase (SOD) and catalase (CAT). 100µM/L H_2O_2 inflicted injury to WJ-MSCs while 1 mM NAC reduced this injury significantly. Results demonstrated that NAC increased cell viability and proliferation, and decreased cell death. NAC also enhanced antioxidant capacity of WJ-MSCs against H_2O_2 induced injury. NAC could be a promising therapeutic approach for improving survival of WJ-MSCs under H_2O_2 induced injury.

KEYWORDS: N-acetylcysteine, Wharton's Jelly derived Mesenchymal stem cells, Oxidative stress.

INTRODUCTION

Stem cell based therapies hold promising role in multiple diseases including cardiovascular diseases, neurological disorders and diabetes.[1-3] WJ-MSCs are attractive candidates for

cellular therapies [4] due to their easy availability, non-invasive procedure of isolation, no ethical concerns and multipotent differentiation potential.[5, 6]

However, one of the challenges faced by stem cells is low survival rate after transplantation in the injured host environment.[7, 8] Therefore, strategies are required to improve stem cells tolerance in injured environment for achieving favorable benefits of stem cell therapy. Different strategies like preconditioning with hypoxia,[9] growth factors[10] and antioxidants[11] enhance stem cells survival in stress conditions. It is documented that N-acetylcysteine (NAC) is one of the most used antioxidant in vivo as well as in vitro.[12-15] It is known as glutathione precursor and prevents injury or oxidative damage by scavenging the free radical species.[16] Therefore, the present study is designed to evaluate the effect of NAC in H_2O_2 induced stress environment to augment the proliferation and survival of WJ-MSCs.

MATERIAL AND METHOD

Procurement of human umbilical cord

Umbilical cords were obtained after full term birth (cesarean section) with the informed consent of the parents using the guidelines approved by the Biosafety Board at The University of Lahore, Pakistan. Donor tested for Hepatitis B and C virus (HBV and HCV) and only HBV and HCV negative donors were selected. Umbilical cord tissue was stored in sterile normal saline (0.9% w/v sodium chloride), until processing.

Isolation and culturing of WJ-MSCs

Isolation of MSCs from WJ was done by enzymatic dissociation with collagenase (Sigma Aldrich, USA) as previously reported.[6] Briefly, the cord sections were incubated in 3 mg/mL collagenase solution (Sigma Aldrich, USA). After 3 hours of incubation, Dulbecco's modified eagle medium low glucose (DMEM LG; Sigma Aldrich) with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NJ) and 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Grand Island, N.J., USA) was added. The medium was renewed after every 3 days. Cells from passages 3 to 4 were used for all experiments.

Experimental design

WJ-MSCs were randomly divided into four experimental groups: (I) control group; (II) 100 µM/L H_2O_2 treated group; (III) 100 µM/L H_2O_2 and 0.1 mM N acetyl cysteine (NAC) group and (IV) 100 µM/L H_2O_2 and 1 mM N acetyl cysteine (NAC) group. All the experiments were performed for 24 and 48 hours.

Cell Viability

The viability of NAC was evaluated by trypan blue exclusion method. Cells were trypsinized after treatment and mixed in equal volume with Trypan blue (Sigma Aldrich) and counted by using a hemocytometer. Cell viability was calculated by using the formula, Viable cells (%) = viable cells/ total number of viable & dead cells×100.

MTT assay

WJ-MSCs of all treatment groups were washed with phosphate buffer saline (PBS) and incubated with 60 µL MTT (0.5 mg/mL, Invitrogen Inc) in 500 µL complete medium for 2 hours at 37°C in 5% CO_2 incubator. Then 150 µL dimethylsulfoxide (DMSO) was added to dissolve the formazen and absorbance was taken at 570 nm.

Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) release was determined by LDH Assay kit (AMP Diagnostics, Austria) according to instructions of the manufacturer. Briefly, 5 µL of the medium from all treatments groups was mixed with 95 µL of working reagent and incubated for 5 minutes. Absorbance values were measured by using Spectra max PLUS 384 (Molecular Devices) at 340 nm.

Determination of superoxide dismutase (SOD) activity

SOD activity was determined by the method of Kakkar et al.[17] Briefly, 100 µL of medium from each treatment group was taken and mixed with 1.2 mL sodium pyrophosphate buffer (52 mM, pH 8.3), 186 µM phenozine methosulphate (PMS), 300 µM nitroblue tetrazolium (NBT). Reaction was started with the addition of 750 µM nicotinamide adenine dinucleotide (NADH) in a total volume of 6.0 mL reaction mixture. The reaction mixture was then incubated at 30°C for 90 seconds. Reaction was stopped by adding 0.1 mL of glacial acetic acid and the content was stirred vigorously with 4.0 mL of n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and butanol layer was separated. The color intensity of chromogen in butanol layer was measured at 560 nm against n-butanol using a spectrophotometer.

Estimation of glutathione (GSH)

Reduced glutathione was assayed according to the method of Beutler et al.[18] Medium from each treatment group was collected and mixed with 2.0 mL of 0.3 M disodium hydrogen phosphate. Then 0.25 mL of 0.001 M freshly prepared DTNB [5, 5`-dithiobis (2-nitrobenzoic

acid) dissolved in 1% w/v sodium citrate] was added. Reduction of DTNB with glutathione (GSH) produces a yellow compound, whose absorbance was noted spectrophotometrically at 412 nm. The reduced chromogen is directly proportional to GSH concentration.

Estimation of catalase (CAT) activity

Activity of CAT was monitored by using method described by Sinha[19] 0.1 mL culture medium was taken and mixed with 1.0 mL phosphate buffer (10 mM, pH 7.0) and 0.4 mL H_2O_2 (0.2 M). Reaction was stopped by adding 2.0 mL dichromate acetic acid reagent. Samples were incubated for 10 minutes in a boiling water bath, cooled and absorbance was measured at 530 nm.

Estimation of malondialdehyde (MDA) level

The level of MDA, a free radical species was evaluated by measuring thiobarbituric acid reactive substances via method of Ohkawa et al.[20] For this, 0.2 mL cell culture medium was added to 0.2 mL SDS (8.1%), 1.5 mL TBA (0.8%), 1.5 mL acetic Acid (20%, pH 3.5) and volume was made up to 4.0 mL with distilled water and incubated at 90˚C for 60 minutes. After cooling, 1.0 mL distilled water, 5.0 mL n-butanol-pyridine mixture (15:1) was added and the mixture was shaken vigorously and centrifuged at 4000 rpm for 10 minutes. The upper n-butanol layer was taken and its absorbance was taken at 532 nm.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). Comparison between groups is done with Bonferroni post hoc test by using 1-way ANOVA or 2-way ANOVA. All data were presented as mean ± standard error of the mean (SEM). A level of $p<0.05$ was considered statistically significant.

RESULTS

The cytoprotective effects of NAC on WJ-MSCs under H_2O_2 injury conditions

Fig. 1 shows the cytoprotective effect of varying concentration (0.1 and 1 mM) of NAC on WJ-MSCs after 24 and 48 hours of incubation. Increased cell viability was observed in 1 mM NAC group NAC at 24 and 48 hours (62% ± 6.05%; 67% ± 4.6%) compared with 100 µM/L H_2O_2 treated group (35% ± 7.1%; 27% ± 4.04%) demonstrated by the trypan-blue exclusion assay Fig. 1A. The cell viability was found to be reduced after 48 hours exposure of H_2O_2. The viability of WJ-MSCs increased after incubation with 1 mM NAC concentration.

Cell proliferation was assessed after NAC treatment. 1mM NAC treatment resulted in significantly higher proliferation at 24 and 48 hours (0.169 ± 0.002; 0.195 ± 0.000) followed by with 100 µM/L H_2O_2 treated group (0.105 ± 0.002; 0.103 ± 0.002) Fig. 1B.

It is evident from Fig.1C that the activity of LDH was significantly elevated after 24 and 48 hours in the 100 µM/L H_2O_2 treated group (0.059 ± 0.00; 0.066 ± 0.002), when compared to 1 mM NAC group (0.045 ± 0.002; 0.066 ± 0.002) and control group (0.037 ± 0.003; 0.033 ± 0.005). The activity of LDH was significantly lower (P<0.01) after 48 hours incubation than 24 hours incubation.

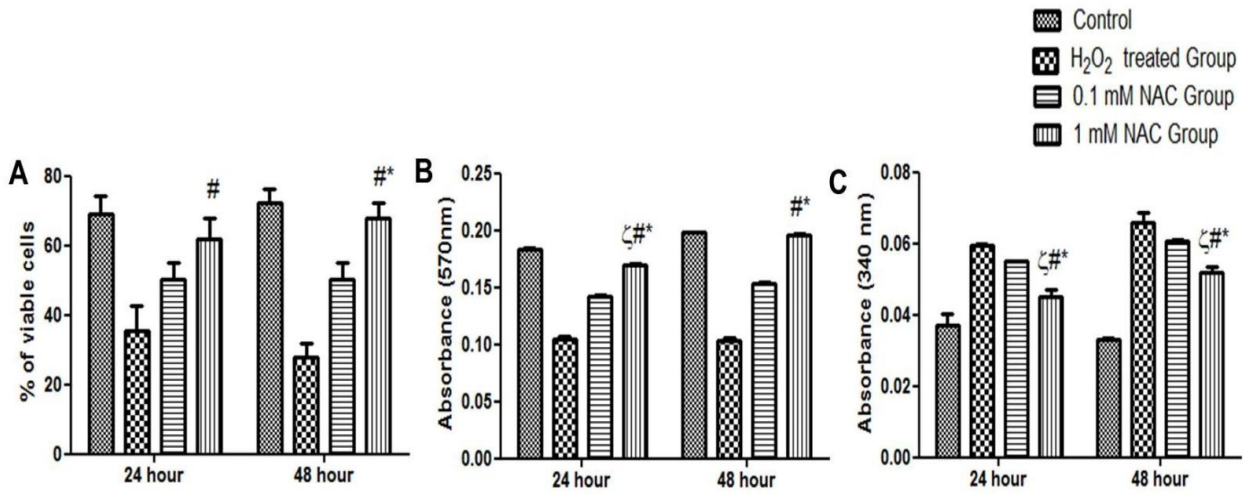

Fig. 1. Cytoprotective effect of NAC treatment on WJMSC. (A) Cell viability assay. (B) Cell proliferation assay. (C) LDH assay. All values were expressed as mean±S.E.M. $^{\zeta}$ p<0.05 for 1 mM NAC group versus Control group; $^{\#}$ p<0.05 for 1 mM NAC group versus H_2O_2 treated group; * p<0.05 for 1 mM NAC group versus 0.1 mM NAC group.

Effects of NAC on WJ-MSCs under H_2O_2 induced oxidative stress

Oxidative stress induced by H_2O_2 in WJMSCs was ameliorated by NAC treatment in a dose-dependent manner. We observed the effects of NAC on antioxidant enzymes i.e. SOD, CAT, GSH and a free radical species, MDA). There was an increase in SOD activity (0.0593 ± 0.001; 0.070 ± 0.000), CAT activity (0.46 ± 0.02; 0.36 ± 0.019), GSH level (0.169 ± 0.002; 0.360 ± 0.01) and decrease in MDA level (0.05 ± 0.000; 0.046 ± 0.008) in 1 mM NAC group compared to 100 µM/L H_2O_2 treated group for SOD (0.051 ± 0.002; 0.055 ± 0.004), CAT (0.81 ± 0.04; 1.02 ± 0.03), GSH (0.105 ± 0.002; 0.102 ± 0.03) and MDA (0.067 ± 0.000; 0.077 ± 0.004) after 24 and 48 hours suggesting significant antioxidant effect of NAC Fig. 2.

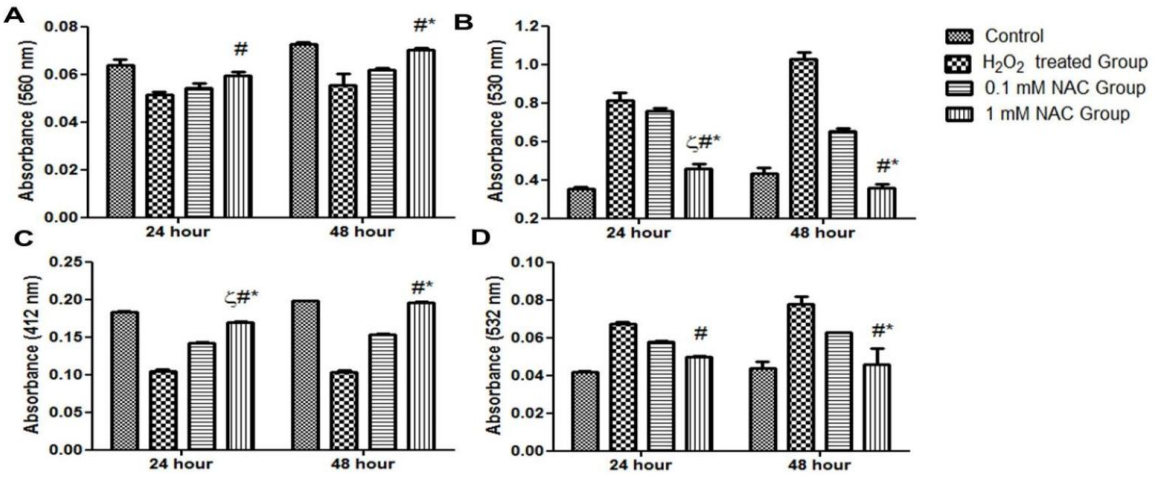

Fig. 2. Effect of NAC treatment on H_2O_2 induced oxidative stress (A) Superoxide dismutase (SOD) activity (B) Catalase (CAT) activity (C) Reduced glutathione (GSH) level (D) Malondialdehyde (MDA) level. All values were expressed as mean±S.E.M. $^{\zeta}$ p<0.05 for 1 mM NAC group versus Control group; $^{\#}$ p<0.05 for 1 mM NAC group versus H_2O_2 treated group; * p<0.05 for 1 mM NAC group versus 0.1 mM NAC group.

DISCUSSION

MSCs derived from umbilical cord (UC) are point of focus for scientists[21-23] due to their non-invasive procedure of isolation, multipotency and most preferably rapid availability.[5] Stem cell based therapies using MSCs have been effectively used for treatment of the damaged organs,[7, 24, 25] however ischemic environment of injured tissue diminished organ repair after stem cells transplantation.[26] The present study demonstrated that supplementation of NAC in H_2O_2 induced injured conditions augments proliferation, survival and reduce oxidative stress in WJ-MSCs.

Oxidative stress is caused by production of reactive oxygen species (ROS), including superoxide (O_2^-), hydroxyl radical (HO^-) and H_2O_2[27] resulting in apoptosis of cells.[28] H_2O_2 is the stable oxidant which causes oxidative injury by modification of proteins, lipids and DNA.[29] Previously, H_2O_2 has been used as a model to study oxidative stress in vitro.[30-32] Therefore, to evaluate the protective effect of NAC on WJMSCs in vitro, we added H_2O_2 in complete medium for short time to mimic the in vitro injury environment. The cytoprotective effect of NAC on WJMSCs was evaluated by calculating viability by trypan blue exclusion assay and by determining survival and cell proliferation. It was observed that cell death was increased in H_2O_2 control compared to cells co-treated with different concentrations of NAC. The increased cell viability and proliferation after supplementation of 1 mM NAC for 48 hours suggested that NAC has a profound effect on the viability and growth of WJMSCs. The current study is in consistent with other reports[6, 33] showing that H_2O_2 treatment reduces the

cell proliferation. Furthermore, NAC reduced the LDH release which indicates the cell membrane integrity and viability.[7] Reduced activity of LDH and improved viability and growth of WJMSCs confirmed the cytoprotective effect of NAC.

Ischemic environment favors the oxidative stress resulting in disturbance between free radicals and antioxidant defense mechanism. One of the important defense enzymes is SOD which catalyzes the dismutation of superoxide radicals[34] whereas CAT catalyzes the reduction of H_2O_2, thus protects from hydroxyl radicals induced injury.[35] Previous study showed that accumulation of superoxide anion and H_2O_2 contributed to unfavorable conditions by decreasing the activity of SOD and CAT.[36] Our results are in accordance with this study as 1 mM NAC group showed significant decrease in the activity of SOD and CAT which is rescued by 1 mM NAC treatment. GSH inhibits lipid peroxidation which is mediated by free radicals. Results shown significant increase in the activity of GSH after 1 mM NAC treatment for 24 and 48 hours suggesting the fact that NAC is precursor of GSH. MDA which is the end products of lipid peroxidation[37] pertained to free radicals mediated injury.[38, 39] Our results showed that H_2O_2 induced oxidative damage increases the MDA level which was reduced significantly after 1mM NAC treatment.

In conclusion, our study demonstrated that the NAC provides an antioxidant effect on H_2O_2 induced injury in WJMSCs in vitro.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman, MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284(5411): -7.
2. Krause DS. Plasticity of marrow-derived stem cells. Gene Ther 2002; 9(11): 754-8.
3. Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells 2007; 25(11): 2739-49.

4. Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DO, Tukun A, Uckan D, Can A. Biology of stem cells in human umbilical cord stroma: in situ and in vitro surveys. Stem Cells 2007; 25(2): 319-31.
5. Mennan C, Wright K, Bhattacharjee A, Balain B, Richardson J, Roberts S. Isolation and characterisation of mesenchymal stem cells from different regions of the human umbilical cord. Biomed Res Int 2013; 2013: 916136.
6. Nagamura-Inoue T and He H. Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility. World J Stem Cells 2014;6(2):195-202.
7. Wajid N, Mehmood A, Khan SN, Riazuddin S. Lovastatin protects chondrocytes derived from Wharton's jelly of human cord against hydrogen-peroxide-induced in vitro injury. Cell Tissue Res 2013; 351(3):433-43.
8. Khan M, Ali F, Mohsin S, Akhtar S, Mehmood A, Choudhery MS. Khan SN, Riazuddin S. Preconditioning diabetic mesenchymal stem cells with myogenic medium increases their ability to repair diabetic heart. Stem Cell Res Ther 2013; 4(3): 58.
9. Rosova I, Dao M, Capoccia B, Link D, Nolta JA. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem Cells 2008; 26(8): 2173-82.
10. Hahn JY, Cho HJ, Kang HJ, Kim TS, Kim, MH, Chung JH, Bae JW, Oh BH, Park YB, Kim HS. Pre-treatment of mesenchymal stem cells with a combination of growth factors enhances gap junction formation, cytoprotective effect on cardiomyocytes, and therapeutic efficacy for myocardial infarction. J Am Coll Cardiol 2008; 51(9): 933-43.
11. Ohshima M, Li TS, Kubo M, Qin SL, Hamano K. Antioxidant therapy attenuates diabetes-related impairment of bone marrow stem cells. Circ J 2009; 73(1): 162- 6.
12. Lan A, Liao X, Mo L, Yang C, Yang Z, Wang X, Hu F, Chen P, Feng J, Zheng D and Xiao L. Hydrogen sulfide protects against chemical hypoxia-induced injury by inhibiting ROS-activated ERK1/2 and p38MAPK signaling pathways in PC12 cells. PLoS One 2011; 6(10): e25921.
13. Lan AP, Xiao LC, Yang ZL, Yang CT, Wang XY, Chen PX, Gu MF, Feng JQ. Interaction between ROS and p38MAPK contributes to chemical hypoxia-induced injuries in PC12 cells. Mol Med Rep 2012; 5(1): 250-5.
14. Smaga I, Pomierny B, Krzyzanowska W, Pomierny-Chamioio L, Miszkiel J, Niedzielska E, Ogorka A, Filip M. N-acetylcysteine possesses antidepressant-like activity through reduction of oxidative stress: behavioral and biochemical analyses in rats. Prog Neuropsychopharmacol Biol Psychiatry 2012; 39(2): 280-7.

15. Xie J, Zhou X, Hu X, Jiang H. H_2O_2 evokes injury of cardiomyocytes through upregulating HMGB1. Hellenic J Cardiol 2014; 55(2): 101-6.
16. Zhang F, Lau SS, Monks TJ. The cytoprotective effect of N-acetyl-L-cysteine against ROS-induced cytotoxicity is independent of its ability to enhance glutathione synthesis. Toxicol Sci. 2011; 120: 87-97.
17. Kakkar P, Das B, Viswanathan PN. A modified spectrophotometeric assay of superoxide dismutase. Toxicol Sci 2011; 120(1): 87-97.
18. Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med 1963; 61: 882-8.
19. Sinha AK. Colorimetric assay of catalase. Anal Biochem 1972; 47(2): 389-94.
20. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95(2): 351-8.
21. Barachini S, Trombi L, Danti S. Morpho-functional characterization of human mesenchymal stem cells from umbilical cord blood for potential uses in regenerative medicine. Stem Cells Dev 2009; 18(2): 293-305.
22. Lee OK, Kuo TK, Chen WM. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 2004; 103(5): 1669-75.
23. Secco M, Zucconi E, Vieira NM. Multipotent stem cells from umbilical cord: cord is richer than blood. Stem Cells 2008; 26(1): 146-50.
24. Masoud MS, Anwar SS, Afzal MZ, Mehmood A, Khan SN, Riazuddin S. Pre-conditioned mesenchymal stem cells ameliorate renal ischemic injury in rats by augmented survival and engraftment. J Transl Med 2012; 10: 243.
25. Nasir GA, Mohsin S, Khan M, Shams S, Ali G, Khan, SN, Riazuddin S. Mesenchymal stem cells and Interleukin-6 attenuate liver fibrosis in mice. J Transl Med 2013; 11: 78.
26. Sun X, Fang B, Zhao X, Zhang G, Ma H. Preconditioning of mesenchymal stem cells by sevoflurane to improve their therapeutic potential. PLoS One 2014; 9(3): e90667.
27. Chen L, Liu L, Huang S. Cadmium activates the mitogen-activated protein kinase (MAPK) pathway via induction of reactive oxygen species and inhibition of protein phosphatases 2A and 5. Free Radic Biol Med 2008; 45(7): 1035-44.
28. Rodrigues M, Turner O, Stolz D, Griffith LG, Wells A. Production of reactive oxygen species by multipotent stromal cells/mesenchymal stem cells upon exposure to Fas ligand. Cell Transplant. 2012; 21(10): 2171-87.
29. Deavall DG, Martin EA, Horner JM, Roberts R. Drug-induced oxidative stress and toxicity. J Toxico, 2012; 2012: 645460.

30. Li J, Wang JJ, Yu Q, Chen K, Mahadev K, Zhang SX. Inhibition of reactive oxygen species by Lovastatin downregulates vascular endothelial growth factor expression and ameliorates blood-retinal barrier breakdown in db/db mice: role of NADPH oxidase 4. Diabetes 2010; 59(6): 1528-38.
31. Li T, Zhang X, Zhao X. Powerful protective effects of gallic acid and tea polyphenols on human hepatocytes injury induced by hydrogen peroxide or carbon tetrachloride in vitro. J Med Plants Res 2010; 4(3): 247-254.
32. Ho, W.P., Chan, W.P., Hsieh, M.S., Chen, R.M. Runx2-mediated bcl-2 gene expression contributes to nitric oxide protection against hydrogen peroxide-induced osteoblast apoptosis. J Cell Biochem 2009; 108(5): 1084-93.
33. Cremers NA, Lundvig DM, van Dalen SC, Schelbergen RF, van Lent, PL, Szarek WA, Regan RF, Carels CE, Wagener FA. Curcumin-Induced Heme Oxygenase-1 Expression Prevents H_2O_2-Induced Cell Death in Wild Type and Heme Oxygenase-2 Knockout Adipose-Derived Mesenchymal Stem Cells. Int J Mol Sci 2014; 15(10): 17974-99.
34. Patel H, Chen J, Das KC, Kavdia M. Hyperglycemia induces differential change in oxidative stress at gene expression and functional levels in HUVEC and HMVEC. Cardiovasc Diabetol 2013; 12: 142.
35. Zhang L, Dong XW, Wang JN, Tang JM, Yang JY, Guo LY, Zheng F, Kong X, Huang YZ, Chen SY. PEP-1-CAT-transduced mesenchymal stem cells acquire an enhanced viability and promote ischemia-induced angiogenesis. PLoS One 2012; 7(12): e52537.
36. Li JL, Wang QY, Luan HY, Kang ZC, Wang CB. Effects of L-carnitine against oxidative stress in human hepatocytes: involvement of peroxisome proliferator-activated receptor alpha. J Biomed Sci 2012; 19: 32.
37. Ramel A, Wagner KH, Elmadfa I. Plasma antioxidants and lipid oxidation after submaximal resistance exercise in men. Br J Sports Med 2004; 38(5): E22.
38. Bandali KS, Belanger MP, Wittnich C. Hyperoxia causes oxygen free radical-mediated membrane injury and alters myocardial function and hemodynamics in the newborn. Am J Physiol Heart Circ Physiol 2004; 287(2): H553-9.
39. Pirinccioglu AG, Gokalp D, Pirinccioglu M, Kizil G, Kizil M. Malondialdehyde (MDA) and protein carbonyl (PCO) levels as biomarkers of oxidative stress in subjects with familial hypercholesterolemia. Clin Biochem 2010; 43(15): 1220-4.