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Anti-inflammatory Influence of Trans-anethole on The Cardiac Regenerative Capacity in Myocardial Ischemia/Reperfusion Injuries

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Abstract

Reperfusion injury of an ischemic heart is considered one of the risk factors of cardiac mortality associated with high inflammation, limiting the innate ability of the body to heal even with the use of external interventions. Trans-anethole (TA) is an efficient anti-inflammatory agent that protects the heart and increases its capacity to regenerate. 3 doses of TA (50, 100, and 200 mg/Kg) were administered 60 minutes before ischemia/reperfusion injury induction. Cardiac left ventricle tissue and blood samples were used for histopathological and immunohistochemical examination and assessment of the expression of mRNA FOXC1- miR-1248 – lncRNA TSIX and cardiac enzymes. TA administration negated biochemical, molecular, and histopathological alterations induced by ischemia-reperfusion injuries. The present findings revealed a reduction in LDH, CK-MB, and cardiac troponin. The microscopic examination revealed regression in the presence of cardiac edema, hemorrhage, cellular inflammatory infiltration, and fibrosis, along with an elevated number of C kit + cells in the cardiac tissue specimens. Also, TA showed an increase in the relative expression of miR-1248 with a decrease in mRNA FOXC1 and lncRNA TSIX. The study's findings suggest that mRNA FOXC1, miR-1248, and lncRNA TSIX possess high potential as diagnostic biomarkers, exhibiting perfect sensitivity and specificity.

Keywords: Trans-anethole, ischemia-reperfusion injury, RNA, rat, progenitor cells

1. Introduction

Ischemic heart disease (IHD) is a leading cause of mortality within the global population among cardiovascular disorders [1]. It is the leading cause of the development of congestive heart failure and cardiac death [2]. IHD occurs due to constriction of the coronary arteries, which supply blood to the myocardium, reducing the supply of oxygen and nutrients to the heart tissue [3]. Reperfusion therapy is the only effective treatment for ischemic myocardium. However, the heart muscle is adversely affected by various factors, including paradoxical pH fluctuation, calcium overload, reactive oxygen species, mitochondrial impairment, inflammation, and disrupted protein phosphorylation. This phenomenon is commonly referred to as ischemia-reperfusion injury (IRI) or reoxygenation injury, which paradoxically inhibits the process of myocardial reperfusion [4], [5]. Following reperfusion, some cardiac myocytes die from necrosis or apoptosis [6]. Numerous researchers have investigated "cardioprotective" interventions that strengthen the heart's resistance to ischemic mortality [7], [8]. An innovative treatment strategy is the recruitment of endogenous cardiac progenitor cells (CPCs). CPCs are a particular kind of stem cells that reside within the heart and can differentiate into cardiomyocytes. It is possible to increase the myocardium's capacity for regeneration and speed up tissue healing by activating and mobilizing these CPCs [9]. However, research has demonstrated that the regeneration capacity of the myocardium declines in the presence of elevated levels of inflammatory mediators [10]–[12]. The immune system and inflammation should be regulated in organisms with the ability to regenerate to create a favorable environment for stem cells and encourage them to replace the missing cells [13]. There is a need for additional research and clinical trials to fully

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comprehend the efficacy and safety of these approaches, but they represent promising developments in the field of cardiac regeneration. TA is a phytochemical compound found in fennel, anise, and star anise, as well as in about 20 other plant species. TA exhibits anti-inflammatory [14]–[18], antioxidant [18]–[20], antimicrobial and antiviral properties [21]. TA is suggested as a neuroprotective agent to recover ischemic neuronal damage, where it shows neuroprotective effects through anti-excitotoxicity, anti-oxidative stress, and mitochondrial protection [22]. It has been proven to be anticarcinogenic [18], [23], anti-metastatic activity [23], and a vasoactive effect [24]. Additionally, experiments have demonstrated that at low levels, TA has no toxicity [25] and it is considered safe because it has no genotoxic or carcinogenic effect [19], [26]. More investigations are required to discover the cardioprotective properties of TA and elucidate its underlying mechanism of action. Competing endogenous RNA (ceRNA) networks have been discovered to fulfill a significant function in ischemic heart diseases. Messenger RNAs (mRNAs), long non-coding RNAs (lncRNAs), and microRNAs interact with one another in ceRNA networks to control gene expression. [27]–[29]. While mRNAs are translated into proteins, non-coding RNAs (ncRNAs), such as miRNAs and lncRNAs, control gene expression at the transcriptional and post-transcriptional levels instead of encoding proteins [30]. Dysregulation of these networks has been implicated in the pathogenesis of various cardiovascular diseases, including ischemic cardiomyopathy [28]. In addition, ncRNAs are emerging players in cardiac regeneration and repair; miRNAs and lncRNAs could be promising targets to enhance cardiac regeneration for designing new potential therapies against cardiovascular diseases [31], [32]. Forkhead box C1 (*FOXC1*) is a transcription factor (TF) that has a substantial role in the processes of cellular proliferation, survival, and the pathogenesis of cardiovascular disorders. [33]. Recent investigations suggest that the *FOXC1* gene activates ELAVL1, which regulates programmed cell death and oxidative stress [33], [34]. miR-1248 has been found to regulate the expression of mRNAs involved in inflammatory pathways (NF-κB) [35]. While the exact role of miR-1248 in cardiovascular diseases is not well-established, microRNAs generally have been recognized as important regulators in cardiovascular pathophysiology [35]. Significantly, the lncRNA TSIX has exhibited functional properties as a ceRNA regulating many cellular behaviors in cancer and inhibiting cardiomyocyte apoptosis [36], [37]. Considering the aforementioned information, we used an IRI rat model to investigate the potential anti-inflammatory role of TA pretreatment in IRI and its related mechanism in modulating the expression of the inflammatory and cardiac-related gene *FOXC1* and its epigenetic regulators (lncRNA-TSIX and miR-1248).

2. Materials and methods

2.1. Chemicals and drugs

Both the TA and urethane used in this experiment were supplied from (Sigma Aldrich, St. Louis, USA).

2.2. Experimental design

The Vaccine and Immunity Organization in Helwan, Egypt, supplied sixty 200±50 g weight male Wistar rats. Animals were kept in a cage with a 12-hr light/dark cycle, running water, and regular rat chow ad libitum (after a week of adaptation). The Institutional Animal Ethics Committee at Ain Shams University's School of Medicine approved all the work done on the animals (approval no. 149/2020), and the protocols followed were consistent with the NIH's recommendations for the care and use of laboratory animals (NIH Publication No. 85-23, revised 1996).

2.2.1. Animal groups

The animals were divided in a random manner into five distinct groups (12 rats/group): (i) sham or naive group, (ii) ischemia-reperfusion (I/R) group, (iii) TA1, (iv) TA2, and (v) TA3 groups. The rats were administered in the last three groups with TA doses of (50mg, 100mg, and 200mg/Kg bwt), respectively, intraperitoneally 60 minutes before coronary ligation.

2.2.2.Induction of ischemia/reperfusion

Intraperitoneal injections of urethane (1.2 mg/kg) were used to induce anesthesia in the rats. Using a rectal thermistor sensor, body temperature was tracked during the experiment. Using the proper heating blanket, the body temperature was kept at 37±1°C. After establishing intubation with a rodent ventilator at 70–80 breaths per minute, the heart was exposed by a median sternotomy, and a 5-0 polyethylene suture was used to tie a ligature around the left anterior descending coronary artery. The ligature's free ends were utilized to create a noose around a syringe plunger that was laid flat on the myocardium. Coronary occlusion was initiated by tightening the noose around the rubber band for 45 minutes [38], confirmed by immediate blanching of the infarcted area. Reperfusion was achieved by releasing the ligature for 120 min, and then animals were euthanized. An electrocardiogram (ECG) tracing was recorded in lead II both before and subsequent to ligation. To record the ECG in lead II, four needle electrodes were put beneath the skin of the anesthetized rat's limb. The purpose of this recording was to assess any changes in the ST segment, which would serve as confirmation of myocardial ischemia. The same technique, but without ligation, was applied to the naive animals.

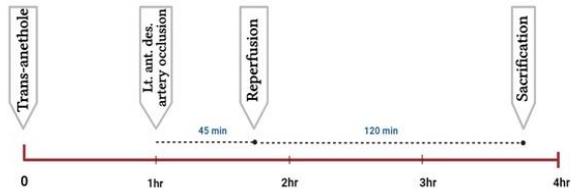

Fig. 1. Experimental design.

2.2.3.Sample collection

Following the sacrifice, blood samples were collected, and the sera were separated by centrifuging the whole blood sample for 10 minutes at a speed of 3000 rpm. The hearts were promptly removed, washed in 0.9% saline solution, collected, and stored for histology, immunohistochemistry, and molecular analysis. Each group's half of the rat heart tissues was used for molecular testing, while for histological and immunohistochemical examinations, the other half was used.

2.3. Biochemical analysis

Spectrophotometer Unico® 1200 was used to assess the levels of lactate dehydrogenase (LDH) and creatine kinase isoenzymatic MB form (CK-MB) using diagnostic kits purchased from Spectrum Company, Cairo, Egypt. The method uses a kinetic spectrophotometric technique to determine serum samples quantitatively and then calculate the activity using an equation for each parameter. Serum cTnT was quantified using a commercial ELISA kit according to the manufacturer's instructions.

2.4. Histopathological examination

The heart was removed after euthanasia, and the tissue was fixed in 10% neutral buffered formalin (pH 7.4) for 24 hours, then washed, treated in various grades of alcohol, cleaned in xylene, and embedded in paraffin wax. Serial sections of these blocks were then cut at a thickness of 5 m, and these sections were routinely stained with hematoxylin and eosin (H&E) [39]. Slides were visualized using an Olympus BX43 light microscope coupled to an Olympus DP 27 digital camera, and photos were transferred into the associated CellSens dimensions software. We examined each tissue section for morphological signs of cell death and fibrosis.

2.5. Immunohistochemical analysis

In brief, endogenous peroxidase activity was suppressed by H_2O_2 to lessen background staining. Slides were cleaned for 2 to 5 minutes in TBS with 0.025% Triton X-100 before being briefly drained. Antibodies were employed at 0.5 to 10 g/mL concentrations and incubated at 4°C overnight. After performing a hematoxylin (blue) counterstain, the slides were finally dried out and cleaned. The morphometric analysis of the stained tissues was conducted in the following manner: A total of six heart sections, stained and obtained from six rats per experimental group, were carefully evaluated. A selection of five high-power fields/sections was made to quantify the proportionate area occupied by collagen fibers in the myocardium stained with Masson's trichrome and to enumerate the c-kit + cells present in the heart. The specimens were analyzed using an image analyzer program called Leica Q win V.3, installed on a computer in the Histology Department of the Faculty of Medicine at Ain Shams University. The computer was linked to a Leica DM2500 microscope manufactured by Leica in Wetzlar, Germany.

2.6. Molecular assays

2.6.1.Molecular markers retrieval

Based on earlier microarray studies, promising RNA-Based biomarkers related to IRI were retrieved using bioinformatics tools. The public microarray databases related to competing endogenous RNA network were used to predict cooperation during I/R. The expression atlas database (https://www.ebi.ac.uk/gxa) (Supplementary **Fig. 1. and 2.**) showed the expected relationship among expression of *FOXC1*, I/R, and stem cell differentiation. Furthermore, the expression of *FOXC1* in heart muscle was verified in Protein atlas database (https://www.proteinatlas.org/), Genatlas database (http://genatlas.medecine.univ-paris5.fr) and The Genotype-Tissue Expression portal (https://gtexportal.org/home/gene/FOXC1) represented in (Supplementary **Fig. 3,4, and 5**), respectively. Non-coding RNAs interacting with *FOXC1* in the heart were retrieved by scanning public microarray databases for the prediction of a set of non-coding RNAs relevant to heart diseases. The Prediction of miRNA targets for *FOXC1* was through (https://mirdb.org/cgi-bin/mining.cgi) and (https://www.targetscan.org/) as shown in (Supplementary **Fig. 6. and 7.**). As regards the lncRNA associated with *FOXC1* mRNA together with its interacting miRNA, they were selected from (http://rtools.cbrc.jp/LncRRIsearch/detail.cgi) and (http://gyanxet-beta.com/lncedb/index.php), as revealed in (Supplementary **Fig. 8. and 9.**).

2.6.2. Extraction of total RNA

Total and miRNAs were extracted from cardiac tissues in accordance with the manufacturer's instructions using a miRNeasy Mini Kit, cat. no. 217004 (Qiagen, Hilden, Germany). Using NanoDrop 2000c Spectrophotometer, the concentration and purity of RNA were estimated, and the RNA purities were ~2.0. Using the miScript II RT Kit, cat no. 218161 (Qiagen, Hilden, Germany), reverse

transcription of the total RNA into cDNA was carried out in accordance with the manufacturer's instructions.

2.6.3. Quantification of the RNA panel using real-time PCR (qPCR)

The Hs_*FOXC1*_1_SG QuantiTect Primer Assay, cat no. 249900, and the QuantiTect SYBR Green PCR Kit, cat no. 204141 (Qiagen, Germany) were used to quantify mRNA-*FOXC1*. The housekeeper gene used is the primer sequence Hs_ACTB_1_SG QuantiTect Primer Assay, cat no. 249900, ID: QT00095431. The miScript SYBR Green PCR Kit, cat no. 218073, and the Hs_miR-1248_1 miScript Primer Assay, cat no. 218300, ID: MS00014238, were used to quantify miRNA-1248. The Hs_SNORD68_11 miScript Primer Assay, cat no. 218300, ID: MS00033712, was used to normalize the gene's expression as a housekeeper gene. The RT^2 qPCR SYBR Green/ROX Master Mix Kit, cat no. 330520, and the RT^2 qPCR Primer Assay for Human TSIX, Primer Assay; cat no. 330701, ID: LPH26832A, were used to quantify the level of LncRNA-TSIX expression. The Hs_ACTB_1_SG QuantiTect Primer Assay, cat no. 249900, ID: QT00095431, was used as a housekeeper gene to normalize the gene's expression. The 5 plex Rotor-Gene PCR Analyzer (Qiagen, Germany) was used to analyze all the samples.

2.7. Statistical analysis

The available statistical software packages of SPSS® software (version 23, SPSS Inc., Chicago, IL) was used to analyze the data. The Post HOC Tukey Test was used for multiple comparisons after one-way analysis of variance (ANOVA) and means ± standard deviation (SD) were used to express the biochemical results. The molecular data were expressed as median and percentile, followed by the Kruskal-Wallis H test for non-parametric variables. Spearman's correlation test was used for correlating non-parametric variables. For molecular data, the ROC curve was used to ascertain the sensitivity and specificity of each variable. The significance of the results was calculated as follows:

P value = level of significance
P value > 0.05 = Not significant
P value ≤ 0.05 = Significant
P value ≤ 0.001 = Highly significant

3. Results

3.1. Effect of TA on cardiac enzymes, LDH, CK-MB, and cTnT

Statistical analysis identified significantly elevated cardiac enzymes LDH, CK-MB, and cTnT levels of the I/R rats compared to the healthy rats ($P<0.001$). TA treatment of IR subjects at different doses has demonstrated significantly lower ($P<0.001$) cardiac enzymes LDH and CK-MB values than the I/R subjects. However, TA1 animals treated with a dose of 50mg/kg insignificantly declined the cardiac cTnT activity compared to the I/R groups. Concomitantly, TA2 (100mg/Kg) and TA3 (200mg/Kg) animals displayed a significant downgrade ($p<0.05$) in the activity of cTnT compared to I/R. The present data indicated a significant difference in the cardiac enzymes activity between all treated animals with different doses of TA except for the cTnT of TA2 (100mg/Kg) animals compared to TA1 (50mg/kg) and TA3 (200mg/kg) groups.

3.2. Histopathological and immunohistochemical c-kit analysis

The naive group's histological analysis using a standard H&E stain revealed a regular arrangement of heart muscle fibers with normal striations and branching. The histological structure of the cardiac myocytes was normal, with central oval nuclei and acidophilic sarcoplasm. Only a few tiny blood capillaries were visible in the intercellular gaps (Fig. 3. A). The histological examination of the ischemia-reperfusion (I/R) group showcased prominent distortion, fragmentation, and deterioration of the cardiac muscle striation. It was observed that myocardial necrosis had occurred, as evidenced by the presence of hypereosinophilia, cytoplasmic vacuolation, and peripheral pyknotic nuclei. The tissue spaces significantly increased, and there was infiltration of inflammatory cells as well as interstitial edema. Blood arteries that were dilated, clogged, and even ruptured were seen. (Fig. 3. B). Rats treated with TA1 (50mg/Kg) significantly preserved the morphology of cardiomyocytes and tissue spaces without signs of focal necrosis. The detected myocardial cells displayed a normal morphology characterized by limited cytoplasm vacuolization. The presence of cellular infiltration was minimal, and there was a notable absence of interfibrillar hemorrhage (Fig. 3. C). Histological examination of the TA2 (100mg/Kg) treated group showed the same finding as the TA1-treated group (Fig. 3. D). TA3 (200mg/Kg) treated rats showed approximately normal histological architecture of the cardiac muscle with congested blood vessels (Fig. 3. E).

C-kit+ cells were primarily detected between cardiac myocytes in the subepicardial region of the heart tissue after immunohistochemical labeling for CPCs by c-kit. TA administration enriched the

presence of C-kit+ cells compared to I/R and naïve animals (Fig. 4. a-e).

3.3. The Effect of TA on the expression of cardiac lnc-TSIX - miR-1248 - *FOXC1* mRNA based on relative gene expression among the different studied groups.

The present data indicated a significant upregulation (P<0.005) of *FOXC1* expression in the I/R animals compared to naïve animals. On the contrary, all treated doses of TA significantly downregulated (P<0.005) the expression of *FOXC1* (Fig. 5.).

Conversely, mir-1248 was significantly downregulated (P<0.005) in the I/R animals compared to naïve groups. Surprisingly, different doses of TA 50mg, 100mg, and 200mg enhanced and successfully increased the expression (P<0.005) of mir-1248 in all treated animal groups (Fig. 5.).

The ischemic reperfusion was able to insignificantly elevate the expression of the lncRNA TSIX compared to the naïve group, while this was significantly reversed (P<0.005) in all TA-treated animals with different doses. However, no significant difference in the expression of *FOXC1* or mir-1248, or TSIX was noted between the different doses of TA-treated animals.

3.4. Correlation and ROC curve for RNA network expressions in cardiac tissues

Correlation among molecular parameters showed a significantly strong negative correlation between *FOXC1* and miR-1248 at (r = -0.678, P≤0.001); also, *FOXC1* showed a significant very strong positive correlation with Lnc-TSIX at (r = 0.967, P≤0.001). Moreover, the long non-coding TSIX showed a significant moderate negative correlation with miR-1206 at (r = -0.598, P≤0.001), as presented in (Fig. 6.). ROC curve analysis revealed that the area under the curve of *FOXC1* mRNA was 1.000 (p< 0.001), miR-1248 was 1.000 (p<0.001), and Lnc-TSIX was 1.000 (p <0.001). This is why the estimation of miR-1248, FOXC1, and Lnc-TSIX molecular parameters may be regarded as a perfect and highly significant test for predicting ischemic heart disease.

4. Discussion

The most effective strategy for reducing myocardial infarction following acute myocardial ischemia (AMI) is rapidly restoring blood supply to the occlusive coronary artery using pharmacological

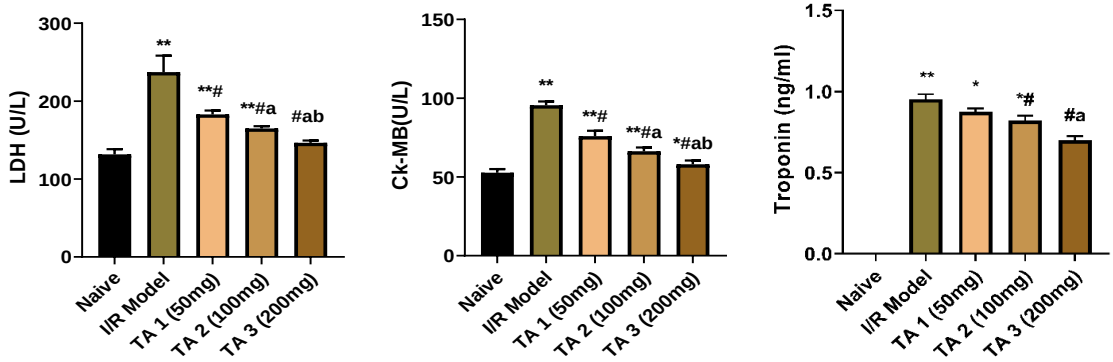

Fig. 2. Effect of ischemic reperfusion and different doses of TA on cardiac enzymes activity. (A) LDH, (B) Ck-MB, and (C) cTnT. Results are displayed as mean ± SD for different studied groups; number of animals = 6 rats/each group.
*P<0.001 compared to naïve group. #P<0.01. ##P<0.001 compared to IR group, aP<0.01 compared to TA1 (50mg/kg). b P < 0.001 compared to TA2 (100mg/Kg).

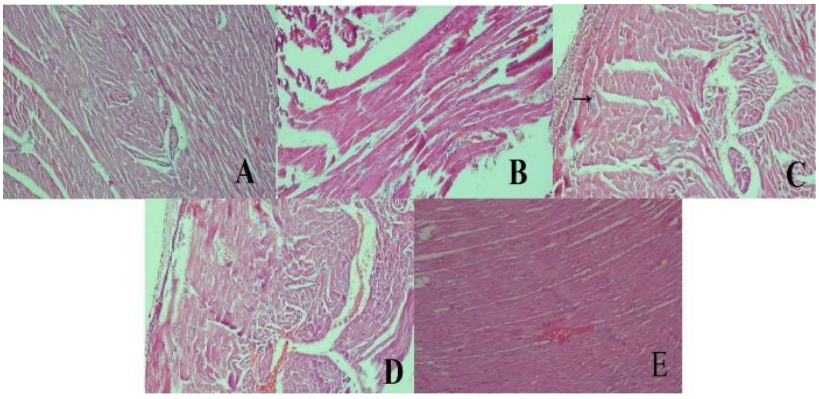

Fig. 3. Photomicrograph of H&E-stained sections of left ventricular tissues:
(A); naive group, (B); I/R group, (C); TA1 (50mg/Kg), (D) TA2 (100mg/Kg), (E); TA3 (200mg/Kg)
(A, B, C, D, E magnification: x100).

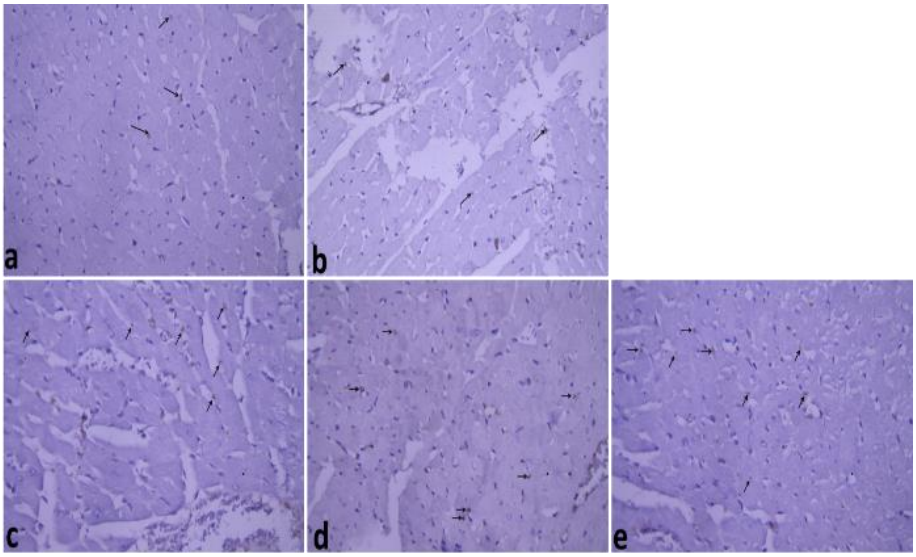

Fig. 4. Photomicrographs of cardiac muscle immunohistochemical stained with c-Kit antibody for demonstrating CPCs in rat myocardium of different groups. a: Naïve group, b: IR group, c: TA1 (50mg), d: TA2 (100mg), e: TA3 (200mg/Kg). Black arrows demonstrate the presence of c-Kit positive cells. Immunohistochemical stained with c-Kit antibody 400x.

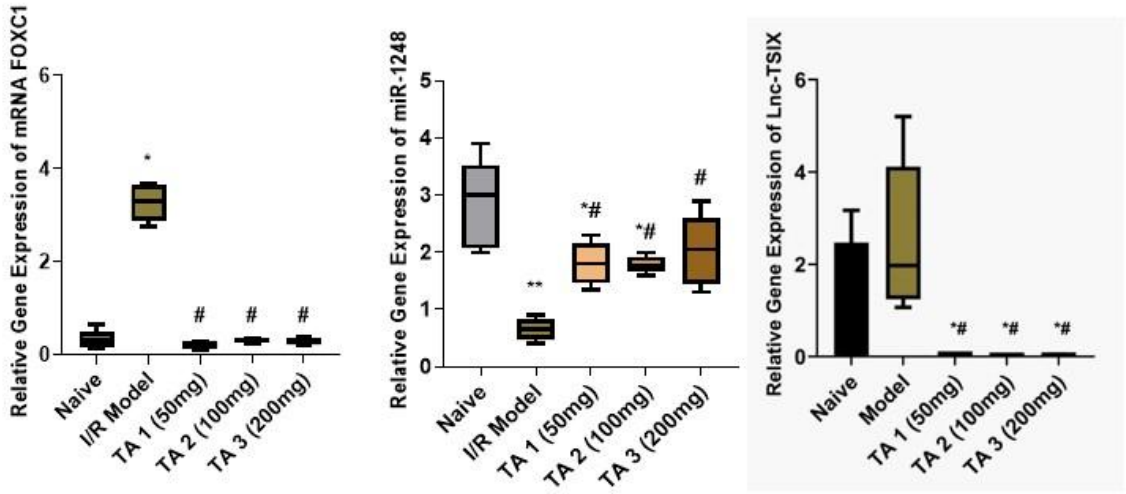

Fig. 5. Relative gene expression of mRNA *FOXC1*, miR-1248, and lnc-TSIX in different studied groups; number of animals = 6 rats/each group. **P<0.001, *P<0.01 compared to naive group. #P<0.001 compared to I/R group, aP<0.05 compared to TA1 (50mg/Kg). bP<0.05 compared to TA2 (100mg/Kg).

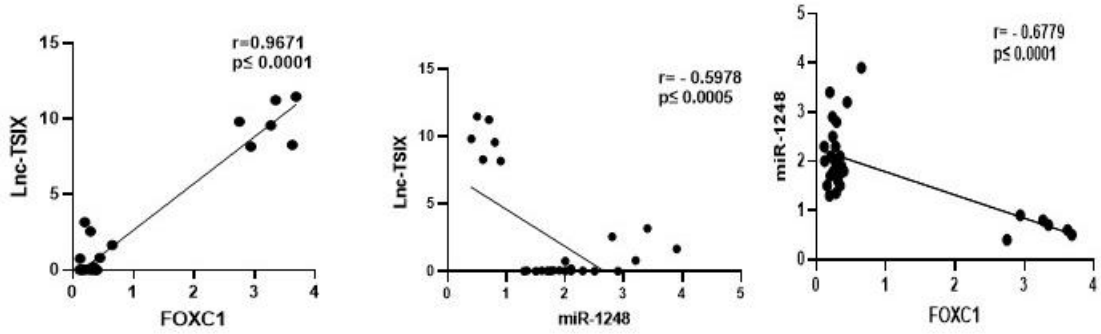

Fig.6. Correlation curve analysis for *FOXC1* mRNA, miR-1248 and Lnc-TSIX gene expression.
P value > 0.05 is not significant.
P value < 0.05 is statistically significant.

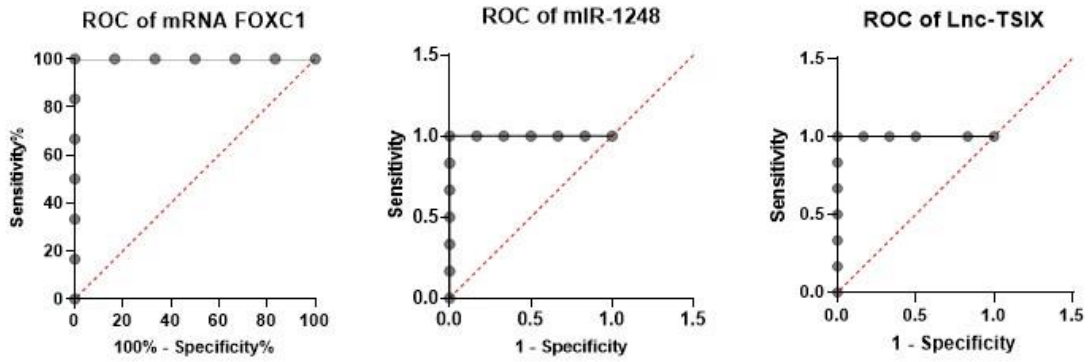

Fig. 7. Roc curve analysis for *FOXC1* mRNA, miR-1248, and lnc_TSIX.
A): Sensitivity and specificity of *FOXC1* gene expression.
B): Sensitivity and specificity of miR-1248 gene expression.
C): Sensitivity and specificity of lnc_TSIX gene expression.

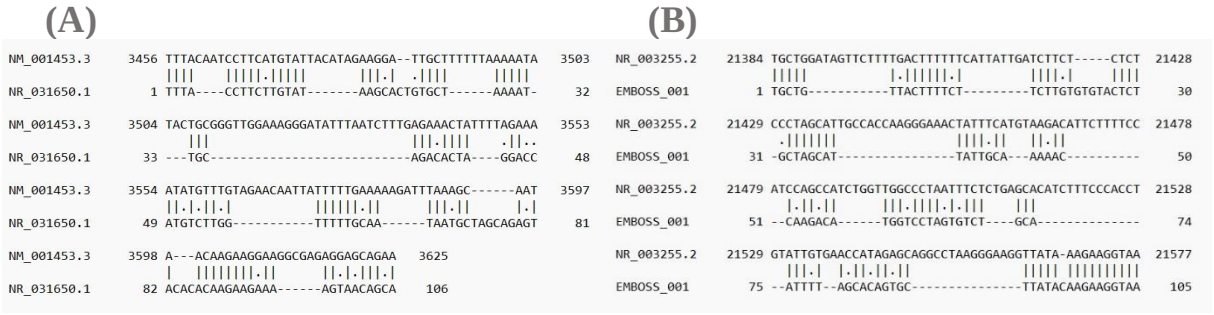

Fig. 8. Predicted complementary binding sites.
(A) *FOXC1* (NM_001453.3) and miR-1248 (NR_031650.1), (B) miR-1248 (NR_031650.1) and TSIX (EMBOSS_001)

or mechanical interventions. However, restoration of oxygen-rich blood to the ischemic tissue itself has the potential to result in myocardial ischemia-reperfusion injury (IRI) and additional complications due to several reasons, including an overload of calcium, reactive oxygen species (ROS), mitochondrial malfunction, PH alteration, and inflammation limiting treatment efficacy. Therefore, effective cardio-protective and treatment approaches that alleviate or reverse I/R-induced biochemical alterations are still required for ischemic heart disease (IHD) individuals [40], [41]. Recruitment of endogenous cardiac progenitor cells (CPCs) is a novel therapeutic strategy that enhances the heart's potential to regenerate and repair itself. It has shown promising results in enhancing neovascularization and blood flow in ischemic tissue, which leads to tissue perfusion and healing [42]–[44]. The ability of stem cells to repair injured myocardium is enormous, but more research is still needed to fully understand CPCs engraftment and survival to improve their clinical usefulness [45]. Previous research revealed that myocardial infarction (MI)-induced ischemia, inflammation, and inadequate vascular supply restrict the function and quantity of stem cells [46]. Furthermore, studies have investigated the negative toll of inflammation, oxidative stress, and hypoxia at the injury site on the quiescent stem cell niches crucial for cellular proliferation and healing [47]–[53]. If early inflammatory responses are reduced, the injured myocardium may respond more quickly to endogenous CPCs recruitment, repair, and regeneration.

Previously a cardioprotective effect of TA has been demonstrated against IRI by inhibiting toll-like receptors TLR4 and the accompanied inflammation and apoptosis [54]–[57]. Previous studies also correlated TA treatment with stem cell proliferation.

At the cellular level, mouse MSCs cultured on polycaprolactone (PCL)/polyvinylpyrrolidone (PVP) fibers loaded with various concentrations of TA were significantly promoted to differentiate into osteoblasts compared to those cultures on PCL/PVP fibers. TA, known for its oestrogenic activity, greatly boosted bone marrow MSC proliferation and osteoblast differentiation [58]. Rats treated with MSCs and fennel oil underwent folliculogenesis, which increased the number of developing ovarian follicles and improved the histological structure and ultrastructure of the rat ovarian tissue [59]. In this study, a rat model of myocardial IRI was used to study the protective

effect of TA by reducing inflammation and, as a result, enhancing the heart's potential to regenerate. In the current investigation, the administration of TA decreased the levels of cardiac enzymes LDH, CK-MB, and cTnT post-IR. This resulted in improved histological appearance of cardiac muscles, a significant reduction in the number of cardiac myocytes destroyed, and the amount of fibrosis that developed. In addition, immunohistochemical staining for the c-kit demonstrated an apparent increase in the total number of cells that stained positive for c-kit. For this work, a panel of promising genetic and epigenetic biomarkers (lncRNA-TSIX, miR-1248, and *FOXC1* mRNA) relevant to IRI after myocardial ischemia; was selected based on previous microarray studies and calculation tools, followed by clinical validation of the chosen genetic panel. Following that, ROC curves was used to distinguish the IRI group from the healthy control group, and our findings suggested that miR-1248, lncRNA TSIX, and *FOXC1* mRNA might be considered efficient biomarkers for distinguishing IRI from healthy subjects. The concurrent assessment of miR-1248, lncRNA TSIX, and *FOXC1* mRNA enhanced the diagnostic accuracy for IRI detection to nearly 100% sensitivity and 100% specificity.

FOXC1 is a potential TF highly responsive to MI and is crucial in heart and cardiovascular development [45], [60]. Some previous studies indicate that *FOXC1* is a hypoxia-activated TF [61]. In response to Hypoxia/Reperfusion, *FOXC1* induces the transcription of ELAVL1 by binding to its promoter region and increasing autophagic ferroptosis [62]. Zhang SP et al. found that TNF-α and IL-6 were dramatically upregulated by *FOXC1* adenovirus in the mouse model of MI. While *FOXC1* siRNA downregulated their expression. Also, they found that *FOXC1* binds and activates the expression of TLR3 and TLR4 after MI. The mRNA and protein levels of TLR3/4 were significantly lowered by *FOXC1* knockdown in heart tissue, resulting in reduced infarct size and enhanced cardiac function [60]. Therefore, all the previously described data can be used to support our findings, which show a considerable increase in the expression level of *FOXC1* mRNA in the I/R group, accompanied by an increase in the levels of the biochemical marker.

These findings were consistent with microscopic findings of myocardial injury and infarction, along with dispersed infiltration of inflammatory cells and a substantial rise in the percentage of collagen fibers in the affected areas through the left ventricle of the I/R rat. Meanwhile, *FOXC1* expression levels started suppressing in cardiac tissues with TA as a protective medication for pathological conditions. Many researchers have discovered that dysregulated ceRNAs gene expression profiles highly contribute to the pathogenesis of different diseases, especially myocardial IRI. CeRNAs have been proposed as potential regulatory networks that might be key players in many disorders [63]. The miRNAs are the main components of the ceRNA network [64]. miR-1248 plays a role in the transport of Stem Loop Binding Protein (SLBP)-independent mature mRNA. Its expression was found to be reduced in elderly and diabetic patients because of age-related systemic chronic inflammation [65], [66]. In miR-1248 overexpressing cells, miR-1248 specifically targets and binds the cytokines IL6 and IL8 involved in inflammatory and signaling pathways related to aging, where these cytokines are reduced significantly. Also, DNA repair pathways were upregulated, suggesting that the decline in miR-1248 expression associated with aging may contribute to older persons' decreased ability to repair DNA damage [65]. Xiao, Shune, et al. discovered that tissue hypoxia in diabetic patients downregulates miR-1248 expression in human adipose-derived stem cells (hADSCs) of DM patients, consequently failing to increase stem cell activity, differentiation, and angiogenesis promotion through activating CITED2, a competitive inhibitor of HIF-1α, thus prevents the activation of growth factors that promote angiogenesis, cellular proliferation, and wound healing such as TGF-β, VEGF-α, and FGF2 [67]–[70]. These data support our results, where levels of miR-1248 were decreased in the IR group compared to the healthy group, accompanied by elevated biochemical markers and myocardial damage in histopathological results. In contrast, miR-1248 expression levels started to increase in the presence of TA as a protective medication, suggesting that decreased serum miR-1248 may potentiate inflammation. The key function of lncRNA is to compete with miRNA to counteract the inhibitory effect of miRNA on target genes. A mechanism that contributes to the development of many diseases. Because of this, lncRNAs have rapidly emerged as a new therapeutic target in various diseases in recent years [71]–[73]. TSIX is a lncRNA that **maintains** the active euchromatin state by preventing Xist accumulation on the active female X chromosome; its dysregulated expression is observed to be linked to some diseases [74]. There have been very few published reports on lncRNA TSIX. A recent study suggested that TSIX may contribute to the pathogenesis of spinal cord injury (SCI), a central nervous system traumatic disease that causes many nerve cell deaths, axonal degeneration, diffuse demyelination, and other pathological changes, resulting in severe spinal cord structural and functional impairment. TSIX was upregulated in SCI mice spinal cord tissues and hypoxia-treated SCI HT22 cells. TSIX knockdown enhanced the functional recovery of SCI mice and inhibited the size of the lesions. Moreover, TSIX knockdown inhibited inflammation

and neuron cell apoptosis through the miR-30a/SOCS3 axis in SCI mice. TSIX silencing notably increased cell viability and decreased the apoptosis portion in hypoxia-treated HT22 cells [75], [76]. Particle-induced osteolysis (PIO) in mice increased TSIX levels and promoted osteoblast apoptosis in vivo and in vitro. Through the miR-30a/Runx2 axis, TSIX re-expression enhances osteoblast apoptosis after PIO. While TSIX knockdown enhanced miR-30a-5p expression, decreased Runx2 expression, and suppressed osteoblast apoptosis in PIO mice, suggesting that TSIX suppression could alleviate PIO development [77]. Weiwei Xu et al. discovered that lncRNA TSIX expression in the plasma of tibia fraction (TF) mice models was significantly upregulated time-dependent after a fracture. When they used osteoblasts as in vitro models, overexpression of lncRNA TSIX dramatically inhibited osteoblastic cells proliferation and promoted their apoptosis. The Knockdown of lncRNA TSIX enhanced the proliferation, inhibited apoptosis, and regulated the osteogenesis-related genes of Col1a1, Col-II, and Col-X expression after tibial fracture [78]. That agrees with our experimental results, where levels of TSIX increased dramatically in the I/R group compared to the healthy group while starting to decrease with the administration of TA as a protective drug.

In the pathogenesis of many diseases, roles of ncRNA interactions, those between miRNAs and lncRNAs, or their interactions with mRNAs were demonstrated by many bioinformatics and/or experimental studies. By specifically complementary binding to their target mRNA, miRNAs can cause mRNA degradation or translational suppression. LncRNAs are RNA transcripts larger than 200 nucleotides that are not translated into proteins, but they can act as a sponge for miRNAs and stop them from acting on target mRNAs. Bioinformatics analysis (Fig. 8.) was applied to predict the binding sites of TSIX or *FOXC1* with miR-1248; results showed that lncRNA TSIX possessed complementary binding sites with miR-1248 (Fig. 8B). Also, results showed that miR-1248 has complementary binding sites with *FOXC1* (Fig. 8A). Additionally, there was a significant, very strong positive correlation between lncRNA-TSIX and *FOXC1* mRNA, a significantly strong negative correlation between *FOXC1* and miR-1248, and a moderate negative correlation between lncRNA TSIX and miR-1248. As a result, we hypothesize that TSIX might regulate *FOXC1* by acting as a sponge to miR-1248 to counteract the inhibitory effect of miR-1248 on the *FOXC1* gene. Since lncRNA-TSIX and *FOXC1* mRNA may compete with one another for microRNA binding sites, this suggests that lncRNA-TSIX may play a role in the epigenetic inhibition of miR-1248 and subsequently relieve microRNA-mediated *FOXC1* repression.

5. Conclusions

TA may potentially play a role in mitigating ischemia-reperfusion injury, enhancing heart function, and increasing the injured heart's regenerative capacity. Measuring the relative gene expression levels of mRNA *FOXC1*, miR-1248, and lncRNA TSIX in cardiac tissue during I/R has a diagnostic importance.

6. Conflicts of interest

The authors declare that there is no conflict of interest".

7. Funding sources

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