

Original Paper

# Thrombopoietin Protects Cardiomyocytes from Iron-Overload Induced Oxidative Stress and Mitochondrial Injury

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## Key Words

Cardiac Failure • Oxidative Stress • Iron-overload • TPO • Fluorometry • Cytometry

## Abstract

**Background/Aims:** Thalassemia accompanied with iron-overload is common in Hong Kong. Iron-overload induced cardiomyopathy is the commonest cause of morbidity and mortality in patients with  $\beta$ -thalassaemia. Chronic iron-overload due to blood transfusion can cause cardiac failure. Decreased antioxidant defence and increased ROS production may lead to oxidative stress and cell injury. Iron-overload may lead to heart tissue damage through lipid peroxidation in response to oxidative stress, and a great diversity of toxic aldehydes are formed when lipid hydroperoxides break down in heart and plasma. **Methods:** Iron entry into embryonic heart H9C2 cells was determined by calcein assay using a fluorometer. Reactive oxygen species (ROS) production in cells treated with  $\text{FeCl}_3$  or thrombopoietin (TPO) was monitored by using the fluorescent probe  $\text{H}_2\text{DCFDA}$ . Changes in mitochondrial membrane potential of H9C2 cells were quantified by using flow cytometry. **Results:** We demonstrated that iron induced oxidative stress and apoptosis in cardiomyocytes, and that iron increased ROS production and reduced cell viability in a dose-dependent manner. Iron treatment increased the proportion of cells with JC-1 monomers, indicating a trend of drop in the mitochondrial membrane potential. TPO exerted a cardio-protective effect on iron-induced apoptosis. **Conclusions:** These findings suggest that iron-overload leads to the generation of ROS and further induces apoptosis in cardiomyocytes via mitochondrial pathways. TPO might exert a protective effect on iron-overload induced apoptosis via inhibiting oxidative stress and suppressing the mitochondrial pathways in cardiomyocytes.

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## Introduction

Thalassaemia accompanied with iron-overload is common in Hong Kong. Iron-overload induced cardiomyopathy is the commonest cause of morbidity and mortality in patients with -thalassaemia. Chronic iron-overload due to blood transfusion can cause cardiac failure. It has been reported that iron-overload can cause toxic effect on heart, bone and nerve cells.

Iron-overload may induce apoptosis in cardiomyocytes, and thrombopoietin (TPO) may exert a protective effect on this toxicity [1, 2]. *In-vivo* studies showed that iron-overload can cause eccentric cardiac hypertrophy, impairing cardiac output and exercise capacity [3, 4]. It is known that free radical species are important for biological functions. Excessive free radical species can induce tissue damage by disrupting lipids, DNA and proteins, leading to severe oxidative damage to cells [5]. Excess iron induced the over-production of superoxide ( $O_2^-$ ) and hydroxyl ( $OH^\cdot$ ) radicals via Fenton and Haber-Weis reactions, leading to cellular damage [5-7]. Decreased antioxidant defence and increased ROS production may lead to oxidative stress and cell injury. Iron-overload may lead to heart tissue damage through lipid peroxidation in response to oxidative stress, and a great diversity of toxic aldehydes are formed when lipid hydroperoxides break down in heart and plasma [8, 9]. These agents are associated with toxic effects on intracellular organelles, and then cause either apoptotic or necrotic cell death [10]. There are many sources of ROS, including mitochondria, xanthine oxidase, uncoupled nitric oxide synthases and NADPH oxidases, all of them contribute to the development of cardiomyocyte hypertrophy [11].

Thrombopoietin (TPO), the c-Mpl ligand, is the primary physiologic regulator of megakaryocyte and platelet development. It can also protect  $CD34^+$  cells and megakaryocytes from undergoing apoptosis [12, 13]. It has been shown that the production of ROS during the oxidative stress response could cause cardiomyocyte apoptosis, [14] playing an important role in ischemic heart disease, and cardiac failure [15]. Thus, in this study, we investigated iron exposure induced oxidative stress and its relation to cardiomyocyte apoptosis. The protective effect of TPO and its mechanism on iron induced cardiotoxicity were also investigated.

## Materials and Methods

### *H9C2 cells*

Embryonic H9C2 cells (American Type Tissue Collection, Manassas, Va) were cultured in IMDM or DMEM supplemented with 10% fetal calf serum in a 5%  $CO_2$  incubator at 37°C. All media and culture reagents were purchased from Gibco (Grand Island, NY). For oxidative stress analysis, H9C2 cells were seeded in 96 well plates at  $5 \times 10^3$  cells per well for 24 hours. For apoptosis analysis, H9C2 cells were seeded in 6 well plates at  $2 \times 10^5$  cells per well for 24 hours. Then, cells were incubated with  $FeCl_3$  (Sigma) or TPO (50 ng/ml [1], R&D) for a further 72 hours.

### *Monitoring iron entry into H9C2 cells*

H9C2 cells were exposed to 2.5  $\mu M$  calcein green (Molecular Probes, Eugene, OR) in DMEM containing 10 mM Na-HEPES (*N*-2-hydroxyethylpiperazine-*N*,-2-ethanesulfonic acid) and 1 mg/mL bovine serum albumin for 10 minutes at 37°C, and washed with preheated HEPES-buffered saline (HBS, pH 7.4). Then, the cells were exposed to  $FeCl_3$  (0.15 mM) in HBS containing 0.5 mM probenecid for 50 minutes at 37°C. Probenecid was added to prevent leakage of the anionic fluorescent probes from cells [14]. To observe the chelating effect, an intracellular iron chelator, ICL 670 was obtained from Roche. One hundred microliters of stock solution was added to each culture at five minutes intervals to give final concentrations of 0.2, 0.6, 1.0, 1.4 and 1.8 mM ICL670. The fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm using a fluorometer (Fusion,  $\alpha$ -FP).

### *Measurement of Oxidative Stress using 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA) fluorescence assay*

ROS production in cells treated with  $FeCl_3$  or TPO was monitored by using  $H_2DCFDA$  as fluorescent probe (Sigma); once inside the cell,  $H_2DCFDA$  is deacetylated by intracellular esterases to become  $H_2DCFH$ . It can

be converted by hydrogen peroxides and hydroxyl radicals in cells to fluorescent 2',7'-dichlorofluorescein (DCF). H9C2 cells were resuspended in DMEM with 2% FCS.  $\text{FeCl}_3$  (0.075-0.6 mM), or  $\text{FeCl}_3$  (0.3 mM) with/without TPO (50 ng/ml) was added to the cells for 24 hours, respectively. Then, the cells were treated with H2DCF-DA (5  $\mu\text{M}$ ) in phosphate buffered saline at 37°C. DCF fluorescence produced by the cells was continuously measured up to 160 minutes or four hours at an excitation wavelength of 485 nm and an emission wavelength of 538 nm using a fluorometer.

#### Detection of apoptosis, and mitochondrial membrane potential in H9C2 cells treated with $\text{FeCl}_3$ and TPO by using flow cytometry

H9C2 cells were cultured in IMDM supplemented with 10% FCS. To investigate the apoptotic effect of  $\text{FeCl}_3$ , cells were resuspended in IMDM with 2% FCS, and then incubated with  $\text{FeCl}_3$  (0.0375 mM to 0.6 mM), or  $\text{FeCl}_3$  (0.3 mM) with/without TPO for 72 hours. Then, the cells were stained with Trypan blue and observed under microscope, and stained with Annexin V/PI or anti-active caspase-3/FITC antibody (BD Biosciences, San Diego) and analyzed by using flow cytometry, respectively.

To investigate the mitochondrial membrane potential changed in H9C2 cells treated with  $\text{FeCl}_3$  and TPO, cells were resuspended in IMDM with 2% FCS, and then incubated with  $\text{FeCl}_3$  (0.3 mM) with/without TPO for 72 hours, and then were stained with JC-1 (5, 5', 6, 6'-tetrachloro-1, 1',3, 3' Tetraethylbenzimidazolcarbocyanine iodide) (BD Biosciences, San Diego) and analyzed by using flow cytometry.

Ten thousand events were acquired for each sample and analyzed by WinMDI2.8 software.

#### Statistical analysis

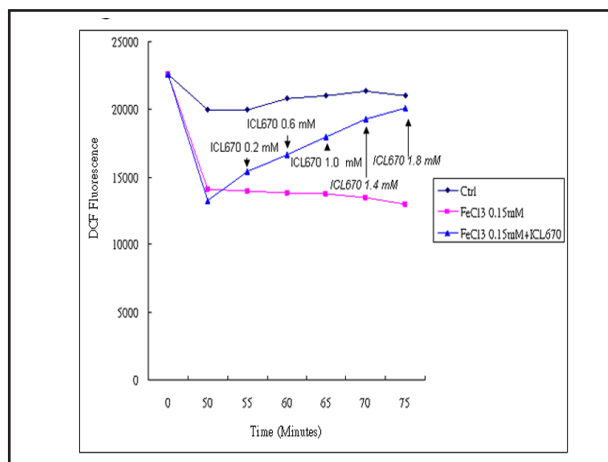
The mean and SEM were computed for each treatment group. Statistical significant differences among treatment groups and control group were detected by the One Way Anova Test with Turkey's Multiple Comparison Test for all pairwise multiple comparisons (Glantz SA. Primer of biostatistics, 3<sup>rd</sup> Edition., New York: McGraw Hill; 1992.). A *P* value of less than 0.05 was considered to be statistically significant. Three levels of significance were denoted as: \*: *P*<0.05, \*\*: *P*<0.01, and \*\*\*: *P*<0.001.

## Results

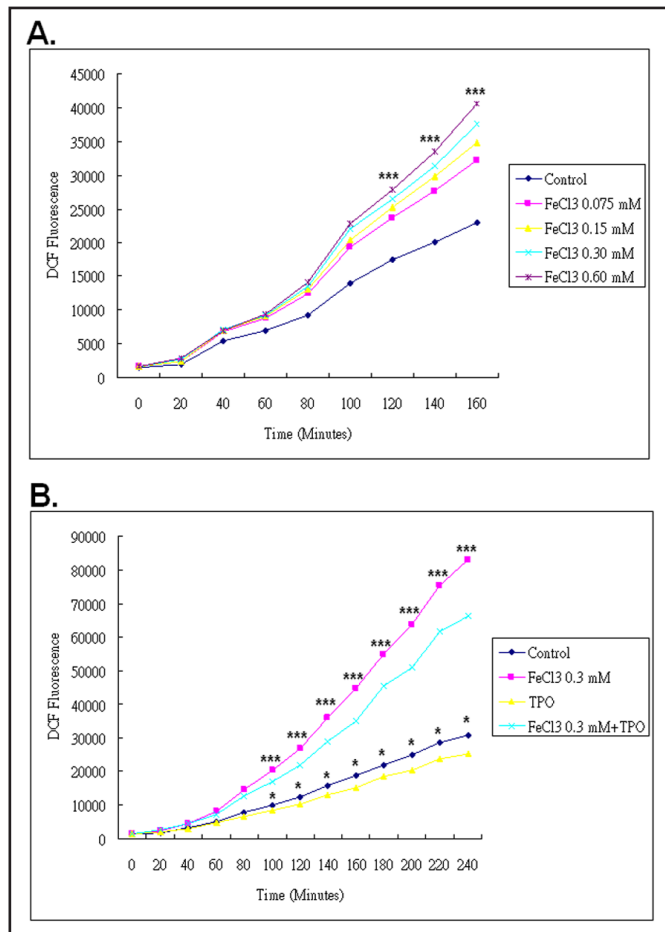
### Iron entry into cardiomyocytes

H9C2 cells were stained with calcein green with high fluorescent signal and then incubated with  $\text{FeCl}_3$  (0.15 mM) at 37°C (Fig. 1). The intracellular calcein green fluorescence signal was quenched by  $\text{FeCl}_3$  and reached a steady state after 50 minutes (Fig. 1). Upon subsequent addition of ICL670, an intracellular iron chelator, the intracellular fluorescence signal underwent a dose-dependent restoration (Fig. 1), indicating that the previous quenching was specifically due to  $\text{Fe}^{3+}$  entry.

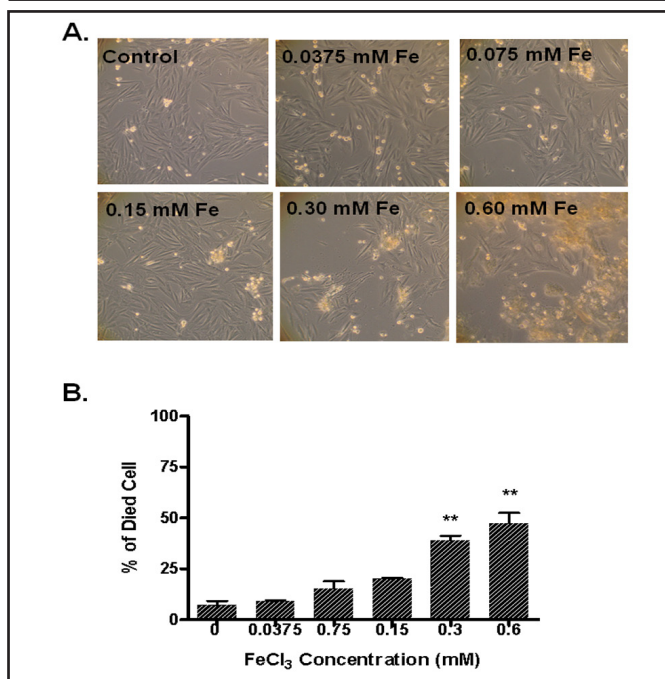
**Fig. 1.** Monitoring iron entry into cardiomyocytes. H9C2 cells labeled with calcein green were exposed to  $\text{FeCl}_3$  (0.15 mM) for 50 minutes. ICL 670 was added at 5-minute intervals to give final concentrations of 0.2, 0.6, 1.0, 1.4 and 1.8 mM. All reactions were carried out at 37°C. The average fluorescence was shown in the Figure (Cells treated with ICL 670 at indicated concentration and time was denoted with an arrow).



**Fig. 2.** Time course of ROS production. (A) H9C2 cells were treated with increasing concentrations of FeCl<sub>3</sub> (0.075-0.6 mM) for 24 hours. After H2DCF-DA staining, DCF fluorescence produced by the cells was continuously measured for up to 160 minutes (N=3, various concentrations of FeCl<sub>3</sub> vs. control group, \*\*\*P<0.001). (B) H9C2 cells were treated with 0.3 mM FeCl<sub>3</sub> with or without TPO for 24 hours. After H2DCF-DA staining, DCF fluorescence produced by the cells was continuously measured for up to 240 minutes (N=3, FeCl<sub>3</sub> vs. FeCl<sub>3</sub>+TPO group, \*\*\*P<0.001; TPO vs. control group \* P<0.05).



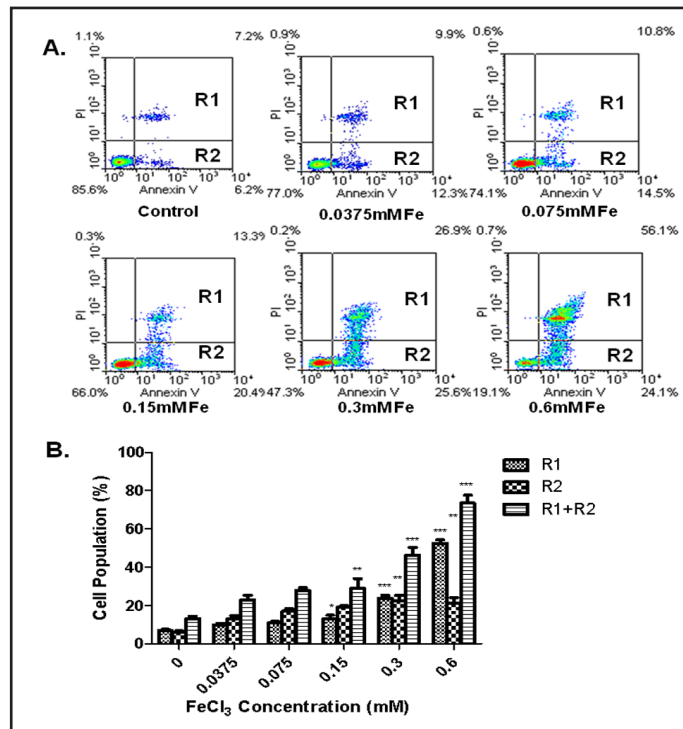
**Fig. 3.** Apoptotic effects of FeCl<sub>3</sub> on H9C2 cells. (A) To investigate the apoptotic effect of FeCl<sub>3</sub>, H9C2 cells were resuspended in serum depleted IMDM medium, and then incubated with different concentrations of FeCl<sub>3</sub> (0.0375mM to 0.60mM) for 72 hours. (A) Samples were investigated under microscope using high-power (\*200) objective. Compared to the normal control group, the number of non-adherent cells increased in a dose-dependent manner. (B) Viability of H9C2 cells were investigated by Trypan Blue Count. The cell viability were decreased in dose-dependent manner (N=6, FeCl<sub>3</sub> vs. control group, \*\*P<0.01).



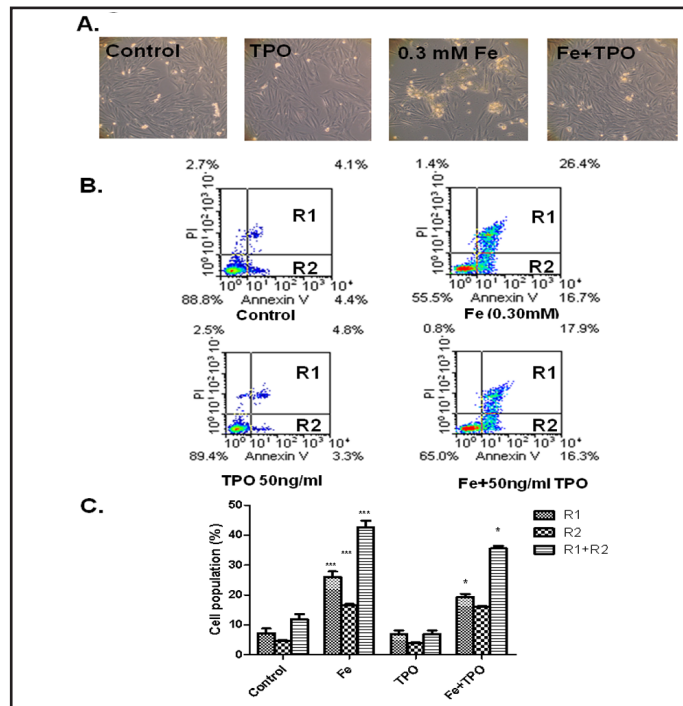
*Iron induced ROS production on cardiomyocytes*

The effect of FeCl<sub>3</sub> on ROS production of H9C2 cell cultures was analyzed (Fig. 2A). A FeCl<sub>3</sub> concentration of 0.075 mM was sufficient to induce high levels of ROS in cell cultures.

**Fig. 4.** FeCl<sub>3</sub>-treated H9C2 cells were stained with AnnexinV/FITC and propidium iodide (PI), and then examined by flow cytometry. Density plot analysis demonstrated that early apoptotic cells (Annexin V positive, PI negative, R2), late apoptotic and necrotic cell (Annexin V positive, PI positive, R1), and total dead cells (R1 + R2) were increased in culture cells treated with FeCl<sub>3</sub> (R1, R2 and R1+R2, N=6, FeCl<sub>3</sub> vs. control group, \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001).



**Fig. 5.** FeCl<sub>3</sub>/TPO-treated H9C2 cells were stained with AnnexinV/FITC and propidium iodide (PI), and then examined by flow cytometry. Early apoptotic cells (Annexin V positive, PI negative, R2), late apoptotic and necrotic cell (Annexin V positive, PI positive, R1), and total dead cells (R1 + R2) were increased in FeCl<sub>3</sub> treated cells (R1, R2 and R1+R2, n=6, FeCl<sub>3</sub> vs. control group, \*\*\*P<0.001). TPO significantly reduced the proportion of late apoptotic and necrotic cells (R1) (n=6, TPO+FeCl<sub>3</sub> vs. FeCl<sub>3</sub> group, \*P<0.05) as well as total dead cells (R1+R2) (n=6, TPO+FeCl<sub>3</sub> vs. FeCl<sub>3</sub> group, \*P<0.05).



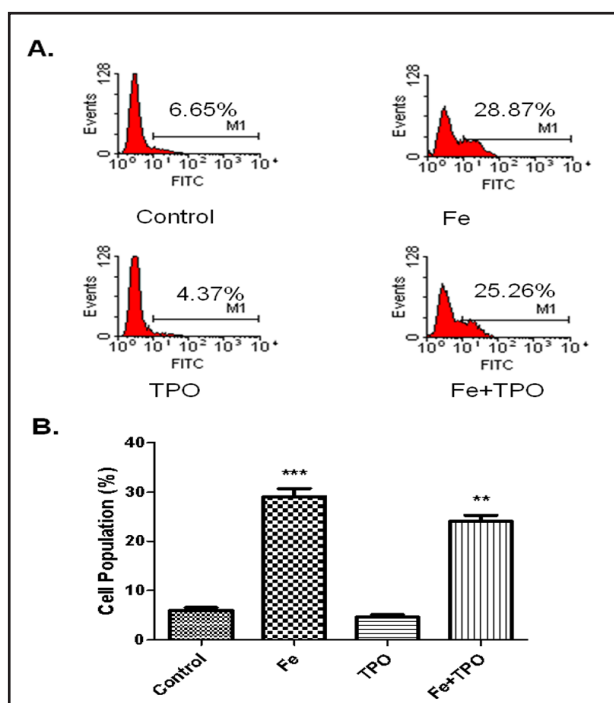
With increasing concentrations of FeCl<sub>3</sub> (0.075-0.6 mM), the levels of ROS in cell cultures were significantly increased (Fig. 2A).

*TPO reduced ROS production from iron-overload cardiomyocytes*

The ability of TPO to attenuate intracellular ROS production was assessed in H9C2 cells. The cells were treated with FeCl<sub>3</sub> (0.3 mM) with or without TPO (50 ng/ml) for 24 hours (Fig. 2B). TPO significantly decreased the FeCl<sub>3</sub>-induced ROS production, and not changed



**Fig. 6.** H9C2 cells under different treatments were stained with anti-active Caspase-3/FITC antibody. The expression of active caspase-3 increased significantly in FeCl<sub>3</sub> treated H9C2 cells (n=6, FeCl<sub>3</sub> vs. control group, \*\*\*P<0.001). Moreover, TPO significantly reduced the expression of caspase-3 of FeCl<sub>3</sub>-treated cells (n=6, TPO+FeCl<sub>3</sub> vs. FeCl<sub>3</sub> group, \*P<0.05).



the baseline level of ROS production in H9C2 cells (Fig. 2B). It was suggested that TPO was effective in reducing the ROS production induced by iron-overload.

#### *Iron-induced apoptosis in Cardiomyocytes was rescued by TPO*

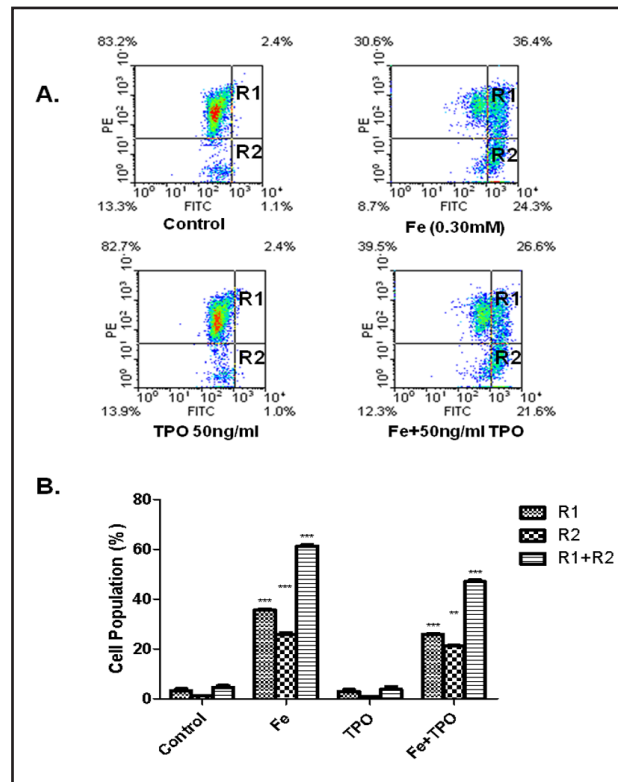
Results of trypan blue assay on H9C2 cells demonstrated that FeCl<sub>3</sub> (0-0.6 mM, 72 hours) significantly reduced cell viability in a dose-dependent manner (n=6) (Fig. 3). These results were also confirmed by flow cytometry with Annexin V/PI double staining. Early apoptotic cells (R2) and total dead cells (R1+R2) were significantly increased in FeCl<sub>3</sub>-treated H9C2 cells in a dose-dependent manner (Fig. 4).

Apoptosis assays were also performed to elucidate whether TPO has anti-apoptotic potential on FeCl<sub>3</sub>-treated H9C2 cells (Fig. 5). Compared to the normal control group, FeCl<sub>3</sub>-treated cells showed a marked increase in the proportions of early apoptotic cells (R2), late apoptotic and necrotic cell (R1) and total dead cells (R1+R2), and it was significantly reduced by TPO (\*P<0.05; n=6) (Fig. 5), while TPO alone had no noticeable effect on apoptosis of H9C2 cells, suggesting TPO has an anti-apoptotic effect on H9C2 cells treated with FeCl<sub>3</sub>.

Caspase-3, which is an effector protein of the caspase family, plays an essential role in apoptosis. Levels of active caspase-3 in FeCl<sub>3</sub>-treated group were significantly increased, compared with control group (n=6, P<0.001). Treatment with TPO with FeCl<sub>3</sub> significantly decreased caspase-3 expression, compared with FeCl<sub>3</sub>-treated group (Fig. 6), suggesting a protective effect of TPO on FeCl<sub>3</sub> treated cells.

JC-1 can be rapidly taken up into mitochondria by the polarized mitochondrial membrane potential to form JC-1 aggregates in living cells. Depolarization of mitochondrial membrane potential indicates altered mitochondrial function. JC-1 fluorescence in these apoptotic cells does not accumulate in mitochondria and remains as monomers (R2). There are transitional subset of apoptotic cells (R1) containing both monomers and aggregates. Our data showed that the proportion of cells containing both JC-1 monomers and aggregates (R1), or JC-1 monomers (R2) increased significantly in FeCl<sub>3</sub>-treated cells, compared with normal control cells (n=6, P<0.001) (Fig. 7), indicating a drop in mitochondrial membrane potential and an increase in the proportion of apoptotic cells. Additional treatment with TPO significantly elevated the mitochondrial membrane potential and reduced the population of apoptotic cells, compared with the FeCl<sub>3</sub>-treated group (Fig. 7). It was suggested that

**Fig. 7.** Mitochondrial membrane potential changed in H9C2 cells treated with FeCl<sub>3</sub> and TPO. Cells under different treatments were stained with JC-1 reagent. The proportion of apoptotic cells contains JC-1 monomers (R2), and a transitional subset of cells contains both monomers and aggregates (R1). The apoptotic cells increased significantly in FeCl<sub>3</sub>-treated group (R1, R2 and R1+R2, n=6, FeCl<sub>3</sub> vs. Control group, \*\*\*P<0.001). And reduced damage in the FeCl<sub>3</sub>+TPO group (R1, R2 and R1+R2, n=6, TPO vs. FeCl<sub>3</sub> group, \*\*P<0.01, \*\*\*P<0.001).



TPO has an anti-apoptotic effect on iron-overload induced apoptosis via suppressing the mitochondrial pathways in H9C2 cells.

## Discussion

Transfusion iron-overload associated with thalassemia leads to the appearance of non-transferrin-bound iron (NTBI) in blood that is toxic and causes morbidity and mortality via tissue damage [16]. Fe<sup>3+</sup> is the common form of iron in the blood circulation of iron-overload patients. In this study, we designed an *in vitro* iron-overload model to study the apoptotic effect of FeCl<sub>3</sub> and the anti-apoptotic effect of TPO on cardiomyocytes. In conclusion, we demonstrated that the protective effects of TPO on iron-induced cardiomyocytes injury *in vitro*, and that this effect was likely to be mediated by reduced ROS production and inhibited anti-apoptotic activity. The inhibition of oxidative stress and apoptosis by TPO might play an important role in the cardiocyte protection.

Our results demonstrated that FeCl<sub>3</sub> could penetrate H9C2 cells to quench intracellular calcein green fluorescence. ICL670 is a cell-permeating and efficient iron chelator which is highly selective for iron as Fe<sup>3+</sup> [15, 17]. It can restore fluorescence by removing the cytosolic iron complexed to calcein green in cell systems, thus we used it in order to generate the high unquenching level. Upon subsequent addition of ICL670, the intracellular fluorescence underwent a dose-dependent restoration. It further confirmed the iron entry into cardiomyocytes.

Activation of mitochondria mediated apoptotic pathway could induce the cardiomyocytes apoptosis [18]. Inhibition of ROS production could protect cardiomyocytes against oxidative stress [19]. According to our data, iron significantly increased ROS production in cardiomyocytes and induced cells apoptosis by activation of mitochondria mediated apoptotic pathway. TPO significantly attenuated ROS production, inhibited collapse of mitochondrial membrane potential, attenuated the expression of active caspase-3, and finally

decreased apoptosis in iron-treated cardiomyocyte. Therefore, our data suggest that TPO inhibits apoptosis by suppressing oxidative stress and inhibiting mitochondria mediated apoptotic pathway. Recently, it has been shown that TPO may induce PI3K/Akt-dependent eNOS phosphorylation and nitric oxide (NO) release in both cardiac [20] and endothelial cells [21]. As far as is known, NO plays important role in cell damage and apoptosis [22]. Thus, the PI3K/Akt-NO pathway was the possible mechanism responsible for the anti-oxidative activity of TPO, it would be further investigated. In addition, the impairment of membrane proteins might prevent the adaptation of cardiomyocytes to intermittent hypoxia [23].

It is known that TPO is the most potent thrombopoietic factor. The second generation thrombopoietic agents, such as TPO peptide mimetics: Romiplostim (AMG 531) and TPO nonpeptide mimetics: Eltrombopag (SB497115, Promacta) have been used to treat patients with thrombocytopenia and the treatment has been suggested to be effective and safe [24, 25]. Recently, we demonstrated that TPO is not only a thrombopoietic agent, but also plays an important role in non-hematopoietic systems, for example, the cardiovascular system. TPO (50 ng/ml and 100 ng/ml) protected cardiomyocytes from doxorubicin-induced injury [1]. In this study, 50 ng/ml TPO was used to treat the cardiomyocytes apoptosis induced by iron-overload. Our group is the first to use TPO as a protective drug to treat iron-overload-induced cardiomyocytes apoptosis. We demonstrated a high potential for this drug in managing iron-overload-induced cardiomyopathy in thalassemia patients.

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## Disclosure Statement

None.

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