RESEARCH ARTICLE

Minocycline pre-treatment up-regulates antioxidant enzymes and enhances the regenerative potential of MSCs in rat myocardial infarction model

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Abstract

Objective: To determine the effect of the pre-treatment of mesenchymal stem cells (MSCs) with minocycline on the expression of antioxidant genes and cardiac repair post myocardial infarction (MI) in rats.

Methods: Rat bone marrow derived MSCs were used in the study. Cytotoxicity of minocycline in MSCs was determined using JC1 assay to identify a safe drug dose for further experiments. The MSCs were pre-treated with 1.0 µM minocycline for 24 hours and then treated with hydrogen peroxide (H2O2), after that mRNA was isolated and the expression levels of antioxidant genes including peroxiredoxin, glutathione peroxidase, and superoxide dismutase were determined. Finally, minocycline pre-treated MSCs were used to treat rats induced with MI by the ligation of left anterior descending coronary artery. The cardiac function was evaluated at two and four weeks post MI using echocardiography.

Results: At 1.0 µM concentration, minocycline was found to be safe for MSCs and used for subsequent experiments. Minocycline pre-treatment was found to up regulate several antioxidant genes in oxidatively stressed MSCs. Furthermore, minocycline pre-treated MSCs displayed greater improvement in cardiac left ventricular function at two and four-weeks post MI as compared to untreated rats.

Conclusion: Pre-treatment of MSCs with minocycline enhances the expression of antioxidant genes and promotes their capability to repair cardiac function after MI.

Keywords: Antioxidants, Minocycline, Glutathione, Peroxiredoxins, Myocardial, Superoxide

(JPMA 73: S-3 [Suppl. 1]; 2023) **DOI: 10.47391/JPMA.AKUS-01**

Introduction

Myocardial infarction (MI) and consequent ischaemia/reperfusion injury is one of the leading causes of death across the globe. The major structural event that follows MI is remodelling of the left ventricle, which ultimately leads to heart failure[1]. The extent of this remodelling is used to predict the mortality of MI patients. The mechanisms for left ventricular remodelling are primarily, local ischaemia, inflammation, and oxidative stress[2]. Increased production of reactive oxygen species (ROS) by cardiac myocytes after MI causes damage to mitochondrial DNA, leading to tissue damage[3].

Several studies have shown that mesenchymal stem cells (MSCs) have the potential to repair cardiac function by regenerating cardiac tissue post MI injury[4]. However, it has been noted that a very low percentage of MSCs survive after implantation due to local ischaemic, oxidative stress, and inflammatory conditions[5]. Conditioning stem cells to increase their ability to resist local oxidative stress and inflammation can enhance their regenerative potential[6].

Minocycline is a commonly available drug that belongs to the 'tetracycline' class of antibiotics. It has been reported to exhibit cyto-protective effects in several cell types. Minocycline reduces tissue infarct size and oxidative stress after being taken up by cardiac cells following MI[7]. It has also been reported as a neuro-protective agent in acute stroke patients[8]. Additionally, minocycline preconditioned neural stem cells enhance neuroprotection in rat ischaemic stroke[9]. The co-administration of MSCs and minocycline leads to enhanced therapeutic effects in rodent models of multiple sclerosis (MS) and cerebral ischaemic injury[10, 11]. The cyto-protective effect of minocycline is potentially exerted by the inhibition of inflammatory cascade in cells[12,13]. Minocycline has also been shown to up regulate antioxidant genes and potentially scavenge reactive oxygen species (ROS)[7,14,15].

Based on previous independent findings which suggest that minocycline displays anti-oxidant and anti-inflammatory activity in various in vitro and *in vivo* models, we hypothesized that it will present similar effects on MSCs in vitro. Moreover, it will improve MSC survival when grafted at the site of MI and consequently enhance cardiac tissue regeneration.

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Materials And Methods

The present study was part of a project undertaken at the Stem Cell laboratory, International Centre for Chemical and Biological Sciences, University of Karachi between 2015 and 2020.

MSC isolation and culture: Bone marrow MSCs were isolated from the femur and tibia of Sprague Dawley rats. Bone marrow was flushed, and, cells were cultured in Dulbecco's Modifies Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were characterised using flow cytometric detection of cell surface markers and multi-lineage differentiation analysis (data not presented). P2-P4 MSCs were used for the experiments.

Cytotoxicity analysis of minocycline, JC-1 mitochondrial membrane potential assay was used to determine cytotoxicity of minocycline at concentrations ranging from 1µM to 30uM. Cells were treated with minocycline for 24 hours, harvested from the plates, stained with JC-1 (Cayman Chemicals Co., USA) according to manufacturer's instructions and subsequently analysed by flow cytometry (FACS Calibur, Becton, Dickinson & Co., USA). A minimum of 10,000 cells were acquired for each sample. The percentage of apoptotic cells in the MSC population was determined.

MSC pre-treatment and oxidative stress induction, MSCs were cultured on T25 flasks until approximately 80% confluence. The cells were divided in to three groups; (1) cultured in basal media, (2) subjected to oxidative stress using basal media containing 400 µM hydrogen peroxide (H2O2) for 2 hours, and (3) pre-treated with basal media containing 1 µM minocycline for 24 hours, followed by 400 µM H2O2 treatment for 2 hours.

RNA extraction and qPCR, RNA was isolated from all MSC samples using SV total RNA isolation kit (Promega, USA) and cDNA was synthesized using revert aid first strand cDNA kit (Promega, USA) according to manufacturer's instructions. Finally, the cDNA was amplified by real time quantitative polymerase chain reaction (RT-qPCR) using Go Taq qPCR master mix (Promega, USA) and specific primers for antioxidant genes (Table). The target gene expression data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The gene expression of minocycline treated and untreated MSCs was determined as fold change relative to MSCs cultured in basal media

Rat model of myocardial infarction and MSC treatment, All experiments were conducted after approval from institutional animal care and use committee (Approved on 28/08/2015). Sprague Dawley rats weighing 180-200 gm were anaesthetized by intraperitoneal administration of xylazine and ketamine HCl at 7 mg/Kg and 60 mg/Kg body weight, respectively. MI was induced by the ligation of left anterior descending coronary artery using the surgical procedure previously described[16]. The animals were divided in to four experimental groups sham, untreated (MI model), MSC treated (MI model), and minocycline pre-treated-MSC treated (MI model)

Table: Forward and reverse primer sequences for rat genes

Gene	Abbreviation	Primer sequence (5'>3')
Nuclear factor (erythroid-derived 2) like 2-	*NRF2*	F-GACAGCTGTAGTCCACATTTCCT R-AGCTTTACACAGGGACAGATCAC
Peroxiredoxin-1	*PRDX1*	F-CAAAGCCACGGCTGTTATGC R-TGGGTCCCAATCCTCCTTGT
Peroxiredoxin-2	*PRDX2*	F-TGTGACTAAAAGCTTGTCCCAGAAT R-TGTTTGGAGAAGTATTCCTTGCTGT
Peroxiredoxin-3	*PRDX3*	F-GACCCTAATGGTGTCATCAAGCA R- CAAAGCCCATGGAGCAGTACTTA
Peroxiredoxin-4	*PRDX4*	F-ATTGGGAAGGAACAGCTGTGATAA R-ATTGATCCAGGCCAAGTGAGTAAA
Glutathione peroxidase 1	*GPX1*	F-TTCCCGTGCAATCAGTTCGG R-TCCAGGAAATGTCGTTGCGG
Glutathione peroxidase 3	*GPX3*	F-GGCTCTTCAGGAAGGAAGCAAA R-GTTTGAGCAGGACCATTGGACT
Glutathione peroxidase 4	*GPX4*	F-GAGGCAGGAGCCAGGAAGTA R-ATAGCACGGCAGGTCCTTCT
Superoxide dismutase 1	*SOD1*	F-GTGGCCAATGTGTCCATTGAAG R-CAATCCCAATCACACCACAAGC
Superoxide dismutase 2	*SOD2*	F-CAGATTGCCGCCTGCTCTAAT R- CAAGAGCAGCTCACACCAAGT
Glyceraldehyde 6-phosphate dehydrogenase	*GAPDH*	F-TTCAACAGCAACTCCCATTCTTC R-CCTCTCTCTTGCTCTCAGTATCC

Cardiac function was assessed at 2 weeks and 4 weeks after induction of myocardial infarction using echocardiography. Rats were anaesthetised and left ventricular function was analysed using echocardiography machine (SV 3500, Aloka, Japan). Left ventricular internal diameter diastolic (LVIDd), left ventricular internal diameter systolic (LVIDs), fractional shortening (FS), end systolic volume (ESV), end diastolic volume (EDV), and ejection fraction (EF) were calculated[16].

Statistical analysis: GraphPad prism software was used for statistical analyses. The results were analysed using Mann-Whitney test and Kruskal Wallis test (post-hoc Dunn's multiple comparison test). Data are presented as mean ± standard deviation and a P value of less than 0.05

represented a statistically significant outcome denoted by asterisk (*).

Results

JC1 mitochondrial membrane potential assay was used to determine the least cytotoxic dose of minocycline for further experiments. It was found that at 1.0 µM minocycline treated MSCs displayed similar cell percentage of apoptotic cells as untreated MSCs, therefore for all subsequent experiments minocycline pre-treatment was performed at 1.0 µM concentration (Figure 1a).

The expression of antioxidant genes was analysed in minocycline pre-treated-MSCs and untreated MSCs subjected to oxidative stress. The expression levels were determined relative to MSCs cultured under normal conditions. The results revealed that minocycline pre-treated-MSCs expressed significantly higher levels of antioxidant genes including PRDX1, PRDX4, SOD2, GPX1, GPX2, and GPX3 relative to untreated MSCs (Figure 1b).

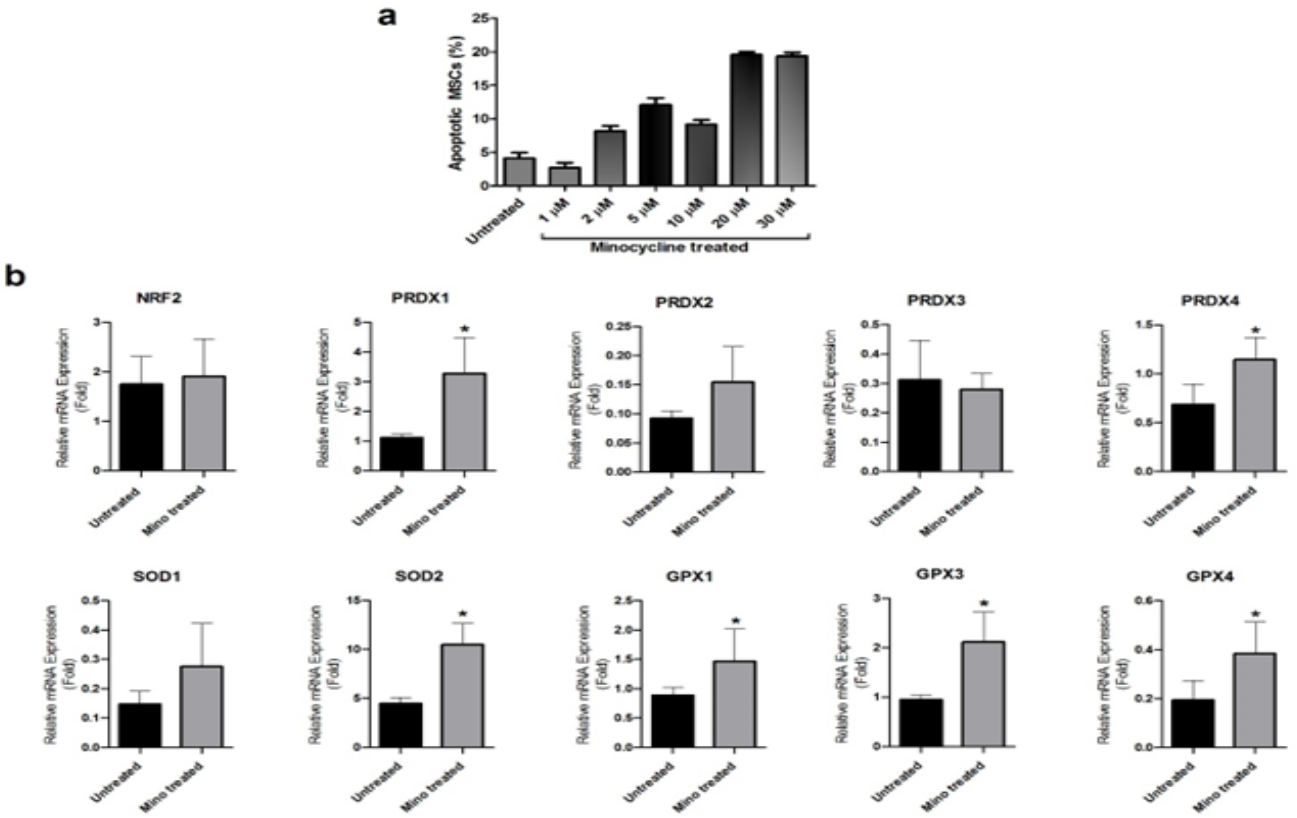

Figure-1: JC-1 mitochondrial membrane potential assay and relative gene expression of antioxidant enzymes. (a) Bar chart shows the percentage of apoptotic MSC in untreated and minocycline treated samples. (b) The gene expression levels in untreated and minocycline (mino) pretreated-MSCs are presented as fold change relative to normal MSCs. Data are expressed as mean ± SD; n=3; (*) represents $p < 0.05$.

The regenerative capability of minocycline pre-treated-MSCs and normal MSCs were analysed in a rat model of MI. At 2 weeks post-MI it was observed that minocycline pre-treated-MSCs significantly improved the fraction shortening and ejection fraction, while other feature of left ventricular function did not display significant improvement relative to the untreated group (Figure 2). Nevertheless, at 4 weeks post MI, the minocycline pre-treated-MSC group displayed significant improvement in left ventricular systolic function along with fraction shortening and ejection fraction relative to the untreated group. The left ventricular function appeared close to sham animals by 4 weeks in the minocycline pre-treated-MSC group, which indicated expedited healing as compared to the MSC treated group (Figure 3).

Discussion

The present study was intended to validate the antioxidant potential of minocycline in MSCs and whether pretreatment of MSCs with minocycline enhances their

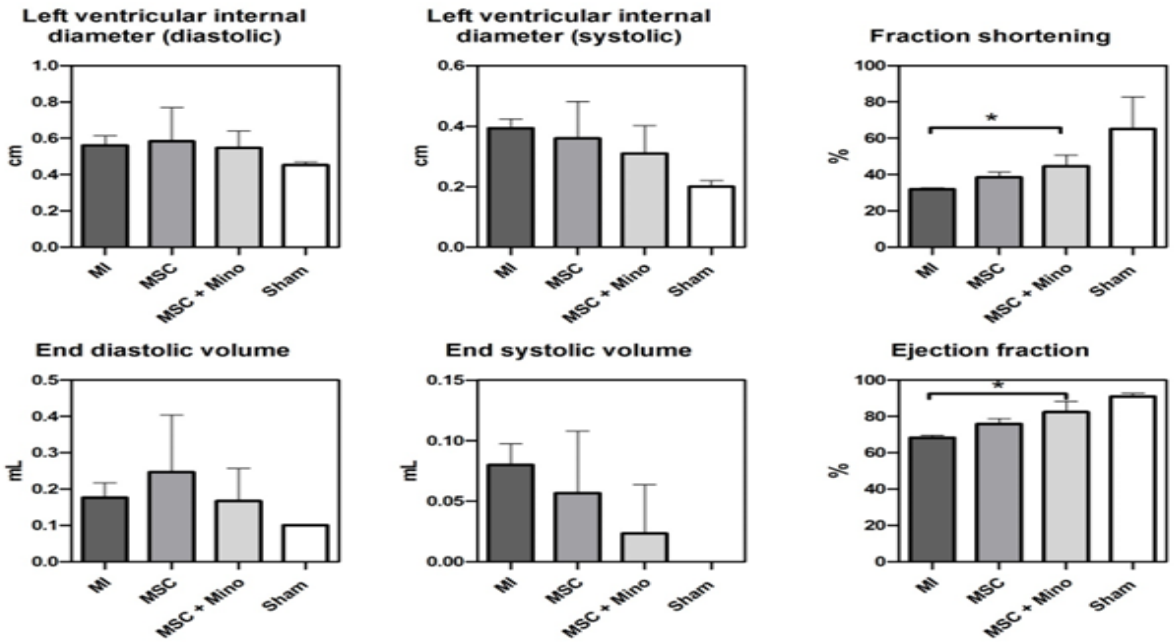

Figure-1: Analysis of left ventricular function at 2 weeks post MI. Bar graphs represents the measurements obtained using echocardiography in untreated, MSC treated, minocycline pretreated-MSC treated (MSC + Mino), and sham control animals. Data are expressed as mean ± SD; n=3; (*) represents $p < 0.05$.

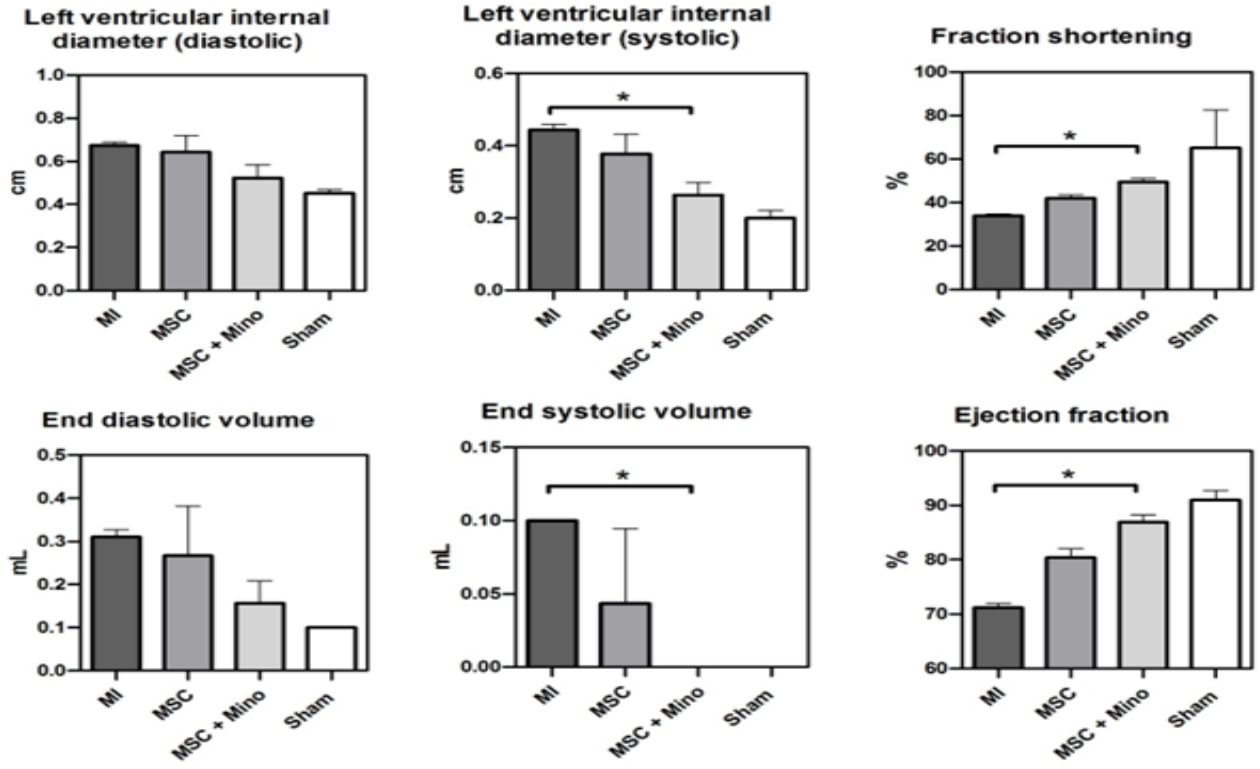

Figure-3: Analysis of left ventricular function at 4 weeks post MI. Bar graphs represents the measurements obtained using echocardiography in untreated, MSC treated, minocycline pretreated-MSC treated (MSC + Mino), and sham control animals. Data are expressed as mean ± SD; n=3; (*) represents $p < 0.05$..

regenerative potential in a rat MI model. The findings confirmed the hypothesis that minocycline upregulates antioxidant genes in rat bonemarrow derived MSCs as previously observed in other cells types. Furthermore, although the cardiac regenerative capability of MSCs is well established, the present data shows that minocycline pretreatment has the potential to enhance the regenerative capability of MSCs.

The antioxidant and cytoprotective effect of minocycline has been reported over the last several years. However, very few studies have focussed on these effects in case of MSCs. A range of doses from 1 to 100 µM concentration of minocycline have been tested in in vitro cell-based experiments[9,17,18]. Sakata et al. observed potent antioxidant effects at 10 µM dose in neural stem cells[9], similarly Wang et al. observed antioxidant activity at 2 µM concetration in kidney-derived cells[19], and Zhang et al. reported that a 4.0 µM concentration of minocycline decreased apoptosis in neuronal cells[17]. In the present study we performed JC1 assay to determine a safe dose of minocycline for MSCs. It was observed that at 1.0 µM concentration the number of apoptotic cells were similar to normal cells, therefore this concentration was used for subsequent gene expression and MI model-based analyses of minocycline pretreated MSC activity.

Minocycline preconditioning enhanced the expression levels in some of the variants of all three anitoxidant enzymes under investigation. Among peroxiredoxins and superoxide dismutases PRDX1,4 and SOD2 were upregulated, respectively, whereas all glutathione peroxidases displayed upregulation in pretreated MSCs. These findings are consistent with other independent studies that have reported that minocycline upregulates SOD and GPX in in vitro cell culture and its treatment results in elevated physiological levels of these enzymes *in vivo*[7,20,21]. At the same time the upregulation of PRDX by minocycline is a relatively novel finding. NRF2, which is a transcription factor that regulates several antioxidant genes was not found to be upregulated, which is in contrast with earlier studies that have shown that minocycline upregulates NRF2[22, 23]. However, those studies were conducted on cells other than MSCs, which could be the potential cause of this variation. Several previous studies have confirmed that minocyline reduces ROS levels[22], although, we did not directly examine ROS, but the upregulation of antioxidant genes provides an indication that minocyline activated the cellular response against H2O2 induced ROS production.

Minocycline has been found to exhibit cytoprotective activity and thus ameliorates the damaging effects of ischaemic events in the brain and heart [7,24]. It has been reported that the administration of minocycline to rodents with myocardial ischaemia reperfusion injury inhibits cardiomycocyte apoptosis and reduces oxidative stress[7,25]. These previous reports lead to the idea that pretreatment of MSCs with minocycline could potentially enhance their ability to survive in the post-infarction tissue environment and exert their regenerative effect at a greater level.

The present findings mandate further investigation of the cytoprotective role of minocycline for MSCs. A dose-response analysis of minocycline will be required to establish its optimal dose. This study specifically focused on echocardiography based evaluation of the rat hearts post MI, in future, a thorough histological assessment will provide greater insights to the impact of pretreated MSCs on the remodeling of infarcted hearts. Finally, the impact of minocycline treatment on the antiiflammatory and immunomodulatory activity of MSCs should be ascertained.

Conclusion

The cytoprotective and antioxidant activity of minocycline has been confirmed in several different cell types and in *in vivo* models of ischaemic injury. However, its effect on MSCs and their regenerative activity was unexplored. The present study confirms that minocycline pretreatment upregulates the antioxidant genes in MSCs subjected to oxidative stress. Moreover, the pretreated MSCs improve the left ventricular function to a greater extent as compared to untreated MSCs.

Disclaimer: None to declare.

Conflict of Interest: None to declare.

Funding Disclosure: None to declare.

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