ORIGINAL ARTICLE

SHP-1/STAT3 Interaction Is Related to Luteolin-Induced Myocardial Ischemia Protection

Donghai Liu[1] , Hong Luo[1] and Chenhui Qiao[1,2]

Abstract——Prevention and management of myocardial ischemia/reperfusion (I/R) injury is a key step in coronary heart disease surgery. Luteolin is a falconoid compound that has an antioxidant effect, but its mechanism in I/R injury *in vivo* and *in vitro* is still under explored. This study attempted to reveal the role of luteolin (Lut) in I/R through mediation of the Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1)/Signal transducer and activator of transcription 3 (STAT3) pathway. To establish I/R rat models, the left anterior descending artery (LAD) was ligated for 30 min and re-perfused for 1 h in Lut-pretreated or nude rats. Comparisons between infarct area, cardiac dysfunction, and myocardial cell death and inflammatory reaction were performed in I/R-induced rats. Hypoxia/reoxygenation (H/R) cell models were established by stimulating H9c2 cells with 95% nitrogen and 5% carbon dioxide. Simultaneously, H/R-related cell death and inflammatory reactions were investigated following Lut treatment. The target protein of Lut was identified using western blotting. Pro-inflammatory cytokines were also measured in serum or Lut-pretreated cell culture medium. The results revealed that compared with the I/R group, Lut treatment could significantly decrease myocardial infarction (MI) area, increase left ventricular ejection fraction (LVEF), and decrease cell death and pro-inflammatory cytokines in the serum. Decreased apoptosis and inflammatory cytokines were also observed in H/R cells after Lut treatment. Lut treatment downregulated SHP-1 expression and subsequently upregulated STAT3 phosphorylation in both I/R rat heart tissue and H9c2 cells. The findings of the current study suggest that Lut can protect the heart and reduce MI area, cell apoptosis rate, and inflammatory level in I/R models.

KEY WORDS: Myocardial Ischemia; Ischemia/Reperfusion Injury; Hypoxia/Reoxygenation Injury; Luteolin; SHP-1; STAT3.

BACKGROUND

Ischemic heart disease (IHD), a main contributor to mortality in patients with cardiovascular problems, is responsible for up to 42% of all deaths [1]. Ischemia/reperfusion injury (I/R) prevention and management are leading clinical problems associated with IHD treatment [2] as crucial factors in cardiovascular surgeries [3]. The

[1]Cardiovascular Surgery II, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, China
[2]To whom correspondence should be addressed at Cardiovascular Surgery II, The First Affiliated Hospital of Zhengzhou University, Henan 450052 Zhengzhou, China. Email: qchenhui@yeah.net

mechanisms underlying I/R are complex and may be related to metabolic energy, free radicals, calcium overload, neutrophil infiltration, and dysfunctional vascular endothelial cell (VEC) [4], finally leading to dead or necrotic cells.

Inflammation in the myocardium is believed to be an important marker for myocardial I/R. Reduction of inflammatory reactions was found to be beneficial in the recovery of patients with I/R-induced myocardial infarction (MI) in previous research [5]. Increasing evidence shows that cell death is critical in myocardial I/R [6]. Protecting myocardial cells from I/R-induced apoptosis could principally promote the survival of myocardial cells, thereby providing current and future benefits to MI cells. Hence, myocardial inflammatory reactions and cell death may represent underlying targets for treating MI and I/R.

Luteolin (Lut), a sickle-like complex present in some medical herbs, has anti-inflammatory [7, 8], anti-oxidant [9], and anti-cancer [10–12] properties. Recently, Lut was used pharmacologically in clinical trials to prevent ischemic heart disease and I/R [13]. A recent study has also demonstrated that Lut at different dose had an inhibitory efficiency on the cardiomyocyte apoptosis in the myocardial I/R-tread rat through TLR4/NF-kappaB/NLRP3 pathway [5, 14]. However, identifying the molecular targets utilized to prevent and treat I/R requires additional research. In addition, Lut greatly increased left ventricular pseudoaneurysm and global and regional coronary flow reserve in Langendorff-perfused rabbits with recurrent MI [15].

Src homology region 2 domain-containing phosphatase-1 (SHP-1) contains an SH region 2 domain and is the non-transmembrane protein tyrosine phosphatase (PTP) primarily produced in hematopoietic progenitor cells (HPC) [16]. SHP-1, a negative-regulator of signaling transduction through receptor phosphorylation, acts as a crucial factor in cell death and/or the repression of survival factors [17]. In mouse models featuring a locally ischemic brain, SHP-1 protein was increased, reaching a peak at 7 days after cerebral ischemia and regulating the multiplication of nerve cells in the ischemic central nervous system (CNS) [18]. Notably, SHP-1 can repress the Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) pathway through the dephosphorylation and inactivation of STAT3 [19], which is widely considered to be the signaling pathway of cell apoptosis and inflammatory reactions [20]. SHP-1-induced STAT3 activation was related to Y-box binding protein 1 (YB1) knockdown in inhibiting hypoxia/reoxygenation (H/R)-mediated myocardial cell death. In I/R mouse models, inactivating SHP-1-induced STAT3 was also highly correlated with improving acute myocardial infarction, reduction of MI area, myocardial cell death, and oxidative stress response.

Based on the abovementioned reports, Lut is indicated as exerting cardioprotective effects on I/R rats by promoting the inactivation of SHP-1-induced STAT3. Using an *in vivo* I/R model with left anterior descending artery (LAD) ligation and an *in vitro* model with H/R-induced H9c2 damage, we investigated the protective functions of Lut in myocardial I/R injury. Lut exerted a myocardial protective role in I/R rats and H/R-treated cells, through mediating SHP-1/STAT3 signal axis. The gain of function experiments with SHP-1 overexpression showed that SHP-1 was involved in the anti-inflammation and anti-apoptosis effect of Lut.

METHODS

Materials

In this research, Lut (pure: 99%, Sigma-Aldrich, stored at concentration of 20 mM) and ELISA kits (Nanjing KeyGEN Biotech, China) were used for detecting IL-1β, IL-18, and TNF-α.

Animals

Adult SD rats (male, 220–250 g) provided by Vitalriver (Beijing, China) were fed in animal houses using a standard configuration requirement for 5 days before this study. All rats could freely drink water and eat standard food. Every procedure was approved by the Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University.

Drug Treatment in in I/R Models

Rats were randomly assigned to three groups ($n = 10$): sham group, I/R group, and I/R + Lut group. Seven-day intragastric administration was performed in the Lut group using Lut (qd, per 40 mg/kg) [21] after operation. Details on the surgery performed are mentioned above [22]. The injury models were established by 24-h myocardial reperfusion following 30-min treatment. After establishing the models, all rats were killed, blood specimens were obtained for biochemical tests, and cardiac tissue specimens were obtained for studying the morphology, biochemistry, and individual molecules.

Tissues and Serum Preparations

Blood specimens (500 µl) taken retro-orbitally were subjected to centrifugation at 1500 g for 10 min at 4 °C. Serum specimens after centrifugation were stored in Eppendorf tubes at -80 °C for downstream analysis [23]. One part from the heart tissue samples (around 1 mm^3) underwent cell fixation with 2.5% glutaraldehyde for ultrastructural examination; another part underwent cell fixation with 4% paraformaldehyde and was embedded in paraffin; the remainder underwent tissue wrapping with AF and was deeply frozen in LIN.

Detection of MI Area

Twenty-four hours after I/R, all rats were killed. The heart was evenly cut into 5 pieces below the ligation and along a line parallel to coronary sulcus after being kept at 20 °C for 15 min. Total sections were weighed again and cultured for 15 min with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, USA) at 37 °C in darkness for pathologic analysis. The MI proportion of total cardiac tissue, including non-infarct size (red, staining part) and infarct size (white/pale, non-stained part), was calculated using the IPP image software (Version 4.1, Media Cybernetics, USA).

Heart Function Test

The size and function of the heart were evaluated by M-mode echocardiography. Left ventricular end-diastolic diameter (LVEDD) and left ventricular end-diastolic and end-systolic diameter (LVESD) were obtained from the results of a left parasternal long-axis section. The mean of 5 continuous cardiac cycles is expressed in our results. Left ventricular end systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV), and left ventricular ejection fraction (LVEF) were obtained using an algorithm. All analysis was blinded.

Cell Culture and H/R Injury Model Establishment

Mouse H9c2 cardiomyocytes were provided by the ATCC (Manassas, USA). H9c2 cells were stored in 10% (v/v) FBS high glucose DMEM (Gibco/Invitrogen), with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco/Invitrogen) under humid conditions including 5% carbon dioxide at 37 °C.

After H9c2 cells were cultured until 70–80% confluence, 20 mM Lut for the treatment group was solubilized in medium containing DMSO and 5% FBS to reach the final concentration of 30 µM, Lut in the control and H/R

groups underwent the same operation in 5% FBS medium for 24 h. Afterward, the above operations in all groups were performed in Krebs–Ringer bicarbonate buffer; pH 7.4). After that, the cells were incubated in oxygen-deficient conditions including 95% nitrogen and 5% carbon dioxide for 6 h, then cultured in DMEM containing 10% FBS and kept in an environment containing 5% CO_2 and 95% air for 5 h to induce reoxygenation damage [24].

The full-length ORF of SHP-1 was cloned into pcDNA3.1(+) to construct SHP-1 express vectors (pcDNA-SHP-1). Transfection of pcDNA-SHP-1/pcDNA-Empty was performed using Lipofectamine 2000 Reagent (Invitrogen, USA).

ELISA

The IL-1β, IL-18, and TNF-α content in serum and supernatant was measured using ELISA kits in accordance with manufacturer instructions. The absorbance at 450 nm was measured using a microplate spectrophotometer. In the end, sample content was calculated using a standard curve.

WB

Proteins were separated from evened heart tissues and cells lysed using Trizol reagent (Invitrogen, Carlsbad, Calif) in accordance with a standard Invitrogen protocol. After quantitative analysis of protein content using the improved Bradford assay (Bio-Rad Laboratories, Calif), proteins were utilized downstream for WB with primary and secondary antibodies. Spots were observed by a chemiluminescence system (Amersham Bioscience, UK). Signal quantification was performed using Image Pro Plus software (Media Cybernetics).

Q-PCR

Total RNA was separated from homogenous cardiac tissues and lysed cells using TRIzol reagent (Invitrogen, USA), then evaluated using a Nanodrop2000 (OD260). The MMLV First-Strand Kit (Invitrogen, USA) and Oligo (dT) 20 primer were utilized in reverse transcription to prepare cDNA. Q-PCR was conducted on cDNA prepared by SYBR Select Master Mix (Invitrogen, USA). mRNA was detected by Q-PCR using the corresponding kits. All operations were carried out according to the manufacturer's instruction. PCR conditions included an initial 10-min denaturation at 95 °C s, then 40-cycles of 15-s denaturation at 95 °C and 40 s of extension at 60 °C. GAPDH mRNA was used as an internal
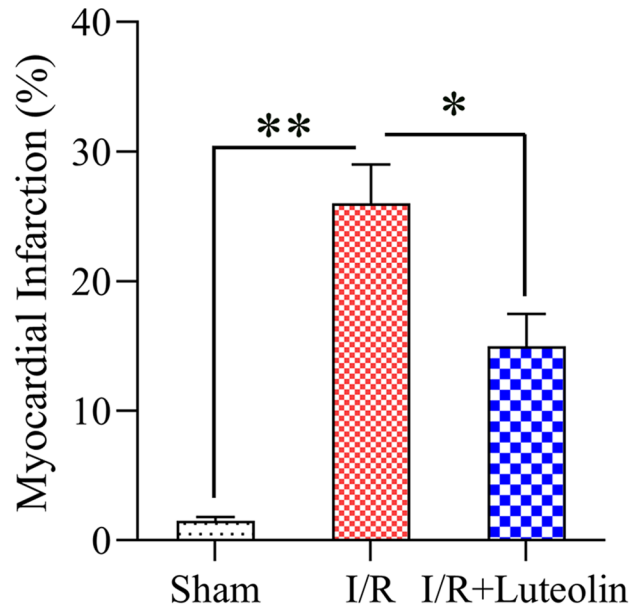
Myocardial infarct size

Fig. 1 Cardiac MI area in I/R. Myocardial necrosis was evaluated by TTC staining. **A** Sham group, **B** model group, **C** Lut group; bar chart represents the ratio of cardiac MI area. $N = 3$. Data are expressed as $M \pm$ SD. Compared with indicated group, the differences were significant ($p < 0.05$; $p < 0.01$).

reference. Target mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method. All tests were conducted in triplicate.

Cell Death Assay

Cell death was assessed using FC and Annexin V-FITC/PI apoptosis detection kits (BD Pharmingen™). Preparation of cell suspensions was performed with 20 μL BB, and then treatment with 10 μL Annexin V-FITC and 5 μL PI. Cell mortality rate was assessed using flow cytometry (FCMs).

Statistical Analyses

All data were analyzed USING SPSS 10.0. Data are represented as $M \pm$ SD. One-way ANOVA was applied in multi-group comparisons (significant, $P < 0.05$); Scheffe's
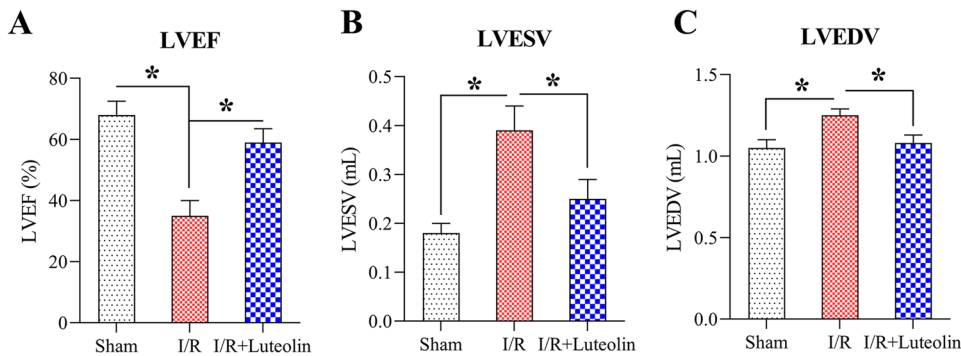

Fig. 2 Lut enhances left ventricular function. **A** Lut treatment significantly increased LVEF, compared to the I/R group. **B**, **C** Lut treatment significantly repressed the increase in LVESV and LVEDV, compared to I/R group. LVEF left ventricular ejection fraction; LVESV left ventricular end-systolic volume; LVEDV left ventricular end-diastolic volume. $N = 3$. Data are expressed as $M \pm$ SD. Compared with indicated group, the difference was significant ($p < 0.05$).

test was performed between individual groups (significant, $P < 0.05$).

RESULTS

Effect of Lut Treatment on MI Area and Left Ventricular Failure in I/R Rat Models

To indicate the efficiency of Lut on the I/R-induced MI and left ventricular failure in I/R rats, TTC staining and electrocardiograph were used. First, myocardial TTC staining results showed that I/R injury was closely related with the progression of I/R-induced irreversible myocardial damage. TTC staining showed no infarct existed in the sham group, and the incidence of MI area in the model group was obviously larger than in the sham group. Meanwhile, MI area expansion was significantly abrogated by Lut treatment (40 mg/kg) (Fig. 1).

The electrocardiograph monitoring of hemodynamic parameters such as LVEF, LVESV, and LVEDV was performed. LVEF was evidently reduced (Fig. 2A), whereas LVESV and LVEDV were greatly increased compared to the sham group (Fig. 2B, C), indicating that the left ventricular failure in the I/R group was inhibited. However, compared to the I/R group, LVEF in the Lut group was obviously higher, while LVESV and LVEDV
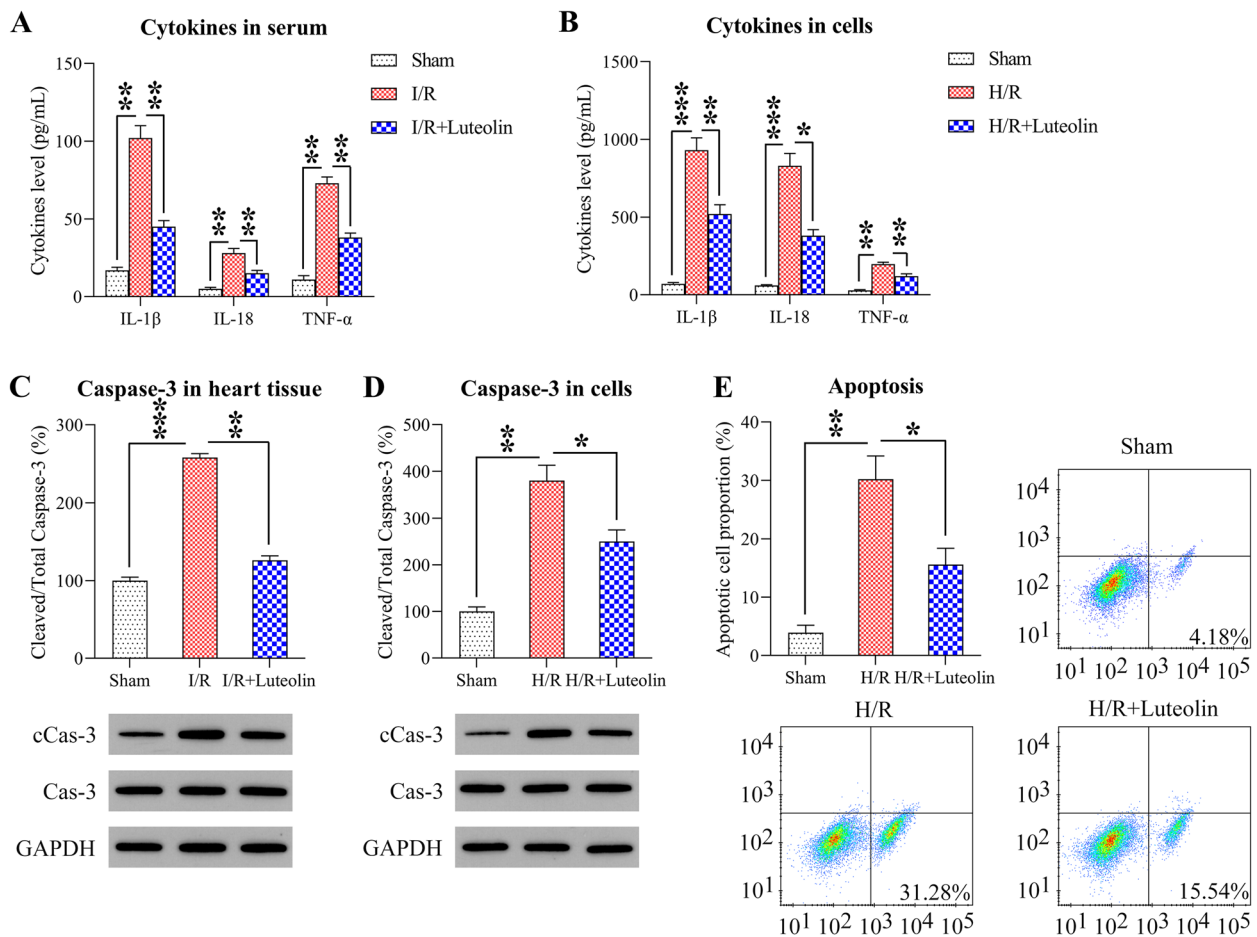

Fig. 3 Effects of Lut on inflammation and apoptosis in I/R-induced rats and H/R-induced cells. **A** Effects of Lut on serum IL-1β, IL-18 and TNF-α (ELISA assay). $N = 3$. **B** Effects of Lut on IL-1β, IL-18 and TNF-α in the supernatant of H/R treated H9c2 cells. $N = 3$. **C** Caspase-3 level and caspase-3 cleavage in heart tissue of rats (WB assay). $N = 3$. **D** Caspase-3 level and caspase-3 cleavage in H/R-induced treated H9c2 cells (WB assay). $N = 3$. **E** Cell mortality of H/R treated H9c2 cells with/without Lut treatment (FCM assay). $N = 3$. Data are expressed as mean ± SD. Compared with indicated group, the differences were significant ($p < 0.05$, $p < 0.01$; $p < 0.001$).

were obviously lower, indicating that Lut could repress left ventricular failure in I/R rats.

Effect of Lut on Inflammatory Response Factors and Cell Death in I/R Rats and H/R-Mediated H9c2 Cells

Inflammatory cytokines are reported to be an important factor in I/R [25]. To verify the role of Lut on the generation of inflammatory cytokines in MI rats, serum levels of IL-1β, IL-18, and TNF-α were measured. ELISA results showed that I/R treatment could significantly increase the expression of IL-1β, IL-18, and TNF-α, while Lut administration could obviously reduce serum content of these cytokines (Fig. 3A). The level of inflammatory cytokines in the supernatant of H9c2 cells affected by H/R was measured. H/R stimulation could increase the levels of IL-1β, IL-18, and TNF-α, while Lut treatment (30 μM) [21] could obviously decrease the expression of inflammatory cytokines (Fig. 3B). The above results indicate that regulation of inflammatory cytokines may be beneficial in the protective effects of Lut on the heart.

The mechanism of myocardial I/R is very complex, but it eventually contributes to myocardial apoptosis [26]. To assess the role of H/R-mediated apoptosis in H9c2
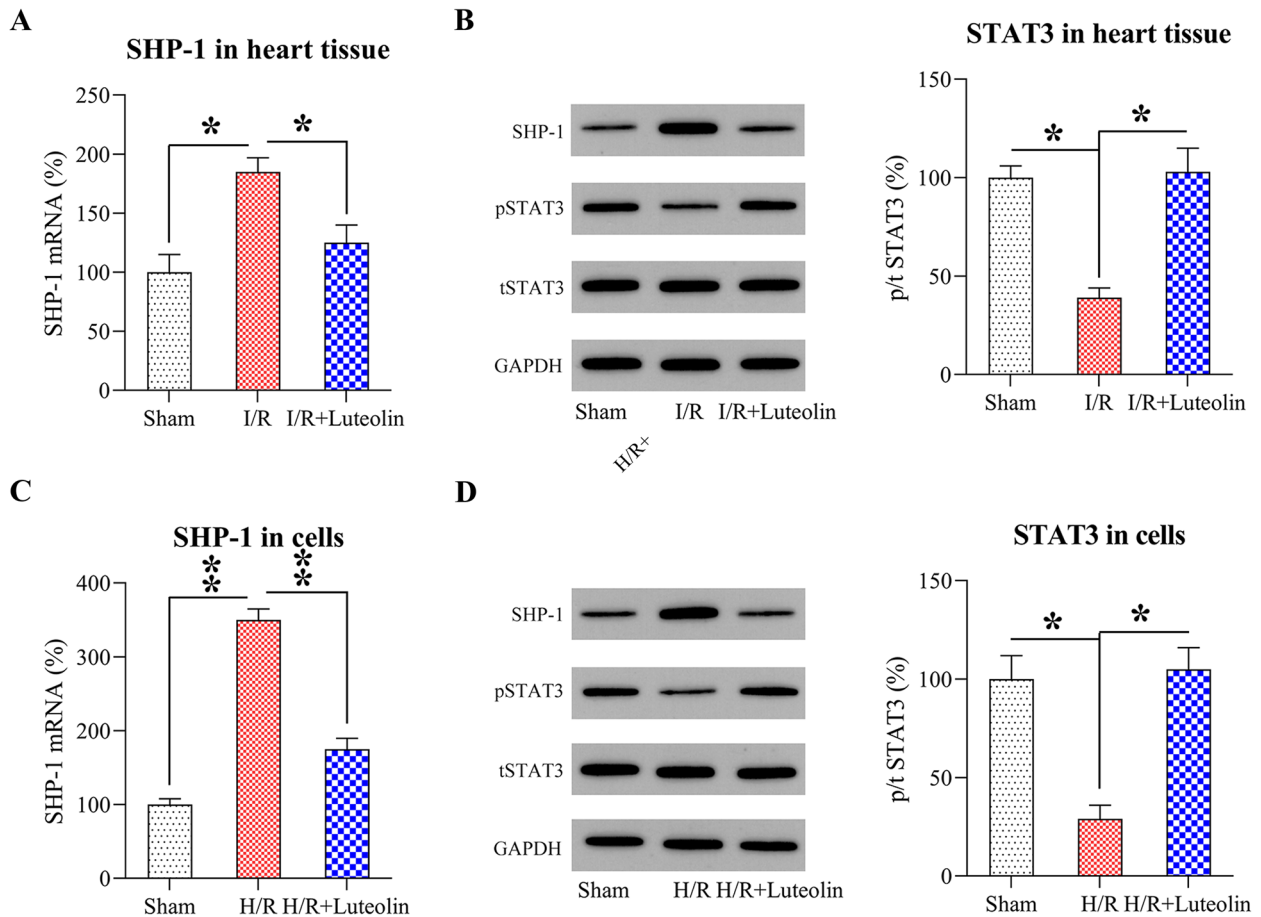

Fig. 4 Effects of Lut on SHP-1/STAT3 pathway activation in I/R-induced rats and H/R-induced cells. **A** Q-PCR detected SHP-1 mRNA level in the heart tissue of rats. $N = 3$. **B** WB analysis of SHP-1 and STAT3 levels, and STAT3 phosphorylation, in the heart tissue of rats. $N = 3$. **C** Q-PCR detected SHP-1 mRNA expression in H/R-induced H9c2 cells. $N = 3$. **D** WB analysis of SHP-1 and STAT3 levels, and STAT3 phosphorylation, in H/R-induced H9c2 cells. $N = 3$. Values are presented as M ± SD. Compared with indicated group, the differences were significant ($p < 0.05$; $p < 0.01$).

cells, WB and FCM assays were conducted. The cleavage level of caspase-3 in the I/R group was obviously increased compared to sham, while Lut administration greatly inhibited the expression of cleaved caspase-3 (Fig. 3C). Meanwhile, we also observed a similar phenotype for caspase-3 cleavage in H/R-induced H9c2 cells (Fig. 3D). FCM data revealed that the apoptotic cell proportion of H9c2 cells was significantly increased after H/R treatment, while Lut treatment repressed the apoptotic level of H9c2 cells (Fig. 3E).

Effects of Lut on Activation of the SHP-1/STAT3 Pathway

As previous observations indicated that the SHP-1/STAT3 pathway was highly relevant to apoptosis and inflammation [27], we therefore tried to investigate whether the SHP-1/STAT3 pathway is mediated by Lut treatment and acts as an important factor in promoting cell inflammation and viability. We measured the levels of SHP-1 and STAT3, and level of phosphorylated STAT3, in I/R-mediated heart tissue and H/R-mediated H9c2 cells. In I/R-treated heart tissue, we observed that SHP-1 was notably increased and phosphorylated STAT3 was decreased in the I/R treatment group, while Lut treatment caused a

decrease in SHP-1 level and increase of STAT3 phosphorylation (Fig. 4A, B). In addition, a similar phenomenon was observed in H/R-induced H9c2 cells. Finally, STAT3 phosphorylation was decreased by H/R induction and increased after Lut administration (Fig. 4C, D).

Effects of SHP-1 on Lut-Mediated Inflammatory Reactions and Mortality of H/R-Mediated H9c2 Cells

To assess the role of SHP-1 in Lut-ameliorated inflammation and apoptosis in H/R-treated cells, SHP-1 was overexpressed in Lut-treated H/R-mediated H9c2 cells, while empty vector was also transfected as control group. The results of Q-PCR and WB showed that transfection with pcDNA-SHP-1 could increase the intracellular content of SHP-1, compared to H/R-induced H9c2 cells (Fig. 5A, B). Meanwhile, phosphorylation of STAT3 was significantly impaired after SHP-1 overexpression as assessed by WB (Fig. 5B).

ELISA was then performed to detect the secretion of inflammatory cytokines from Lut-treated H/R-induced H9c2 cells with or without SHP-1 overexpression. Our findings indicated that SHP-1 overexpression reduced the production of secreted inflammatory cytokines in the
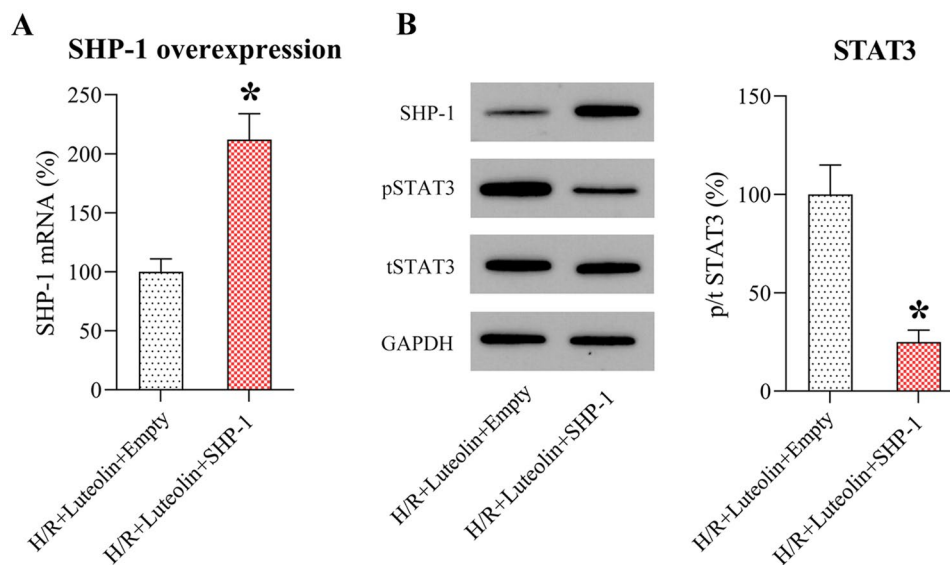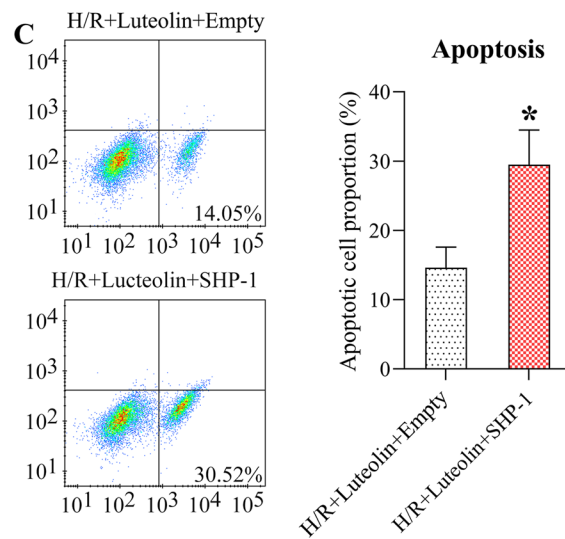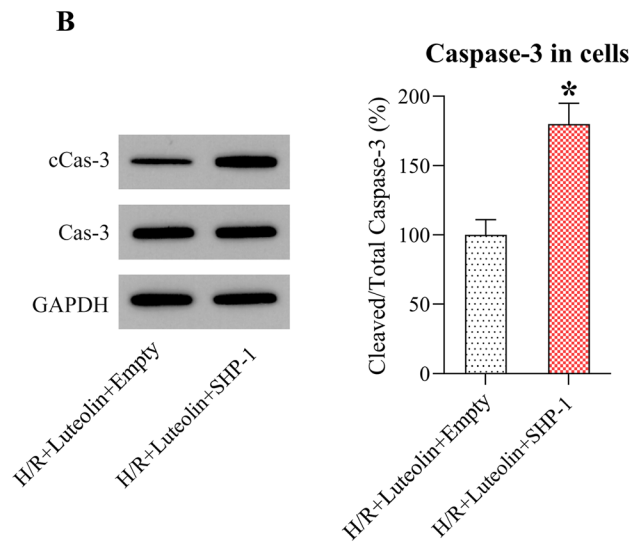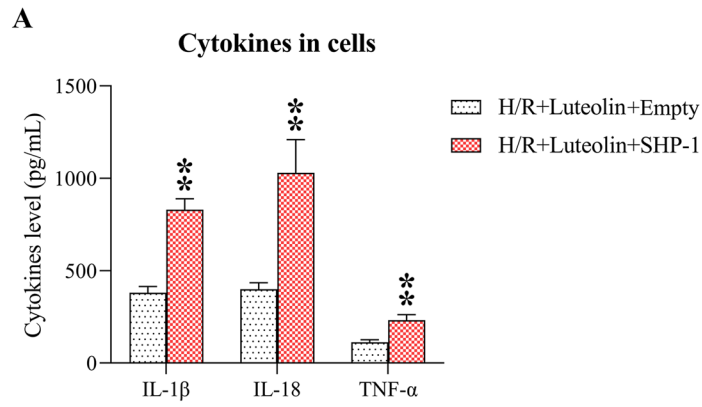

Fig. 5 Overexpression of SHP-1 in Lut-treated H/R treated cells. H9c2 cell transfection was performed with pcDNA-SHP-1/pcDNA-Empty, followed by Lut and H/R treatment. **A** Q-PCR detected SHP-1 mRNA content in H/R-induced H9c2 cells. $N = 3$. **B** WB analysis of SHP-1 and STAT3 levels, and STAT3 phosphorylation, in H/R treated H9c2 cells. $N = 3$. Results are presented as M ± SD. Compared with indicated group, the difference was significant ($p < 0.05$).

A

Cytokines in cells

B

Caspase-3 in cells

C

Apoptosis

cell supernatant (Fig. 6A), suggesting that SHP-1 exerts a positive role on H/R-induced inflammation.

WB then showed that cleavage of caspase-3 was obviously improved in the SHP-1 overexpression group, comparing to Lut-treated H/R-induced H9c2 cells without overexpression (Fig. 6B). FCM data further indicated an upregulation in apoptotic cell proportion after SHP-1 over-expression, indicating that SHP-1 is a key modulator of H/R-induced apoptosis (Fig. 6C).

DISCUSSION

Lut, a naturally occurring polyphenol flavonoid, has demonstrated to exert myocardial protection effects. Due to the newly emerged COVID-19 threat, Lut has been found as a dietary molecular to inhibit the SARS-COV-2 [28]: corticosteroid dexamethasone can reduce mortality of severe COVID-19 patient through limiting the production and damaging effect of the cytokines and inhibiting the protective function of T cells and block B cells from making antibodies [29]. Dexamethasone could be given together with the Lut due to its antiviral and anti-inflammatory properties, especially its ability to inhibit Mast cell activation syndrome, which are the main source of cytokines in the lungs of COVID-19 patients [29, 30]. The *in vitro* study in the present study demonstrated that myocardial I/R injury activated myocardial SHP-1/STAT3 signal transduction pathways and induced inflammatory reactions and apoptotic death, but Lut treatment decreased inflammatory cytokines and apoptotic cell proportion and affected the SHP-1/STAT3 pathway. *In vivo* experiments confirmed that Lut administration ameliorated the I/R-induced MI lesion and left ventricular failure through mediating SHP-1/STAT3 pathway. In brief, the protection provided by Lut in I/R injury may be due to inhibiting inflammatory reactions and cell apoptosis by modulating the SHP-1/STAT3 pathway.

A major indicator of I/R is the large production of inflammatory cytokines. Ischemic damage is generated by temporary insufficiency of blood supply in the tissues, but reperfusion damage is caused by rapid inflammation after blood reperfusion [31]. The production of inflammatory cytokines is an important process for the progression of inflammatory reactions in the heart [32]. For instance, TNF-α had a negative inotropic effect on myocardium contraction and blood pressure control. TNF-α could also expand the MI area during I/R injury and is involved in adverse ventricular remodeling [33]. IL-18 also participated in the pathophysiological process of I/R-associated inflammatory diseases by abrogating myocyte necrosis and immunity reactions [34]. IL-1β is an important and early inflammatory mediator in I/R injury [35]. Our results confirmed that IL-1β, IL-18, TNF-α, and other inflammatory cytokines were obviously increased in I/R models and H/R-treated H9c2 cells. However, significant inhibition of the cardiac inflammatory response *in vivo* and *in vitro* was found after Lut treatment, due to the inhibition of these three inflammatory cytokines in I/R-mediated rats and H/R-mediated H9c2 cells.

Cardiomyocyte apoptosis is another important representation in heart failure during MI. Inhibition of cell death represses damage in contractile cells, reduces I/R injury-induced heart injuries, and thus attenuates the incidence of heart failure [36, 37]. A recent study has demonstrated that Lut at different dose had an inhibitory efficiency on the cardiomyocyte apoptosis in the myocardial I/R-tread rat [14]. The present study indicates an elevation in I/R-induced cell death in the I/R treated rats, which was evidenced by the cleavage of caspase-3. *In vitro* results also showed that H/R obviously promoted the apoptotic death of H9c2 cells. Apoptosis in both *in vivo* and *in vitro* model was inhibited in the Lut treatment.

The non-receptor PTPs, SHP-1 (aka PTPN6), and SHP-2 (aka PTPN11) play crucial regulatory roles in basic cellular processes, including cell proliferation, cell death, cell migration, cytodifferentiation, inflammatory reactions, and intermediate metabolisms [38]. The expression of SHP-1 in hematopoietic and epithelial cells is extensively considered to play a role in the negative regulation of inflammatory reactions [39]. SHP-1 is also closely related to cell death and can bind with tumor necrosis factor receptor 1, Fas receptor, and other death receptors to block anti-apoptosis signals in neutrophils. Therefore, SHP-1 is believed to act as a crucial factor in cell death and/or the blocking of survival-related factors. STAT3 is an oncogenic

transcription factor that could play a significant role through the phosphorylation dimerization of tyrosine residues and translocation to the nucleus [40, 41]. Constitutive activation of STAT3 in various malignancies is closely related to disease progress [42, 43]. Phosphorylated STAT3 was involved in inflammatory reactions and cell death in rat models with Cd-induced testis toxicity [44]. Various evidences have shown that SHP-1 protein level is upregulated in rats with cerebral ischemia and MI, resulting in the increase of cerebral ischemia and MI [45]. The mechanism regarding how SHP-1 regulated STAT3 phosphorylation is well-documented in many previous studies [35, 46–51]; however, the influence and mechanism of SHP-1/STAT3 axis in MI was not elucidated. As far as we know, this is the first attempt to assess the role of SHP-1/STAT3 axis in MI. In this study, our findings showed that the expression of SHP-1 in cardiac tissue and cells was upregulated in rats after I/R and H9c2 cells with H/R treatment, with deactivated STAT3 sensor. Lut treatment significantly reduced SHP-1/STAT3 signal transduction by inhibiting the expression of SHP-1. SHP-1 overexpression restored both inflammation and apoptosis which were downregulated accompanying SHP-1 upregulation in Lut-treated and H/R-induced H9c2 cells. These data suggest that the SHP-1/STAT3 axis is highly associated with Lut-mediated inflammation and apoptotic death in an H/R cell model, as well as I/R rats.

CONCLUSIONS

In conclusion, this research indicates that Lut provides myocardial protection during I/R by improving myocardial contraction function and inhibiting inflammatory reactions and cell death. Meanwhile, activating the SHP-1/STAT3 signaling pathway is related to the protective effects of Lut on the MI-injured heart tissue.

AUTHOR CONTRIBUTION

Study design/planning: Donghai Liu, Hong Luo, Chenhui Qiao. Data collection/entry: Donghai Liu, Hong Luo, Chenhui Qiao. Data analysis/statistics: Donghai Liu, Hong Luo, Chenhui Qiao. Data interpretation: Donghai. Liu, Chenhui Qiao. Preparation of manuscript: Chenhui Qiao. Literature analysis/search: Donghai Liu, Hong. Luo, Chenhui Qiao.

DECLARATIONS

Ethics Approval Every procedure was approved by the Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University.

Informed Consent Informed consent was obtained from all individual participants included in the study.

Conflict of Interest The authors declare no competing interests.

REFERENCES

1. Mendis, Shanthi, Pekka, Puska, Bo, Norrving, and World Health Organization. 2011. *Global atlas on cardiovascular disease prevention and control*. World Health Organization.
2. Rodriguez-Porcel, Martin, Xiang-Yang. Zhu, Alejandro R. Chade, Beatriz Amores-Arriaga, Noel M. Caplice, Erik L. Ritman, Amir Lerman, and Lilach O. Lerman. 2006. Functional and structural remodeling of the myocardial microvasculature in early experimental hypertension. *American Journal of Physiology-Heart and Circulatory Physiology* 290 (3): H978–H984.
3. Monassier, Jean Pierre. 2008. Reperfusion injury in acute myocardial infarction. From bench to cath lab. Part I: Basic considerations. *Archives of cardiovascular diseases* 101 (7–8): 491–500.
4. Turer, Aslan T., and Joseph A. Hill. 2010. Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy. *The American journal of cardiology* 106 (3): 360–368.
5. Zhang, Xu, Du. Qianming, Yan Yang, Jianing Wang, Shuai Dou, Chao Liu, and Junguo Duan. 2017. The protective effect of Luteolin on myocardial ischemia/reperfusion (I/R) injury through TLR4/NF-κB/NLRP3 inflammasome pathway. *Biomedicine & Pharmacotherapy* 91: 1042–1052.
6. Lee, Young Soo, Young Jin Kang, Hye Jung Kim, Min Kyu Park, Han Geuk Seo, Jae Heun Lee, Hye Sook Yun-Choi, and Ki Churl Chang. 2006. Higenamine reduces apoptotic cell death by induction of heme oxygenase-1 in rat myocardial ischemia-reperfusion injury. *Apoptosis* 11 (7): 1091–1100.
7. Palombo, Ramona, Isabella Savini, S. Luciana Avigliano, Andrea Cavani Madonna, C. Albanesi, G. Alessandro Mauriello, and Melino, and A Terrinoni. . 2016. Luteolin-7-glucoside inhibits IL-22/STAT3 pathway, reducing proliferation, acanthosis, and inflammation in keratinocytes and in mouse psoriatic model. *Cell death & disease* 7 (8): e2344–e2344.
8. Taliou, Anilia, Elias Zintzaras, Lefteris Lykouras, and Kostantinos Francis. 2013. An open-label pilot study of a formulation containing the anti-inflammatory flavonoid luteolin and its effects on behavior in children with autism spectrum disorders. *Clinical therapeutics* 35 (5): 592–602.
9. Manju, Vaiyapuri, Vairappan, Balasubramaniyan, and Namasivayam, Nalini. 2005. Rat colonic lipid peroxidation and antioxidant status: The effects of dietary luteolin on 1, 2-dimethylhydrazine challenge. *Cellular and Molecular Biology Letters* 10 (3): 535.

10. Stefano, De. Sabrina Caporali Alessandro, Nicola Di Daniele, Valentina Rovella, Carmine Cardillo, Francesca Schinzari, Marilena Minieri, Massimo Pieri, Eleonora Candi, and Sergio Bernardini. 2021. Anti-Inflammatory and Proliferative Properties of Luteolin-7-O-Glucoside. *International journal of molecular sciences* 22 (3): 1321.

11. Lin, Yong, Ranxin Shi, Xia Wang, and Han-Ming. Shen. 2008. Luteolin, a flavonoid with potential for cancer prevention and therapy. *Current cancer drug targets* 8 (7): 634–646.

12. Palombo, Ramona, Sabrina Caporali, Mattia Falconi, Federico Iacovelli, Blasco Morozzo Della. Rocca, Alessandro Lo Surdo, Elena Campione, Eleonora Candi, Gerry Melino, and Sergio Bernardini. 2019. Luteolin-7-O-β-d-Glucoside Inhibits Cellular Energy Production Interacting with HEK2 in Keratinocytes. *International journal of molecular sciences* 20 (11): 2689.

13. Qi, Lingling, Huanjun Pan, Dongye Li, Fang Fang, Dan Chen, and Hong Sun. 2011. Luteolin improves contractile function and attenuates apoptosis following ischemia–reperfusion in adult rat cardiomyocytes. *European journal of pharmacology* 668 (1–2): 201–207.

14. Li, J.P., R. Yan, P.L. Ma, P. Fu, H.T. Tian, and L.L. Wang. 2020. Effects of luteolin in different doses on the cardiomyocyte apoptosis in rats with myocardial ischemia reperfusion. *Journal of Biological Regulators and Homeostatic Agents* 34 (6).

15. Rump, A.F.E., M. Schüssler, D. Acar, A. Cordes, M. Theisohn, R. Rösen, W. Klaus, and U. Fricke. 1994. Functional and antiischemic effects of luteolin-7-glucoside in isolated rabbit hearts. *General Pharmacology: The Vascular System* 25 (6): 1137–1142.

16. Wlodarski, Pawel, Qian, Zhang, Xiaobin, Liu, Monika, Kasprzycka, Michal, Marzec, and Mariusz A. Wasik. 2007. PU. 1 activates transcription of SHP-1 gene in hematopoietic cells. *Journal of Biological Chemistry* 282 (9): 6316–6323.

17. Daigle, Isabelle, Shida Yousefi, Marco Colonna, Douglas R. Green, and Hans-Uwe. Simon. 2002. Death receptors bind SHP-1 and block cytokine-induced anti-apoptotic signaling in neutrophils. *Nature medicine* 8 (1): 61–67.

18. Wishcamper, Celine A., Diane M. Brooks, J. Douglas Coffin, and Diana I. Lurie. 2003. Focal cerebral ischemia upregulates SHP-1 in reactive astrocytes in juvenile mice. *Brain research* 974 (1–2): 88–98.

19. Han, Yajun, Hesham M. Amin, Bevin Franko, Christine Frantz, Xinzhe Shi, and Raymond Lai. 2006. Loss of SHP1 enhances JAK3/STAT3 signaling and decreases proteosome degradation of JAK3 and NPM-ALK in ALK+ anaplastic large-cell lymphoma. *Blood* 108 (8): 2796–2803.

20. Chen, Kuen-Feng., Su. Jung-Chen, Chun-Yu. Liu, Jui-Wen. Huang, Kuei-Chiu. Chen, Wei-Lin. Chen, Wei-Tien. Tai, and Chung-Wai. Shiau. 2012. A novel obatoclax derivative, SC-2001, induces apoptosis in hepatocellular carcinoma cells through SHP-1-dependent STAT3 inactivation. *Cancer letters* 321 (1): 27–35.

21. Yu, Dongsheng, Mengwen Li, Youqing Tian, Jun Liu, and Jing Shang. 2015. Luteolin inhibits ROS-activated MAPK pathway in myocardial ischemia/reperfusion injury. *Life Sciences* 122: 15–25.

22. Bhindi, Ravinay, Paul K. Witting, Aisling C. McMahon, Levon M. Khachigian, and Harry C. Lowe. 2006. Rat models of myocardial infarction. *Thrombosis and haemostasis* 96 (11): 602–610.

23. Wu, Hua-Li, Si-Lin, Pang, Qiong-Zhen, Liu, Qian, Wang, Min-Xuan, Cai, and Jing Shang. 2014. 5-HT1A/1B receptors as targets for optimizing pigmentary responses in C57BL/6 mouse skin to stress. *PloS one* 9 (2): e89663.

24. Chiu, Po Yee, Ka Fai Luk, Hoi Yan Leung, Ka Ming Ng, and Kam Ming Ko. 2008. Schisandrin B stereoisomers protect against hypoxia/reoxygenation-induced apoptosis and inhibit associated changes in Ca2+-induced mitochondrial permeability transition and mitochondrial membrane potential in H9c2 cardiomyocytes. *Life sciences* 82 (21–22): 1092–1101.

25. Sivarajah, Ahila, Massimo Collino, Mohammed Yasin, Elisa Benetti, Margherita Gallicchio, Emanuela Mazzon, Salvatore Cuzzocrea, Roberto Fantozzi, and Christoph Thiemermann. 2009. Anti-apoptotic and anti-inflammatory effects of hydrogen sulfide in a rat model of regional myocardial I/R. *Shock* 31 (3): 267–274.

26. Zhao, Zhi-Qing, Masanori Nakamura, Ning-Ping. Wang, Josiah N. Wilcox, Steven Shearer, Russell S. Ronson, Robert A. Guyton, and Jakob Vinten-Johansen. 2000. Reperfusion induces myocardial apoptotic cell death. *Cardiovascular research* 45 (3): 651–660.

27. Hodge, David R., Elaine M. Hurt, and William L. Farrar. 2005. The role of IL-6 and STAT3 in inflammation and cancer. *European journal of cancer* 41 (16): 2502–2512.

28. Shawan, Mohammad Mahfuz, Ali Khan, Sajal Kumar Halder, and Md Ashraful Hasan. 2021. Luteolin and abyssinone II as potential inhibitors of SARS-CoV-2: An in silico molecular modeling approach in battling the COVID-19 outbreak. *Bulletin of the National Research Centre* 45 (1): 1–21.

29. Theoharides, TC, and P Conti. 2020. Dexamethasone for COVID-19? Not so fast. *J Biol Regul Homeost Agents* 34 (3): 10.23812.

30. Theoharides, TC, and P Conti. 2020. COVID-19 and multisystem inflammatory syndrome, or is it mast cell activation syndrome? *Journal of Biological Regulators and Homeostatic Agents* 34 (5).

31. Kaisho, Tsuneyasu, and Shizuo Akira. 2006. Toll-like receptor function and signaling. *Journal of Allergy and Clinical Immunology* 117 (5): 979–987.

32. He, He., Xiayun Chang, Jin Gao, Lingpeng Zhu, Mingxing Miao, and Tianhua Yan. 2015. Salidroside mitigates sepsis-induced myocarditis in rats by regulating IGF-1/PI3K/Akt/GSK-3β signaling. *Inflammation* 38 (6): 2178–2184.

33. Tsai, Hsin-Ju, Shiang-Suo Huang, Meng-Ting Tsou, Hsiao-Ting Wang, and Jen-Hwey Chiu. 2015. Role of opioid receptors signaling in remote electrostimulation-induced protection against ischemia/reperfusion injury in rat hearts. *PloS one* 10 (10): e0138108.

34. Gu, Haifeng, Minghua Xie, Xu. Liqian, Xiaojun Zheng, Yunmei Yang, and Xueying Lv. 2015. The protective role of interleukin-18 binding protein in a murine model of cardiac ischemia/reperfusion injury. *Transplant International* 28 (12): 1436–1444.

35. Li, B.L., D.Y. Zhao, P.L. Du, X.T. Wang, Q. Yang, and Y.R. Cai. 2021. Luteolin alleviates ulcerative colitis through SHP-1/STAT3 pathway. *Inflammation Research*. https://doi.org/10.1007/s00011-021-01468-9.

36. Fu, Jiajia, Heqing Huang, Jiajun Liu, Rongbiao Pi, Jianwen Chen, and Peiqing Liu. 2007. Tanshinone IIA protects cardiac myocytes against oxidative stress-triggered damage and apoptosis. *European journal of pharmacology* 568 (1–3): 213–221.

37. Song, Jun-Qiu, Xu. Teng, Yan Cai, Chao-Shu. Tang, and Yong-Fen. Qi. 2009. Activation of Akt/GSK-3β signaling pathway is involved in intermedin 1–53 protection against myocardial apoptosis induced by ischemia/reperfusion. *Apoptosis* 14 (11): 1299.

38. Chong, Z.Z., and Kenneth Maiese. 2007. The Src homology 2 domain tyrosine phosphatases SHP-1 and SHP-2: Diversified

control of cell growth, inflammation, and injury. *Histology and histopathology* 22 (11): 1251.

39. Fish, Jason E., Massimo M. Santoro, Sarah U. Morton, Yu. Sangho, Ru-Fang. Yeh, Joshua D. Wythe, Kathryn N. Ivey, Benoit G. Bruneau, Didier YR. Stainier, and Deepak Srivastava. 2008. miR-126 regulates angiogenic signaling and vascular integrity. *Developmental cell* 15 (2): 272–284.

40. Lim, Cheh Peng, and Xinmin Cao. 2006. Structure, function, and regulation of STAT proteins. *Molecular biosystems* 2 (11): 536–550.

41. Schindler, Christian, David E. Levy, and Thomas Decker. 2007. JAK-STAT signaling: From interferons to cytokines. *Journal of Biological Chemistry* 282 (28): 20059–20063.

42. Santoni, Matteo, Francesco Massari, Marzia Del Re, Chiara Ciccarese, Francesco Piva, Giovanni Principato, Rodolfo Montironi, Daniele Santini, Romano Danesi, and Giampaolo Tortora. 2015. Investigational therapies targeting signal transducer and activator of transcription 3 for the treatment of cancer. *Expert opinion on investigational drugs* 24 (6): 809–824.

43. Subramaniam, Aruljothi, Muthu K. Shanmugam, Ekambaram, Perumal, Feng Li, Alamelu, Nachiyappan, Xiaoyun, Dai, Shivananju Nanjunda Swamy, Kwang Seok Ahn, Alan Prem Kumar, and Benny KH Tan. 2013. Potential role of signal transducer and activator of transcription (STAT) 3 signaling pathway in inflammation, survival, proliferation and invasion of hepatocellular carcinoma. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* 1835 (1): 46–60.

44. Andreadou, Ioanna, Panagiotis Efentakis, Evangelos Balafas, Gabriele Togliatto, Constantinos H. Davos, Aimilia Varela, Constantinos A. Dimitriou, Panagiota-Efstathia. Nikolaou, Eirini Maratou, and Vaia Lambadiari. 2017. Empagliflozin limits myocardial infarction *in vivo* and cell death *in vitro*: Role of STAT3, mitochondria, and redox aspects. *Frontiers in Physiology* 8: 1077.

45. Sugano, Masahiro, Keiko Tsuchida, Tomoji Hata, and Naoki Makino. 2005. RNA interference targeting SHP-1 attenuates myocardial infarction in rats. *The FASEB journal* 19 (14): 2054–2056.

46. Bian, Y., L. Yuan, X. Yang, L. Weng, Y. Zhang, H. Bai, and J. Chen. 2021. SMURF1-mediated ubiquitylation of SHP-1 promotes cell proliferation and invasion of endometrial stromal cells in endometriosis. *Annals of Translational Medicine* 9 (5): 362. https://doi.org/10.21037/atm-20-2897.

47. Ding, X., D. Lu, and J. Fan. 2021. A natural product phillygenin suppresses osteosarcoma growth and metastasis by regulating the SHP-1/JAK2/STAT3 signaling. *Bioscience, Biotechnology, and Biochemistry* 85 (2): 307–314. https://doi.org/10.1093/bbb/zbaa007.

48. Han, Y., J. Zhang, Y. Pang, Y. Wang, X. Zhang, and H. Zhang. 2021. The role of Src homology region 2 domain-containing phosphatase-1 hypermethylation in the classification of patients with myelodysplastic syndromes and its association with signal transducer and activator of transcription 3 phosphorylation in skm-1 cells. *Journal of International Medical Research* 49 (3): 300060521999550. https://doi.org/10.1177/0300060521999550.

49. Teng, R., Y. Wang, N. Lv, D. Zhang, R.A. Williamson, L. Lei, P. Chen, et al. 2021. Corrigendum to "Hypoxia Impairs NK Cell Cytotoxicity through SHP-1-Mediated Attenuation of STAT3 and ERK Signaling Pathways." *Journal of Immunology Research* 2021: 3625084. https://doi.org/10.1155/2021/3625084.

50. Teng, R., Y. Wang, N. Lv, D. Zhang, R.A. Williamson, L. Lei, P. Chen, et al. 2020. Hypoxia Impairs NK Cell Cytotoxicity through SHP-1-Mediated Attenuation of STAT3 and ERK Signaling Pathways. *Journal of Immunology Research* 2020: 4598476. https://doi.org/10.1155/2020/4598476.

51. Yang, M.H., I.J. Ha, J.Y. Um, and K.S. Ahn. 2021. Albendazole Exhibits Anti-Neoplastic Actions against Gastric Cancer Cells by Affecting STAT3 and STAT5 Activation by Pleiotropic Mechanism(s). *Biomedicines* 9 (4). https://doi.org/10.3390/biomedicines9040362.