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The effect of N-acetyl cysteine on H_2O_2 mediated oxidative stress in Wharton's jelly derived mesenchymal stem cells

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Abstract

Background: Hypoxic stress is a crucial factor for retaining the cell survival in injured tissue. Overcoming this issue is the key for successful cellular regenerative therapy. Therefore the purpose of this study was to investigate whether the *in-vitro* pretreatment of Wharton's Jelly (WJ) derived Mesenchymal stem cells (WJ-MSCs) with an antioxidant, namely N-acetylcysteine (NAC), can improve the efficacy of WJ-MSCs for transplantation purpose.

Methods: WJ-MSCs were cultured with or without NAC at different concentrations (0.1mM, 1mM and 10mM). To simulate oxidative stress conditions, cultures were exposed to hydrogen peroxide (H_2O_2) 100 µM for 1 hour. Cytoprotective effect of NAC was evaluated by determining cell injury, viability, and proliferation. The oxidative stress is assessed by measuring the activity of glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malodialdehyde (MDA).

Results: Pretreatment of WJ-MSCs with NAC increased their viability and proliferation in concentration-dependent manner. Furthermore, 10 mM NAC significantly reduced the H_2O_2 induced oxidative stress by enhancing the activity of GSH, SOD, and CAT and reduced the level of MDA

Conclusion: The study results indicate that NAC may abrogate H_2O_2 induced oxidative-stress of WJ-MSCs. This study provides basis to explore NAC effect on WJ-MSCs survival without cytotoxicity.

Introduction

Wharton's jelly derived mesenchymal stem cells (WJ-MSCs) have great potential in regenerative medicine and cellular therapy [1]. Cell therapy is based on transplantation of stem cells to repair damaged tissue but the transplanted cells face hypoxic environment in the tissue which may lead to apoptosis of cells [2,3]. To overcome this situation, *in vitro* preconditioning of stem cells is well established strategy [2,4-6]. In this study WJ-MSCs are *in vitro* preconditioned with N-acetylcysteine (NAC). NAC is a cysteine analogue drug used as a glutathione (GSH) precursor [4]. GSH acts as an antioxidant to reduce the oxidative stress. Recent studies showed that the oxidative stress is increased in injury condition due to overproduction of reactive oxygen species (ROS) [7].

ROS include hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and superoxide (O_2^-), these may cause deleterious effects on living systems [8]. The damaging effect of ROS termed as oxidative stress decreases the efficiency of both enzymatic and non-enzymatic antioxidants. Recent studies have underlined the protective effect of NAC by reducing the ROS and preventing the damage to many organs [9-11].

It is hypothesized that pretreatment of WJMSCs with NAC could be a promising therapeutic approach to improve cell survival. Therefore, the purpose of this study was to investigate the role of pretreatment of WJ-MSCs with NAC to improve the efficacy of WJ-MSCs.

Methods

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. Donors were 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.

WJ-MSCs cell culture

Isolation of MSCs from WJ was done by explant culturing method (Sigma Aldrich, USA) as previously reported [12]. 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. The cells of passage 3 were used in the study.

Experimental design

WJ-MSCs were randomly divided into five experimental groups: untreated with both H_2O_2 and NAC (Untreated; UNTR), 100 µM H_2O_2 treated control group (H_2O_2 control group; H_2O_2); WJ-MSCs treated with 0.1mM concentration of NAC (Sigma Aldrich, USA) (0.1mM NAC group); with 1mM concentration of NAC (1mM NAC group); and 10 mM concentration of NAC (10mM NAC group) for 48 hours [16] and subsequently treated with 100 µM H_2O_2 for 1 hour (NAC group). 100 µM H_2O_2 dose was selected in this investigation based on a series of preliminary test experiments. All experiments were performed in serum free DMEM for 48 hours (Figure 1).

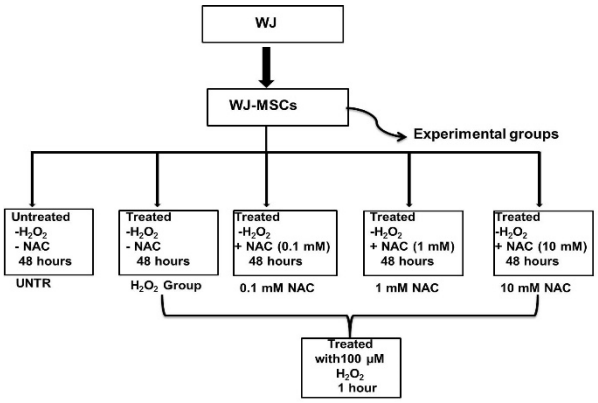

Figure 1: Experimental Design.

Cell viability assay

After treatment cells were washed with PBS and then stained with crystal violet (Sigma Aldrich., USA) for 20 minutes which were subsequently lysed with SDS after three washings with PBS. Absorbance was taken at 595 nm.

Cell proliferation assay

To compare the proliferative potential of experimental groups, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed. Monolayer of cells was first washed with phosphate buffer saline (PBS) (Invitrogen Inc., USA). 500 µL complete medium along with 60 µL MTT solution (Invitrogen Inc., USA) was added to cells and

incubated for 2 hours at 37°C. Purple color crystals formed within cells were solubilized with DMSO and absorbance was taken at 570 nm.

LDH assay

LDH assay was performed using 5μL medium from each group at the end of treatment using LDH assay kit (AMP Diagnostics, Austria) according to manufacturer's instructions. Briefly, cell culture medium (5 μL) of both groups was mixed with working reagent (95 μL), incubated for 5 minutes and then absorbance was recorded at wavelength of 340 nm.

Determination of oxidative stress

The oxidative stress markers, including activities of glutathione reductase (GSH), catalase (CAT), and superoxide dismutase (SOD), and level of Malondialdehyde (MDA) were determined, according to procedure previously described [13].

Determination of SOD activity

Homogenate was prepared by mixing serum and trichloroacetic acid (50%) in 1:1 ratio and centrifuged at 13,000 rpm for 10 minutes at 25°C. 15 μL supernatant was added to 120 μL sodium pyrophosphate buffer (52 mM, pH 8.3), 12 μL phenazine methosulphate, 36 μL nitroblue tetrazolium. Reaction was started by addition of 24 μL nicotinamide adenine dinucleotide. After incubation at 37°C for 90 seconds, reaction was stopped by addition of 12 μL of glacial acetic acid. The reaction mixture was stirred vigorously with 400 μL of n-butanol. The mixture was incubated for 10 minutes and then centrifuged at 2000 rpm for 5 minutes at 25°C and butanol layer was separated. The color intensity of chromogen in n-butanol layer was measured at 560 nm against n-butanol using a spectrophotometer.

Estimation of reduced GSH

Homogenate was prepared by mixing serum and trichloroacetic acid (10%) in 1:1 ratio and centrifuged at 1000 rpm for 10 minutes at 25°C. 40 μL supernatant was mixed with 150 μL of 0.3 M disodium phosphate buffer. Then 25 μL of 0.001 M freshly prepared DTNB [5,50-dithiobis (2-nitrobenzoic acid) dissolved in 1% sodium citrate] was added. Reduction of DTNB with GSH produced a yellow compound, whose absorbance was noted spectrophotometrically at 412 nm. The reduced chromogen is directly proportional to GSH concentration.

Estimation of catalase activity

Serum (40 μL) was mixed with 360 μL phosphate buffer (10 mM, pH 7.0) and centrifuged at 13,000 rpm for 10 minutes at 25°C. 21 μL of the supernatant and 180 μL phosphate buffer (10 mM, pH 7.4) were mixed. Reaction was started by addition of freshly prepared 75 μL H_2O_2 (0.2 M). 360 μL potassium dichromate acetic acid reagent (5%) was added to reaction mixture and incubated for 10 minutes in boiling water, cooled, and absorbance was measured at 530 nm.

Estimation of MDA level

For this, 40 μL of serum was taken and a homogenate was prepared in 360 μL phosphate buffers (10 mM, pH 7.4) and centrifuged at 13,000 rpm for 10 minutes at 25°C. 15 μL supernatant was mixed with 15 μL SDS (8.1%), 96 μL TBA (0.8%), 96 μL acetic acid (20%) and 18 μL distilled water and incubated at 90°C for 1 hour. Afterwards, 60 μL distilled water and 300 μL n-butanol-pyridine mixture (15:1) was added and the mixture was shaken vigorously and centrifuged at 4000 rpm at 25°C for 10 minutes. The upper n-butanol layer was separated and its absorbance was taken at 532 nm.

Statistical analysis

The experimental data were expressed as the mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, San Diego, CA). The mean data of all groups were compared with one-way ANOVA, followed by Scheffe's post hoc test for multiple comparisons. The $P<0.05$ value was considered as statistically significant.

Results

Cytoprotective effect of NAC on WJ-MSCs

Cells' viability

The crystal violet assay was performed to check the cells' viability. Preconditioning with 0.1 mM and 1 mM NAC concentrations resulted in increased cell viability (0.34 ± 0.010 nm; 0.35 ± 0.002 nm) but not more than 10 mM NAC group (0.44 ± 0.018 nm). Figure 2 shows that cell viability level is significantly lower in H_2O_2 group (0.23 ± 0.004 nm) compared with 10 mM NAC group.

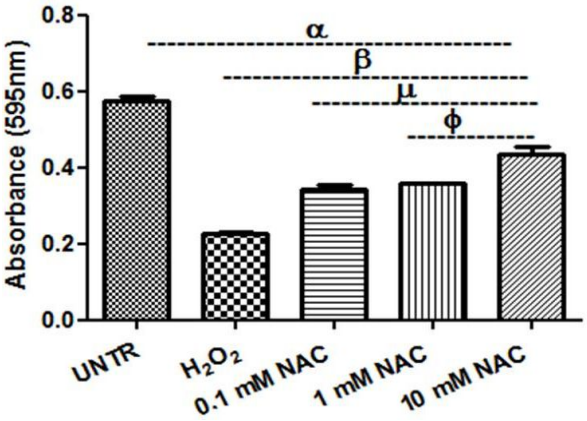

Figure 2: Cytoprotective effect of NAC treatment on WJMSC by cells' viability assay. All values were expressed as mean ± S.E.M. α $p<0.05$ for 10 mM NAC group versus UNTR group; β $p<0.05$ for 10 mM NAC group versus H_2O_2 treated group; μ $p<0.05$ for 10 mM NAC group versus 0.1 mM NAC group; ϕ $p<0.05$ for 10 mM NAC group versus 1 mM NAC group.

Cells' proliferation

Cells' proliferation potential of treatment groups was assessed by performing MTT assay. Preconditioning with 0.1 mM and 1 mM NAC concentrations resulted in increased cell proliferation (0.49 ± 0.002; 0.55 ± 0.000) but 10 mM NAC group (0.62 ± 0.000) showed even better results. Figure 3 shows that proliferation is significantly low in H_2O_2 group (0.40 ± 0.009) compared with 10 mM NAC group.

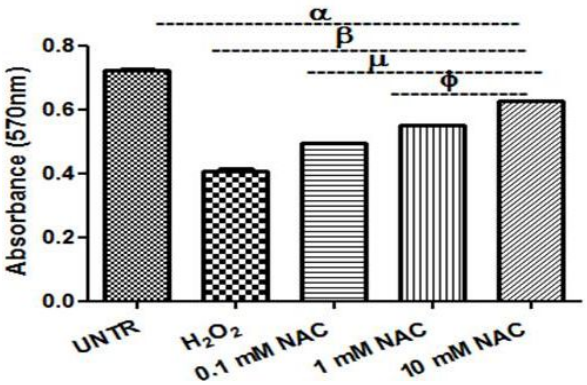

Figure 3: Cells' proliferation assay. All values were expressed as mean ± S.E.M. α $p<0.05$ for 10 mM NAC group versus UNTR group; β $p<0.05$ for 10 mM NAC group versus H_2O_2 treated group; μ $p<0.05$ for 10 mM NAC group versus 0.1 mM NAC group; ϕ $p<0.05$ for 10 mM NAC group versus 1 mM NAC group.

Lactate dehydrogenase (LDH) release

The membrane integrity of cells was determined by performing LDH cytotoxicity assay. As shown in Figure 4, LDH release was significantly decreased in 0.1 mM and 1 mM NAC groups (0.77 ± 0.001; 0.57 ± 0.008) while the best results were observed with 10 mM NAC group (0.38 ± 0.004). Figure 4 shows that LDH release was significantly high in H_2O_2 group (0.86 ± 0.000) compared with 10mM NAC group.

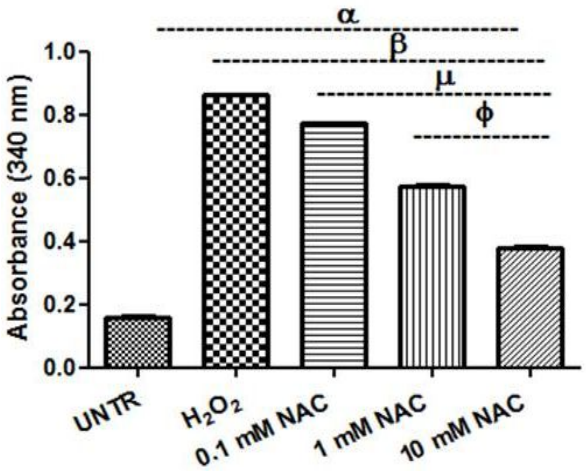

Figure 4: Lactate dehydrogenase assay. All values were expressed as mean ± S.E.M. α $p<0.05$ for 10 mM NAC group versus UNTR group; β $p<0.05$ for 10 mM NAC group versus H_2O_2 treated group; μ $p<0.05$ for 10 mM NAC group versus 0.1 mM NAC group; ϕ $p<0.05$ for 10 mM NAC group versus 1 mM NAC group.

Oxidative stress

Antioxidant enzymes status was evaluated by spectrophotometric assays (Figure 5). Figure 5A showed that pre-incubation of cells with 0.1 mM and 1 mM NAC concentraion resulted in increased SOD level (0.06 ± 0.000; 0.07 ± 0.001) but not more than 10 mM NAC group (0.12 ± 0.001). SOD level is significantly less in H_2O_2 group (0.05 ± 0.004) compared with10 mM NAC group.

Figure 5B showed that preconditioning of cells with 0.1 mM and 1 mM NAC concentration resulted in increased GSH level (0.96 ± 0.018; 1.19 ± 0.048) which is even better with 10 mM NAC group (1.33 ± 0.025). GSH level is significantly decreased in H_2O_2 group (0.59 ± 0.000) compare with10 mM NAC group.

Figure 5C showed that preconditioning of cells with 0.1 mM and 1 mM NAC concentration which resulted in an increased CAT activity (0.58 ± 0.004; 0.44 ± 0.000) which is significantly increased in 10 mM NAC group (0.29 ± 0.004). CAT activity is significantly decreased in H_2O_2 group (0.64 ± 0.010) compared with10 mM NAC group.

Figure 5D showed that preconditioning of cells with 0.1 mM and 1 mM NAC concentration resulted in increased MDA level (0.31 ± 0.077; 0.29 ± 0.002) which is better with 10 mM NAC group (1.79 ± 0.002). MDA level is significantly increased in H_2O_2 group (0.59 ± 0.002) compared with10 mM NAC group.

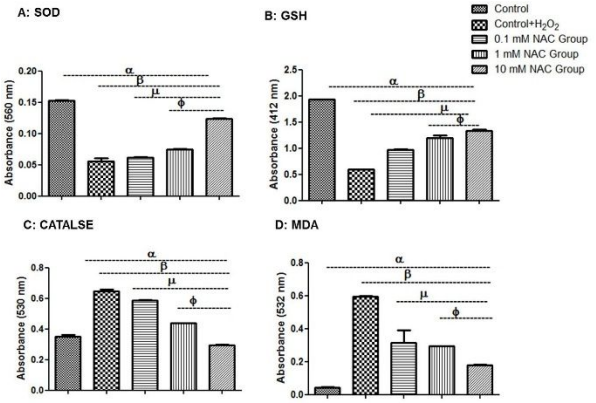

Figure 5: Effect of NAC treatment on oxidative stress. (A) SOD assay (B) GSH assay (C) Catalase assay (D) MDA assay. All values were expressed as mean ± S.E.M. α $p<0.05$ for 10 mM NAC group versus UNTR group; β $p<0.05$ for 10 mM NAC group versus H_2O_2 treated group; μ $p<0.05$ for 10 mM NAC group versus 0.1 mM NAC group; ϕ $p<0.05$ for 10 mM NAC group versus 1 mM NAC group.

Discussion

Oxidative stress acts as a major contributor to morbidity and mortality in patients as it hampers the repair potential of stem cells [14,15]. The functional capability of stem cells to regenerate injured tissue is critical to repair the organ. WJ-MSCs are pluripotent therefore; they are used in cell based therapies [5,16,17]. Hypoxic stress affects the MSCs' proliferation and function which warrants a strategy to increase functioning of affected MSCs for stem cell-based therapies [18]. Thus, different strategies are required which can augment the functioning of stem cells in hypoxic microenvironment [6,19]. Preconditioning of MSCs with antioxidant is one of the potential strategies to address this issue [20]. So, we devised a strategy to improve the behavior of WJMSCs.

Therefore, the aim of present study was to devise a preconditioning strategy to improve the cell survival of WJMSCs in hypoxic condition. In this study the effect of NAC on the behavior of WJMSCs was evaluated. In the current study, H_2O_2 stimulation resulted in reduced cell viability (Figure 2), cell proliferation (Figure 3), and increased LDH release (Figure 4) in accordance with previous studies [21,22]. NAC resulted in enhanced cell viability, cell proliferation and reduced LDH release and it conferred protection against H_2O_2-induced cellular injury.

NAC preconditioned WJMSCs were subjected to hypoxic injury to mimic the hypoxic tissue microenvironment. In injury condition ROS production elicit the disease progression due to reaction with DNA, protein and lipids all these events are reported in many cases [23,24]. It has been previously shown that the antioxidant activity is dependent on ROS level [6,25]. Previous studies have shown that ROS affect the antioxidant defense mechanisms by decreasing the activity of SOD and CAT [26], and also reducing the intracellular concentration of GSH [27]. As the level of hypoxic stress increases, it decreases the antioxidant level, this may explain the observed low antioxidant level (Figure 5A-D). The study results showed that low antioxidant enzyme level was significantly improved after NAC treatment as evidenced in Figure 5A-C. Pirincciogl u *et al.* have demonstrated that ROS cause peroxidation of polyunsaturated lipids and produces polyunsaturated precursors such as MDA, which is used as an oxidative stress biomarker [28]. Figure 5D coincides with similar findings as it demonstrates high level of lipid peroxidation product MDA in hypoxic group.

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Conflict of interest

The authors declare no conflict of interest.

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