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Protective effect of antioxidant Tempol on cardiac stem cells in chronic pressure overload hypertrophy

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ABSTRACT

Aims: Cardiac hypertrophy, an independent risk factor for cardiac failure; is associated with oxidative stress. Decline in the proportion of healthy cardiac stem cells (CSCs), possibly mediated by oxidative stress can lead to cardiac failure. The present study was carried out to examine the hypothesis that reduction of oxidative stress restores CSC efficiency and prevents progressive cardiac remodelling.

Materials and methods: Six-month old Spontaneously hypertensive rats (SHR) were supplemented with the antioxidant Tempol (20 mg/kg/day) for 14 days. The effect of Tempol on blood pressure and heart were assessed in SHR. Cardiac stem cells were isolated from atrial explants and expanded in culture for assessment of stem cell characteristics. Intracellular reactive oxygen species (ROS), proliferation, migration and senescence were evaluated in cultured atrial CSCs.

Key findings: Tempol treatment reduced blood pressure, regressed cardiac hypertrophy and reduced oxidative stress in SHR. Compared to Wistar rat, the efficiency of CSCs was significantly compromised in SHR. Tempol reduced intracellular ROS and restored migration potential and proliferative capacity along with reduction of senescent CSCs and expression of senescence proteins $p16^{ink4a}$ and p21.

Significance: Restoration of functional efficiency of CSCs by antioxidants signifies the role of oxidative stress in deterioration of stem cell attributes in the hypertrophic heart. The observations envisage the use of antioxidants as adjuvant medication for maintaining a healthy stem cell population, which can in-turn prevent progressive cardiac remodelling, a major determinant of cardiac failure.

1. Introduction

Resident cardiac stem cells play a vital role in the maintenance of a healthy myocardium [1]. The importance of maintaining a healthy stem cell pool for preventing adverse cardiac remodelling has not received much attention. Hypertension induced progressive cardiac remodelling is a leading cause for cardiac failure [2]. In pathological remodelling and physiological aging, CSCs are adversely affected [3]. Cardiac remodelling is associated with the generation and release of reactive oxygen species (ROS), that play an important role in the pathogenesis of heart failure. Oxidative stress is also implicated in myocardial ischemia and cardiac hypertrophy; as well as age associated cardiac failure.

The impact of myocardial oxidative stress on cardiac stem cells in the pathological heart has not drawn the attention of researchers. Embryonic stem cells and mesenchymal stem cells were found to be sensitive to oxidative stress and is also implicated in the progenitor and stem cell dysfunction in cardiovascular diseases. Life span of stem cells and endothelial progenitor cells (EPCs) are adversely affected in the presence of oxidative stress. This leads to the assumption that deterioration of stem cell attributes mediated by reactive oxygen species (ROS) can contribute to progressive cardiac remodelling in pathological hypertrophy. Treatment with antioxidants at an early stage is reported to be beneficial to the ailing heart [4]. However, information regarding the dynamic effects of antioxidant therapy on the characteristics of CSCs is lacking. Tempol is a redox-cycling, metal-independent, membrane permeable antioxidant. Antioxidant therapy with Tempol has been reported to increase the survival in rats with hemorrhagic shock and chronic heart failure [5,6]. Tempol treatment reversed dexamethasone induced hypertension in rat [7]. When delivered to the myocardium during cell transplantation, Tempol improved the survival of skeletal myoblasts and cardiomyoblasts [8]. Given the potential link between CSC efficiency and oxidative stress in the pathological heart, it is logical to hypothesize that antioxidants can restore the stem cell attributes. This hypothesis is based on the premise that maintaining a

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healthy stem cell population can prevent progressive cardiac remodelling in chronic pressure overload. The study was designed with the objective of examining whether supplementation with the antioxidant Tempol can restore the stem cell attributes in the myocardium of spontaneously hypertensive rat. This is the first study to report the compromised efficiency of CSCs in pathological hypertrophy and the restoration of the functional efficacy on supplementation with antioxidants.

2. Materials and methods

All animal procedures for this study were approved by the Institutional Animal Ethics Committee of Sree Chitra Tirunal Institute for Medical Sciences and Technology, according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Guidelines.

Spontaneously hypertensive rat (SHR) was used as the experimental model as the cardiac remodelling resembles the clinical course of hypertension. The breeding stock was obtained from Animal Resource Center, Perth, Australia. Six-month-old animals were selected as this represents the stable phase of hypertrophy in SHR [9]. Myocardial oxidative stress was also significantly high in SHR compared to Wistar rat [10]. The animals were housed in independent cages at 22 °C, maintained on a 12 h light-dark cycle, fed with regular Rat Chow and had free access to drinking water.

2.1. Treatment

To examine the effect of the antioxidant Tempol on CSCs cells, 12 male SHR (6 months old) were randomly assigned into two groups of six rats each. One group received intra peritoneal injection of 20 mg/kg/day Tempol (4-hydroxy Tempol, Sigma, #176141) for 2 weeks. Untreated SHR served as hypertensive control. Six age-matched male Wistar rat served as normotensive control. Untreated SHR and Wistar rat received the vehicle. Following the treatment, blood pressure was determined non-invasively by the tail-cuff method

(NIBP system, IN125/R, AD Instruments) and LV function was assessed by 2-D echocardiography (GE 2D Echo Machine, 10 mhz non-linear probe).

2.2. Isolation of cardiac stem cells (CSCs)

CSCs were isolated from atria, as a major proportion of CSCs reside in hypoxic niches of the atrium [11]. C-kit$^+$ CSCs were isolated as previously reported with minor modifications [12]. Briefly, atria were minced into bits and seeded onto the surface of 2% gelatin coated plates containing Iscove's Modified Dulbecco's Medium (IMDM) along with the supplements. Within 2-weeks of culture, small, round, phase bright cells were seen over a layer of fibroblast like outgrowth from the explants. C-kit$^+$ cells were sorted immunomagnetically using Easy Sep magnet (Stem Cell Technologies, #18000) and FITC positive selection kit (Stem Cell Technologies, #17682). Cultured CSCs from passage 3 were used for evaluation of stem cell attributes.

2.3. Colony forming unit assay

Cells were seeded at a density of 500 cells per 60 mm culture plate. After 2 weeks, colonies larger than 2 mm were counted.

2.4. Growth kinetics, growth rate and population doubling time

10,000 cells were seeded in 35 mm dishes. Cell numbers were determined every 48 h using a Neubauer improved haemocytometer up to the 10th day. Growth rate was calculated using the formula, $GR = \ln(N_t/N_0)/T$ and population doubling time was calculated using the formula, $PDT = \ln(2)/GR$, where T is the incubation time, N_0 is the cell number at the beginning of the incubation time and N_t is the cell number at the end of the incubation time.

2.5. Determination of cellular ROS levels

ROS levels were determined by DCFDA (Sigma, #D6883) fluorescence assay [13]. The fluorescence intensity was measured using ELISA plate reader. It was done in cultured CSCs and was repeated thrice for each of the animals.

2.6. Cell migration assay

For trans-well migration assay, 1×10^4 cells in 300 µL of serum free IMDM were seeded on to the upper chamber of trans-wells (BD Falcon inserts, 8 µm pore size). Cells were allowed to migrate towards 10% serum containing IMDM for 18 h following which, the cells on the upper surface of the membrane was wiped away and migrated cells were fixed and stained. The mean cell count from six random fields was used as a measure of cell migration.

2.7. Proportion of senescent cells

Senescence-associated β-galactosidase staining (Abcam, #102534) was used as a biomarker of senescence in CSCs. The proportion of stained cells was expressed as percentage of senescent cells. The expression levels of senescence associated proteins; $p16^{ink4a}$ (Abcam, #54210) and p21 (Abcam, #ab80633) were detected by Western blot analysis.

2.8. Statistical analysis

Values are presented as mean ± SD. Comparisons between groups were analyzed by ANOVA followed by Student's *t*-test (two-tailed). Results were considered statistically significant for p values < 0.05.

3. Results

3.1. Isolation, culture, and characterization of CSCs

Atrial explants were established in culture; and within 14 days, small, round, phase bright cells were seen loosely attached over a layer of fibroblast like cells. The sorted CSCs were expanded in culture to get sufficient number of cells for the evaluation of different variables. Flow cytometric analysis and immunocytochemistry confirmed the purity of cells in the 3rd passage. The cells were positive for c-kit and negative for CD34 and CD45. Approximately 96 ± 2% of the cells that adhered to the culture surface formed colonies, further confirming the stemness. Cultures in the third passage were therefore used for assessment of stem cell attributes.

3.2. Cardiovascular response to Tempol

Reduction of oxidative stress was confirmed following 14 days of supplementation with Tempol (Fig.1). Reduction of blood pressure as assessed by tail-cuff method and regression of hypertrophy as determined by 2-D echocardiography was observed in response to the treatment (Tables 1 and 2).

3.3. Effect of Tempol on cellular ROS levels of CSCs

Cellular ROS levels were significantly higher in CSCs from SHR ($p < 0.01$ when compared with WST rat). As expected, ROS levels decreased significantly on treatment with the antioxidant Tempol, the values being comparable with that of normotensive control ($p = 0.6$) (Fig. 2).

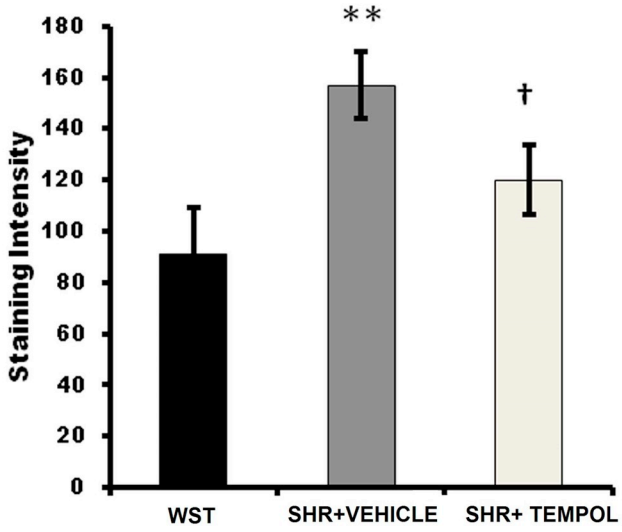

Fig. 1. Effect of Tempol on myocardial 3-nitrotyrosine levels determined by immunohistochemistry (Representative photomicrographs and Graphical representation of staining intensity).
Data presented as mean ± SD. Variation was analyzed by ANOVA followed by *t*-test. **p < 0.01 SHR Vs WST; ##p < 0.01 SHR + vehicle Vs SHR + Tempol. One way ANOVA p < 0.01 (n = 6 animals/group, 3 images were analyzed per animal).

Table 1
Effect of Tempol treatment on blood pressure as assessed by Tail cuff method.

	Wistar	SHR + vehicle	SHR + Tempol
SBP	115.2 ± 12.2	165 ± 14.8††	131 ± 11.5*
DBP	76 ± 9.8	114 ± 14.3††	94 ± 10.1*

SBP: Systolic Blood Pressure in mm Hg.
DBP: Diastolic Blood Pressure in mm Hg.
Data presented as mean ± SD. (n = 6) Variation was analyzed by one-way ANOVA (p < 0.01) followed by Student *t*-test.
†† p < 0.01 SHR+ Vehicle Vs WST.
* p < 0.05 SHR + Vehicle Vs SHR+ Tempol.

Table 2
: Effect of Tempol treatment on LV function as assessed by 2D-echocardiography.

	Wistar	SHR + vehicle	SHR + Tempol
LV mass	495 ± 21	684 ± 37†	593 ± 22*
RWT (Relative Wall Thickness)	0.68 ± 0.05	0.83 ± 0.06†	0.78 ± 0.04
E/A ratio	2.1 ± 0.34	1.12 ± 0.27†	1.46 ± 0.22

LV-Left Ventricle, E/A ratio - ratio of the maximal velocities of E and A waves.
Data presented as mean ± SD. (n = 6) Variation was analyzed by one-way ANOVA (p < 0.01) followed by Student *t*-test.
† p < 0.05 SHR+ Vehicle Vs WST.
* p < 0.05 SHR + Vehicle Vs SHR+ Tempol.

3.4. Effect of Tempol on self- renewal capacity, growth kinetics, population doubling time (PDT) and growth rate of CSCs

SHR exhibited a significant decrease in CFU when compared with Wistar rat (Fig. 3A). However, treatment with Tempol enhanced the ability of CSCs to form colonies (p < 0.01 when compared to untreated

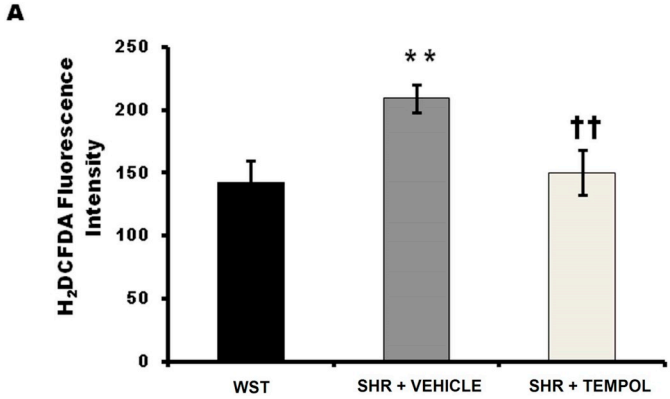

Fig. 2. Effect of Tempol on cellular ROS levels in CSCs assessed by H_2DCFDA fluorescence intensity.
The fluorescence intensity was measured using ELISA plate reader. It was carried out on cultured CSCs and was repeated thrice for each of the animals (n = 6 animals/group).
Data presented as mean ± SD. Variation was analyzed by ANOVA followed by *t*-test. **p < 0.01 SHR Vs WST; ##p < 0.01 SHR = vehicle Vs SHR + Tempol. One way ANOVA p < 0.01.

control). The extent of CFU was comparable with that of WST (p = 0.08).

Following 10 days in culture, the yield of $134.33 \pm 14.04 * 10^4$ cells per dish was obtained from WST rat compared to $65.33 \pm 6.5 * 10^4$ cells from untreated SHR (Fig. 3B). The mean cell yield in cultures from treated SHR, $111 \pm 8.5 * 10^4$ was comparable with that of WST. CSCs from SHR exhibited a significantly decreased growth rate (p < 0.01) and significantly increased PDT (p < 0.01) when compared with CSCs from WST. However, consequent to the

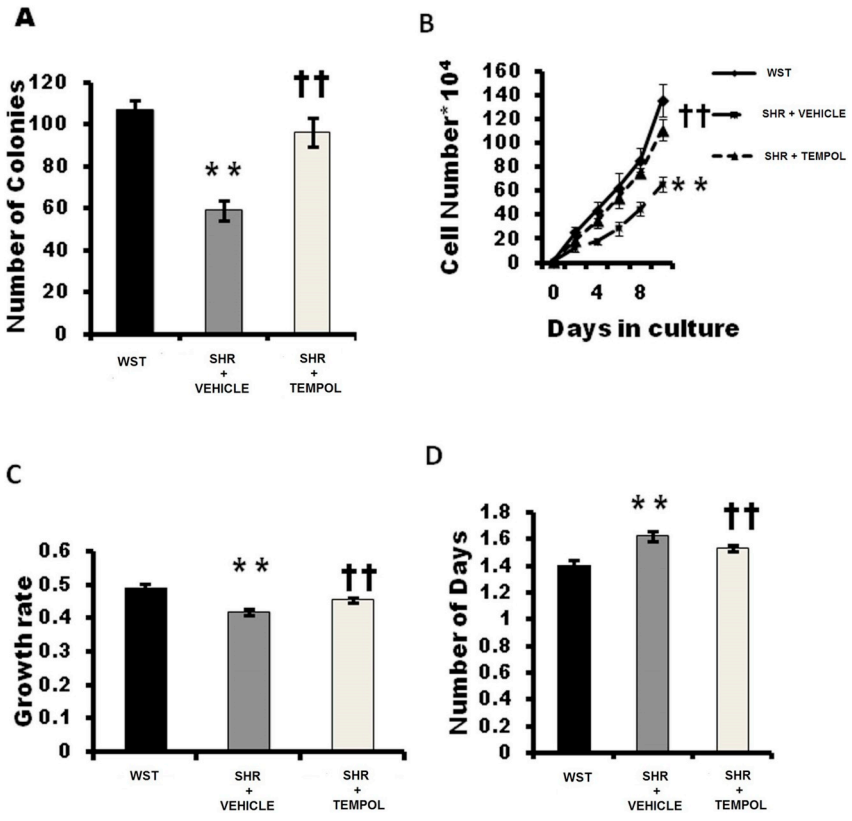

Fig. 3. Effect of Tempol on self-renewal capacity, growth kinetics, growth rate and population doubling time of CSCs: A) Self-renewal capacity assessed by colony formation, represented as number of colonies/ culture plate (60 mm diameter) B) growth kinetics of CSCs represented as cell number $* 10^4$, C) growth rate of CSCs calculated as Log (N) of the ratio of cell number at two fixed time points, D) population doubling time represented as number of days.
Data presented as mean ± SD. Variation was analyzed by ANOVA followed by *t*-test. **p < 0.01 SHR Vs WST; $^{\#\#}$p < 0.01 SHR + vehicle Vs SHR + Tempol, ANOVA p < 0.01(A-D) (n = 6 animals/group).

treatment, GR and PDT of CSCs improved; and the values were comparable with that of normotensive control and significantly different when compared to CSCs from untreated SHR (p < 0.01 for both GR and PDT).

3.5. Effect of Tempol on migration potential of CSCs

Migratory capacity of CSCs was evaluated by Trans-well migration assay. CSCs from SHR exhibited 37% decline in the number of migrated cells when compared to the WST rat (p < 0.01) (Fig. 4A). A two fold increase was observed on treatment with Tempol, with the migration rate being comparable to Wistar rat and significantly higher compared to untreated SHR (p < 0.01).

3.6. Effect of Tempol on senescence of CSCs

A four-fold increase in the proportion of senescent cells was observed in CSCs of SHR when compared with that of WST (p < 0.01) (Fig. 4B). In response to the treatment, the number decreased significantly and was comparable with that of WST. The expression of nuclear proteins involved in cellular aging - $p16^{ink4a}$ and p21 were significantly higher in SHR compared to WST. However, on treatment with Tempol, the expression levels of both proteins decreased and were comparable with WST (Fig. 4C, D).

4. Discussion

The role of cardiac stem cells in pathological remodelling is acknowledged, but its modulation by antioxidants has not been reported. Hypertension induced hypertrophy is associated with oxidative stress, [10] and the latter is also implicated in left ventricular remodelling in Spontaneously hypertensive rat [10,14]. Although the adverse effect of reactive oxygen species and free radicals on pathological remodelling is acknowledged, its effect on CSCs has not received much attention. Deterioration in efficiency of mesenchymal stem cells in lung disorders has been reported [15]. To our knowledge, the effect of antioxidants on CSCs in chronic pressure overload has not been studied earlier. Reduction of oxidative stress by the use of anti-hypertensive like metoprolol has been reported to be beneficial in restoring cardiac stem cell function along-side protecting the heart [16]. Tempol has been shown to attenuate oxidative stress in endothelial cells [17–20]. With this background, the study was carried out to examine whether Tempol administration benefits the cardiac stem cell population during hypertensive stress. This study for the first time demonstrates that the efficiency of cardiac stem cells can be restored by supplementation with antioxidants, thereby preventing progressive cardiac remodelling.

Six-month old spontaneously hypertensive rats were treated with the SOD mimetic Tempol for 14 days. Age matched Wistar and untreated SHR served as controls. Measurement of blood pressure and echocardiographic analysis, established the cardioprotective effect of Tempol, marked by reduction of blood pressure, decrease in left ventricular mass and improvement in function (Tables 1, 2). Myocardial tissue oxidative stress was also reduced on supplementation with Tempol (Fig. 1). Previous studies also show similar results supporting our findings of Tempol being a anti-hypertensive drug [21] and a potent antioxidant [7,22]. Meta-analysis studies revealed the potential role played by ROS in inducing hypertension and the beneficial role of Tempol in reducing it [21]. These studies reiterate the importance of reducing the oxidative stress for maintaining a healthy heart. An earlier study reported that compromised antioxidant defense and an increase in oxidative damage contributed to the development of hypertension in 2 K-1C rats, and that Tempol treatment prevented these effects [23].

Reduction of blood pressure and restoration of LV function in response to Tempol was as expected. Therefore, CSCs were immunomagnetically isolated from atrial explant culture and used for further analysis.

Tempol treatment enhanced the growth kinetics of CSCs of SHR (Fig. 3). Restoration of the proliferative capacity implies that the antioxidant has a protective effect on CSCs, which can in turn generate new myocytes and vascular cells in the event of a pathological insult.

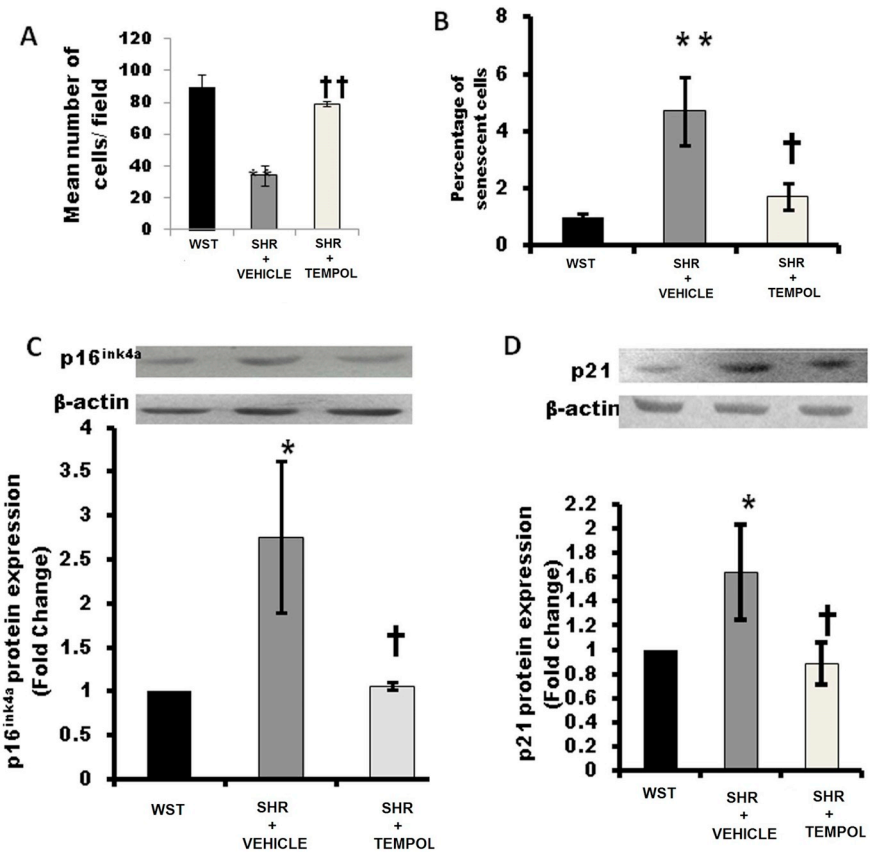

Fig. 4. Effect of Tempol on the migration potential and senescence of Cardiac stem cells.
A) Migration ability of CSCs represented as mean number of cells/field. B) Proportion of senescent cells determined by senescence associated β-galactosidase staining, expressed as percentage C) representative blot and graphical representation of $p16^{ink4a}$ protein expression by CSCs as evaluated by Western blotting, D) representative blot and graphical representation of p21 protein expression as evaluated by Western blotting.
Data presented as mean ± SD. Variation was analyzed by ANOVA followed by *t*-test (**$p < 0.01$ SHR Vs WST; $^{\#\#}p < 0.01$ SHR + vehicle Vs SHR + Tempol) ANOVA $p < 0.01$ (n = 6animals/group).

Though no reports are available on CSCs, studies have shown that addition of antioxidants enhanced the growth rate of adipose derived mesenchymal stem cells [24].

Redox homeostasis is critically important in the regulation of stem cell self-renewal and differentiation [25,26]. This study has also demonstrated that, diminished self renewal capacity in hypertensive heart disease as determined by CFU assay was enhanced on treatment with the antioxidant, establishing the improvement in efficiency of CSCs (Fig. 3A). The positive impact of Tempol on self-renewability of CSCs (Fig. 3A–D) was also accompanied by the maintenance of stemness.

Migration facilitates the homing of stem cells to the site of injury for tissue repair and is an indicator of stem cell efficacy. Stress is known to promote homing of stem cells to heart by upregulation of SDF [27,28]. In adipose derived stem cells, reactive oxygen species were shown to play a key role in the proliferation and migration [29,30]. The compromised migration potential of CSCs from SHR at 6 months of age implies impairment of function of CSCs at the early stages of hypertrophy (Fig. 4A). The decline in migratory capacity of stem cells in SHR was restored upon treatment with the SOD mimetic, reiterating the role of ROS in stem cell dysfunction.

Our study has demonstrated for the first time, the reduction of cellular ROS levels in CSCs upon supplementation with antioxidants (Fig. 2). Tempol has been reported to act as a potent intracellular antioxidant [31]. Antioxidant supplementation is expected to retain the stem cells in an undifferentiated state and prevent depletion of the stem cell pool. Senescence or cell cycle growth arrest can be driven by oxidative stress and can affect the typical behavior of stem cells [32]. The presence of senescent CSCs in SHR (Fig. 4B) represents the consequence of the prevailing adverse microenvironment compared to the WST. The decrease in the proportion of senescent β-galactosidase positive cells implies that decrease in oxidative stress facilitated the recovery of CSCs in treated SHR. Expression of senescent markers $p16^{ink4a}$ and p21 were also in concordance with the beta-galactosidase staining (Fig. 4 C,D), reiterating the positive effects of Tempol. Reduction of oxidative stress in the microenvironment (Fig.1) is presumed to modulate the stem cell characteristics. In the senescent rat heart, long term Tempol treatment restored pharmacological preconditioning, providing a new protective strategy for the ischemic myocardium in the elderly [33].

The antihypertensive property of Tempol [21] has been established; and in human hypertension, biomarkers of systemic oxidative stress are elevated [34–37]. Being a superoxide dismutase mimetic, Tempol facilitates hydrogen peroxide metabolism and limits formation of toxic hydroxyl radicals. In-vivo and in-vitro studies have shown that Tempol detoxifies reactive oxygen species, thereby modulating various intracellular signaling pathways [38]. Hypertensive end-organ damage is attributed to vascular oxidative stress [39,40]. By virtue of the fact that Tempol confers beneficial effect on many cell-types, the cardioprotective response can be attributed to the concerted outcome of multiple anti-oxidative mechanisms.

5. Conclusion

This study has demonstrated that, the functional efficiency of cardiac stem cells was compromised in SHR and that the antioxidant Tempol restored the stem cell characteristics; comparable to that in normotensive rat heart. The results suggest that ROS plays an important role in modulating stem cell characteristics in hypertension induced hypertrophy, which can lead to cardiac failure. The beneficial effects of Tempol has been attributed to its antioxidant capacity. The finding of improvement in stem cell efficiency in accompaniment with reduction of cardiac hypertrophy underscores the importance of maintaining a healthy stem cell reserve in the management of hypertensive heart disease by supplementation with antioxidants. Maintenance of stem cell homeostasis is expected to prevent progressive cardiac remodelling as a measure for prevention of cardiac failure.

Author contribution

S Saheera - designed the study, performed experiments, analyzed data and prepared the manuscript.

A G Potnuri - designed and performed the animal experiments.

R R Nair - conceived the study and edited the manuscript.

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Conflict of interest

The Authors declare no “Conflict of interest.”

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