Human Trophoblasts-derived Exosomes Attenuate Doxorubicin Induced Cardiac Injury via Regulating Mir-200b and Downstream Zeb1

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Research

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Abstract

Purpose: Human trophoblast stem cells (TSC)have been confirmed to play a cardioprotective role in heart failure. However, whether trophoblast stem cell derived exosomes (TSC-Exos) can protect cardiomyocytes from doxorubicin (Dox) induced injury remains unclear.

Methods: In the present study, TSC-Exos were isolated from the supernatant of Human Trophoblasts using the ultracentrifugation method and characterized by transmission electron microscope and western blotting.In vitro, primary cardiomyocytes subjected to Dox were treated with TSC-Exos, miR-200b mimic or miR-200b inhibitor. Cell apoptosis was observed by flow cytometry and immunoblotting. In vivo, mice were intraperitoneally injected into Dox to establish a heart failure model. Then they received a tail injection of either PBS, adeno-associated virus (AAV)-vector, AAV-miR-200b-inhibitor or TSC-Exosfor different groups. Then cardiac function, cardiac fibrosis and cardiomyocyte apoptosis among groups were evaluated and downstream molecular mechanism was explored.

Results: TSC-Exos and miR-200b inhibitor both decreased primary cardiomyocytes apoptosis. Similarly, mice receivingTSC-Exos andAAV-miR-200b-inhibitor have improved cardiac function, accompanied by reduced apoptosis and inflammation. Bioinformatic prediction and luciferase reporter results confirmed that Zeb1 was a downstream target of miR-200b, which had an antiapoptotic effect.

Conclusion:TSC-ExosattenuatedDoxorubicin induced cardiac injury by playing an antiapoptosis and antiinflammation role. The underlying mechanism could be increased expression of Zeb1 by inhibiting miR-200b expression, due to the TSC-Exos treatment. This study sheds newlight on the application of MSC-Exo as a potential therapeutic tool for heart failure.

Introduction

Cancer mortality has decreased during the last decades due to the widely use of chemotherapeutic drugs. However, the increased number of patients were suffering from the long-term effects of cardiotoxicity. Several agents used in cancer treatment, such as doxorubicin (Dox), can adversely affect the cardiac function and lead to congestive heart failure, a leading causes of disability in long-term survivors of cancer[1, 2], which is a growing problem in cardio-oncology.

Dox is an anthracycline cytostatic agent, which has been in clinical use for almost a half century[3–5]. However, cumulative doses of Dox were found to be harmful to the myocardium [6], which leads to left ventricular dysfunction and development of heart failure, namely Dox-induced cardiotoxicity. The underlying mechanisms include mitochondrial iron accumulation and reactive oxygen species (ROS) burst, [7–10], eventually leading to cell apoptosis or cell necrosis [11–15] However, the exact mechanisms have not been fully established, and optimal cardioprotective therapeutics remain undefined[5].

Various types of stem cells have been proposed for cardiac cell therapy[16, 17], such as skeletal myoblasts, mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), and embryonic stem cells

(ESCs). Trophoblast stem cells (TSCs) are a population of stem cells originating from a single layer of blastocysts when the earliest cell differentiation occurs[18]. Our previous results [19] illustrated that TSCs could improve cardiac function and attenuate myocardial adverse remodeling, which could be attributed to a lower level of miRNA-200b-3p. However, the stem cell therapy was limited to an inconsistent supply, infusion toxicity, low survival and immune rejection. Besides, researchers confirmed that the cardioprotective effect of the administrated stem cells was not derived from the direct trans-differentiation into cardiomyocytes. Conversely, it was mediated by paracrine effects of exosomes secreted by stem cells [20, 21]. Therefore, in our study, we explored the effect of trophoblast cell-derived exosomes on Dox-induced cardiac injury and the underlying mechanism.

Materials And Methods

1. Animal experimental

C57BL/6 mice (male, 8 weeks) were purchased from the Model Animal Research Center of Nanjing University. Animals were fed a standard laboratory diet with free access to food and water, and kept in a temperature (22 ± 1°) and humidity (65–70%) controlled room, with a 12-h light–dark cycle. After study, all animals were anesthetized by isoflurane inhalation (1.5%-2%) and then euthanized by cervical dislocation. All procedures with animals were approved by the Institutional Ethics Committee of Nanjing Drum Tower Hospital (Approval No. 20011141) and performed in accordance with Care and Use of Laboratory Animals published by the National Institutes of Health (Eighth Edition).

Doxorubicin (Dox; Sigma; USA) was administered intraperitoneally into mice at a dose of 5 mg/kg once a week for 4 weeks (Dox groups). Dox + Exo groups received additionally intramyocardial injection of 25μL of PBS containing 50 μg TSC-Exo. Dox + AAV groups received tail vein injection of 25 ul of adeno-associated virus (AAV; Keygen; Jiangsu), which carried miR-200b inhibitor (a small RNA to silence the expression of miR-200b), while Dox + Vector received an equivalent empty virus. All groups were anesthetized by isoflurane inhalation (1.5%-2%), ventilated with a rodent ventilator and subjected to thoracotomy for intramyocardial injection at 3 different points. Each group has 5 mice.

2. Exosome Isolation And Identification

TSCs (Novobio; shanghai) were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin solution under 5% CO2 at 37 °C. When reached 70–80% confluency, it was cultured with fresh DMEM containing 5% exosome-depleted fetal bovine serum (System Biosciences Inc; USA) and cultured for 48 h. Exosomes were isolated using differential centrifugation based on previously described methods with slight modifications[22, 23]. Briefly, cells are removed by centrifugation at 300 x g for 10 min; the supernatant is then cleared of apoptotic bodies by centrifugation at 2000 x g for 20 min; MVs are preferentially pelleted at 10 000 x g for 30 min; exosome are then purified from the supernatant by

ultracentrifugation at 100 000 x g for 60 min. After isolation, 10 μl exosomes were diluted in 1 ml of filtered PBS and stored at -80 ℃.

The morphology of exosomes was observed using transmission electron microscope (JEM1011; Japan) and size distribution was measured using nanoparticle tracking analysis (NTA). For electron microscopy, we used 0.5% uranyl acetate to stain the exosomes and calculated their diameter from 5 to 15 images. For NTA, samples were loaded into the sample chamber of an NS500 unit (Nanosight, Amesbury, UK) and five 1 min videos of each sample were recorded. Data analysis was performed with NTA 2.3 software (Nanosight) and the size and concentration of particle were calculated. The exosome marker (CD9, Alix and Flotillin) was detected by immunoblotting. To facilitate tracking in vitro, exosomes were labelled with PKH67 Green Fluorescent Cell Linker Kit ((Sigma; USA).

3. **Cardiomyocyte culture and treatment.**

Primary cardiomyocytes were isolated from neonatal mice (post 1–2 days; Science of Chinese Academy of Medical Sciences; Beijing). In brief, hearts were enzymatically digested in HEPES-buffered saline solution containing 1.2 mg/ml pancreatin and 0.14 mg/ml collagenase at 37 °C. After centrifugation, cells were collected and resuspended in DMEM. The dissociated cells were pre-plated at 37 °C for 1 h and then diluted to $1*10^6$ cells/ml, and plated in 10 mg/ml laminin-coated culture dishes according to the specific experimental requirements. Dox group received Doxorubicin (1 uM) for 24 hours. Dox + Exo group received additional 20 ug of exosomes. Besides, cells were treated with miR-200b mimic (50 nM) (Dox + Mimic) and miR-200b inhibitor (50 nM) (Dox + Inhibitor), which were synthesized to overexpress miR-200b expression and inhibit miR-200b expression respectively.

4. Echocardiography

Cardiac function was assessed by transthoracic echocardiography (VEVO2100; visual sonics). Mice were anesthetized with isoflurane (1.5% in air) and monitored of respiratory frequency and temperature. End diastole was measured at the time of the apparent maximal LV diastolic dimension, and end systole at the time of the most anterior systolic excursion of the posterior wall. Mice were measured at pre- 1 and post-1, 2, 3 and 4 weeks following TSC-Exos injection, respectively. Left ventricular internal dimensions at end-diastole (LVIDd) and at end-systole (LVIDs) were measured digitally on the M-mode tracings from 3 cardiac cycles. Left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS) were calculated accordingly.

5. RNA extraction and real-time RT-PCR

Total mRNA was extracted using a commercial kit (TaKaRa; Japan) according to the manufacturer's instruction. Quantitative analyses were carried out on a real-time system (Applied Biosystems; USA) using SYBR Green PCR Master Mix (TaKaRa; Japan) for mRNA or miRNA Universal SYBR qPCR Master Mix for miRNA (Vazyme, China). The miRNA primers were obtained from Genescript (Nanjing). The relative expression level for each mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers were listed as

following: ANP F: GCTTCCAGGCCATATTGGAG; R: GGGGGCATGACCTCATCTT; βMHC F: ACTGTCAACACTAAGAGGGTCA; R: TTGGATGATTTGATCTTCCAGGG. Collagen I F: GCTCCTCTTAGGGGCCACT; R: CCACGTCTCACCATTGGGG.

6. Western blotting

Heart tissues, exosomes and cells were lysed using RIPA buffer (Beyotime; Jiangsu). Protein concentration was measured by the BCA method (Thermo; USA). Equal amounts of protein were loaded on SDS-PAGE gels, separated and then transferred onto PVDF membranes (Millipore; USA). Then the membranes were incubated with primary rabbit anti-mouse antibodies against CD9 (ab92726) Flotillin (ab41927), Alix (ab186429), Cleved-caspase3 (ab2302), Bcl-2 (ab32124), Zeb1 (ab81972), and GAPDH (ab181602) (Abcam; USA) respectively at a dilution of 1:1000. After overnight at 4 °C, the membranes were subsequently incubated with HRP-conjugated rabbit anti-mouse IgG (1:10000) for 1 h at room temperature. The immunobands were visualized using an enhanced chemiluminescence (ECL) detection kit (Beyotime; China).

7. Annexin V/PI Staining for cell apoptosis

Primary cardiomyocytes were digested and collected at a concentration of 1*10^6 cells/ml. Then cells were resuspended with 200 μl binding buffer and added 5 μl Annexin-FITC and PI (Keygen; China). After incubating for 30 minutes in the dark, they were analyzed by flow cytometry (BD; USA).

8. Enzyme-Linked Immunosorbent Assay (ELISA)

Murine blood was collected from the orbit in and centrifugated at 3000 rpm for 5 minutes at 4 °C to acquire serum. The serum IL-1β and IL-6 were measured using commercial enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (R&D; UK).

9. Luciferase reporter measurement

The luciferase reported clones with 3'-untranslated region (UTR) of Zeb1 were purchased from GeneCopoeia (Rockville; MD). The clones included predicted miR-200b target sites acquired from online database (www.mirdb.org) and a mutated sequence of 3'UTR of Zeb1. The plasmids were transfected into HEK293 cells (200 ng per well) using Lipo 2000 (Life Technologies; USA) with 10 nM of miR-200b mimic. After 24 hrs, the cell medium was collected for luciferase assay.

10. Histology assay

Heart tissues were harvested after 4 weeks. Then the heart tissues were fixed in 4% paraformaldehyde (pH 7.4). After fixation and paraffin embedding, the cardiac tissues were cut into 5 μm-thickness slices. The sections were stained with hematoxylin and eosin (Servicebio; China) to analyze the global heart morphology and inflammatory cell infiltration. And slices were stained with Masson's trichrome (Servicebio; China) for cardiac fibrosis evaluation. The slices were scanned at 20-fold magnification on a high-resolution microscope (Leica; Japan).

11. Statistical analysis

Data are presented as the mean ± standard derivation. All statistical analyses were performed with SPSS (23.0). Differences were analyzed with one-way analysis of variance (ANOVA) for multiple groups and Student's t-test for only two groups. $P < 0.05$ was considered statistically significant.

Results

1.Characterization of TSC-derived exosome

Utilizing ultracentrifugation, we obtained exosomes from TSC, as shown in TEM (Fig. 1A). And immunoblotting results (Fig. 1B) confirmed their expression of exosomal marker (CD64, CD9, TSG101). Besides, DLS illustrated they have an average diameter of 101 nm (Fig. 1C).

2.TSC derived exosomes protected cardiomyocyte from Dox-induced apoptosis.

As shown in Fig. 2A, labelled exosomes were taken in by primary cardiomyocytes. Previous studies showed that TSC-Exos could decrease the expression of miR-200b in cardiac tissues. So we treated primary cardiomyocyte with TSC-Exos, as well as miR-200b mimic and inhibitor. The flow cytometry results (Fig. 2B) showed that exosomes and inhibitor both inhibited cell apoptosis. Besides, exosomes and inhibitor treated cardiomyocyte had lower C-caspase 3 expression and higher Bcl-2 expression compared with other groups (Fig. 3).

3.TSC-Exos improved cardiac function of Dox-induced heart failure.

Dox-treated mice were administrated with or without exosomes to determine the effect of exosomes in vivo. After 4 weeks, echocardiographic results (Table 1) showed that exosome-treated mice had a higher EF and FS (Fig. 4A, B), suggesting a favorable role in the cardiac remodeling. Also, the heart failure associated marker (ANP, β-MHC, and Collagen I) showed a decreased expression in the exosome group (Fig. 4C).

4. TSC-Exos decreased miR-200b expression for a protective role in vivo.

Both in exo-treated cardiac tissues and primary cardiomyocytes, miR-200b was downregulated (Fig. 5A, B), suggesting miR-200b involved in the protective role of TSC-Exos. To further confirmed the role of miR-200b, miR-200b was inhibited by transfecting miR-200b inhibitor AAV. Exosome group and inhibitor group had a decreased level of serum IL-1β and IL-6 (Fig. 6A) and cardiac p65 (Fig. 6B), suggesting a decreased systemic inflammation and local inflammation. What's more, exosomes and miR-200b inhibitor both attenuated the heart size (Fig. 7A) and heart weight to body weight ratio (Fig. 7B), as well as cardiac fibrosis (Fig. 7C), suggesting a favorable role in cardiac remodeling.

5. Zeb1 was a target of miR-200b involved in the protective role of TSC exosome.

Previous studies reveled that Zeb1 was a downstream target of miR-200b and played an antiapoptotic effect. So, we examined Zeb1 expression in the abovementioned groups. In addition to decreased

apoptosis, exosome and overexpressed group both had an increased expression of Zeb1 (Fig. 8A). The luciferase reporter also confirmed the direct combining of miR-200b to Zeb1 (Fig. 8B).

Discussion

Our studies revealed for the first time that TSC-Exo can protect murine heart from Dox-induced injury by regulating miR-200b expression. Specially, TSC-Exos had an antiapoptotic and antiinflammation effect by decreasing Zeb1 expression and some inflammatory cytokines, which provided an alternative way to reverse chemotherapy induced heart failure.

miRNAs are endogenous, single-stranded molecules consisting of approximately [24]noncoding nucleotides, which were reported to regulate multiple biological processes[25]. miRNAs are involved in cell proliferation, differentiation, metastasis, apoptosis, and immune responses[26].Doxorubicin could cause cardiac injury by inducing the expression some miRNAs. For example, a study confirmed a strong association of miR-34a-5p and miR-451a to Dox-induced cardiotoxicity[27]. miR-21 suppression or miR-130a prevented cardiac dysfunction induced by doxorubicin[28, 29].Altered miRNAs could be as a potential biomarker or early toxicity signature, which was reviewed in some publications[30, 31]. In our study, the expression of miR-200b was upregulated in Dox-induced cardiomyocyte and was negatively correlated with Bcl2, indicating that miR-200b was a proapoptotic mediator. Our results showed that TSC-Exos decreased miR-200b expression in cardiomyocytes and inhibit cardiomyocyte apoptosis by upregulating anti-apoptotic Bcl-2 protein. miR-200 members were reported to promote cell apoptosis in cancer[23, 32], which was in consistent with our result that miR-200 mimic aggravated cardiac injury suffering from Dox injection. Korpal et al had previously reported that miR-200b could target Zeb1[33]. A luciferase reporter was constructed to confirm the direct combining of miR-200b and Zeb1.

Recent findings illustrated that Dox activated NF-κB pathway to cause inflammatory effects on the myocardium and the vasculature[34]. Conversely, blocking NF-κB pathway in cardiac microvascular endothelial cells could weaken inflammatory damage[35]. Our data showed that IL 1b, IL-6 and p65 were increased in Dox and miR-200b mimic group, while decreased in the exosome and inhibitor group. At the same time, we monitored echocardiography in animal models and found that cardiac function improved significantly in animal models treated with exosomes. These results suggested that exosomes could inhibit inflammatory response, thereby protecting cardiomyocytes and improving cardiac function subjected to Dox injury.

Conclusions

In summary, our study elucidated the antiapoptotic role of TSC-Exos in injured cardiomyocytes, which was achieved by inhibiting miR-200b while increasing the expression of Zeb1. This gives us a new strategy to treat heart failure in the future.

Abbreviations

TSC
trophoblast stem cells
TSC-Exos
trophoblast stem cell derived exosomes
Dox
doxorubicin
AAV
adeno-associated virus
ROS
reactive oxygen species
MSCs
mesenchymal stem cells
HSCs
hematopoietic stem cells
ESCs
embryonic stem cells
LVIDd
left ventricular internal dimensions at end-diastole
LVIDs
left ventricular internal dimensions at end-systole
EF
ejection fraction
FS
fractional shortening
ELISA
Enzyme-Linked Immunosorbent Assay
UTR
3'-untranslated region
ANOVA
one-way analysis of variance

Declarations

Availability of data and materials

All the data can be obtained upon the reasonable request.

Consent for publication

Not applicable

Acknowledgements

Not applicable

Competing interests

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Authors' contributions

N J and L YH draft the article; W K, Z WF, and H ZL perform experiments; G R and X B design the study and approve the manuscript.

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Figures

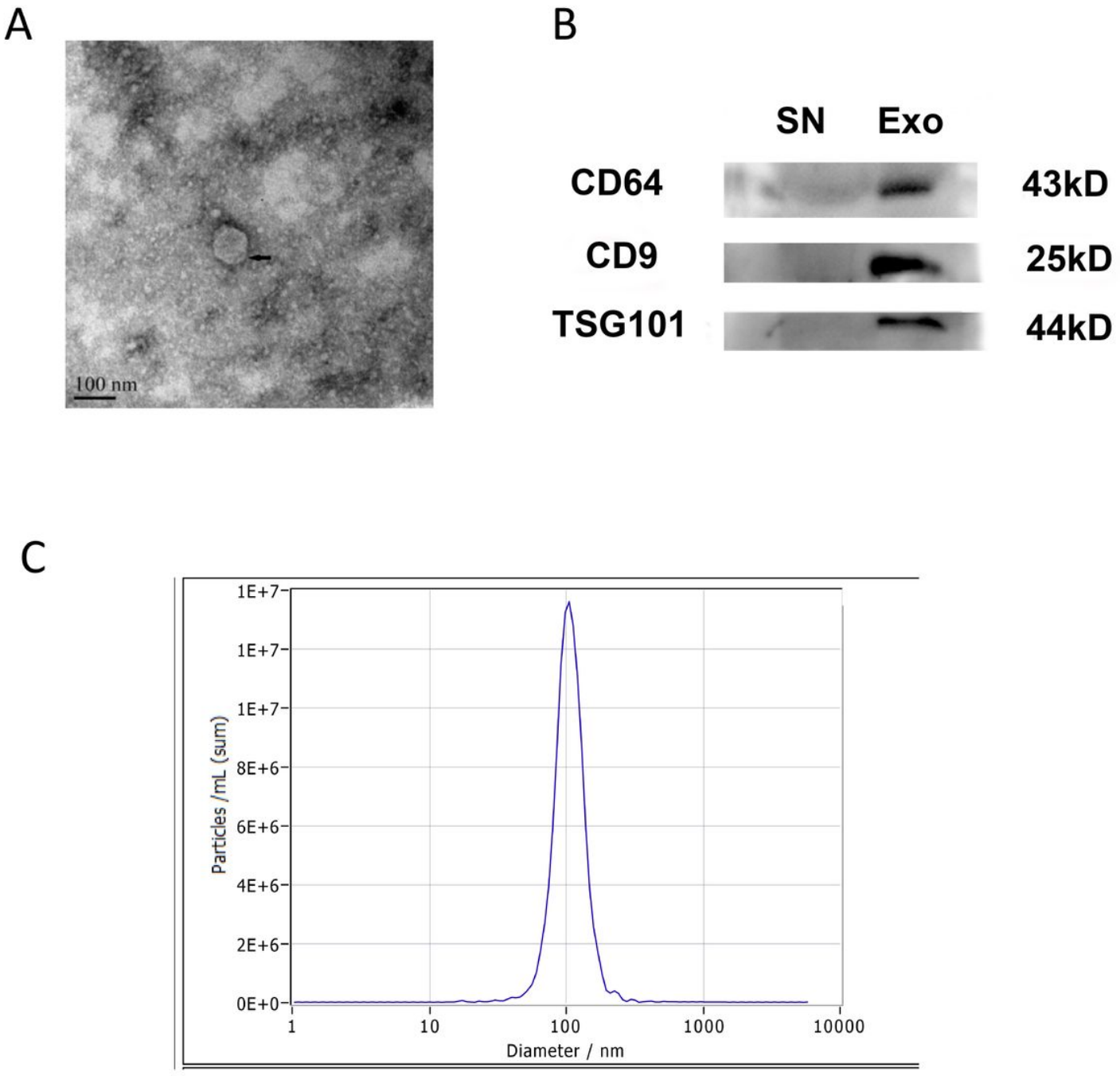

Figure 1

Characterization of TSC-derived exosomes. (A) The ultrastructure of exosomes was analyzed by transmission electron microscopy. (B)Western blot showed the expression pf protein markers of exosomes such as CD64, CD9 and TSG101. (C)Representative DLS number distribution measurement of isolated exosome population demonstrated an average diameter of 101nm.

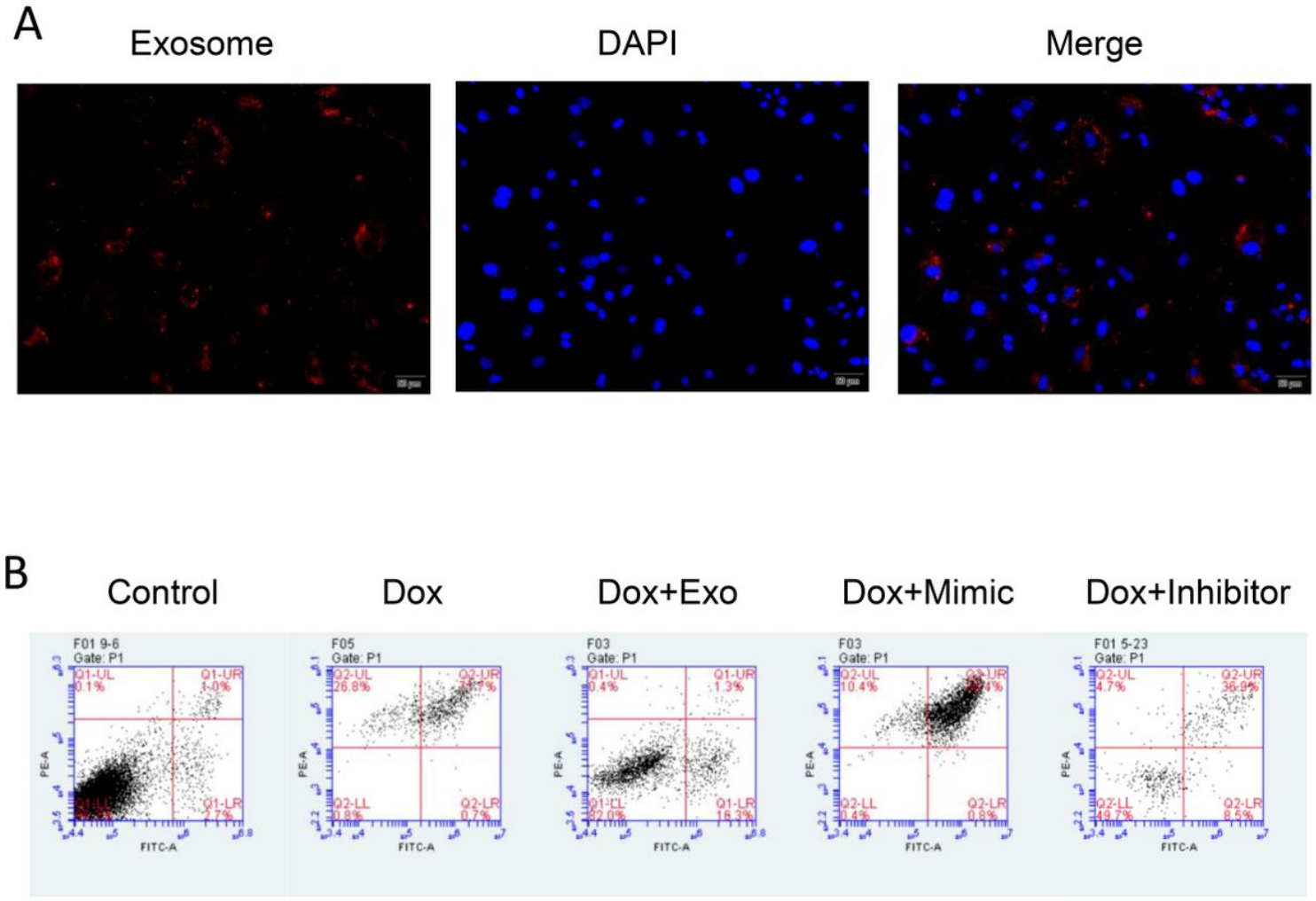

Figure 2

(A) The uptake of PKH67-labeled exosomes (red) by cardiomyocytes (blue). (B) The cardiomyocyte apoptosis level among groups was shown in flow cytometry.

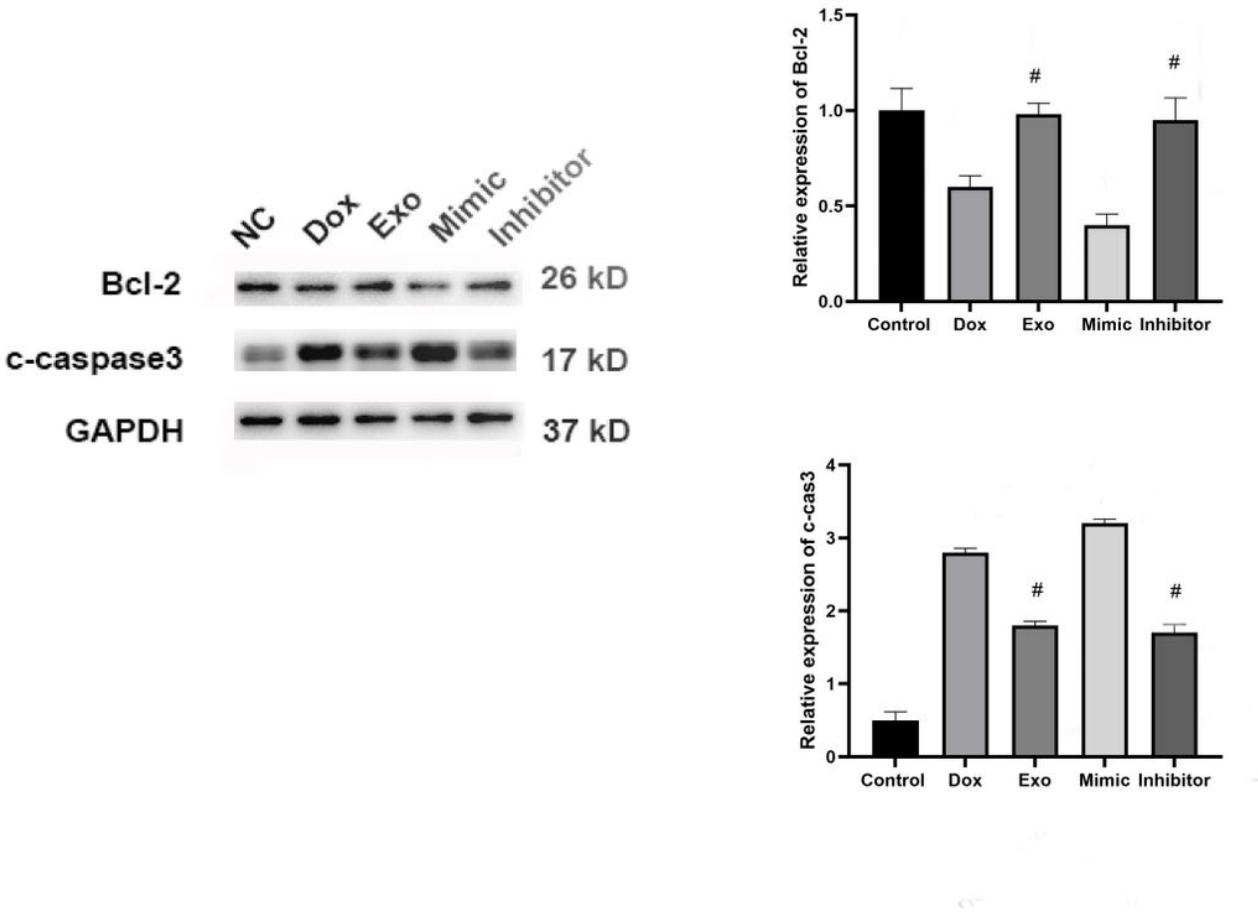

Figure 3

(A)TSC-Exos exertedantiapoptotic effect in Dox-treated cardiomyocytes. The expression of Bcl-2 and caspase3 by western blotting (left), and quantitative analysis (right). # $p<0.05$ compared with Dox.

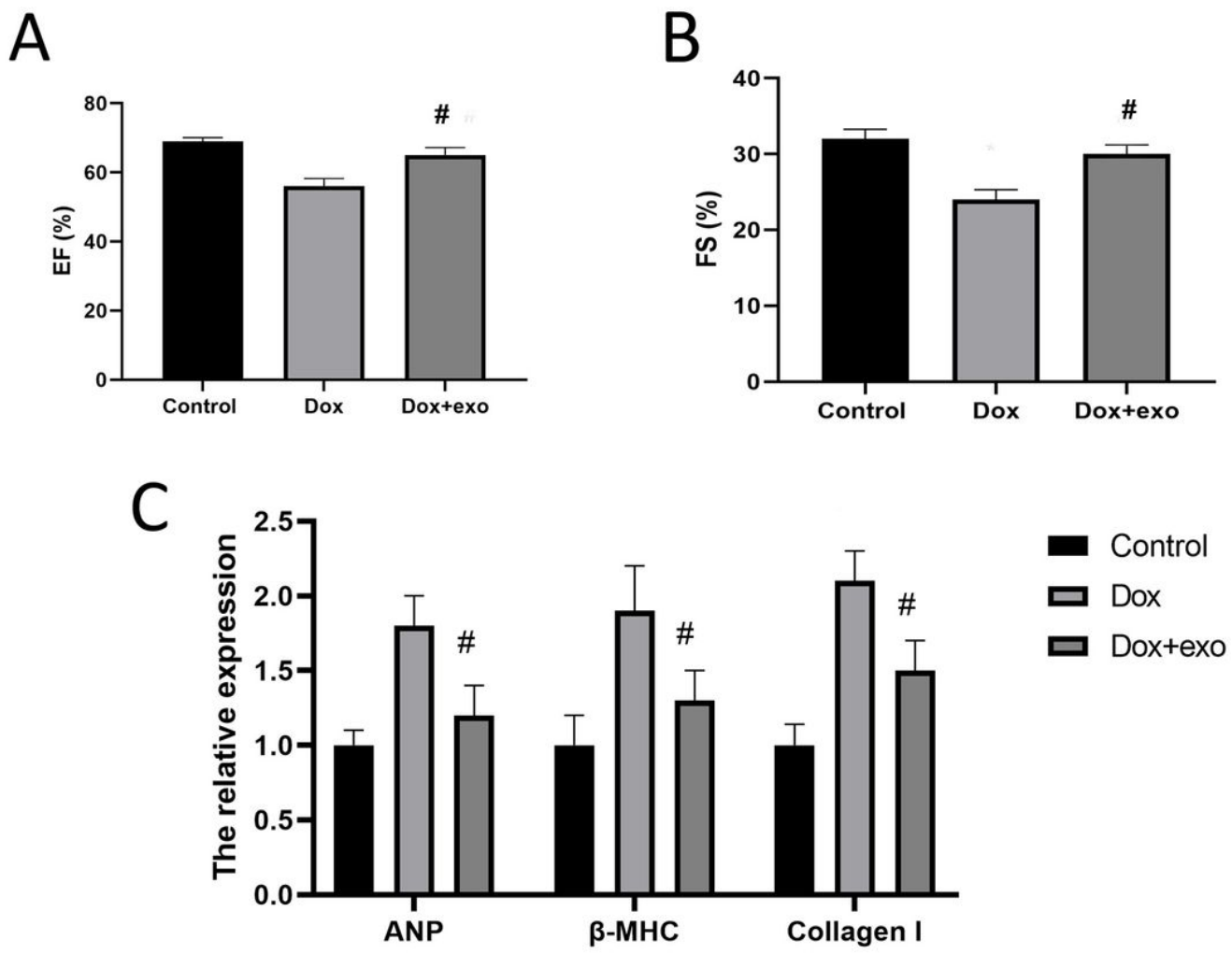

Figure 4

Echocardiographic data of different groups after 4 weeks. (A) left ventricular ejection fraction(EF); (B) left ventricular fraction shortening (FS); (C) the mRNA expression of heart failure marker, such as ANP,MHC and Collagen I. #p<0.05 compared with Dox.

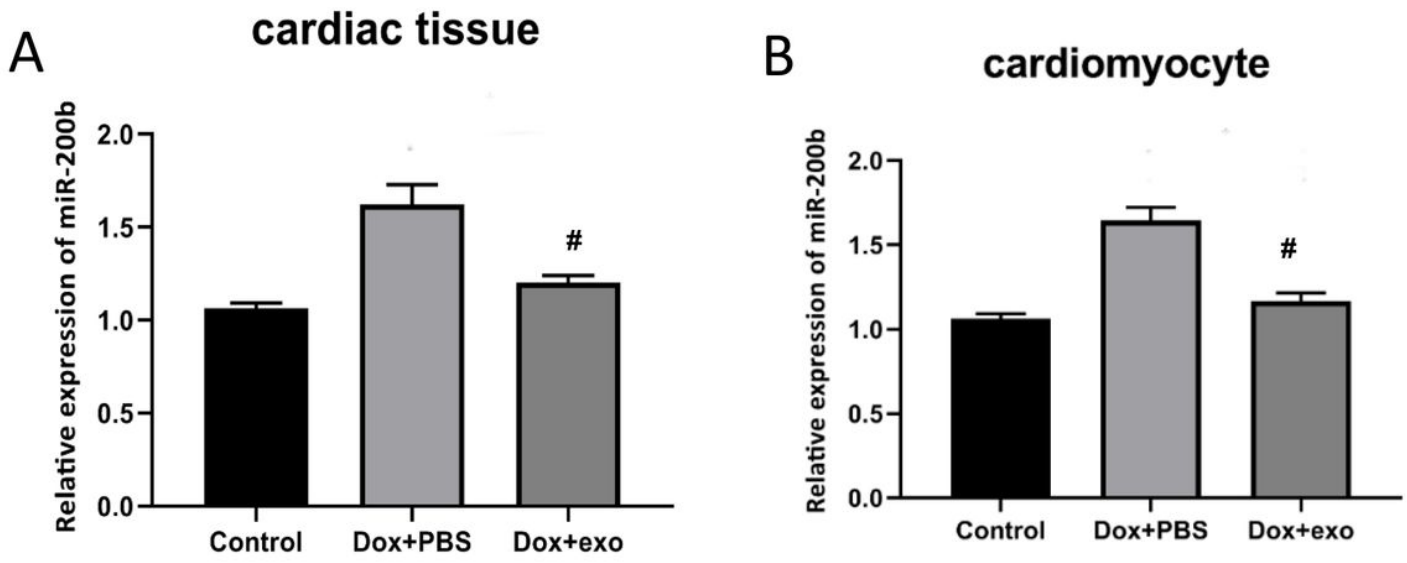

Figure 5

(A) The expression of miR-200b in Dox-treated heart tissues; (B) the expression of mir-200b in Dox-treated cardiomyocytes.#p<0.05 compared with Dox.

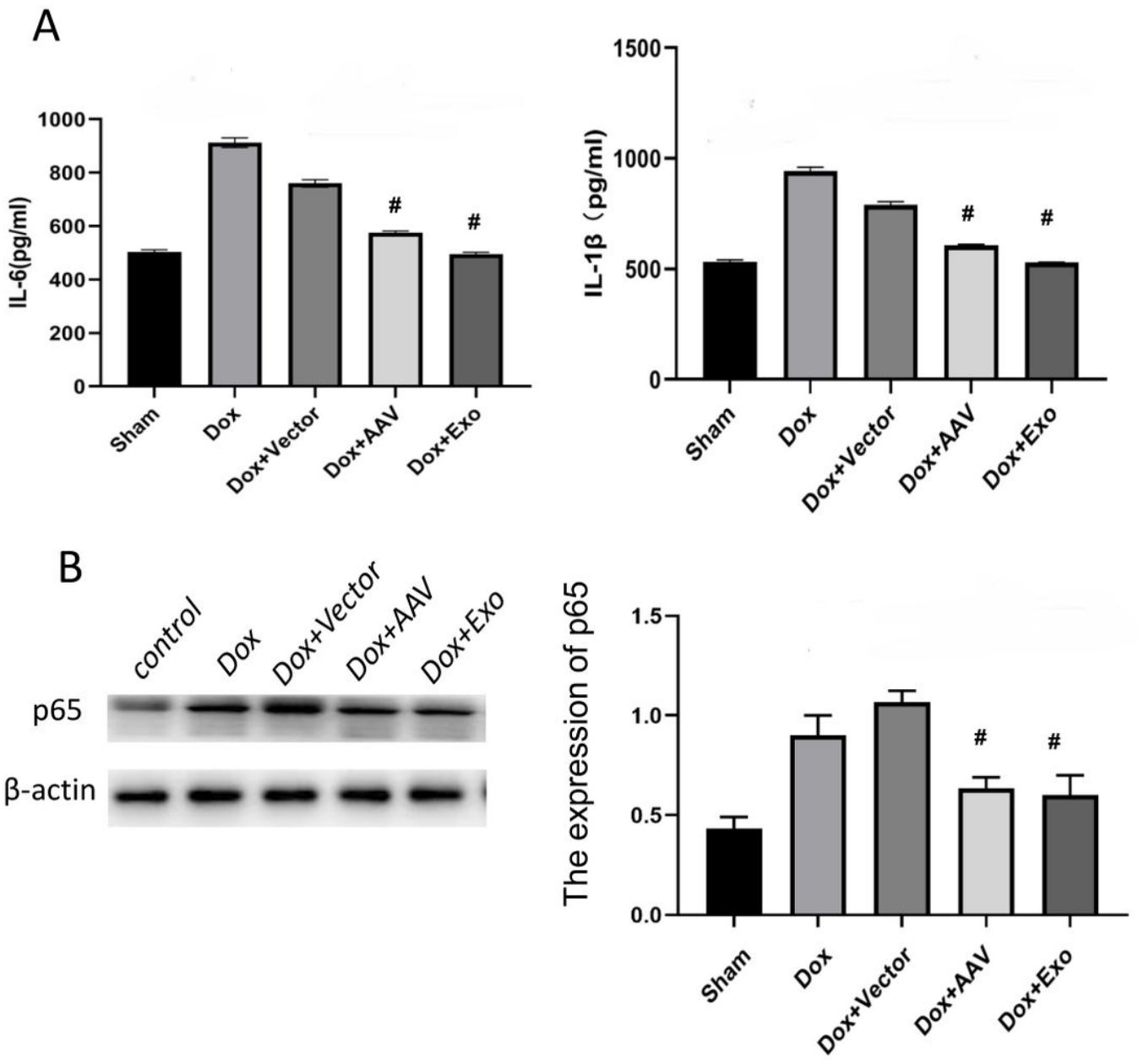

Figure 6

TSC-Exos exerts anti-inflammation function in Dox-induced cardiac injury. (A) ELISA analysis of inflammatory factors IL-1β and IL-6; (B) The expression of NF-kB by western blotting. #p<0.05 compared with Dox+Vector.

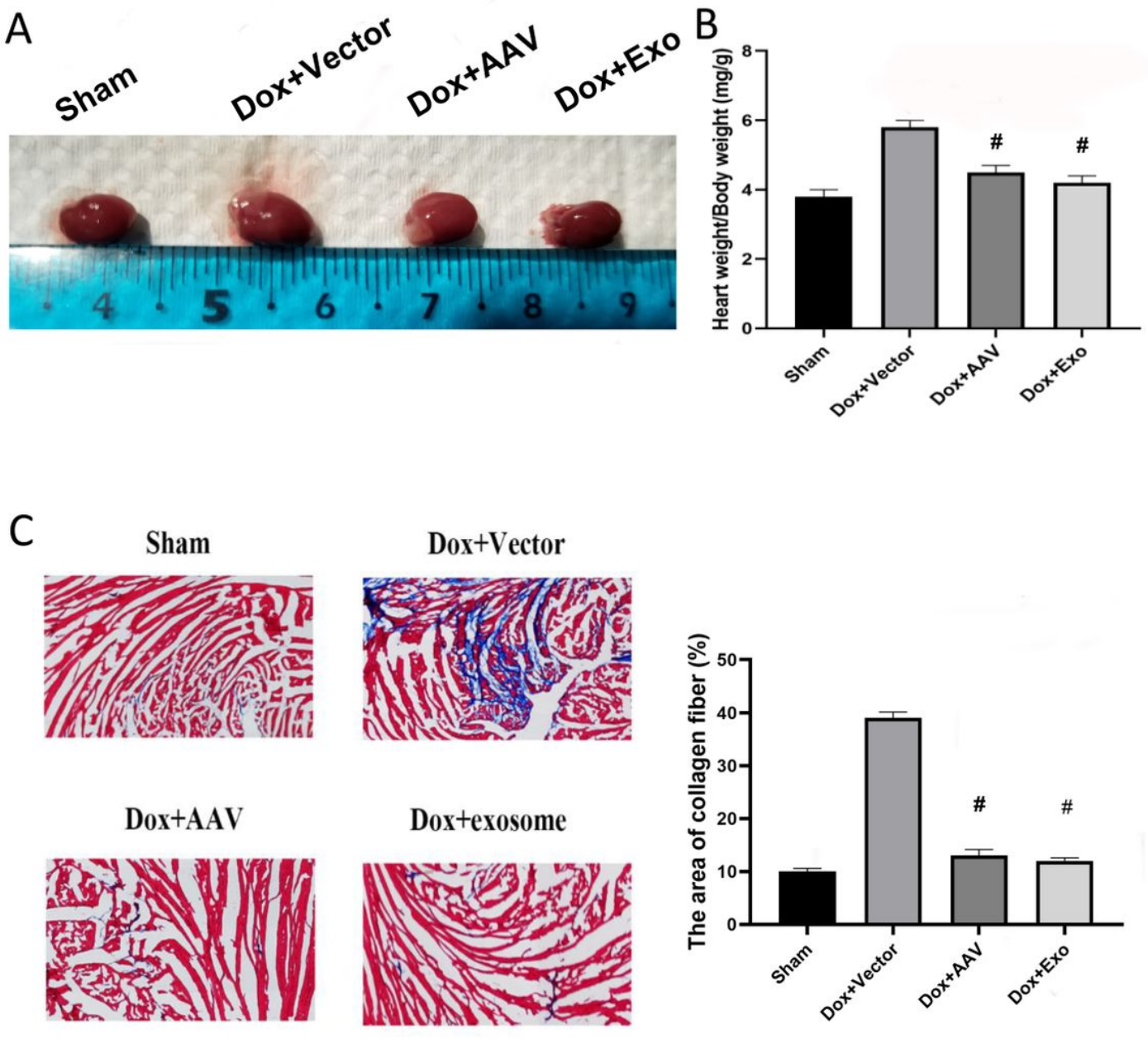

Figure 7

(A) The morphology of heart of different groups; (B) the heart weight to body weight ratio between groups; (C) the fibrotic areas between groups.#p<0.05compared with Dox+Vector.

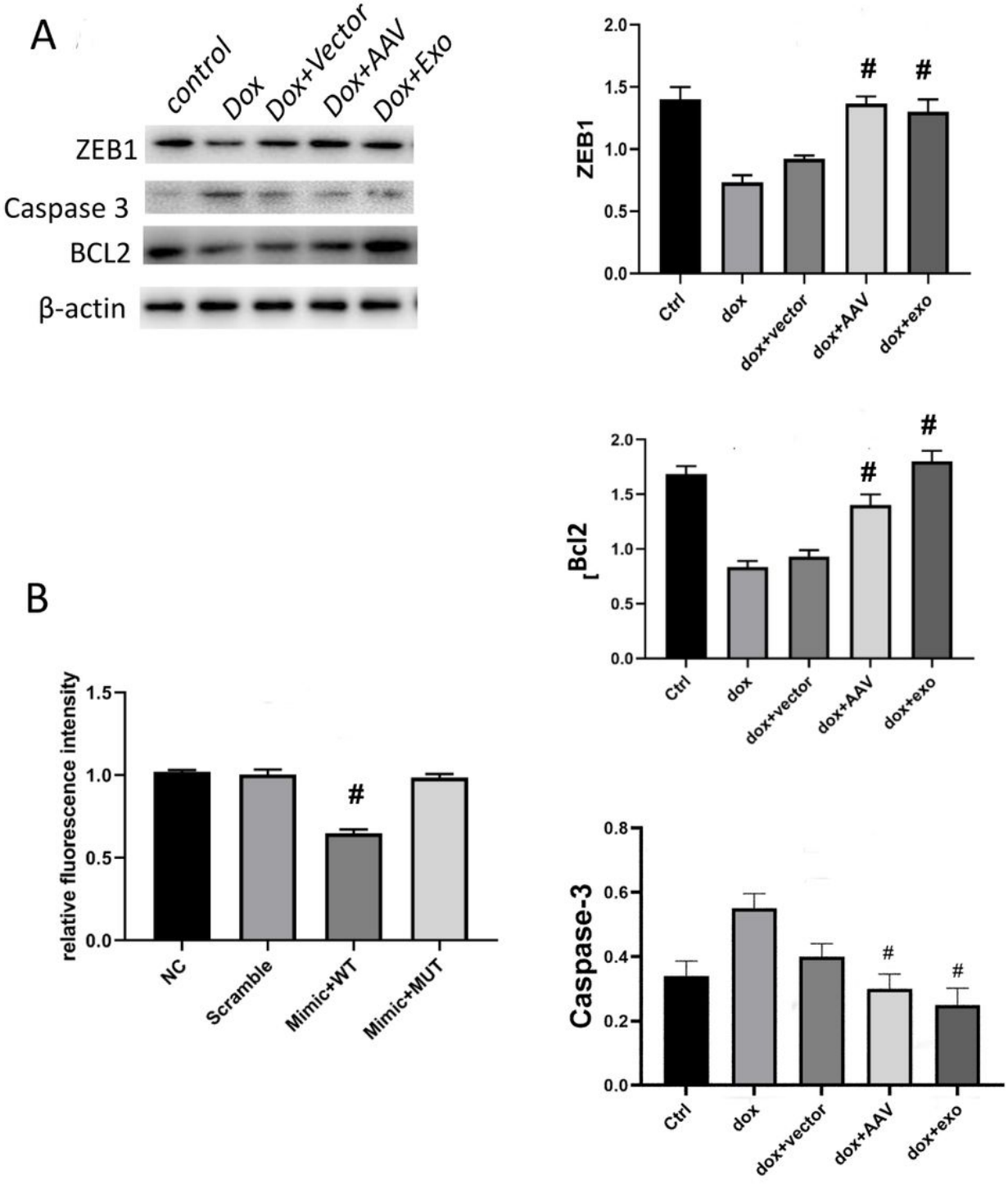

Figure 8

TSC-Exos exert anti-apoptosis effects on Dox-induced cardiac injury tissues. (A) The expression of Zeb1,Caspase3 and Bcl-2 by western blotting; (B) the luciferase results of miR-200b binding to Zeb1. #p<0.05compared with Scramble.