The Dual Effect and Mechanism of (-)-epicatechin on Hypoxic-induced Proliferation and Apoptosis of Cardiac Fibroblasts

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Research article

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Abstract

Objective: Cardiovascular diseases impose a considerable economic burden on health services and remain a threat to human being. (-)-Epicatechin [(-)-EPI], a traditional Chinese medicine is applied to treatment against the cardiovascular diseases. Herein, we aimed at investigating the underlying mechanism whereby (-)-EPI affects myocardial fibrosis (MF).

Methods: The efficacy of (-)-EPI was determined in mouse models of acute myocardial infarction (AMI) and hypoxia-treated fibroblasts. Western blot and RT-qPCR analyses were conducted to determine TGF-β1, SMAD2 and SMAD3 expression, as collagen content was detected. CCK-8 assay and flow cytometry were carried out to detect fibroblast proliferation and apoptosis. HE and Masson staining reflected the histological change of myocardial tissues

Results: Compared to sham-operated mice, AMI group exhibited MF and hypoxia-treated cardiac fibroblasts proliferation was restrained and apoptosis was increased. Treatment with (-)-EPI significantly attenuated the MF condition and restored fibroblast proliferation and apoptosis, whereas these effects were abrogated by the TGF-β1 agonist HY-100347A. (-)-EPI administration caused a decline in TGF-β1, SMAD2 and SMAD3 expression. Mechanistically, (-)-EPI targeting TGF-β signaling inhibits collagen deposition and attenuates MF.

Conclusion: Collectively, (-)-EPI could improve MF following AMI through down-regulation of TGF-β1 signaling, providing a novel insight into treatment against the cardiovascular diseases.

Introduction

With the increase in the aging population, cardiovascular disease has become a major disease seriously threatening human health (1). The elderly are susceptible to cardiovascular diseases such as hypertension, diabetes, stroke (2). It is estimated that millions of people die from cardiovascular disease every year, where heart failure and myocardial infarction are the main causes (3-6). From the perspective of the pathogenesis, MF is common feature of cardiovascular diseases such as hypertension and MI (7,8). MF characterized by significant increase in collagen content or accumulation of extracellular matrix (ECM) of the myocardium, affects cardiovascular functions (9). MF accelerates the progression of heart failure, even leading to sudden death (10). However, current clinical measures still fail to prevent this disease. The main reason is that the pathogenesis of MF is still unclear, restraining the administration of appropriate and timely measures. Therefore, investigating the underlying basis of MF and effective preventive and therapeutic measures are the focus of current researches.

Traditional Chinese medicines are widely applied to the treatment for cardiovascular diseases, such as Myricetin (11), Scrophularia and Catechins (12). (-)-EPI, a kind of catechin compound, has antioxidant (13), enhances immune defense (14) and anti-tumor activity (15). The impact of (-)-EPI on oxidative damage has been studied previously and evidence notes that for cardiovascular system, administration of EPI reduces blood lipids, lowers blood pressure and inhibits thrombosis (12,16,17). Yamazaki KG et al.

found that (-)-EPI alleviates myocardial ischemic damage through potentially protecting mitochondria (18). Also, treatment with (-)-EPI reduced the MI size by 50% in the rat model of ischemia-reperfusion injury. The protective effects are not related to hemodynamics changes and maintain over time, accompanied by a decrease in tissue damage indicators (19). Furthermore, Yamazaki KG has demonstrated that administration of (-)-EPI in advance in MI mice reduces the MI area and restrains left ventricular remodeling (20). A study by Prince P et al. unveils that (-)-EPI exerts anti-oxidative stress effect by scavenging free free radicals, thereby protecting the myocardial tissue of the isoproterenol-induced MI model (21,22). However, the mechanism underlying (-)-EPI preventing and treating MF remains unclear.

Transforming growth factor beta (TGF-β) is a multifunctional cytokine promoting or inhibiting cell proliferation and differentiation as its effect depends on the target cells (23,24). TGF-β1 is known to associate with ECM deposition, and is recognized as a therapeutic target for organ fibrosis (25). In recent years, the role of TGF-β1 in myocardial tissue has received increasing attention. TGF-β1 and its receptors are expressed in cardiomyocytes and non-cardiomyocytes, whilst TGF-β1 is mainly produced by cardiac fibroblasts and myofibroblasts (26). Addition of TGF-β1 to cultured rabbit myocardial fibroblasts coincided with an increase in type I and type III collagen, and fibronectin mRNA and protein levels, as indicated by Eghbali et al. in 1991 (27). TGF-β1 inhibits the production of collagenase and elastase and stimulates the expression of plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitors of matrix metalloproteinases (TIMP), thereby delaying degradation of ECM components such as type I collagen and promoting the process of MF (28). Collectively, the TGF-β/Smads signaling pathway is responsible for MF and TGF-β has been confirmed as an important factor for fibrosis, despite that the causality of TGF-β in MF has not been clarified. Therefore, in-depth studies on TGF-β/Smads signaling pathway will not only further elucidate the pathogenesis of MF, but also contribute to a novel treatment.

Our study is the first to combine (-)-EPI with TGF-β signaling to explore the mechanism of (-)-EPI regulating MF after AMI.

Methods

Animal model

A total of 24 healthy male C57BL/6 mice (8 weeks old, weight 25-30g), were maintained under a 12h/12h dark/light regime (7:00 a.m.-7:00 p.m.) at 21 ± 1°C and at a humidity of 55%-60% with free access to food and water. After 7 days of adaptive feeding, all animals were randomly divided into four groups: (1) The sham-operated group (sham group, n = 6), mouse left anterior descending (LAD) coronary artery was subjected to suture without ligation; (2) AMI group, n = 6, the suture on needle was passed through mouse LAD coronary artery followed by ligation; (3) AMI + (-)-EPI group (n = 6), mouse received injection of (-)-EPI (1 mg/kg/day) for continuous 10 days followed by ligation of LAD coronary artery; (4) AMI + (-)-EPI + HY-100347A group (n = 6), mice were administrated (-)-EPI (1 mg/kg/day) for continuous 10 days after intraperitoneal injection of HY-100347A (10 mg/kg/day) for 30 min followed by LAD occlusion through ligation.

Hematoxylin and eosin (HE) staining

The sections were dewaxed with xylene □ for 10 min and xylene □, □ for 5 min, followed by incubation in absolute ethanol and gradient ethanol (95%, 85%) for 1 min, respectively. After rinsing with tap water, sections were stained with hematoxylin for about 1-5 min, differentiated with 1%/0.5%/0.25% hydrochloric acid for 3-5 s, and stained with eosin for 20 s-2 min. After that, the sections were dehydrated with gradient ethanol from 85% to 100%, immersed in xylene □and □, blocked with neutral gum, and finally sealed. hematoxylin stains the cell nuclei are stained with a blue or purplish-blue color, muscle, cytoplasm, red blood cells, and connective tissue with a pink color, and eosinophilic particles in cytoplasm with a bright red color; collagen fibers are stained light pink; elastic fibers are stained bright pink.

Masson staining

Myocardial tissues were cut into sections and deparaffinized in distilled water. The sections were stained with hematoxylin for 5-10 min, differentiated with hydrochloric acid and alcohol, and rinsed in running water. After washing, the sections were stained in acid fuchsin solution for 5-8 min, washed in distilled water, and stained with 1% phosphomolybdic acid for 1-3 min and with aniline blue solution or bright green solution for 5 min. Then the samples were dried in a 60°C incubator, cleared with xylene, and sealed. Collagen fibers were counterstained with aniline blue (blue) or bright green (green), cytoplasm, muscle fibers and red blood cells were stained red, and nuclei were stained blue-brown.

Fibroblast cell culture

1-2 day-old neonatal mouse hearts were extracted upon disinfection with 70% alcohol and placed on a DMEM petri dish. After removing blood and cutting off the aorta and atrium, the hearts were cut into pieces (2 mm). The sections were digested with 0.25% pancreatin at 37°C for 5 min, 6 to 8 times, and subjected to differential adhesion to to obtain fibroblasts. The cells were seeded in 30 mm culture flasks, 6-well plates or 96-well plates at a density of 10^6 cells/ml. The flasks and plates were placed in an incubator (37°C, 95% air and 5% CO_2) for 48-72 h and then transferred to serum-free DMEM for culturing for 24 h. The fibroblasts then were randomly divided into groups for various experiments.

Cell Counting Kit-8 (CCK-8) assay

Adherent cardiac fibroblasts in the logarithmic growth phase were seeded in a 96-well plate with 100 μl/well (5×10^3 cells), and cultured under normoxia or hypoxia for 24 h. The fibroblasts were incubated with different concentrations of (-)-EPI for 72 h and 10 μM CCK-8 solution was added to the cells every 24 h and cultured for 2 h. A microplate reader was used to detect optical density (OD) at 450 nm for subsequent analysis of viability.

Flow cytometry

Following washing cells in cold PBS buffer, the cells were suspended in Binding Buffer to prepare a suspension of 1 × 10^6 cells/ml. 100 μl of cell suspension was taken to mix with 5 μg of purified recombinant Annexin V-FITC, and incubated for 15 min at room temperature in the dark. Then the mixture was centrifuged at 1000 rpm for 5 min with the supernatant removed. 200 μl Binding Buffer was added to resuspend the cells with 5 μl PI, and a flow cytometer was used to evaluate the cell apoptosis.

Reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cell and tissues using Trizol reagent (15596026, Invitrogen, USA) and reverse transcribed into cDNA according to the procedure of PrimeScript RT reagent Kit (RR047A, Takara, Japan). Then the cRNA was subjected to RT-qPCR using Fast SYBR Green PCR kit (Applied biosystems) and ABI PRISM 7300 RT-PCR system (Applied biosystems) to quantitatively analyze RNA in triple. With GAPDH as an internal reference, gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and the experiment was repeated for at least three times. The primer sequences were listed in table 1.

Western blot

Cardiac fibroblasts were incubated with 100 μl RIPA and 1 μl PMSF, and sonicated. Mouse myocardial tissues were grinded in 200 μl cell lysate and lysed on ice for 30 min. The above cell and tissue samples were centrifuged at 4°C, 13000 r/min for 30 min and the supernatant was collected, with protein concentration determined by Bicinchoninic Acid method. 100 μg of protein was mixed with 5 × loading buffer and denatured at 100°C for 5 min. After centrifugation, proteins were separated through SDS-PAGE and transferred to polyvinylidene fluoride membrane, blocked with 5% skim milk. The membrane was then incubated with the corresponding primary and secondary antibodies, and detected by an infrared fluorescence scanning system after incubation. With GAPDH as an internal reference, the samples were photographed, and analyzed the image analysis software odyssey1.2.

Statistical analysis

All statistics were analyzed using the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Measurement data was presented as mean ± standard deviation. Comparisons those among three or more groups were made by one-way analysis of variance (ANOVA), with Tukey post-hoc test. All tests were two-sided and $P < 0.05$ was considered statistically significant.

Results

(-)-EPI regulates MF following AMI in mice

To investigate whether (-)-EPI regulates malignant arrhythmia, a mouse AMI model was established and the modeled mice were administered with (-)-EPI. Mouse heart samples were collected and subjected to HE and Masson staining to evaluate pathological changes in myocardial interstitium (Figure 1A, B). The results showed that compared with the sham-operated mice, the AMI mice had obvious MF in the marginal myocardial infarct tissue. But upon the treatment with (-)-EPI, the MF was significantly attenuated, indicating that (-)-EPI could improve MF in AMI mice.

(-)-EPI increases hypoxia-induced cardiomyocyte proliferation and decreases apoptosis

Apart from the *in vivo* function of (-)-EPI, myocardial fibroblasts from neonatal rats were taken for further identifying its impact on AMI *in vitro*. The fibroblasts were cultured under hypoxia to induce MI and establish cell model of AMI (Figure 2A). CCK-8 assay was initially performed to determine the effect of different concentrations of (-)-EPI treatment on cell proliferation. Our results indicated that hypoxia significantly inhibited cell proliferation, while (-)-EPI treatment promoted hypoxia-induced fibroblast proliferation (Figure 2B). Besides, (-)-EPI was indicated to attenuate hypoxia-induced apoptosis, according to the results from flow cytometry (Figure 2C). Taken altogether, these results indicate that (-)-EPI could enhance fibroblast proliferation and restrain apoptosis, prolonging the survival of cardiomyocytes.

(-)-EPI down-regulates TGF-β1 expression

We then further explored the mechanism of (-)-EPI in the process of MF. TGF-β signaling is known to play a large role in MF. Herein, we examined the specific effect of (-)-EPI on TGF-β expression under different conditions by RT-qPCR and Western blot. Compared to control treatment, hypoxia treatment significantly promoted the expression of TGF-β1, SMAD2 and SMAD3, while (-)-EPI treatment restored their expression levels to that of the control group. Additionally, as HY-100347A is an agonist of TGF-β signaling, addition of HY-100347A only offset the effect of (-)-EPI to a certain extent (Figure 3A and B). As for the the impact of (-)-EPI on the collagen content in cells, the imaging of Sirius Red staining depicted increased collagen content in hypoxia-treated cells and that compared with the hypoxia group, (-)-EPI administration resulted in a decline in collagen content which could be reversed by addition of HY-100347A (Figure 3C). These above results elucidate that (-)-EPI could improve MF by down-regulating the expression of TGF-β1.

(-)-EPI attenuates MF following AMI in mice by down-regulating the expression of TGF-β1

To study whether and how (-)-EPI regulates MF in AMI mice through TGF-β1 signaling *in vivo*, (-)-EPI and HY-100347A were administered to AMI mice and control mice through intraperitoneal injection. (-)-EPI treatment alone inhibited the process of MF, but HY-100347A aggravated MF (Figure 4A, B). In a word, (-)-EPI could impair MF after AMI in mice by down-regulation of TGF-β1.

Discussion

MF, a typical symptom of myocardial remodeling, could contribute to increasing myocardial stiffness and deterioration of ventricular diastolic function, and a decline in coronary artery reserve (29,30). It has been proved that myocardial infarction always results in heart failure and both are often accompanied by severe MF (31). Previous studies mainly focused on the prevention and reversal of structural and functional myocardial abnormality. Recent investigators have gradually realized the importance of MF in the heart failure, so MF becomes a new target of cardiovascular disease.

(-)-EPI has a wide range of effects. Its antioxidant and anti-inflammatory activity as well as regulatory impact on cardiovascular diseases have been elucidated in recent years (16,32). Accumulating evidence has reported that (-)-EPI directly chemically reacts with reactive oxygen species (ROS), or interact with signal pathways or enzymes regulating ROS to eliminate ROS S(13,33). In addition, pancreatic inflammatory cells infiltrate and release IL-1β during diabetes, whereas (-)-EPI inhibits the expression of IL-1β and iNOS through blocking the nuclear localization of the p65 subunit of NF-κB (34). In cardiovascular diseases, (-)-EPI could mitigate platelet function to allow fibrin clot dissolution, and play multifaceted roles in thromboembolic diseases (35,36). A short-term study on healthy people demonstrated that consumption of high-flavonoid dark chocolate containing 46 mg (-)-EPI for 2 weeks strengthens endothelial function and improves vascular endothelial-dependent blood flow-mediated dilation (37). Furthermore, (-)-EPI is confirmed to protect the heart against damage and promote heart growth. One study pointed out that (-)-EPI induces physiological cardiac growth through activation of PI3K/Akt pathway (38). Another study by Li JW et al. notices that (-)-EPI attenuates ischemia-induced heart damage targeting PTEN to regulate PI3K-AKT signaling (39). Besides, treatment with (-)-EPI effectively reduces cardiac fibrosis size in the sarcoglycan null mice (40). However, in spite of these findings, the interaction between (-)-EPI, MF and MI is still unclear, and therefore, we managed to further unravel the mechanism underlying (-)-EPI in MF after MI.

Our findings depict the role of (-)-EPI in regulating MF and its underlying mechanism. (-)-EPI administration could significantly improve MF following AMI *in vivo* and effectively prolong the survival of cardiac fibroblast *in vitro.* Mechanistically, (-)-EPI targeting TGF-β signaling exerts its regulatory activity and thereby inhibits collagen deposition and MF.

In cardiovascular disease, TGF-β signaling is closely related to several catechin compounds such as Epigallocatechin gallate (EGCG) which is reported to attenuate myocardial damage in mice with heart failure through the TGF-β1/Smad3 signaling pathway (41). EGCG effectively interferes with TGF-β1 signaling to decrease fibroblast proliferation and collagen production (42). But the relationship between (-)-EPI and TGF-β has not been fully elucidated, especially in cardiovascular diseases. Our study elucidates the TGF-β signaling mechanism how (-)-EPI regulates MF, serving to further understand the (-)-EPI and its function in MF.

Limitations, certainly, still exist in the present study. We have not introduced how (-)-EPI is located on the TGF-β signal to perform its function. In addition, whether (-)-EPI also could effectively regulate the MF following MI in other animal models, such as pig models still requires more experiments, which are direction of our following investigation.

Conclusion

In conclusion, our study illustrates that (-)-EPI attenuates MF following MI by targeting TGF-β signals and underlies the causality of (-)-EPI in improving MF.

Abbreviations

(-)-EPI: (-)-Epicatechin

MF: myocardial fibrosis

AMI: acute myocardial infarction

ECM: extracellular matrix

TGF-β: Transforming growth factor beta

PAI-1: plasminogen activator inhibitor-1

TIMP: tissue inhibitors of matrix metalloproteinases

LAD: left anterior descending

HE: Hematoxylin and eosin

CCK-8: Cell Counting Kit-8

RT-qPCR: Reverse transcription polymerase chain reaction

ROS: reactive oxygen species

EGCG: Epigallocatechin gallate

Declarations

Ethics approval

The study protocol was approved by the institutional ethics committee and research board of Harbin Medical University.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Disclosure statement

The authors declare that they have no competing interests.

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Authors' contributions

JWL wrote and submitted the manuscript. XCZ and ZMG contributed to the conception and design of the study; JWL, YF and FT performed the experiments and collected the data; TL and QP performed the statistical analysis; FYL and XL completed data interpretation. All authors contributed to revising the manuscript and approved the submitted version.

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Not applicable.

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Tables

Table 1 Sequence of primers for real-time PCR

Primer	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')
GAPDH	CAGAAGGGGCGGAGATGAT	AGGCCGGTGCTGAGTATGTC
TGFβ1	CTAATGGTGGACCGCAACAAC	GCTTCCCGAATGTCTGACGTA
SMAD2	ACGTTAACCGAAATGCCACT	ATGTAATACAAGCGCACTCCC
SMAD3	ACTGTCCAATGTCAACCGGAA	ATGTAGTAGAGCCGCACACC

Figures

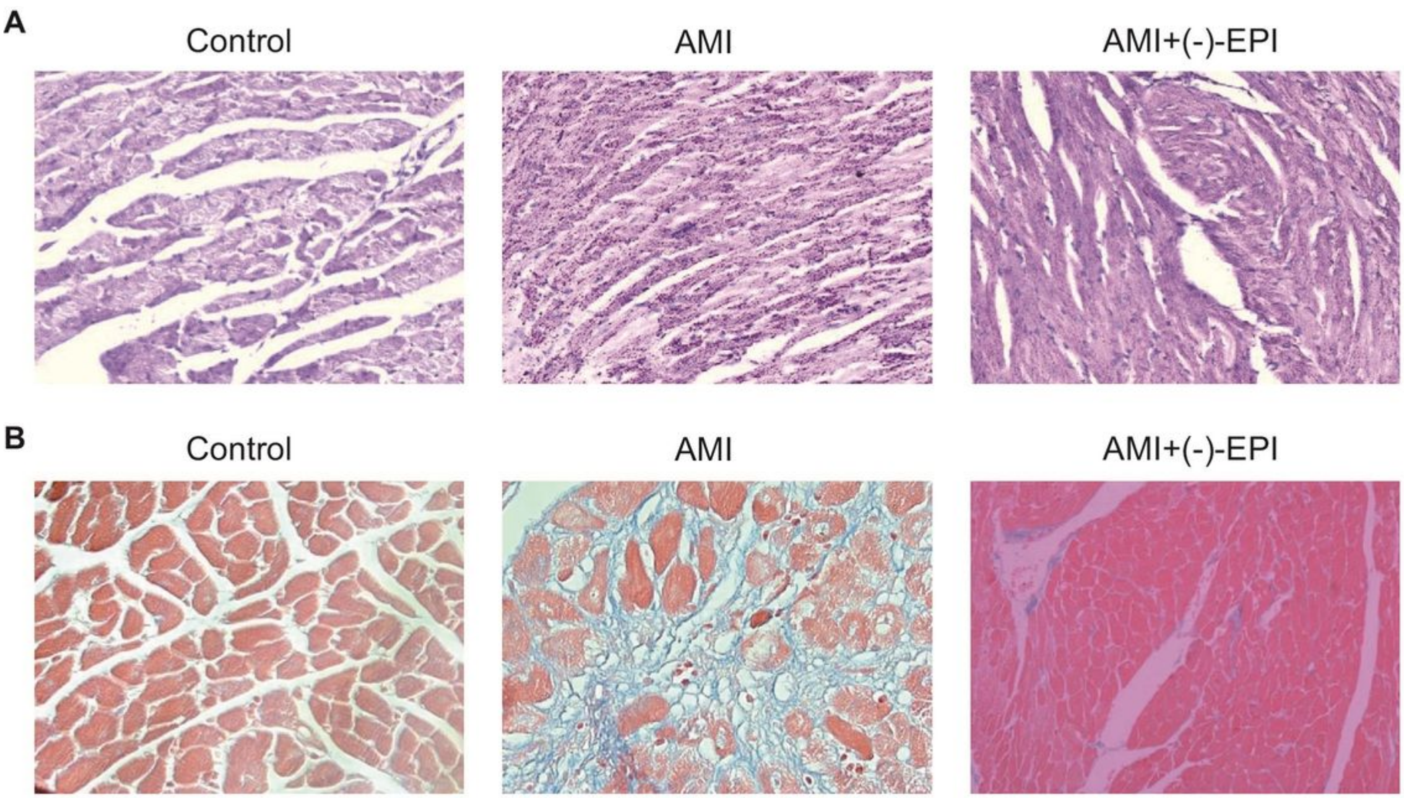

Figure 1

(-)-EPI attenuates MF of AMI mice. Mice were gavaged with or without (-)-EPI (1 mg/kg/day) for 10 days, and subjected to LAD occlusion to induce AMI models. After that, the mice were sacrificed and their hearts were taken out for staining. (A, B) Representative imaging of HE staining (A) and Masson staining (B) to assess the severity of MF (100 ×).

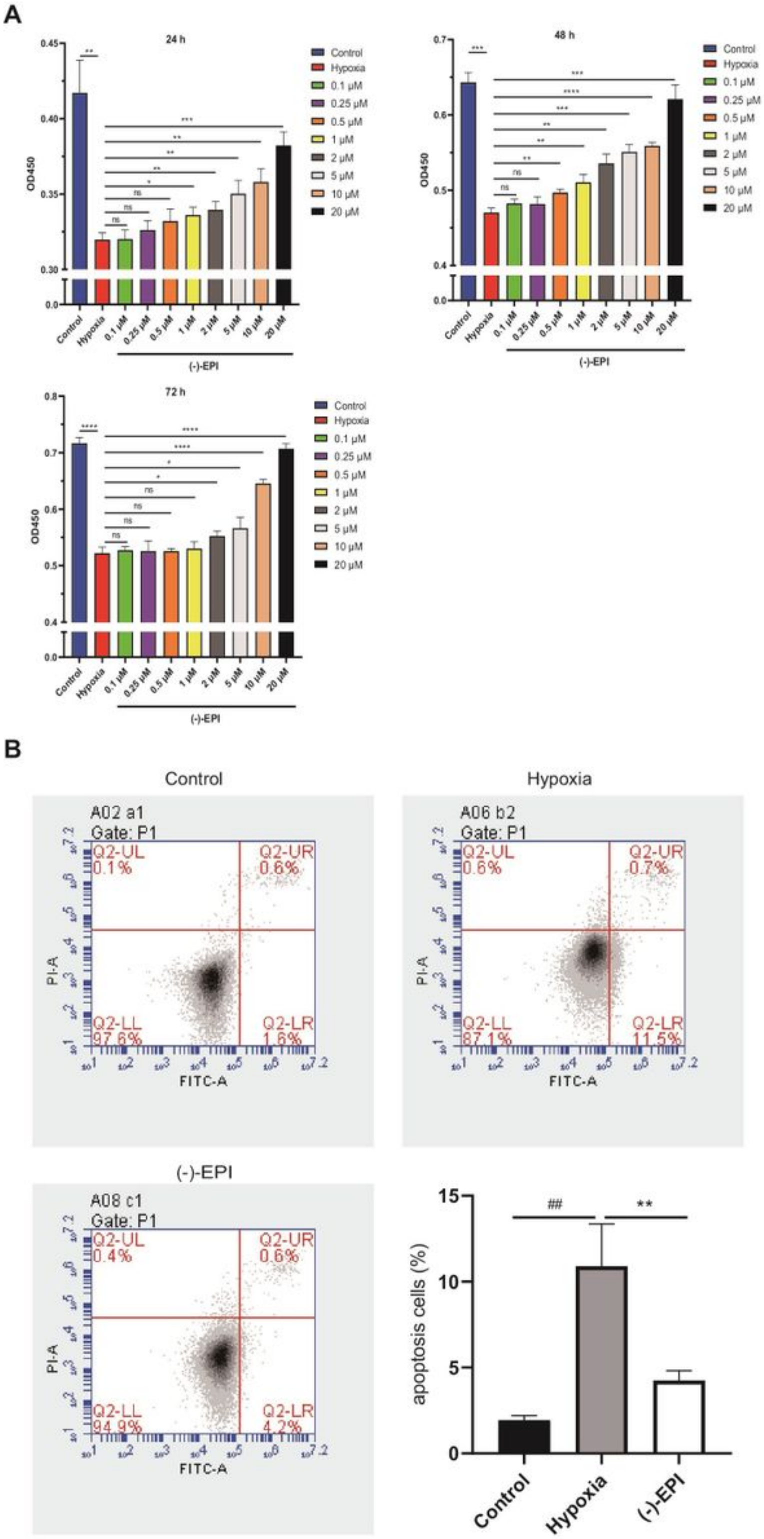

Figure 2

(-)-EPI regulates myocardial fibroblast proliferation and apoptosis. (A) Myocardial fibroblasts were cultured in hypoxic condition (2% O2) for 24 h. (B) For CCK8 assay, cells were treated with different concentrations of (-)-EPI and were placed back in the 5% carbon dioxide incubator for 72 h, and taken out every 24 h to detect cell proliferation; (C) For apoptosis assay, cells were treated with 20 μM (-)-EPI for 48 h and then used to calculate apoptotic ratio. Data represent the results of three independent experiments

± SD; ns non-significant, ##p < 0.01, ###p < 0.001, ####p < 0.0001 vs. control; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. hypoxia.

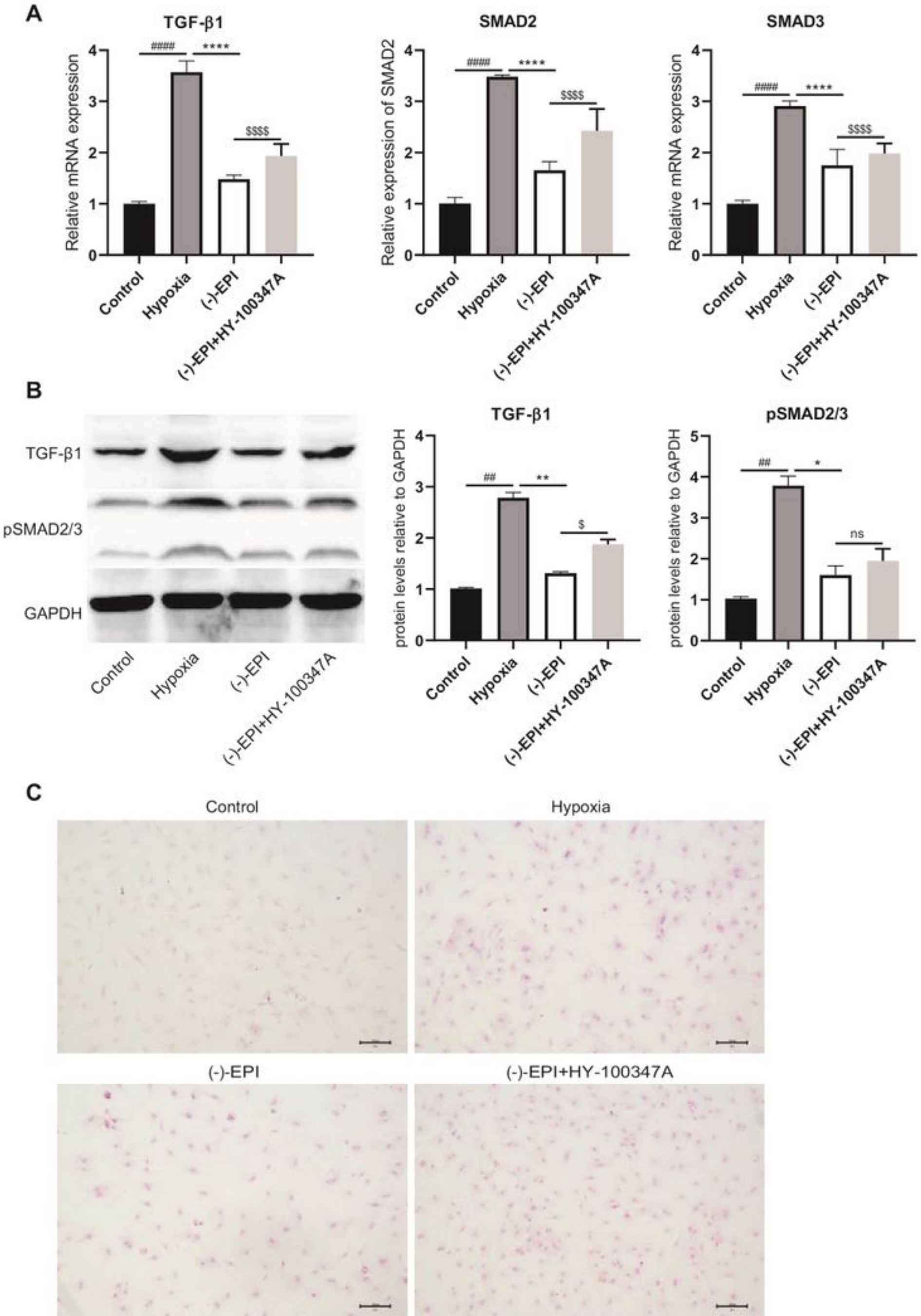

Figure 3

(-)-EPI down-regulates TGF-β signaling and inhibits collagen deposition. (A, B) RT-qPCR and Western blot of mRNA and protein expression of TGF-β1, SMAD2 and SMAD3 under normoxia or hypoxia with (-)-EPI and HY-100347A treatment; (C) Representative images of Sirius Red staining to reflect the collagen content under normoxia or hypoxia with (-)-EPI and HY-100347A treatment and corresponding quantification. Data represent the results of three independent experiments ± SD; ##$p < 0.01$, ####$p < 0.0001$ vs. control; *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ vs. hypoxia; ns non-significant, \$$p < 0.05$,

$p < 0.0001$ vs. (-)-EPI.

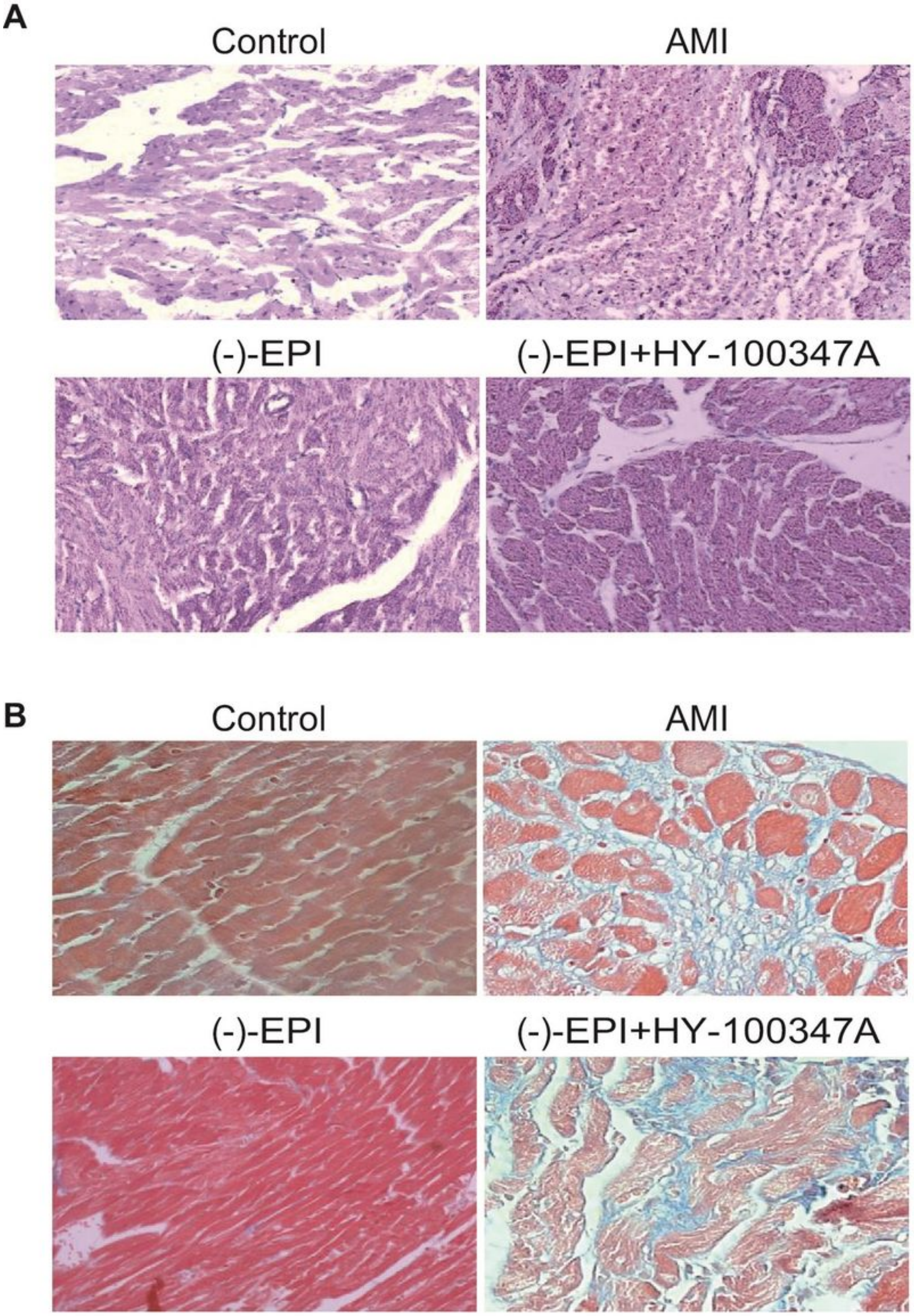

Figure 4

(-)-EPI inhibited MF of AMI mice by inactivation of TGF-β signaling. Mice were gavaged with or without HY-100347A (10 mg/kg/day) or (-)-EPI (1 mg/kg/day) for 10 days, and then used to construct AMI models. After that, the mice were sacrificed and their hearts were taken out for staining. (A, B) Representative imaging of HE staining (A) and Masson staining (B) to assess the severity of MF (100 ×)