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Original Article

N-Acetyl cysteine protects diabetic mouse derived mesenchymal stem cells from hydrogen-peroxide-induced injury: A novel hypothesis for autologous stem cell transplantation

Fatima Ali [a,b,*], Mohsin Khan [b,c], Shaheen N. Khan [b], Sheikh Riazuddin [a,b,d]

[a] *The University of Lahore, Defence Road Campus, Lahore, Pakistan*
[b] *National Center of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan*
[c] *Center for Translational Medicine, Temple University, Lewis Katz School of Medicine, Philadelphia, PA, USA*
[d] *Allama Iqbal Medical College/Jinnah Hospital Complex, University of Health Sciences, Lahore, Pakistan*

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Abstract

Background: Stem cell transplantation is one of the therapeutic options available to repair damaged organs. However, transplanted cells entail several challenges including their survival in diabetes-affected injured tissue. This study was designed to determine the effects of preconditioning of mesenchymal stem cells (MSCs) with N-acetyl cysteine (NAC), a widely used antioxidant drug.
Methods: Diabetic-mouse-derived MSCs (blood glucose $\geq$ 300 mg/dL) were preconditioned with 30mM NAC for 1 hour followed by oxidative injury with 100μM hydrogen peroxide (H_2O_2) for 1 hour.
Results: Gene expression analysis showed marked upregulation of prosurvival genes (*Akt* and *Bcl-2*) and significantly downregulated expression of proapoptotic and stress genes (*Capase-3*, *Bax*, *Bak*, *p53*, *p38*, and *NF-κB)* in the 30mM-NAC-treated group when compared with those cells treated with H_2O_2 alone. NAC preconditioning improved cell viability, decreased lactate dehydrogenase release, β-galactosidase activity, and Annexin-V-positive cells. Also, amelioration of oxidative stress, as shown by a decrease in malondialdehyde level and an increase in superoxide dismutase and catalase activities and glutathione level, was observed in the 30mM-NAC-treated group in comparison to cells treated with H_2O_2 alone.
Conclusion: This study demonstrates the potential benefits of pharmacological preconditioning of diabetic-mouse-derived MSCs with NAC for amelioration of apoptosis and oxidative stress in H_2O_2 induced injury.

Keywords: apoptosis; caspase-3; N-acetyl cysteine; nuclear factor-κB; survival

1. Introduction

Diabetes is a major risk factor for the development of various complications, and is associated with increased morbidity and mortality in patients.[1,2] Diabetes elicits the production of hyperglycemia, which initiates the generation of reactive oxygen species (ROS),[3] contributing to oxidative-stress-mediated tissue injury.[4] In these circumstances, the antioxidant defense mechanism is affected, which ultimately makes cells unable to combat ROS-induced injury.[5] This can hamper the survival of stem cells for patients with diabetes.

Many studies have shown that mesenchymal stem cells (MSCs) can regenerate damaged organs, resulting in restoration of organ functions.[6,7] However, the functioning of MSCs is impaired in terms of their proliferation and paracrine ability for patients in a diabetic condition, so it counteracts the effects of autologous MSC transplantation.[8] Different strategies such

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* Corresponding author. Dr. Fatima Ali, Institute of Molecular Biology and Biotechnology, The University of Lahore, Defence Road Campus, Lahore, Pakistan

E-mail address: Fatemei.ali@gmail.com (F. Ali).

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as preconditioning with growth factors, hypoxic shock and antioxidants[9−11] have been used for the improvement of stem cell potential. Several studies have demonstrated the beneficial effects of antioxidant therapy, suggesting it could be a promising approach for scavenging the excessive ROS production and thereby enhance cell survival.[11,12] Preconditioning of MSCs with antioxidants has been found to be cytoprotective.[13,14] N-acetyl cysteine (NAC) is a free radical scavenger and it has been demonstrated to protect various cell types against hypoxic-stress-induced injury.[15−17]

The present study hypothesized that NAC preconditioning may promote the survival of MSCs under hydrogen peroxide (H_2O_2)-induced injury, thereby attenuating apoptosis. Diabetic-mouse-derived MSCs were preconditioned with NAC and subsequently evaluated for survival under H_2O_2-induced injury conditions. The present study reports that NAC treatment of diabetic-mouse-derived MSCs improved cell viability, attenuated apoptosis in H_2O_2-induced injury condition, and improved oxidative stress.

2. Methods

2.1. Animals

The animals used in this study were treated within the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and the study was approved by the Institutional Review Committee at the National Center of Excellence in Molecular Biology, Lahore, Pakistan.

2.2. Diabetic mouse model

Initially, diabetes was induced in C57BL/6 6−8-week old male mice. Intraperitoneal injection of streptozotocin (55 mg/kg body weight) was given to mice for five consecutive days, as streptozotocin is metabolized within 24 hours. Mice were housed with absorbent bedding and food and water were provided *ad libitum*.[18] At Day 16 after initial injection, the mice were weighed, and their blood glucose level was measured by using Accu-Check blood glucose meter (Roche Diagnostics, Indianapolis, IN, USA). At that point, mice with blood glucose level $\geq$ 300 mg/dL were considered for further study.

2.3. Isolation, culture, and characterization of diabetic MSCs

The manner in which MSCs were isolated and cultured has been previously reported.[18] MSCs were extracted from tibias and femurs of C57BL/6 mice after 60 days of diabetes induction. MSCs were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Sigma−Aldrich, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (Sigma−Aldrich), streptomycin (100 μg/mL), and penicillin (100U/mL; Gibco, Grand Island, NJ, USA). The cells were incubated at 37°C in a humidified atmosphere in the presence of 5% CO_2, and the medium was changed after every 3 days. The diabetic-mouse-derived MSCs were characterized using a florescence-activated cell sorting system as described previously.[9]

2.4. Experimental design

Diabetic-mouse-derived MSCs were seeded in six-well plates (Corning, Tewksbury, MA, USA) at a density of 10^5 cells. The cells were randomly divided into five groups: non-preconditioned MSCs (control group); non-preconditioned MSCs incubated with 100μM H_2O_2 for 1 hour (H_2O_2-treated group); and MSCs preconditioned with different concentrations of NAC (Sigma−Aldrich; 10−30mM) for 1 hour and subsequently treated with 100μM H_2O_2 for 1 hour (NAC group). All experiments were performed in serum-free IMDM.

2.5. Measurement of cell viability, lactate dehydrogenase, and apoptosis

Cell viability was assessed using the trypan blue exclusion method. The cells in different experimental groups were washed with Hanks' Balanced Salt Solution (Sigma−Aldrich) and dispersed with 0.025% trypsin in 0.02% EDTA. The cells were centrifuged (18*g* for 10 minutes; Eppendorf 5810R, Thomas Scientific, Swedesboro, USA), washed with Hanks' Balanced Salt Solution and then mixed with trypan blue solution using a 1:1 ratio. Thereafter, cells were counted by using a hemocytometer. The percentage of viable cells was calculated by dividing the number of trypan-blue-negative cells by the total number of cells examined and then multiplied by 100. Lactate dehydrogenase (LDH) release was analyzed using cell supernatants by a commercially available kit (Sigma−Aldrich). Annexin-V staining was used to measure apoptosis in different treatment groups. Cultured cells were stained with Phycoerythrin Annexin-V kit (Abcam, Cambridge, MA, USA). The Annexin-V^+ cells were then counted at random in five fields per triplicate well for each treatment.

2.6. β-Galactosidase assay

Senescence-associated β-galactosidase activity was measured using a senescent staining kit (Sigma−Aldrich), and the treatment regimen for each group was the same as described above. Diabetic-mouse-derived MSCs were washed twice with phosphate-buffered saline and fixed for 15 minutes in a fixative solution. After washing with phosphate-buffered saline, fresh β-galactosidase staining solution was added and cells were incubated overnight at 37°C without CO_2. Senescent cells displayed a β-galactosidase positive blue color in the cytoplasm.

2.7. Gene expression profiling

Total RNA was extracted from all experimental groups using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed with 1 μg RNA using a Revert Aid H-Minus First Strand cDNA synthesis kit (Invitrogen).

Amplification was performed for the genes *caspase-3*, *Bax*, *Bak*, *p53*, *p38*, *Akt*, *Bcl-2*, and *NF-κB*. Real-time polymerase chain reaction (PCR) was carried out for *caspase-3, p38*, *Bcl-2*, and *NF-κB* gene using Maxima SYBR Green qPCR Master Mix (Fermentas International Inc., Glen Burnie, MD, USA). *β-actin* was used as an internal control. The sequences (5′ to 3′) and product lengths for the primer pairs are mentioned in Table S1.

2.8. Western blot analysis

Western blot analysis was performed to assess the expression of caspase-3. Protein was extracted with RIPA buffer (Sigma−Aldrich) from all experimental groups. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The membrane was then incubated overnight at 4°C with anti-caspase-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA USA) and anti-β-actin (Abcam, Cambridge, MA, USA). After incubation, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies (Abcam) for 1 hour on a shaker, then washed, and finally developed with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Amersham, Piscataway, NJ, USA).

2.9. XTT assay

To check the effect of NAC preconditioning on the proliferation of diabetic-mouse-derived MSCs, a proliferation assay was performed using the commercially available XTT (sodium 3-[1{phenylaminocarbonyl}-3, 4-tetrazolium]-bis {4-methoxy-6-nitro} benzene sulfonic acid hydrate) cell proliferation kit (Roche Diagnostics, Indianapolis, IN, USA). A 96-well plate (Corning) was seeded with 4×10^3 cells/well in IMDM supplemented with 20% fetal bovine serum. Absorbance was taken at 450 nm using an ELISA plate reader Spectra max PLUS 384 (Molecular Devices, Sunnyvale, CA, USA), with 650 nm designated as the reference wavelength.

2.10. Evaluation of oxidative parameters

Cells were cultured in six-well plates (Corning) at a density of 10^5 cells. Medium from all treatment groups was removed after treatment as described above and centrifuged at 250*g* for 4 minutes (Eppendorf 5810R, Thomas Scientific, Swedesboro, U.S.A) to remove the cell debris. This medium was used for analysis of oxidative stress parameters, for example, lipid peroxidation (malondialdehyde; MDA), superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) levels. The extent of cellular lipid peroxidation was determined by measuring MDA as described previously.[19] Additionally, reduced GSH was assayed according to the method of Beutler et al,[20] and SOD activity was determined by the method of Kakkar et al.[21] CAT activity was assayed following the method of Sinha et al.[22] All assays perform in triplicate according to our previously published protocol.[23]

2.11. Statistical analysis

All data values were presented as mean ± standard error of the mean and statistical analysis were carried out with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and SPSS statistical software version 16 (Chicago, IL, USA). Comparison between more than two parameters was evaluated by analysis of variance followed by application of the Bonferroni *post hoc* test. The difference between two groups was assessed using Student's *t* test. A p value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Cytoprotective effect of NAC on diabetic-mouse-derived MSCs

NAC preconditioning markedly protected the diabetic-mouse-derived MSCs against H_2O_2-induced injury as indicated by enhanced cell viability evaluated by trypan blue exclusion assay. Results showed more viable cells in the 30mM NAC group versus control versus H_2O_2-treated groups ($84 \pm 5.63\%$ vs. $48 \pm 6.14\%$ vs. $35 \pm 7.13\%$, respectively; Fig. 1A). Similarly, reduced LDH release was observed in the 30mM NAC group versus control versus H_2O_2-treated groups ($24 \pm 2.88\%$ vs. $36 \pm 1.15\%$ vs. $63 \pm 2.3\%$, respectively; Fig. 1B). β-Galactosidase assay documented a similar pattern of cell viability, as a significantly higher expression of senescence associated β-galactosidase was observed in the H_2O_2-treated group versus control versus 30mM NAC concentration ($68 \pm 1.73\%$ vs. $46 \pm 1.12\%$ vs. $21 \pm 1.15\%$, respectively; Figs. 1C−H). A high percentage of apoptotic cells, that is, Annexin-V-positive cells were observed in the H_2O_2-treated group versus control versus 30 mM NAC concentration, ($71 \pm 6.91\%$ vs. $43 \pm 4.46\%$ vs. $11 \pm 2.39\%$; Fig. 1I).

3.2. Effect of NAC on gene expression profiling

Gene expression profiling was done on the control, H_2O_2-treated and NAC-treated groups. The results of this profiling showed decreased levels of *caspase-3*, *Bax*, *Bak*, *p53*, *p38* (apoptotic genes), and *NF-κB* (inflammation gene), and elevated levels of *Akt* and *Bcl-2* (survival genes) in the 30mM-NAC-treated group as compared with the H_2O_2-treated group (Figs. 2A and 2B).

Caspase-3, *p38*, *Bcl-2*, and *NF-κB* expression was also evaluated by real-time PCR. Furthermore, NAC preconditioning was observed to have a more pronounced effect in the 30mM NAC group. The higher concentration of NAC minimized the effect of H_2O_2-induced injury in diabetic-mouse-derived MSCs, as *Caspase-3* and *NF-κB* expression was upregulated while *Bcl-2* expression was downregulated in the H_2O_2-treated group (Fig. 2C).

Caspase-3 expression was elevated in H_2O_2-induced injury but NAC preconditioning inhibited the activation of caspase-3 in diabetic-mouse-derived MSCs after H_2O_2-induced injury (Figs. 2D and 2E).

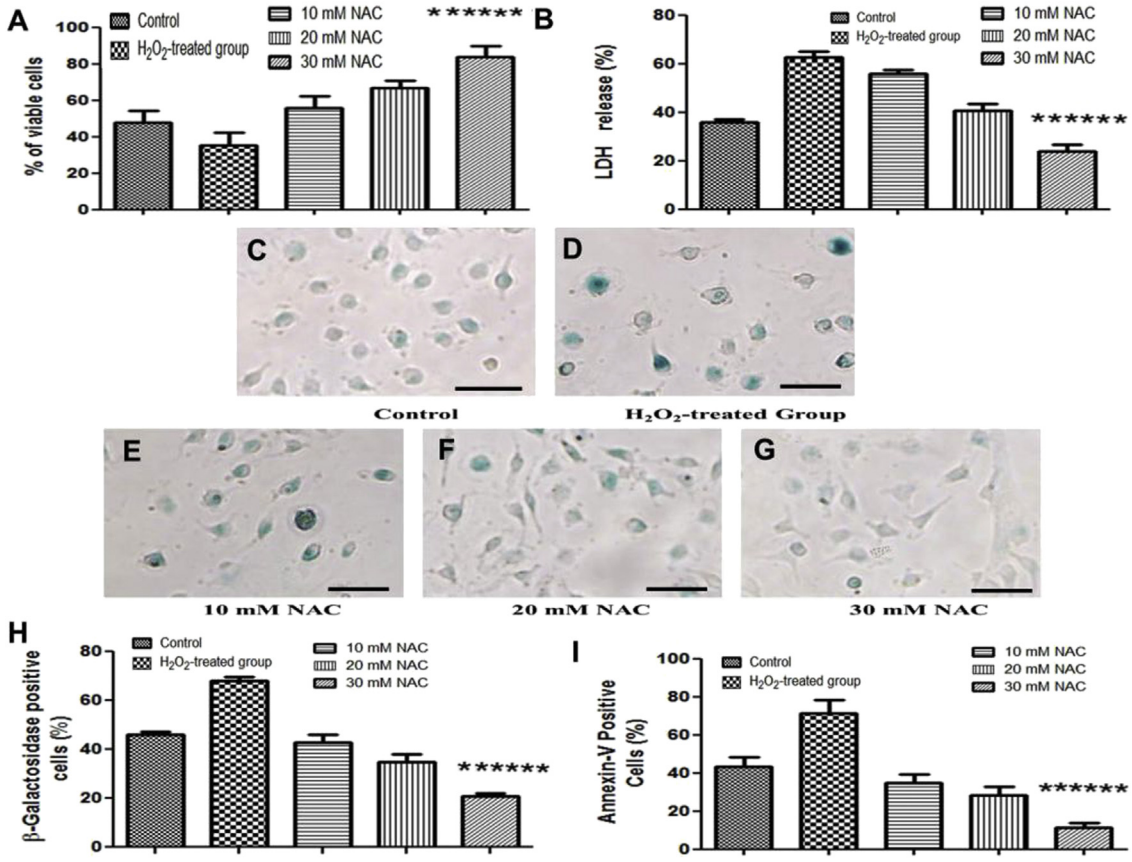

Fig. 1. Effects of NAC preconditioning on cell viability against H_2O_2 induced injury. (A) Cell viability assay. (B) Lactate dehydrogenase release was measured in the cell supernatant. (C−G) β-Galactosidase assay showing less expression of β-galactosidase activity at 30mM NAC concentration. (H) Bar graph showing percentage of β-galactosidase-positive cells in different treatment groups. (I) Quantification of annexin-V cells in different treatment groups with quantification. 200×, bar ~50 μm. All values were expressed as mean ± standard error of the mean. * $p < 0.05$ for 30mM NAC group versus control group. ** $p < 0.05$ for 30mM NAC group versus H_2O_2-treated group. *** $p < 0.05$ for 30mM NAC group versus other NAC groups. H_2O_2 = hydrogen peroxide; NAC = N-acetyl cysteine.

3.3. Effect of NAC on cell proliferation

Cell proliferation determined by XTT assay was higher in the 30mM NAC preconditioned MSCs group versus control versus H_2O_2-treated group, (1.74 ± 0.064 vs. 0.94 ± 0.032 vs. 0.37 ± 0.023, respectively; Fig. 3A). Furthermore, reverse transcriptase PCR analysis for proliferating cell nuclear antigen (*PCNA*) expression demonstrated a similar pattern (Figs. 3B and 3C).

3.4. Effect of NAC preconditioning on oxidative stress parameters

To investigate the effect of NAC preconditioning on oxidative damage and antioxidant status, MDA and GSH levels as well as SOD and CAT activities were assessed in all treatment groups (Fig. 4). H_2O_2-induced hypoxic injury increased MDA level and decreased GSH level and SOD and CAT activities in the H_2O_2-treated group. A reduced level of MDA was found in the 30mM NAC preconditioned MSCs groups versus H_2O_2-treated group versus control group ($0.38 \pm .002$ vs. 0.60 ± 0.004 vs. $0.47 \pm .000$, respectively; Fig. 4A). Increased GSH level was found in the 30mM NAC preconditioned MSC groups versus H_2O_2-treated group versus control group (0.113 ± 0.004 vs. 0.073 ± 0.002 vs. 0.078 ± 0.003; Fig. 4B). Increased SOD and CAT activities were found in the 30mM NAC preconditioned MSC groups versus H_2O_2-treated group versus control group (0.070 ± 0.000 vs. 0.051 ± 0.004 vs. 0.064 ± 0.001, respectively) and (0.87 ± 0.039 vs. 1.26 ± 0.135 vs. 0.91 ± 0.004, respectively; Figs. 4C and 4D).

4. Discussion

Diabetes is associated with enhanced ROS production, which is potentiated by hyperglycemia.[4,23] It has been shown that cultured MSCs exposed to high glucose produce ROS, which significantly contributes to cellular injury.[24] ROS induce a variety of abnormalities including cell death and apoptosis.[25] Therefore, the aim of this study was to investigate whether NAC can inhibit apoptosis and reduce oxidative stress in diabetic-mouse-derived MSCs subjected to H_2O_2-induced injury.

To determine whether diabetic-mouse-derived MSCs become tolerant to H_2O_2-induced injury, the viability and apoptosis of treated cells were evaluated with trypan blue,

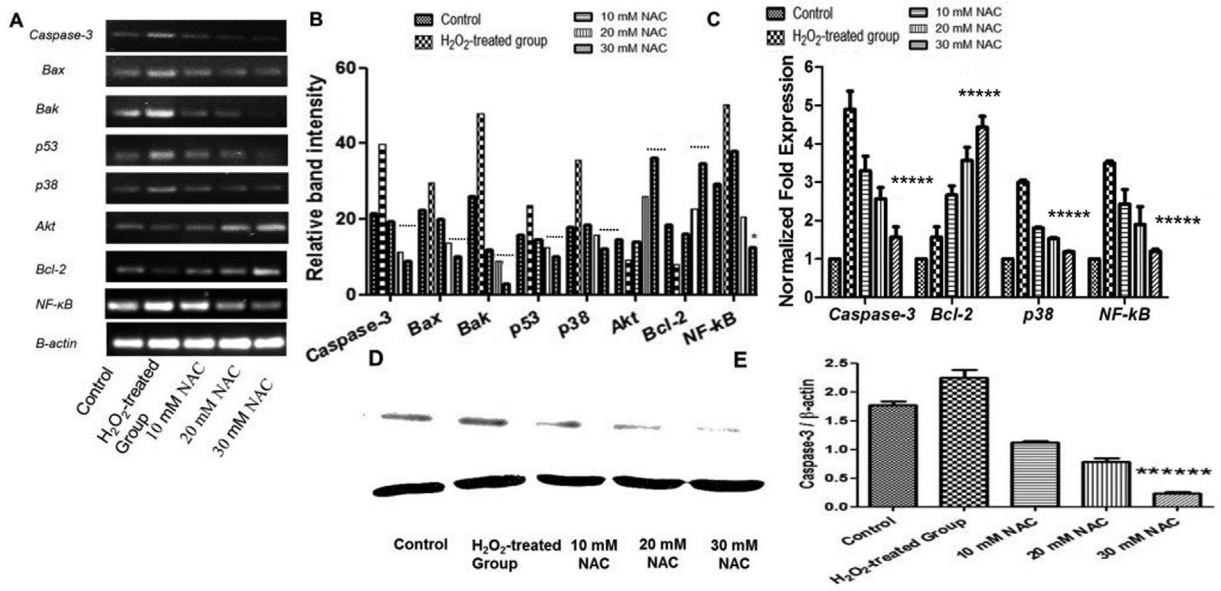

Fig. 2. Gene expression profiling of diabetic-mouse-derived mesenchymal stem cells after NAC treatment. (A, B) Reverse transcriptase PCR analysis for genes of apoptosis (*caspase-3*, *Bax*, *Bak*, *p53*, and *p38*), survival (Akt and Bcl-2) and inflammation (*NF-κB*). (C) Real-time PCR for *Caspase-3*, *Bcl-2*, and *NF-κB* revealed that *caspase-3* and *NF-κB* were significantly reduced by 30mM NAC, while expression of *Bcl-2* was increased compared to the other NAC concentrations. (D) Western blot analysis. (E) Ratio of caspase-3/β-actin after gel quantification by Image J. All values were expressed as mean ± standard error of the mean. * $p < 0.05$ for 30mM NAC group versus control group. ** $p < 0.05$ for 30mM NAC group versus hydrogen-peroxide-treated group. *** $p < 0.05$ for 30mM NAC group versus other NAC groups. NAC = N-acetyl cysteine; PCR = polymerase chain reaction.

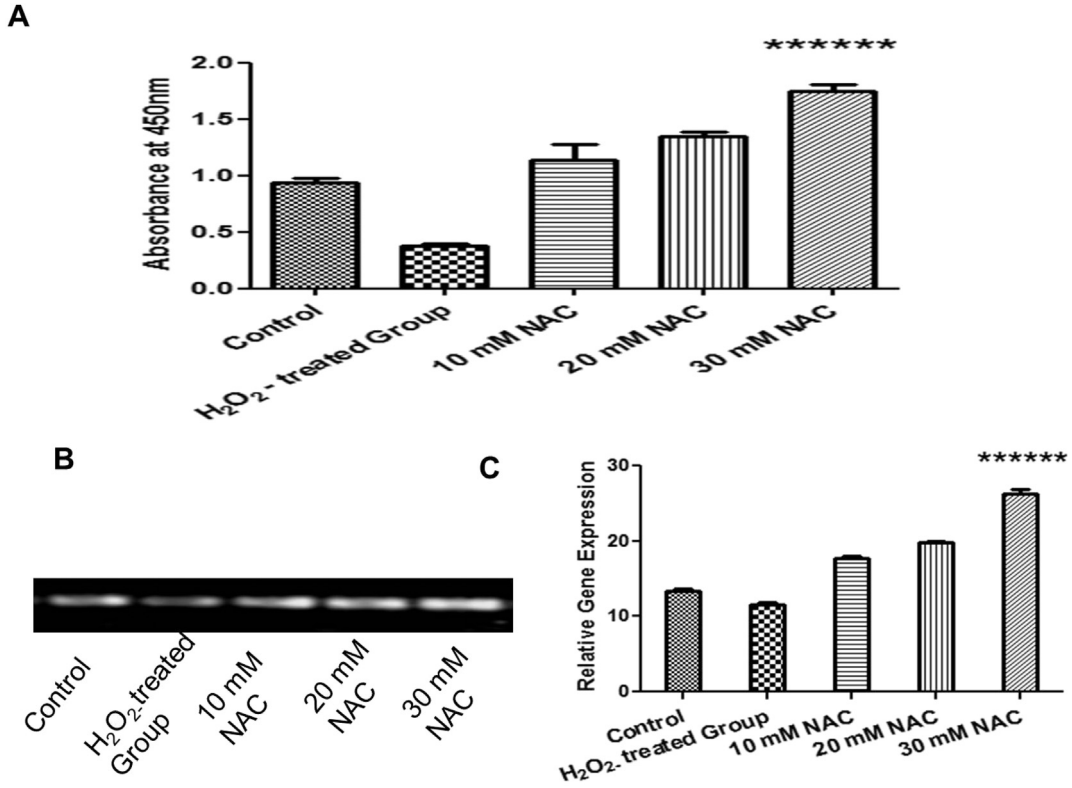

Fig. 3. Proliferative potential of diabetic-mouse-derived mesenchymal stem cells after NAC preconditioning. (A) XTT assay data. (B) Reverse transcriptase polymerase chain reaction analysis of *PCNA* in different treatment groups. (C) Quantification of *PCNA* gel bands by using Image J software. All values are expressed as mean ± standard error of the mean. * $p < 0.05$ for 30mM NAC group versus control group; † $p < 0.05$ for 30mM NAC group versus hydrogen-peroxide-treated group. *** $p < 0.05$ for 30mM NAC group versus other NAC groups. NAC = N-acetyl cysteine; XTT = sodium 3-[1{phenylaminocarbonyl}-3, 4-tetrazolium]-bis {4-methoxy-6-nitro} benzene sulfonic acid hydrate.

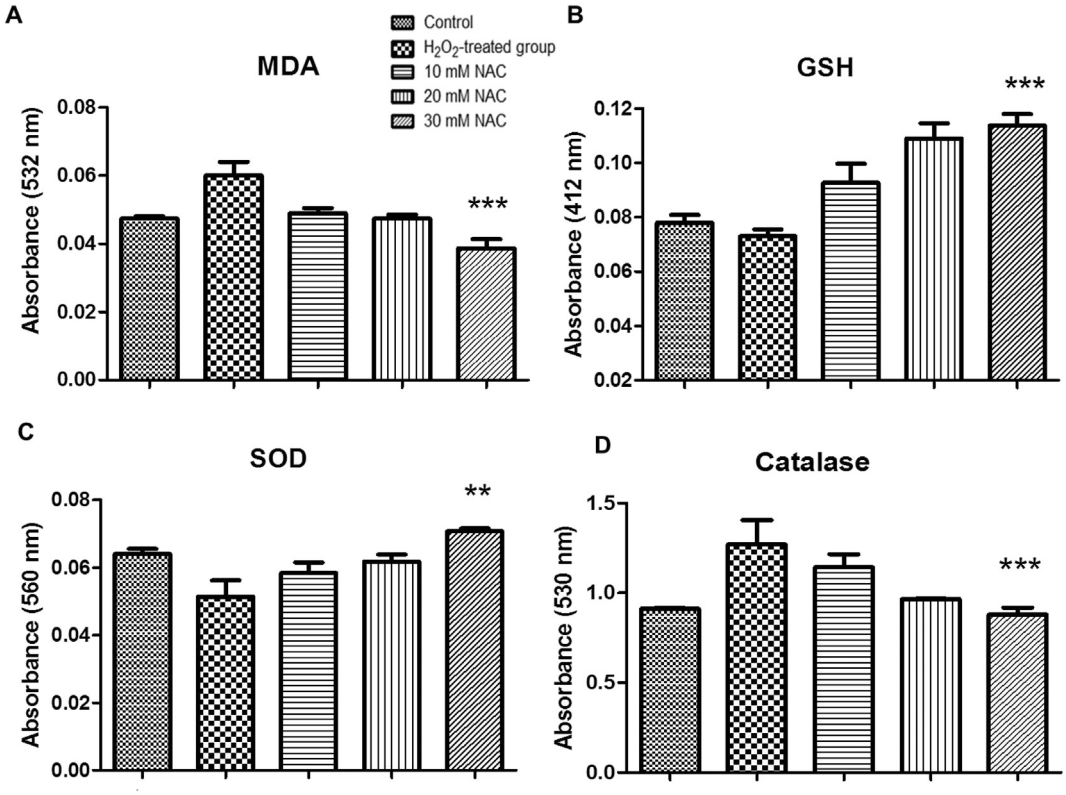

Fig. 4. Assessment of NAC preconditioning on oxidative stress and oxidative stress markers against H_2O_2-induced injury. (A) Lipid peroxidation was assessed by measuring malondialdehyde generation in different treatment groups. (B) Reduced glutathione; (C) superoxide dismutase; (D) catalase. All values are expressed as mean ± standard error of the mean. * $p < 0.05$ for 30mM NAC group versus control group. ** $p < 0.05$ for 30mM NAC group versus H_2O_2-treated group. *** $p < 0.05$ for 30mM NAC group versus other NAC groups. H_2O_2 = hydrogen peroxide; NAC = N-acetyl cysteine.

LDH release and β-galactosidase activity. Cell viability was assessed by using trypan blue assay[26] in the presence of graded concentrations of NAC (10mM, 20mM, and 30mM). The results indicated that 30mM NAC significantly minimized H_2O_2-induced cell death as compared to lower concentrations. The leakage of LDH into culture medium from treatment groups was monitored; LDH has been regarded as an indicator of the degree of cell death.[27] The level of LDH was significantly increased after H_2O_2 treatment, whereas when 30mM NAC was added to the culture medium before H_2O_2 treatment, LDH leakage from the treatment groups was suppressed. NAC treatment exhibited an overall cytoprotective effect on the diabetic-mouse-derived MSCs, reducing the number of senescent cells and Annexin-V-positive cells after treatment. These findings indicate that, in our experimental setting, 30mM NAC is adequate for inhibition of H_2O_2-induced injury.

Caspase-3, is involved in the apoptotic process.[28] In this study, we analyzed the effects of H_2O_2-induced injury and NAC on caspase-3 activation by reverse transcriptase PCR and Western blot analysis. Decrease in mitochondrial GSH level leads to enhanced caspase-3expression in acute diabetic condition,[29] and our findings are in accordance with this evidence. Moreover, 30mM NAC blocked caspase-3 activation, suggesting that NAC preconditioning enhances the survival of diabetic-mouse-derived MSCs. It is evident that *Bax*, *Bak*, *p53*, and *p38* are markers of apoptosis,[30,31] and it was observed that expression of these markers was upregulated in the H_2O_2-treated group as compared to the NAC-treated groups. Prosurvival markers *Akt* and *Bcl-2*[18,30] were upregulated after NAC treatment. Additionally, increased production of ROS activates *NF-κB*, which leads to organ hypertrophy and dysfunction.[32] So, it has been elucidated that the pathophysiology of organ damage is associated with *NF-κB*-induced inflammation. To optimize the best concentration of NAC to overcome H_2O_2-induced injury, we tested the effect of NAC on diabetic-mouse-derived MSCs and its relationship with *NF-κB*. The results indicated that NAC reduced expression of *NF-κB*. Real-time PCR data suggest that 30mM NAC is the best concentration to enhance survival and reduce H_2O_2-induced injury. The current results are consistent with previous reports that H_2O_2-induced injury threatens the survival of MSCs.[33] The results of XTT assay and *PCNA* gene expression also revealed that 30mM NAC promotes survival of diabetic-mouse-derived MSCs.

As previously reported, hyperglycemia can cause ROS production, which further induces auto-oxidation of glucose and can cause DNA, protein and lipid damage.[34,35] Previous studies have shown that ROS affect the antioxidant defense mechanisms by decreasing the activity of SOD and CAT,[36] and also reducing the intracellular concentration of GSH.[37]

ROS cause peroxidation of polyunsaturated lipids and produces polyunsaturated precursors such as MDA, which is used as an oxidative stress biomarker.[38] Previous studies have demonstrated that an increased level of MDA indicates cellular oxidative damage.[39,40] Therefore, we determined the oxidative damage in diabetic-mouse-derived MSCs by measuring the MDA level. Our results showed that H_2O_2-induced oxidative damage is rescued by the antioxidant NAC.

We determined the antioxidant ability of NAC by measuring the SOD, GSH and CAT levels. SOD is an important antioxidant defense enzyme that catalyzes the dismutation of superoxide (O_2^-) into oxygen and H_2O_2.[41] This H_2O_2 can then be detoxified into water by GSH and CAT.[42]

Our results are consistent with previous studies, which demonstrated that H_2O_2-induced injury decreased SOD and CAT activities and depleted GSH content.[36,43] This could be reversed by NAC preconditioning and in this study the 30mM-NAC-treated group showed an improvement in GSH level as well as SOD and CAT activities when compared with those cells treated with H_2O_2 alone.

An important limitation of our study was the lack of evaluation of differentiation potency of diabetic-mouse-derived MSCs after NAC treatment and associated *in vivo* study. Further studies involving transplantation of NAC-pretreated cells in animal models of heart infarction are required to elucidate its role *in vivo*.

The results of the present study suggest that the beneficial effects of NAC are consistent with other studies demonstrating that NAC preconditioning of stem cells reverses H_2O_2-induced cell death.[13,44] In conclusion the study revealed that NAC can potentially minimize the adverse effects of oxidative injury to MSCs derived from diabetic mice, not only by reducing apoptosis, but also by enhancing the viability and survival of cells.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcma.2015.09.005.

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