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Effects of infliximab on oxidative stress and inflammation of H9c2 cells induced by H_2O_2

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ABSTRACT

This study investigated the effects of infliximab (INF) on oxidative stress and inflammation in H9c2 cardiomyocytes, aiming to address the damage caused by myocardial infarction (MI). H9c2 cells were divided into three groups: control, H2O2 treatment, and H2O2+INF. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay. Protein expression of SOD1, SOD2, TNF-α, and IL-1β was examined through Western blot, while mRNA expression was analyzed via polymerase chain reaction (PCR). Reactive oxygen species (ROS) levels were measured, and IL-1β immunofluorescence was utilized to observe inflammation. The expression of IκB-α and IκKα was evaluated to investigate the mechanism of action. INF significantly improved H9c2 cell viability and reduced LDH and MDA levels in the supernatant. Moreover, INF enhanced the expression of SOD1 and SOD2, reducing ROS production. In comparison to the H2O2 group, TNF-α and IL-1β expression markedly decreased in the H2O2+INF group. Additionally, the fluorescence intensity of IL-1β immunofluorescence was higher in the H2O2+INF group. INF treatment decreased TNF-α and IL-1β expression and reduced IL-1β fluorescence intensity. Furthermore, INF increased IκB-α expression and decreased IκKα expression, suggesting inhibition of the nuclear factor-κB (NF-κB) pathway. In summary, INF effectively suppressed H2O2-induced oxidative stress and inflammation in H9c2 cells by targeting the NF-κB pathway.

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Introduction

Myocardial infarction (MI) is a severe cardiovascular condition primarily caused by coronary atherosclerosis, which leads to the occlusion of coronary arteries due to plaque rupture, hemorrhage, thrombosis, or stenosis. This acute occlusion results in a significant interruption or reduction in coronary blood flow, leading to sustained and severe myocardial ischemia. Eventually, this ischemic insult leads to myocardial necrosis (1). Myocardial infarction often occurs during periods of intense physical activity, excessive emotional agitation, and other triggers. It is believed to be associated with factors such as increased cardiac load, elevated myocardial oxygen consumption, enhanced blood viscosity, slow blood flow, heightened stress response, and vasospasm (2,3).

Following myocardial infarction, multiple mechanisms are triggered, activating the immune system and initiating an inflammatory response along with oxidative stress. Infiltration of inflammatory cells in the myocardium leads to the release of numerous cytokines involved in the inflammatory process. These cytokines stimulate cell death, cell infiltration, and extracellular remodeling (4). Excessive immune system activation and a persistent inflammatory response following MI can worsen myocardial damage, promote ventricular remodeling, and accelerate cardiac deterioration (5,6). Therefore, regulating the inflammatory response post-MI and restoring the balance of the cytokine network are key factors in delaying ventricular remodeling and the deterioration of cardiac function.

Infliximab (INF) is a human-mouse chimeric IgG1 monoclonal antibody that specifically binds to the pro-inflammatory factor transforming growth factor-α (TNF-α). INF exhibits a potent binding ability to TNF-α produced by various cells, thereby inhibiting inflammation (7,8). Currently, INF is primarily used in the treatment of conditions such as rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, and others (9). However, the effects of INF on cardiomyocytes have not been extensively studied.

In this study, we aimed to investigate the potential therapeutic role of INF in myocardial injury. We utilized an in vitro model of H9c2 cells, a well-established cell line used for studying cardiac function and disease. We examined the effects of INF on oxidative stress and inflammation in the myocardium to assess its potential cardioprotective function.

By examining the impact of INF on H9c2 cell injury models, we sought to evaluate its effects on myocardial oxidative stress and inflammation. These cellular processes play significant roles in the progression of myocardial infarction and subsequent cardiac remodeling. Investigating the influence of INF on these key pathological factors may provide valuable insights into its potential as a therapeutic intervention in the context of myocardial infarction.

Overall, this study aims to explore the effects of INF on myocardial injury, particularly its impact on oxidative

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stress and inflammation. By shedding light on the potential cardioprotective properties of INF, we hope to contribute to the understanding of its therapeutic relevance in the context of myocardial infarction.

Materials and Methods

Cell culture

H9c2 cells (Procell, Wuhan, China) were cultured in Dulbecco's modified eagle medium (DMEM) (Life Technology, Wuhan, China) containing 10% fetal bovine serum (FBS) (Life Technology, Wuhan, China) and 1% penicillin/streptomycin (Life Technology, Wuhan, China). H_2O_2 (100 μM, 4 h) was utilized to construct the damage model of H9c2 cells. After that, the infliximab (Sigma, St. Louis, MO, USA) was added in for 24 hours.

RNA extraction and Real-time polymerase chain reaction (PCR)

TRIzol kit (MCE, Nanjing, China) was utilized to extract total RNA from the cells according to the instructions. Add 1 mL TRIzol reagent per 1×10^6 cells, and gently blow the cell masses evenly with a pipette. Add chloroform in a ratio of 5:1 of TRIzol: chloroform, shake vigorously for 15 seconds on a shaker, and let stand for 3 minutes at room temperature; then centrifuge the mixture for 15 minutes at 4°C, 12000 rpm. Pipette the supernatant water to another new 1.5 mL EP tube, add an equal volume of isopropanol, mix and let stand at room temperature for 20 minutes. After centrifugation at 12,000 rpm for 10 minutes at 4°C, the supernatant was discarded, then 1 mL of 75% ethanol was added and centrifuged the tubes at 7500 rpm for 10 minutes, and the supernatant was discarded. After drying at room temperature, the precipitate was dissolved in 20 μL of ribonuclease-free water. RNA concentration was measured using NanoDropTM 8000 (Thermo Fisher Scientific, Waltham, MA, USA).

mRNA reverse transcription was performed using a reverse transcription kit (MCE, Nanjing, China) according to the protocols.

SYBR Green qPCR Mix (KeyGen, Shanghai, China) was used to perform PCR in accordance with the protocols. All the primers were listed in Table 1.

Reactive oxygen species (ROS) quantification

Quantification of ROS was performed in accordance with the protocol of the DHR-ROS test kit (MCE, Nanjing, China).

Western blot

The total protein was obtained using a protein extraction kit (KeyGen, Shanghai, China). The concentration was measured in line with the instructions of the bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Waltham, MA, USA). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was made using an SDS-PAGE gel preparation kit (Beyotime, Shanghai, China). A total of 20 μg of protein was added to the wells of SDS-PAGE for electrophoresis. Then the electrophoresed protein was transferred to the polyvinylidene fluoride (PVDF, EpiZyme, Shanghai, China) membrane. Then 5% skim milk was utilized to block the non-specific antigen on the protein bands. After that, the protein bands were incubated by the primary antibodies (SOD1, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; SOD2, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; TNF-α, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IL-1β, Abcam, Cambridge, MA, USA, Rabbit, 1:5000; IκKα, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; IκB-α, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; GAPDH, Proteintech, Rosemont, IL, USA, 1:3000) overnight. The next day, the protein bands were incubated using the secondary antibody and then washed using tris buffered saline-tween (TBST). Finally, Image Lab™ Software was used to expose the bands.

Superoxide dismutase (SOD) activity assay

SOD levels of H9c2 cells were detected in accordance with the protocol of the SOD assay kit (KeyGen, Shanghai, China).

Lactate dehydrogenase (LDH) levels

The content of LDH in the H9c2 cell supernatant was measured by Cytotoxicity LDH Assay Kit (Dojindo, Kumamoto, Japan).

Malondialdehyde (MDA) levels

The content of MDA in the H9c2 cell supernatant was detected by MDA Assay Kit (Beyotime, Shanghai, China).

Cell counting kit-8 (CCK-8) assay

Cell Counting Kit-8 (MCE, Nanjing, China) was utilized to detect cell viability in line with the protocol.

IL-1β immunofluorescence

H9c2 cells in a 24-well plate were fixed using 4% paraformaldehyde, then the goat serum (CWBIO, Beijing, China) was added in for 1 hour. After that, the cells were

Table 1. Real time PCR primers.

Gene name	Forward (5'>3')	Reverse (5'>3')
SOD1	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
SOD2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
Ikkα	GTCAGGACCGTGTTCTCAAGG	GCTTCTTTGATGTTACTGAGGGC
IκB-α	GGATCTAGCAGCTACGTACG	TTAGGACCTGACGTAACACG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

incubated with the primary antibody IL-1β overnight. The next day, the fluorescent secondary antibody was added for 1 hour in the dark. And DAPI (KeyGen, Shanghai, China) was added to stain the nucleus. Finally, the immunofluorescence was observed by a Confocal Laser Scanning Microscope (CLSM).

Statistical analysis

Data were expressed as $\chi\pm s$. All data were plotted using GraphPad Prism5 software (La Jolla, CA, USA). Differences between the two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using a One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). Test level $\alpha=0.05$.

Results

INF inhibited the injury of H9c2 cells induced by H_2O_2.

First, H9c2 cells were added with different concentrations of H_2O_2, and after 4 hours CCK-8 was utilized to detect cell viability. We can see that a 100 μM concentration of H_2O_2 can reduce the viability of H9c2 cells by half (Figure 1A) and this is why we chose this concentration for follow-up experiments. Then, we treated H9c2 cells which treated with 100 μM H_2O_2 with different concentrations of INF. 10 μg/mL of INF can maximize H9c2 cell viability (Figure 1B). So, we chose 10 μg/mL of INF to treat H9c2 cells. We then tested the levels of LDH and MDA in the supernatants of the three groups and found that INF greatly decreased the content of LDH and MDA (Figures 1C and 1D).

INF inhibited oxidative stress in H9c2 cells

First, Western blot was utilized to detect the expression of SOD1 and SOD2 proteins in the three groups. As can be seen from Figure 2A~2C, H_2O_2 can greatly reduce the expression of these two proteins, but when treated with INF, the expression of both proteins is reversed. The same result appeared on the expression of SOD1 and SOD2 mRNA detected by PCR (Figures 2D and 2E). Then we further proved the above results with an SOD activity assay (Figure 2F). We also used a DHR-ROS test kit to detect ROS production. The ROS production in the H_2O_2 group increased significantly, but when INF was added, the ROS product decreased (Figure 2G). These results indicated that INF could inhibit the oxidative stress of H9c2 cells.

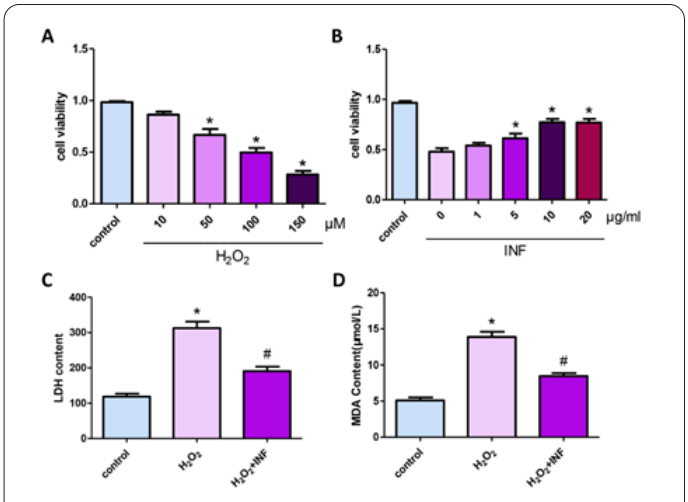

Figure 1. INF inhibited the injury of H9c2 cells. (A) CCK-8 assay showed H9c2 cell viability at different concentrations of H_2O_2 ("$_*$" $P<0.05$ *vs.* control, n = 3). (B) CCK-8 assay showed cell viability after the addition of different concentrations of INF in H_2O_2-treated H9c2 cells ("$_*$" $P<0.05$ *vs.* 0, n = 3). (C) The LDH content decreased significantly in the H_2O_2+INF group ("$_*$" $P<0.05$ *vs.* control, "$_\#$" $P<0.05$ *vs.* H_2O_2, n = 3). (D) The MDA content decreased significantly in the H_2O_2+INF group ("$_*$" $P<0.05$ *vs.* control, "$_\#$" $P<0.05$ *vs.* H_2O_2, n = 3).

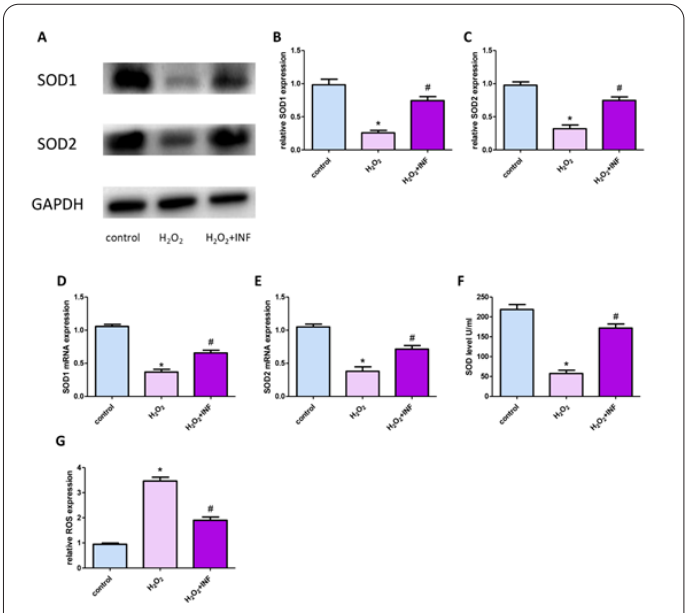

Figure 2. INF inhibited oxidative stress induced by H_2O_2. *(*A) The expression of SOD1 and SOD2 in the H_2O_2 treatment group decreased significantly, and the expression of SOD1 and SOD2 increased in the H_2O_2+INF group. (B) Statistical results of protein level of SOD1 ("$_*$" $P<0.05$ *vs.* control, "$_\#$" $P<0.05$ *vs.* H_2O_2, n=3). (C) Statistical results of protein level of SOD2 ("$_*$" $P<0.05$ *vs.* control, "$_\#$" $P<0.05$ *vs.* H_2O_2, n=3). (D) The expression of SOD1 mRNA in the H_2O_2 treatment group decreased significantly, and the expression increased in the H_2O_2+INF group ("$_*$" $P<0.05$ *vs.* control, "$_\#$" $P<0.05$ *vs.* H_2O_2, n=3). (E) The expression of SOD2 mRNA in the H_2O_2 treatment group decreased significantly, and the expression increased in the H_2O_2+INF group ("$_*$" $P<0.05$ *vs.* control, "$_\#$" $P<0.05$ *vs.* H_2O_2, n=3). (F) SOD activity assay showed that H_2O_2 can significantly reduce SOD levels, while INF can reverse SOD levels ("$_*$" $P<0.05$ *vs.* control, "$_\#$" $P<0.05$ *vs.* H_2O_2, n=3). (G) The expression of ROS increased in the H_2O_2 treatment group but decreased significantly in the H_2O_2+INF group ("$_*$" $P<0.05$ *vs.* control, "$_\#$" $P<0.05$ *vs.* H_2O_2, n=3).

INF inhibited inflammation in H9c2 cells

To explore the role of INF in inhibiting cardiomyocyte inflammation, we first examined the expression of IL-1β and TNF-α in three groups. Western blot results showed that INF could significantly inhibit the expression of these two pro-inflammatory factors (Figure 3A~3C). And the expression of SOD1 and SOD2 mRNA also decreased after the treatment of INF (Figures 3D and 3E). At the same time, IL-1β immunofluorescence showed that the IL-1β fluorescence density of the H_2O_2+INF group decreased markedly compared with the H_2O_2 group (Figure 3F). These results demonstrated that INF can inhibit pro-inflammatory factors in myocardial cell injury models.

INF inhibited the NF-κB pathway

The NF-κB pathway has important functions in many

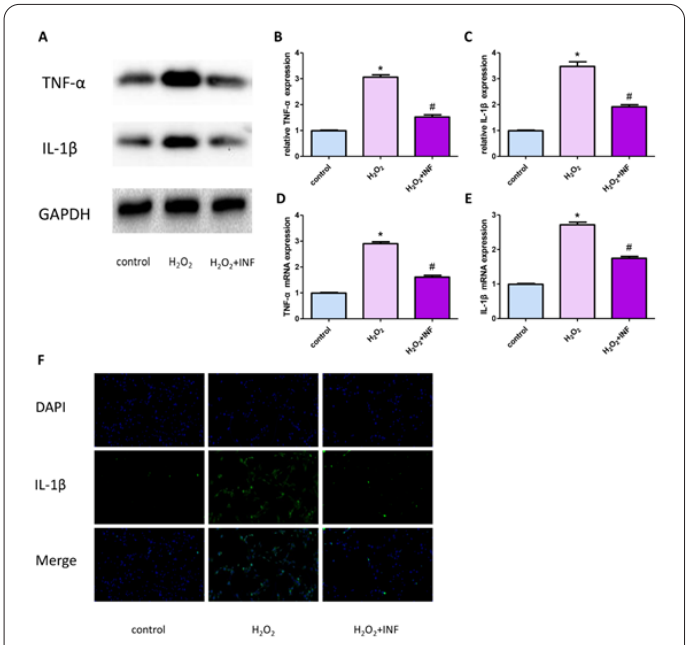

Figure 3. INF inhibited inflammation induced by H_2O_2. (A) The expression of IL-1β and TNF-α in H_2O_2 treatment group increased significantly but decreased in H_2O_2+INF group. (B) Statistical results of expression of TNF-α ("*" $P<0.05$ *vs.* control, "#" $P<0.05$ *vs.* H_2O_2, n=3). (C) Statistical results of expression of IL-1β ("*" $P<0.05$ *vs.* control, "#" $P<0.05$ *vs.* H_2O_2, n=3). (D) TNF-α mRNA expression was similar to the results of Western blot ("*" $P<0.05$ *vs.* control, "#" $P<0.05$ *vs.* H_2O_2, n=3). (E) IL-1β mRNA expression was also similar to the results of Western blot ("*" $P<0.05$ *vs.* control, "#" $P<0.05$ *vs.* H_2O_2, n=3). (F) Immunofluorescence showed that INF significantly reduced the H_2O_2-mediated elevation of IL-1β (magnification: 200×).

aspects such as cellular inflammation. Therefore, we examined the expression of important regulatory proteins IκKα and IκB-α in this pathway. In the H_2O_2 group, the expression of IκKα was greatly increased, and the expression of IκB-α was greatly decreased, while in the H_2O_2+INF group, the expression of both proteins was reversed (Figure 4A~4C). The mRNA levels of IκKα and IκB-α detected by PCR were consistent with the results of Western blot (Figures 4D and 4E). These results suggested that INF can inhibit NF-κB pathway activation.

Discussion

Oxidative stress and inflammation have important functions in MI. There is a large number of inflammatory reactions during the development of MI. The damaged myocardium and other inflammatory cells produce a large number of inflammatory cytokines and inflammatory mediators, which in turn cause ventricular remodeling. Studies have shown that activation signals such as hypoxia and oxidative stress after MI can activate nuclear factor-κB, and then up-regulate the expression of inflammatory cytokines TNF-α, interleukin-1, etc. (10,11). TNF-α is the major pro-inflammatory cytokine (12). TNF-α can disrupt the stability of endothelial cell-constitutive NO synthase (eNOS) mRNA, down-regulate the expression of NO synthase, and induce the expression of immune-inducible NO synthase (iNOS), which produces cardiotoxicity, inhibits myocardial contraction, induces cardiomyocyte apoptosis and negative chronotropic effects (13,14). TNF-α can also mediate the increase of myocardial matrix metalloproteinase activity, change the myocardial matrix, increase collagen fiber content, myocardial fibrosis, and cause enlargement of the lumen and thinning of the wall (15). In the process of inflammatory reaction, lipid peroxidation is often accompanied (16). In the case of myocardial ischemia and hypoxia, due to the influence of inflammatory reaction, endothelial cell damage, neutrophil activation and infiltration of cardiomyocytes cause an increase in reactive oxygen species and microcirculation disturbance (17). It leads to the imbalance between the pro-oxidation system and the antioxidant system in the body, the reduction of the active oxygen scavenging function, and the accumulation of oxygen free radicals, further damaging the cell membrane and organelles, leading to cell necrosis and apoptosis, and further strengthening the chain reaction between oxidation and inflammation (18).

INF is a synthetic antibody (19). Mouse-derived antibodies were originally extracted from mice. When a mouse-derived protein enters the body, it stimulates the body to produce an immune response, so the common mouse-derived domain is replaced with a similar human antibody. This antibody is split into a clonal population from the same cell and is, therefore, a monoclonal antibody (20). As a combination of a murine antibody and a human antibody amino acid sequence, INF is a chimeric monoclonal antibody. INF is a specific IG1 monoclonal antibody directed against TNF-α, the Fab segment of which has a murine sequence (21). INF inactivates TNF-α by binding to soluble and transmembrane TNF-α, thereby achieving the goal of blocking the cytotoxic effect of TNF-α (22,23).

In this article, we used H_2O_2 to create an H9c2 cell injury model to explore the effects of INF on myocardial inflammation and oxidative stress.

It was found that INF can significantly inhibit the expression of IL-1β and TNF-α in H9c2 cells and increase the expression of SOD1 and SOD2. And INF can also reduce the content of LDH, MDA and ROS. These results suggest that INF can inhibit inflammation and oxidative stress after MI. Therefore, our research has brought new potential methods for the treatment of MI.

Our study sheds light on the potential therapeutic ef-

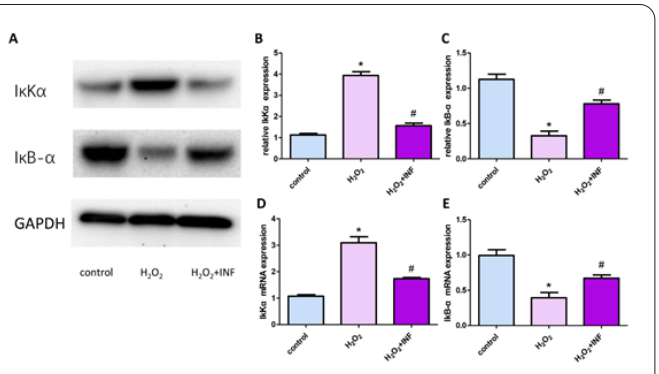

Figure 4. INF inhibited the NF-κB pathway. (A) In the H_2O_2 treatment group, the expression of IκKα increased, and the expression of IκB-α decreased. In the H_2O_2+INF group, the expression of IκKα decreased, and the expression of IκB-α increased. (B) Statistical results of protein level of IκKα ("*" $P<0.05$ *vs.* control, "#" $P<0.05$ *vs.* H_2O_2, n=3). (C) Statistical results of protein level of IκB-α ("*" $P<0.05$ *vs.* control, "#" $P<0.05$ *vs.* H_2O_2, n=3). (D) Expression of IκKα mRNA in three groups ("*" $P<0.05$ *vs.* control, "#" $P<0.05$ *vs.* H_2O_2, n=3). (E) Expression of IκB-α mRNA was consistent with the results of Western blot ("*" $P<0.05$ *vs.* control, "#" $P<0.05$ *vs.* H_2O_2, n=3).

fects of infliximab (INF) on oxidative stress and inflammation induced by H2O2 in H9c2 cells. By investigating the underlying mechanisms, we have demonstrated that INF exerts its beneficial effects by inhibiting the NF-κB pathway. Oxidative stress and inflammation are key contributors to the pathogenesis of various diseases, including cardiovascular disorders. H9c2 cells, a widely used model for studying cardiac function and disease, were subjected to H2O2-induced oxidative stress to mimic the conditions of cellular damage. Our findings indicate that INF effectively attenuated oxidative stress and subsequent inflammation in H9c2 cells, providing valuable insights into its potential cardioprotective role. NF-κB is a crucial transcription factor involved in the regulation of inflammatory responses. In this study, we observed that INF treatment inhibited the activation of NF-κB signaling in H9c2 cells exposed to H2O2. By doing so, INF disrupted the inflammatory cascade, leading to a reduction in the production of pro-inflammatory mediators and cytokines. Moreover, INF's ability to inhibit NF-κB signaling also contributed to the attenuation of oxidative stress in H9c2 cells. Activation of NF-κB is known to promote the generation of reactive oxygen species (ROS) and impair antioxidant defense systems. By suppressing NF-κB activation, INF effectively reduced ROS production and enhanced cellular antioxidant capacity, thereby mitigating oxidative stress. These findings highlight the potential of INF as a therapeutic agent for combating oxidative stress and inflammation-related pathologies, particularly in the context of cardiac diseases. By targeting the NF-κB pathway, INF holds promise for attenuating the detrimental effects of oxidative stress and inflammation on cardiac cells. Further studies are warranted to validate these findings in *in-vivo* models and to explore the clinical implications of INF in the management of cardiovascular disorders.

Conclusions

In summary, our study provides evidence that INF inhibits the NF-κB pathway, effectively suppressing oxidative stress and inflammation induced by H2O2 in H9c2 cells. These findings contribute to our understanding of the molecular mechanisms underlying the beneficial effects of INF and pave the way for potential therapeutic interventions aimed at mitigating oxidative stress and inflammation-associated cardiac pathologies.

Ethical Compliance

Not applicable.

Conflict of Interest

We have no interest in competing with others.

Acknowledgements

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