



## N-ACETYLCYSTEINE PREVENTS CORD DERIVED STEM CELLS FROM H<sub>2</sub>O<sub>2</sub> 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  $\mu$ M H<sub>2</sub>O<sub>2</sub> 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), malondialdehyde (MDA) as well as by activity of superoxide dismutase (SOD) and catalase (CAT). 100 $\mu$ M/L H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> induced injury. NAC could be a promising therapeutic approach for improving survival of WJ-MSCs under H<sub>2</sub>O<sub>2</sub> 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.<sup>[1-3]</sup> WJ-MSCs are attractive candidates for

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

However, one of the challenges faced by stem cells is low survival rate after transplantation in the injured host environment.<sup>[7, 8]</sup> 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,<sup>[9]</sup> growth factors<sup>[10]</sup> and antioxidants<sup>[11]</sup> 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.<sup>[12-15]</sup> It is known as glutathione precursor and prevents injury or oxidative damage by scavenging the free radical species.<sup>[16]</sup> Therefore, the present study is designed to evaluate the effect of NAC in H<sub>2</sub>O<sub>2</sub> 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.<sup>[6]</sup> 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<sub>2</sub>O<sub>2</sub> treated group; (III) 100 µM/L H<sub>2</sub>O<sub>2</sub> and 0.1 mM N acetyl cysteine (NAC) group and (IV) 100 µM/L H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub> incubator. Then 150 µL dimethylsulfoxide (DMSO) was added to dissolve the formazan 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.<sup>[17]</sup> 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 phenazine 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.<sup>[18]</sup> 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<sup>[19]</sup> 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<sub>2</sub>O<sub>2</sub> (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.<sup>[20]</sup> 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  $\pm$  standard error of the mean (SEM). A level of  $p < 0.05$  was considered statistically significant.

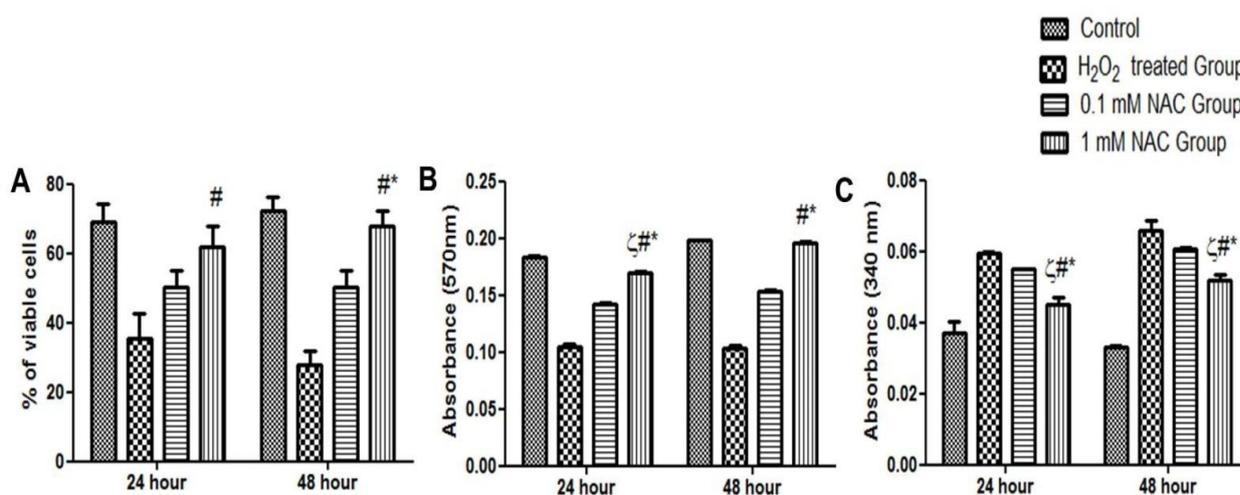
## **RESULTS**

### **The cytoprotective effects of NAC on WJ-MSCs under H<sub>2</sub>O<sub>2</sub> 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 ( $62\% \pm 6.05\%$ ;  $67\% \pm 4.6\%$ ) compared with 100  $\mu$ M/L H<sub>2</sub>O<sub>2</sub> treated group ( $35\% \pm 7.1\%$ ;  $27\% \pm 4.04\%$ ) demonstrated by the trypan-blue exclusion assay Fig. 1A. The cell viability was found to be reduced after 48 hours exposure of H<sub>2</sub>O<sub>2</sub>. 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 ( $0.169 \pm 0.002$ ;  $0.195 \pm 0.000$ ) followed by with 100  $\mu\text{M/L}$   $\text{H}_2\text{O}_2$  treated group ( $0.105 \pm 0.002$ ;  $0.103 \pm 0.002$ ) Fig. 1B.

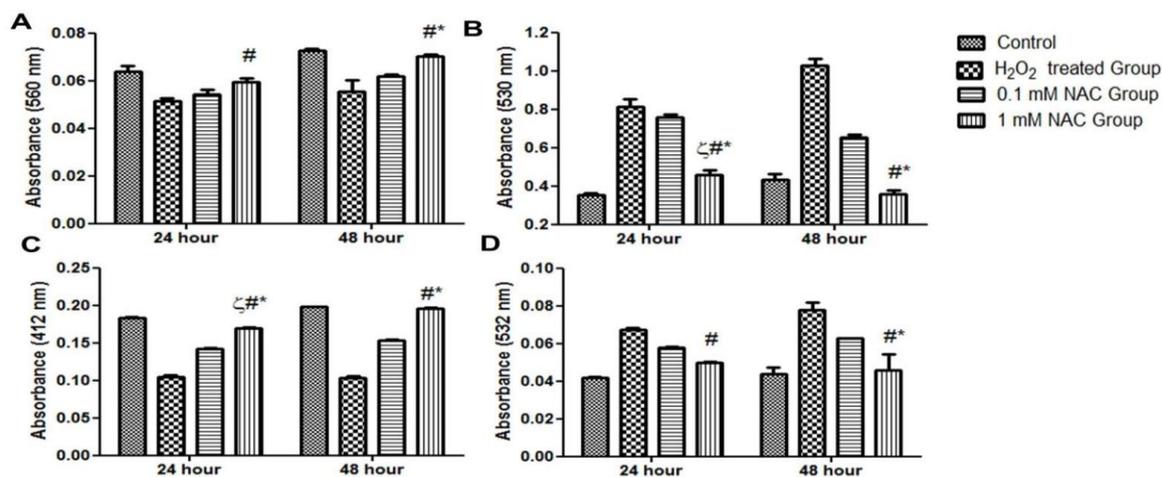
It is evident from Fig.1C that the activity of LDH was significantly elevated after 24 and 48 hours in the 100  $\mu\text{M/L}$   $\text{H}_2\text{O}_2$  treated group ( $0.059 \pm 0.00$ ;  $0.066 \pm 0.002$ ), when compared to 1 mM NAC group ( $0.045 \pm 0.002$ ;  $0.066 \pm 0.002$ ) and control group ( $0.037 \pm 0.003$ ;  $0.033 \pm 0.005$ ). 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  $\pm$  S.E.M.  $\zeta$   $p < 0.05$  for 1 mM NAC group versus Control group;  $\#$   $p < 0.05$  for 1 mM NAC group versus  $\text{H}_2\text{O}_2$  treated group;  $*$   $p < 0.05$  for 1 mM NAC group versus 0.1 mM NAC group.**

#### Effects of NAC on WJ-MSCs under $\text{H}_2\text{O}_2$ induced oxidative stress

Oxidative stress induced by  $\text{H}_2\text{O}_2$  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 ( $0.0593 \pm 0.001$ ;  $0.070 \pm 0.000$ ), CAT activity ( $0.46 \pm 0.02$ ;  $0.36 \pm 0.019$ ), GSH level ( $0.169 \pm 0.002$ ;  $0.360 \pm 0.01$ ) and decrease in MDA level ( $0.05 \pm 0.000$ ;  $0.046 \pm 0.008$ ) in 1 mM NAC group compared to 100  $\mu\text{M/L}$   $\text{H}_2\text{O}_2$  treated group for SOD ( $0.051 \pm 0.002$ ;  $0.055 \pm 0.004$ ), CAT ( $0.81 \pm 0.04$ ;  $1.02 \pm 0.03$ ), GSH ( $0.105 \pm 0.002$ ;  $0.102 \pm 0.03$ ) and MDA ( $0.067 \pm 0.000$ ;  $0.077 \pm 0.004$ ) after 24 and 48 hours suggesting significant antioxidant effect of NAC Fig. 2.



**Fig. 2.** Effect of NAC treatment on H<sub>2</sub>O<sub>2</sub> 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. § p<0.05 for 1 mM NAC group versus Control group; # p<0.05 for 1 mM NAC group versus H<sub>2</sub>O<sub>2</sub> 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<sup>[21-23]</sup> due to their non-invasive procedure of isolation, multipotency and most preferably rapid availability.<sup>[5]</sup> Stem cell based therapies using MSCs have been effectively used for treatment of the damaged organs,<sup>[7, 24, 25]</sup> however ischemic environment of injured tissue diminished organ repair after stem cells transplantation.<sup>[26]</sup> The present study demonstrated that supplementation of NAC in H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub><sup>-</sup>), hydroxyl radical (HO<sup>-</sup>) and H<sub>2</sub>O<sub>2</sub><sup>[27]</sup> resulting in apoptosis of cells.<sup>[28]</sup> H<sub>2</sub>O<sub>2</sub> is the stable oxidant which causes oxidative injury by modification of proteins, lipids and DNA.<sup>[29]</sup> Previously, H<sub>2</sub>O<sub>2</sub> has been used as a model to study oxidative stress in vitro.<sup>[30-32]</sup> Therefore, to evaluate the protective effect of NAC on WJMSCs in vitro, we added H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sup>[6, 33]</sup> showing that H<sub>2</sub>O<sub>2</sub> treatment reduces the

cell proliferation. Furthermore, NAC reduced the LDH release which indicates the cell membrane integrity and viability.<sup>[7]</sup> 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<sup>[34]</sup> whereas CAT catalyzes the reduction of H<sub>2</sub>O<sub>2</sub>, thus protects from hydroxyl radicals induced injury.<sup>[35]</sup> Previous study showed that accumulation of superoxide anion and H<sub>2</sub>O<sub>2</sub> contributed to unfavorable conditions by decreasing the activity of SOD and CAT.<sup>[36]</sup> 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<sup>[37]</sup> pertained to free radicals mediated injury.<sup>[38, 39]</sup> Our results showed that H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> induced injury in WJMSCs in vitro.

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#### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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