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MECHANISM STUDY OF SHENFU INJECTION REGULATING MAPK PATHWAY TO ALLEVIATE MYOCARDIAL ISCHEMIA-REPERFUSION INJURY IN RATS BASED ON BIOINFORMATICS AND EXPERIMENTAL VERIFICATION

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Abstract: To elucidate SFI's protective mechanism against MIRI, this study identified the MAPK pathway via bioinformatics. Experimental validation in MIRI rats confirmed that SFI improves myocardial injury by suppressing MAPK activation and inflammatory factors (IL-1β, IL-6, TNF-α), supporting its clinical potential.
Keywords: Shenfu Injection (SFI); Myocardial Ischemia-Reperfusion Injury (MIRI); Mitogen-Activated Protein Kinase (MAPK) signaling pathway; Bioinformatics; Inflammatory factors

1 INTRODUCTION

Myocardial is chemia-reperfusion injury (MIRI), a serious post-revascularization complication, is characterized by oxidative stress, inflammation, and cell death[1-2], The outbreak of reactive oxygen species (ROS) and calcium ion (Ca^2) overload in the early stage of reperfusion trigger the opening of mitochondrial permeability transition pore (mPTP) and energy imbalance[3], subsequently, damage-associated molecular patterns (DAMPs) mediate the activation of pathways including Toll-like receptor-nuclear factor-kappa B (TLR-NF-kB), nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3), and mitogen-activated protein kinase(MAPK), leading to the increase of pro-inflammatory factors (IL-1β, IL-6, TNF-α) and further aggravation of tissue damage[4]. Shenfu Injection's documented multi-faceted pharmacological profile aligns with the network pathology of MIRI, suggesting its potential as a comprehensive therapeutic agent [5]. However, its upstream molecular targets and signaling networks have not been systematically clarified, which restricts the establishment of mechanistic and translational evidence. Among the multiple parallel pathways in MIRI, the MAPK pathway (p38/JNK/ERK) plays a core role [6]: p38 and c-Jun N-terminal kinase (JNK) dominate the stress and inflammatory transcriptional programs, while extracellular signal-regulated kinase (ERK) is associated with cell survival/ repair [7]. Since re-perfusion often over-activates pro-inflammatory/ apoptosis p38/JNK, ideal cardio protection requires their inhibition while maintaining ERK activity. SFI's ability to remodel these pathways at this hub provides a unified mechanism for its therapeutic effect.
Integrating bioinformatics prediction with in vivo validation, this study demonstrated that SFI alleviates MIRI by inhibiting the MAPK pathway and subsequent inflammation.

2 MATERIALS AND METHODS

2.1 Bioinformatics Analysis

2.1.1 GEO data mining and biofunctional analysis

This study obtained the GSE6381 dataset from the GEO database, applying log 2transformation and normalization. Probes were mapped to gene symbols, redundant records were removed, and mean values were calculated. Batch effects were addressed using the limma package, while differential expression analysis was performed with GEO2R based on the criteria $|logFC|>1.0$ and adjusted $p<0.05$. Target genes were integrated to construct miRNA-mRNA networks, and GO and KEGG analyses were conducted using DAVID with adjusted $p<0.05$ as the significance threshold.

2.1.2 Animal modeling and grouping

Male SD rats (SPF grade,8-10 weeks old,250-300 g) were acclimatized for 7 days under standard SPF conditions (22±2°C,50±5% humidity,12-h light/ dark cycle) with free access to food and water. All procedures were approved by the Institutional Animal Ethics Committee and designed as terminal experiments. Rats were randomly divided into five groups (n=5): Sham, I/R, SFI-L, SFI-M, and SFI-H.

2.1.3 Drug administration

Following a 7-day pretreatment with SFI or saline, I/R was induced by LAD clamping. Investigators were blinded to the treatment groups throughout the experiment. Myocardial I/R injury was induced by 30- minute occlusion of the left anterior descending coronary artery followed by60- minute reperfusion, with sham rats undergoing identical surgery

without occlusion.

2.2 Detection of Molecular Mechanisms and Inflammatory Indicators

Sampling and Time Points: According to the animal experimental protocol, the left ventricular anterior wall ischemic zone and plasma were collected immediately after 60 minutes of reperfusion for the following assays.

2.3 qRT-PCR (p38, JNK, ERK mRNA)

The mRNA expression of MAPK pathway genes (p38/JNK/ERK) was analyzed by qPCR in triplicate and calculated relative to Sham controls using the 2^- Δ△Ct method.

2.4 Western Blot (p-p38、p-JNK、p-ERK)

Phosphorylation levels of MAPK proteins (p38, JNK, ERK) were determined by Western blot analysis. Protein extracts were separated by SDS-PAGE, transferred to PVDF membranes, and probed with specific antibodies. The p-MAPK/ total-MAPK ratio was quantified using ImageJ, with technical replicates for validation.

2.5 ELISA (IL-1β、IL-6、TNF-α)

Plasma analyte levels were measured in duplicate using validated kits (CV ≤ 10%) and are expressed in pg/mL.

3 STATISTICAL ANALYSIS

Data are presented as the mean±standard deviation. Intergroup comparisons were performed using one-way analysis of variance (ANOVA), with a P-value of less than 0.05 considered statistically significant.

4 RESULTS

4.1 Bio-informatics Prediction Results

Enrichment analysis highlighted key processes: in BP, hypoxia response and immune activation; in MF, kinase/ ubiquity-related activities; in CC, secretary and nuclear structures. This collectively suggests crucial roles in immune secretion, energy metabolism, and transcriptional regulation during MIRI.

Significantly enriched KEGG pathways included the MAPK signaling pathway, Rap1 signaling, Apoptosis, and pathways related to cardiovascular-metabolic injury, such as ' Fluid shear stress and atherosclerosis', 'AGE-RAGE signaling in diabetic complications',' Lipid and atherosclerosis', and' Diabetic cardiomyopathy'. Neurodegenerative disease pathways (e.g., Alzheimer's, Parkinson's, Huntington's) and several pathogen infection pathways (e.g., Epstein-Barr virus infection, Hepatitis B, Human cytomegalovirus infection) were also significantly enriched.

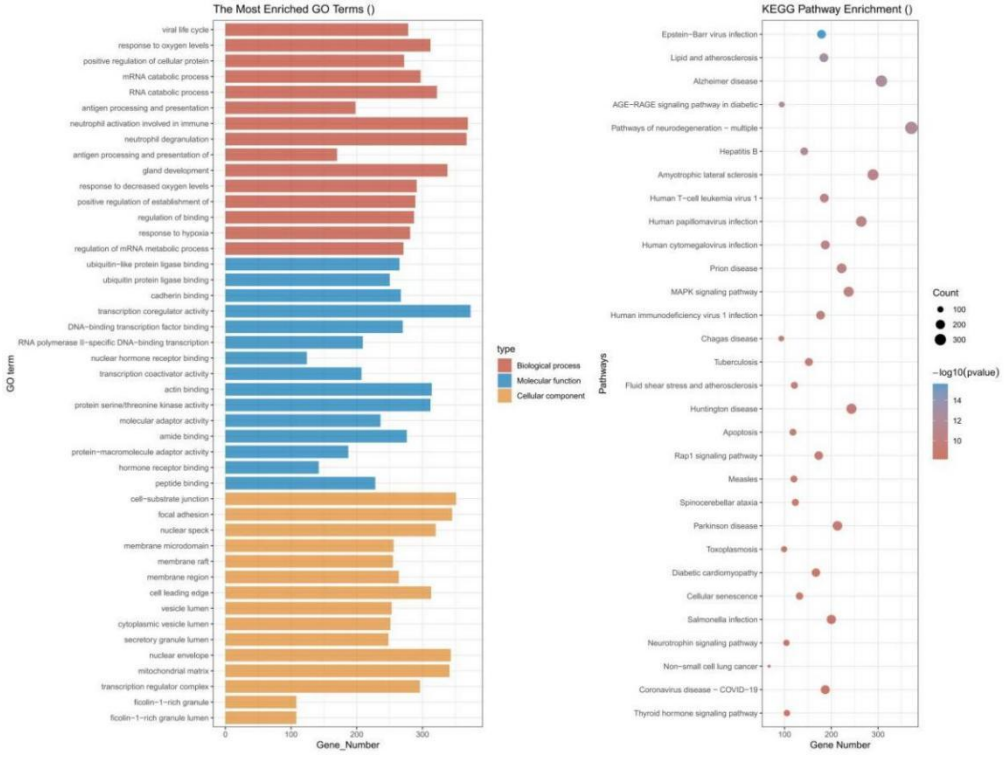

Figure 1 Functional Enrichment Analysis of Differential Expressed Genes

Collectively, the enrichment profile points to stress-inflammatory cascades and metabolic/ mitochondrial imbalance as key pathological hubs. This bio-information prediction aligns with the in vivo observation of MAPK axis activation and its suppression by Shenfu Injection (SFI), as illustrated in Figure 1.
GO/KEGG enrichment was analyzed (cluster Profiler v3.18.0) and visualized as bar/ bubble plots showing significance (-log10(P/FDR)) and gene count. Significant terms/ pathways met P/FDR <0.05.

4.2 Experimental Verification Results

4.2.1 MAPK pathway regulation

The MAPK pathway was activated: phosphorylation levels of p-p38, p-JNK, and p-ERK were significantly higher in the I/R group compared to the Sham group. Shenfu Injection (SFI) treatment resulted in a dose-dependent reduction (High> Medium> Low) in phosphorylation, consistent with the enriched MAPK signaling pathway. At the mRNA level, expressions of p38, JNK, and ERK were up-regulated in the I/R group, while SFI treatment induced dose-dependent suppression, with the high-dose group approaching baseline levels (See Table 1).

Table 1 Shenfu Injection (SFI) Attenuates Ischemia-Reperfusion (I/R)-Induced MAPK Pathway Activation in a Dose-Dependent Manner: Results from qRT-PCR and Phosphoprotein Quantification

Group	p38mRNA	JNKmRNA	ERKmRNA	p-p38	p-JNK	p-ERK
I/R	1.98±0.1	2.39±0.18	1.84±0.31	5198±323	4719±500	5228±260
SFI-H	1.12±0.10	1.15±0.08	1.18±0.21	1869±40	1804±233	1633±193
SFI-L	1.63±0.11	1.80±0.19	1.53±0.16	3967±207	3527±229	3766±544
SFI-M	1.41±0.12	1.38±0.10	1.27±0.11	2915±322	2571±216	2664±286
Sham	1.01±0.06	0.91±0.07	1.00±0.11	1227±96	1010±138	1029±96

4.2.2 Changes in Inflammatory Cytokines

Table 2 Plasma Inflammatory Factor Levels (ELISA, pg/mL, mean ± SD)

Group	IL-1β	IL-6	TNF-α
Sham	11.8±1.2	22.2±2.2	14.7±1.5
I/R	83.2±8.1	197.3±19.6	128.3±12.6
SFI-L	57.7±5.5	160.5±16.0	92.6±9.3
SFI-M	42.7±4.3	93.5±9.4	62.5±6.3
SFI-H	25.4±2.5	55.4±5.5	40.1±4.0

Compared to the Sham group, the I/R group exhibited significant increases in IL-1β, IL-6, andTNF-α levels. All doses of Shenfu Injection (SFI) significantly reduced the levels of these three inflammatory cytokines in a dose-dependent manner (High > Medium > Low) compared to the I/Rgroup. One-way ANOVA revealed significant intergroup differences(IL-1β:$F=387.41$, $p<10^{-15}$;IL-6:$F=259.57$, $p<10^{-14}$; TNF-α: $F=143.35$, $p<10^{-12}$). Post-hoc Bonferroni tests indicated that the differences between each SFI dose group (Low, Medium, High) and the I/R group were statistically significant (most $p<0.001$; e. g., for IL-6: SFI-L $p=0.028$, SFI-M $p=2.2\times10^{-4}$, SFI-H,$p=1.6\times10^{-4}$. Specifically, compared to the I/R group, SFI Low, Medium, and High doses reducedIL-1β levels by 30.7%,48.7%, and 69.4%; The levels of IL-6 were reduced by 18.6%,52.6%,ahd 71.9%; and TNF-αby 27.8%,51.3%, and 68.8%, respectively (Bonferroni correction following one-way ANOVA, all $P<0.05$). These results suggest that SFI effectively attenuates"I/R-induced amplification of the inflammatory response (See Table 2).

5 DISCUSSION

Myocardial ischemia-reperfusion injury (MIRI) is driven by early MAPK axis activation,which amplifies inflammatory damage. This study demonstrates that Shenfu Injection (SFI)addresses this mechanism. Employing a bioinformatics-to-experimental approach, we found that SFI significantly suppressed I/R-induced activation of the p38/JNK/ERK pathway and dose-dependently reduced key inflammatory cytokines(IL-1β,IL-6, TNF-α) by up to 69.4%, 71.9%,and 68.8%, respectively.
Bioinformatic enrichment analysis highlighted hypoxic stress and innate immunity activation in MIRI, as evidenced by key GO-BP terms including" response to hypoxia" and" granulocyte activation. "[8]; GO-MF terms including" protein serine/ threonine kinase activity" and" transcription factor binding" directly reflected core molecular functions of the MAPK pathway, while GO-CCtérms like" secretory granule lumen" and" mitochondrial matrix" suggested organelle dysfunction and enhanced immune secretion. KEGG analysis not only confirmed the central role of the MAPK signaling pathway but also revealed enriched pathways such as" Apoptosis" and" Lipid and atherosclerosis, " collectively depicting MIRI's pathological network involving energy metabolism disruption, inflammation, and cell death. Notably, the enrichment of neurodegenerative disease pathways may imply share stress-inflammatory mechanisms between MIRI and neurological disorders [9]. Overall, the enrichment profile consistently highlights stress-inflammatory cascades and metabolic/ mitochondrial imbalance as key pathological hubs, which aligns with in

vivo observations of MAPK axis activation and its suppression by SFI, providing multi-omics evidence for SFI's cardio-protective role via MAPK pathway modulation.

The observed synchronous inhibition of p38/JNK and reduction in inflammation align with established understanding that early stress pathways drive inflammatory amplification in MIRI. The dose-dependent suppression of pro-inflammatory cytokines by SFI is consistent with its reported anti-inflammatory properties [10]. While some studies suggest that ERK may mediate survival/ repair signals during early re-perfusion [11], while some studies suggest that ERK may mediate survival/ repair signals during early re-perfusion [12]. High-dose Shenfu Injection (SFI)significantly and simultaneously reduced phosphorylation levels of p38(p-p38) and JNK (p-JNK) in myocardial tissue and decreased plasma concentrations of key inflammatory cytokines includingIL-1β, IL-6, and TNF-α in the MIRI model. This indicates that one of the primary mechanisms underlying SFI's cardio-protective effect is through effectively suppressing the excessive activation of the p38 and JNK branches within the stress-responsive MAPK pathway [13-14]; Thereby, SFI interrupts the key upstream circuit of inflammatory signal amplification, preventing the vicious cycle of inflammatory response and subsequent tissue damage [15].

In summary, high-dose SFI treats MIRI by coordinately modulating the MAPK pathway: suppressing the pro-inflammatory p38/JNK branches while preserving pro-repair ERK activity, thereby integrating anti-inflammatory and reparative effects.

6 CONCLUSION

This study demonstrates that SFI alleviates myocardial injury by inhibiting the MAPK pathway and its associated inflammation, providing mechanistic support for its clinical use.

COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

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