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Jing-Si Herbal Tea Suppresses H_2O_2-Instigated Inflammation and Apoptosis by Inhibiting Bax and Mitochondrial Cytochrome C Release in HIG-82 Synoviocytes

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ABSTRACT

Inflammation is an intrinsic protective mechanism against various forms of cellular injuries in humans; however, its undesired activation results in tissue damage and cell death. The onset of chronic inflammation and oxidative stress are the key characteristics of autoimmune inflammatory diseases such as rheumatoid arthritis (RA), for which an effective treatment is yet to be developed. Therefore, in this study, we investigated the protective effects and molecular mechanisms of a novel herbal preparation, Jing-Si herbal tea (JS), against H_2O_2-induced inflammation and cellular damage in HIG-82 synoviocytes. We found that JS did not show any significant alterations in cell viability at <188 μg/mL; however, a cytotoxic effect was observed at 188–1883 μg/mL concentrations tested. We found that expressions of inflammation associated extracellular matrix (ECM)-degrading proteases MMP-13, ADAMTS-2, -8, and -17 were abnormally enhanced under H_2O_2-induced pathological oxidative stress (ROS) in HIG-82 cells. Interestingly, JS treatment not only reduced the ROS levels but also significantly repressed the protein expressions of collagen degrading proteases in a dose-dependent manner. Treatment with JS showed enhanced cell viability against H_2O_2-induced toxic ROS levels. The expressions of cell protective aggrecan, Collagen II, and Bcl-2 were increased, whereas MMP-13,

ADAMTS-2, Cytochrome C, and cleaved Caspase 3 were decreased by JS under inflammatory agents H_2O_2, MIA, LPS, and TNF-α treatment, respectively, in HIG-82 cells. Interestingly, the cytoprotective effect of JS treatment was attributed to a decreased mitochondrial localization of Bax and a reduction of Cytochrome C release into the cytoplasm of H_2O_2-treated HIG-82 cells. Collectively, our results suggest a novel protective mechanism of JS for RA treatment, which could be potentially applied as a complementary treatment or as an alternative therapeutic approach to mitigate inflammatory diseases.

1 | Introduction

Inflammation is a protective response by the human body against various forms of acute injuries. that recognize many diverse stimuli, such as stresses, toxins, microbial infections, etc. Inflammasomes (a complex of multiple proteins) trigger the release of pro-inflammatory cytokines and the activation of proteases, leading to tissue damage and cell death (pyroptosis) [1]. In that context, rheumatoid arthritis (RA), an inflammatory and autoimmune disease, causes painful swelling in the affected parts of the body, mainly the lining of articular structures, which become inflamed, causing the destruction of joint tissues and impairing mobility and quality of life. Patients are usually prescribed prolonged disease-modifying anti-rheumatic drugs (DMARDs) to reduce synovial tissue inflammation, minimize joint damage, and preserve joint function [2]. Multiple risk factors, including both genetic and environmental, may be required to trigger RA, which comprises asymptomatic synovitis and the instigation and propagation of self-immunity against transformed auto-proteins that occurs several years before clinical symptoms become evident [3]. RA is characterized by increased cellularity of joint membranes (hyperplasia) and migration and invasion by inflammatory cells that release pro-inflammatory cytokines and tissue degrading proteases. The major signaling pathways implicated in RA include janus-activated kinase (JAK) signal transduction and activator of transcription (STAT), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3 kinase (PI3K)-AKT (also known as PKB), Wnt/β-catenin, and Notch, along with a variety of transcription factors such as nuclear factor erythroid 2-related factor 2 (Nrf2), hypoxia-inducible factor (HIF), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and activator protein-1 (AP-1), which are closely associated with the pathogenesis of RA [4]. Owing to the abnormal activation of the above pathways, there is an enhancement in the expression of inflammatory mediators, including Interleukin 1 beta (IL-1β), matrix metalloproteinases (MMPs), tumor necrosis factor alpha (TNF-α), and a disintegrin and metallopeptidase with thrombospondin (ADAMTS). These factors further instigate the propagation of fibroblast-like synoviocytes within the synovial membrane, culminating in cartilage destruction [4, 5].

Inflammation and oxidative stress are two intertwined biological phenomena with mutually interconnected responses during cellular stresses. Oxidative stress (also known as redox imbalance) is a condition in which the sum of all reactive oxygen species (ROS) intensifies over time, either by their amplified generation, the decrease in antioxidant defenses, or a combination of both [6]. Immune cells produce ROS during the inflammatory process, contributing to oxidative stress that encourages the stimulation of additional signaling pathways and the production of tissue-damaging inflammatory factors. The abnormal oxidative stress in individuals with RA results in chronic inflammation, sustained synovitis, and irreparable destruction of the joint tissues [7]. Mitochondria are the main sites of superoxide (O_2^-) production at complex I and III in the ETC. O_2^- produced by mitochondria generates hydrogen peroxide (H_2O_2) by reacting with manganese SOD (MnSOD) in the mitochondrial matrix, which can permeate the mitochondrial outer membrane to react with cytosolic proteins or other targets. The mitochondrial ROS production exacerbates the intracellular oxidative stress which could result in cell death (apoptosis) if uncontrolled by antioxidant defenses. There is increased mitochondrial outer membrane permeability (MOMP), which causes release of mitochondrial Cytochrome-c, apoptosis-inducing factor (AIF), endonuclease G, and other factors which instigate apoptosis through formation of apoptosome in either a caspase-dependent or -independent way. The proapoptotic proteins Bax and Bak of the Bcl-2 family are the two key members which adapt from harmless monomers into noxious oligomers that create pores in the MOM under cellular stress conditions. In healthy cells, pro-survival Bcl-2 proteins directly bind and sequester proapoptotic Bax and Bak in the cytoplasm which prevent their activation and dimerization [8].

Due to a lack of effective medications to cure RA, the current treatment regimen applies to accelerate diagnosis and achieve a mild disease state as early as possible. The current pharmacologic therapy with nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids could alleviate stiffness and pain but not halt disease progression, often accompanied by side effects linked to their long-term use [2]. Therefore, there is an unmet medical need for a novel or complementary therapeutic strategy to effectively manage and avert the onset of RA. Traditional herbal preparations are gaining attention as an emerging treatment modality for RA. Research indicates that herbal extracts are abundant in naturally occurring bioactive components. Numerous biological effects are exhibited by these chemicals, including vasodilatory, anticarcinogenic, anti-inflammatory, antimicrobial, antioxidant, and antiaging properties [9, 10]. Recognizing the advantages of herbal preparations, a novel herbal mixture known as JS Herbal Tea was recently formulated by combining eight different local Taiwanese herbs, namely: *Ophiopogon japonicas, Perilla frutescens, Houttuynia cordata, Anisomeles indica* (L.) Kuntze, *Artemisia argyi*, *Glycyrrhiza glabra*, *Platycodon grandifloras*, and *Chrysanthemum morifolium* Ramat. It has already been documented that the components of JS protect against various diseases such as genitourinary, neurological, cardiovascular, and metabolic impairments, in addition to having antiaging, anti-COVID, and anticancer properties [11].

In a recent clinical study, in patients with mild-to-moderate COVID-19, co-treatment with JS decreased systemic inflammation, lessened lung infiltration, and reduced the SARS-CoV-2

viral load [12]. On the other hand, in severe COVID-19 patients, the death rate and recovery time upon JS co-administration were reduced to 20% and 40%, respectively [13]. Furthermore, JS treatment demonstrated antiaging effects by maintaining stem cell preservation and cytoprotection in adipose-derived mesenchymal stem cells (ADMSC), regulating the blood sugar metabolism in type II diabetic mice, improving the autophagy process, attenuating the 1-methyl-4-phenylpyridinium ion (MPP^+)-induced neurotoxicity in SH-SY5Ycells, and alleviating doxorubicin-induced DNA damage and hypertrophic effects in H9c2 cardiomyoblast cells [14]. Previous research has suggested that the individual constituents of JS exert multiple protective effects against various diseases [11]. However, the current health-related information and molecular mechanisms of JS herbal tea formulation against damages inflicted by pathological inflammation and oxidative stress in RA is inadequate, and its clinical importance has not been sufficiently investigated. Therefore, through this study, we explored the molecular protective mechanisms of treatment with JS herbal formulation, against H_2O_2-induced inflammation, oxidative stress, and cellular damages in HIG-82 synoviocytes.

2 | Materials and Methods

2.1 | Preparation of JS Herbal Tea

The JS herbal tea included leaves of *O. japonicas*, *A. argyi*, *P. frutescens*, *Ohwia caudate*, flowers of *Chrysanthemum* × *morifolium*, and roots of *P. grandifloras*, *Glycyrrhiza uralensis* (*Glycyrrhizae radix*), *H. cordata* (*Ophiopogonis Radix*). The JS herbal tea was procured from the local herbal store (Taiwan), which had already been studied and characterized by HPLC analysis as described previously [13, 15].

2.2 | Cell Culture

HIG-82 cells derived from rabbit synovium tissue were procured from Bioresource Collection and Research Center, Taiwan (BCRC) (# 60242) and cultured in Ham's F-12 Nutrient Mixture (Hyclone, Logan, UT, USA) with 10% charcoal-treated fetal bovine serum (FBS) and penicillin–streptomycin (100 units/mL penicillin and 100 μg/mL streptomycin) in an incubator with 5% CO_2 at 37°C. Upon attainment of confluent cultures for experimental requirements, synoviocytes were treated with H_2O_2 (100 μM) for 24 h, followed by various doses of JS (24 h), as indicated for respective experiments.

2.3 | Antibodies and Reagents

The following antibodies were used in current study: ADAMTS-2 (sc-393 562), ADAMTS-8 (sc-514 717), ADAMTS-17 (sc-100 480), BAX (sc-7480), Cytochrome C (sc-13 560), GAPDH (sc-32 233), MMP13 (GTX100665), Bcl-2 (BD, #610539), and cleaved Caspase-3 (cst #9664). Chemicals/reagents included: MTT reagent (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide), (H_2O_2), monosodium iodoacetate (MIA), and lipopolysaccharide (LPS) was obtained from Sigma-Aldrich, TNF-α, and Bax activator-1 were from MedChemExpress, CM-H2DCFDA and ProLong Diamond Antifade Mountant with DAPI (Invitrogen), and PageRuler Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA). All other chemicals utilized were of the highest grade commercially available acquired from either Sigma-Aldrich or Invitrogen.

2.4 | Cell Viability Assay

The MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) assay was used to measure the cell viability. To determine the cell viability of HIG-82 synoviocytes using MTT assay, 1×10^3 cells/well were seeded in 96-well plates and cultured overnight at 37°C in a humidified 5% CO_2 incubator. The procedure was followed as described previously [16]. After various experimental treatments, HIG-82 cells were incubated with MTT (0.5 mg/mL) at 37°C for 4 h. The purple MTT formazan crystals that formed were solubilized in dimethyl sulfoxide (DMSO). The cell viability was estimated by measuring absorbance at 570 nm using an automated microplate reader, and expressed as the percentage relative to control (as 100%).

2.5 | Western Blot Analysis

The cell lysates were prepared and proteins were extracted using RIPA (radioimmunoprecipitation assay) lysis buffer (Thermo Fisher Scientific), added with protease and phosphatase inhibitors. Cell pellets were lysed for 30 min at 4°C, followed by centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant was collected and stored at −80°C for further use. The mitochondrial and cytosolic protein fractions were isolated using commercially available reagent (Mitochondria Isolation Kit, Thermo Fisher Scientific) as described previously [17]. To run the SDS-PAGE, equal amount (30 μg/well) of protein was loaded into each well and western blotting was executed. The western blot was performed using earlier reported procedures [18].

2.6 | H2DCFDA (2′,7′-Dichlorodihydrofluorescein Diacetate) Staining

The intracellular ROS generation in HIG 82 cells was determined using the cell-permeable cationic reagent CM-H2DCFDA (Thermo Fisher Scientific), according to the manufacturer's instructions. 1×10^5 cells per well were grown in a 24-well plate. At the end of treatments, cells were incubated with 10 μM H2DCFDA in the dark for 30 min at room temperature and rinsed once with PBS. ROS production was detected as described earlier [19] by measuring DCF fluorescence (as green) using a fluorescence microscope.

2.7 | Statistical Analysis

All experiments were performed in triplicates, and analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test, to conclude the significant differences between groups, using GraphPad Prism software (San Diego, CA, USA). $p < 0.05$ was considered as statistically significant.

3 | Results

3.1 | JS Treatment Protects Against the H_2O_2-Induced Decreased Cell Viability in HIG-82 Cells

The clinical manifestation of RA is largely driven by chronic inflammation and associated oxidative stress. H_2O_2 is known to exacerbate the production of ROS, trigger inflammation, and cause cell death [20]. To determine the optimal concentration of JS treatment for the cytoprotective effect in inflammation, we established an in vitro HIG-82 synoviocyte cell line model and examined the cytotoxicity of JS toward HIG-82 cells by exposing them to increasing concentrations (ranging from 38 to 1883 μg/mL) for a 24-h period. The results from the MTT assay revealed that JS treatment did not show any significant alterations in cell viability at concentrations below 188 μg/mL; however, a cytotoxic effect was observed at concentrations 188–1883 μg/mL tested (Figure 1A). Next, we assessed the protective effect of JS over H_2O_2-induced cellular stresses. Our results showed that H_2O_2 (100 μM) significantly decreased HIG-82 cell viability, which was reverted by JS treatment and exhibited a dose-dependent increase in cell viability against H_2O_2 alone-treated cells (Figure 1B). The above results implicate that JS exerts protective effects against H_2O_2-triggered diminished cell viability in HIG-82 cells.

3.2 | JS Suppresses ECM-Degrading Proteases and ROS Production Under H_2O_2-Elicited Inflammation in Synoviocytes

The occurrence of inflammation has a close link with the intracellular levels of ROS, while exposure to oxidative stress is associated with increased expression of extracellular matrix (ECM)-degrading enzymes [21]. Therefore, we examined the expressions of ECM-degrading proteases and levels of ROS upon H_2O_2 treatment in HIG-82 cells. Our results demonstrated that exposure to H_2O_2 increased the protein expressions of the ECM degrading enzymes MMP-13, ADAMTS-2, -8, and -17 in HIG-82 cells. Interestingly, posttreatment with JS opposed the H_2O_2-induced enhanced proteases, resulting in a significant decline of MMP-13, ADAMTS-2, -8, and -17 expressions in HIG-82 cells (Figure 2A,B). Not only that, as shown by H_2DCFDA staining, the ROS induction upon H_2O_2 treatment was also attenuated by JS in a dose-dependent manner (70 and 100 μg/mL) (Figure 2C,D). Together, the above results suggest that JS contributes to amelioration of inflammatory mediators and ROS generation in H_2O_2-treated synoviocytes.

3.3 | JS Inhibits Pro-Inflammatory Factors and Enhances Survival Markers in HIG-82 Cells Under Diverse Exogenous Inflammatory Stimuli

In continuation with the above results, we also tested the anti-inflammatory and cytoprotective efficacy of JS against various inflammatory agents, including H_2O_2 (100 μM), MIA (2 μM), LPS (10 μg/mL), and TNF-α (20 ng/mL) treatment for 24 h, respectively, in HIG-82 cells. The western blot results showed that the protein expressions of MMP-13, ADAMTS-2, Cytochrome C, and cleaved Caspase 3 were increased, whereas cell-protective aggrecan, Collagen II, and Bcl-2 were decreased under H_2O_2, MIA, LPS, and TNF-α treatment, respectively. Contrastingly, post-incubation with JS significantly antagonized the above effects. The expressions of aggrecan, Collagen II, and Bcl-2 were increased, whereas MMP-13, ADAMTS-2, Cytochrome C, and cleaved Caspase 3 were decreased by JS under H_2O_2, MIA, LPS, and TNF-α treatment, in HIG-82 cells (Figure 3A,B). The above results suggest that JS inhibits inflammation and improves survival in synoviocytes under diverse inflammatory conditions.

3.4 | JS Regulates Mitochondrial-Associated Apoptosis Markers Under H_2O_2-Induced Inflammation in HIG-82 Cells

As pathological inflammation and increased levels of ROS are accompanied by enhanced expressions of mitochondrial apoptosis markers, we interrogated the expressions of Bcl-2, Bax, Cytochrome C, and cleaved Caspase-3 in H_2O_2-treated HIG-82 cells in the presence or absence of JS. The results showed that mitochondria-associated apoptosis markers Bax, Cytochrome

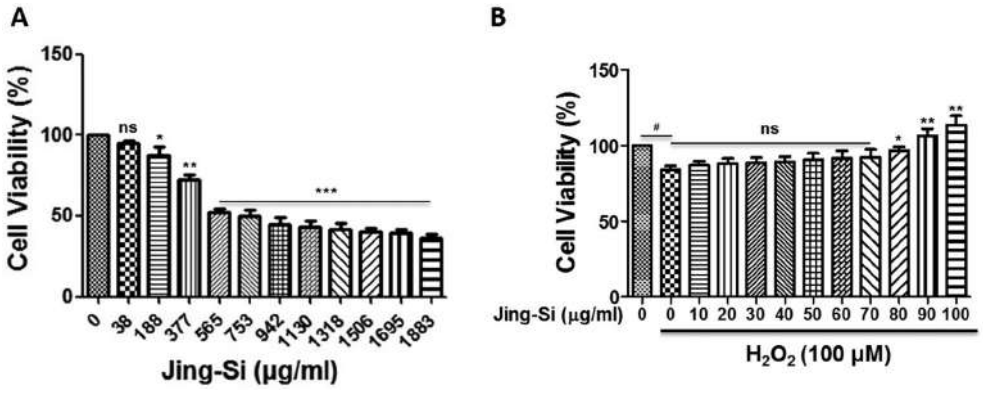

FIGURE 1 | Effect of JS treatment on cell viability in H_2O_2-stimulated HIG-82 cells. (A) MTT assay showing the dose-dependent effect of JS treatment (0–1883 μg/ml) for 24 h on the cell viability of HIG-82 cells. (B) Cells were pretreated with H_2O_2 (100 μM) for 24 h, followed by various concentrations of JS (0–100 μg/mL) for another 24 h. The viability of HIG-82 cells treated with different concentrations of JS and/or H_2O_2 was determined using MTT assay. (ns: not significant, $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

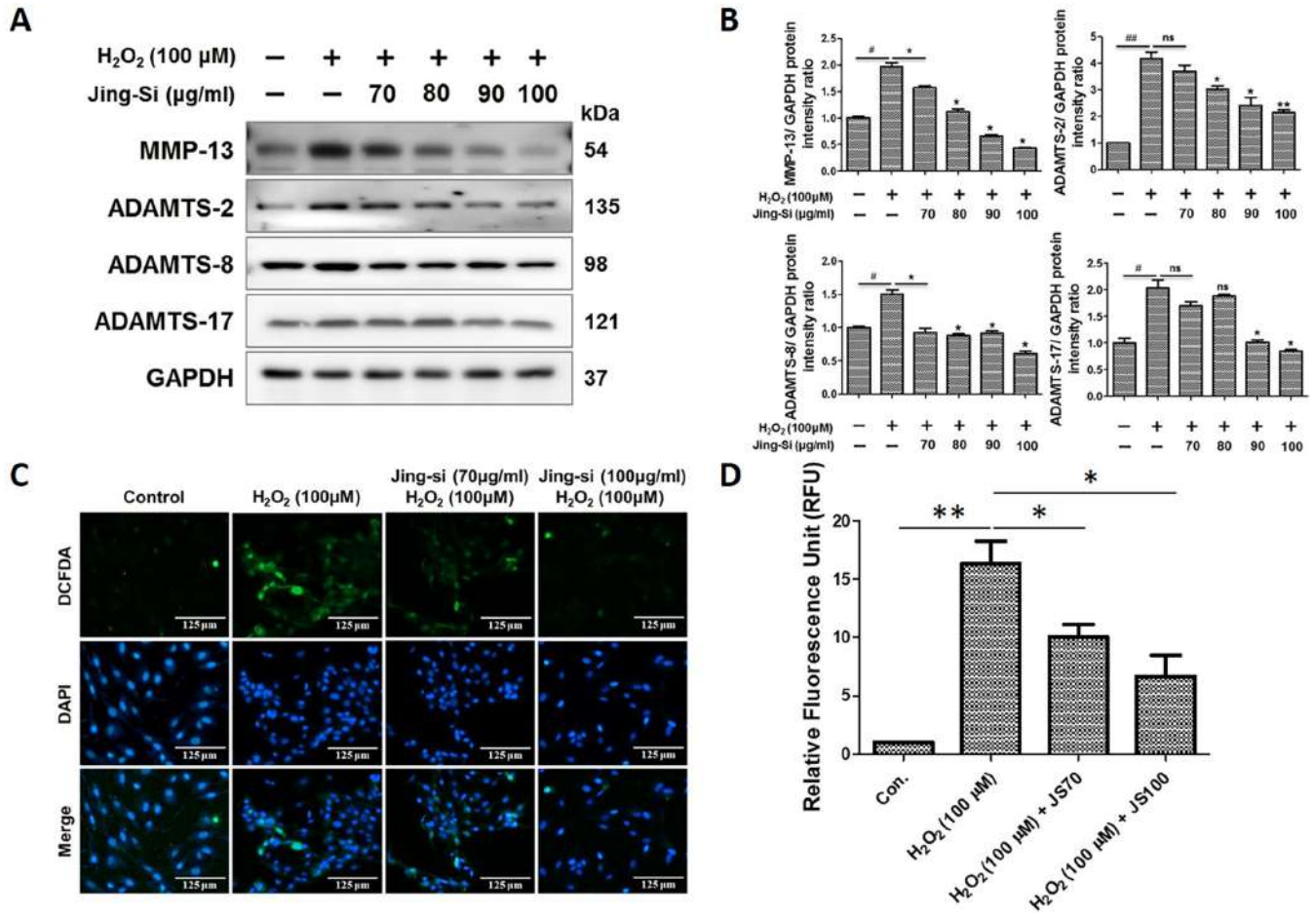

FIGURE 2 | Effect of JS treatment on H_2O_2-induced inflammation and ROS in HIG-82 cells. (A) Immunoblotting results showing the protein expression levels of MMP-13, ADAMTS-2, -8, and -17 in HIG-82 cells. Cells were pretreated with 100 μM H_2O_2 for 24 h, followed by treatment with JS (70–100 μg/mL) for another 24 h. (B) Quantification of protein expression levels using GAPDH as the internal control. The relative fold change in protein expression levels compared to controls has been depicted in adjacent graphs. (ns: not significant, $*p<0.05$, $**p<0.01$, $\#p<0.05$, and $\#\#p<0.01$). (C) DCFDA staining showing ROS release in HIG-82 cells pretreated with 100 μM H_2O_2 for 24 h, and then treated with JS (70 and 100 μg/mL) for another 24 h. (D) The relative fold change in DCF fluorescence (RFU) indicating ROS levels is presented in graphs. ($*p<0.05$, $**p<0.01$).

C, and cleaved Caspase-3 were upregulated, whereas survival factor Bcl-2 was downregulated in H_2O_2-treated HIG-82 cells. However, posttreatment with JS displayed a cytoprotective effect by increasing the expression of Bcl-2 and diminishing the levels of Bax, Cytochrome C, and cleaved Caspase-3 in a concentration-dependent manner in HIG-82 cells under H_2O_2 treatment (Figure 4A,B). This result was further corroborated by the TUNEL assay, which showed that H_2O_2 exposure led to a significant cell death, which was conspicuously reverted by JS (Figure 4C,D). The above results suggest that JS regulates inflammation-associated mitochondria arbitrated apoptosis markers in H_2O_2-treated HIG-82 cells.

3.5 | JS Rescues Inflammation-Associated Intrinsic Apoptosis in HIG-82 Synovial Cells by Regulating Mitochondrial Localization of Bax and Cytochrome C Release

To further investigate the effect of JS treatment over H_2O_2-induced inflammation and mitochondrial-mediated apoptosis in HIG-82 cells, the mitochondrial and cytosolic protein fractions were isolated after treatment with H_2O_2 (150 μM) for 24 h and then, JS treatment (70 and 100 μg/mL) for another 24 h. The results showed that Bax was accumulated in mitochondrial protein fractions, whereas Cytochrome C was enriched in the cytoplasm of H_2O_2-treated HIG-82 cells. Remarkably, treatment with JS displayed a cytoprotective effect by diminishing the mitochondrial localization of Bax and reducing the mitochondrial leakage of Cytochrome C into the cytoplasm of H_2O_2-treated HIG-82 cells (Figure 5A,B). The role of Bax and Cytochrome C leakage from mitochondria in JS-mediated protective effect was further confirmed by using Bax activator. The results showed that posttreatment with Bax activator prominently antagonized the JS-elicited protective effects, resulting in enhanced mitochondrial Bax expression as well as increased Cytochrome C release in the cytoplasm of H_2O_2-treated HIG-82 cells (Figure 5C,D). Together, the above results suggest that JS treatment plays a protective role against H_2O_2-triggered inflammation and associated intrinsic apoptosis by regulating mitochondrial Bax localization and the release of Cytochrome C in the cytoplasm of HIG-82 synoviocytes.

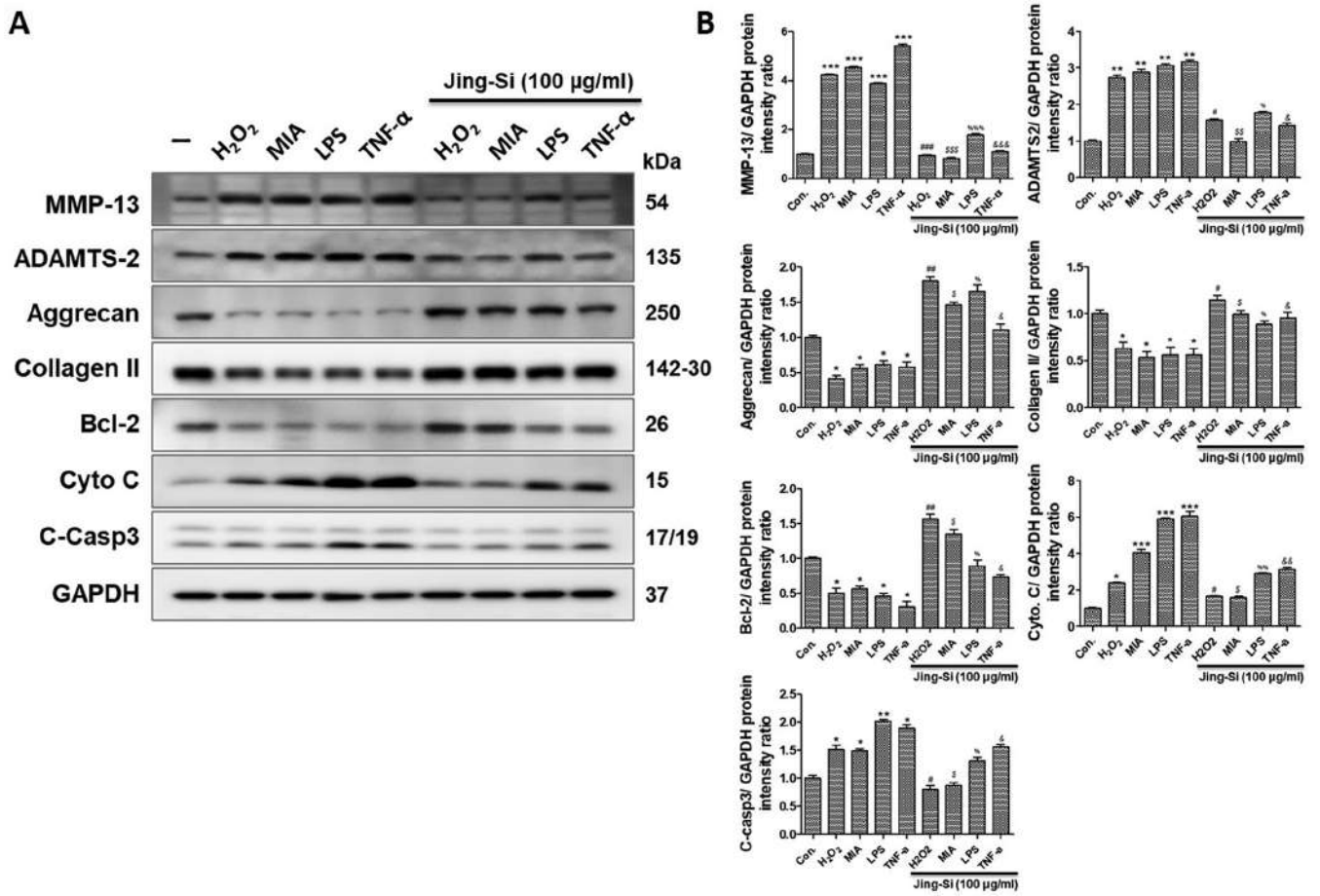

FIGURE 3 | Effect of JS treatment on ECM degrading proteases and survival markers under diverse exogenous inflammatory stimuli. (A) Immunoblotting showing the protein expression levels of MMP-13, ADAMTS-2, -8, -17, Bcl-2, Cytochrome C, and cleaved Caspase-3 in HIG-82 cells. HIG-82 cells were pretreated with H_2O_2 (100 μM), MIA (2 μM), LPS (1 μg/mL), and TNF-α (10 ng/mL) for 24 h, respectively. Next, the cells were treated with JS (100 μg/mL) for another 24 h. (B) The graphs represent the quantification of protein expression relative to GAPDH as the internal control (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$; %$p < 0.05$, %%$p < 0.01$, and %%%$p < 0.001$; \$$p < 0.05$, \$\$$p < 0.01$, and \$\$\$$p < 0.001$; &$p < 0.05$, &&$p < 0.01$, and &&&$p < 0.001$).

4 | Discussion

The advent of pathological inflammation accompanied by oxidative stress is one of the key features of autoimmune inflammatory illnesses such as RA, for which an effective treatment has not yet been found. Therefore, in this study, the cytoprotective effects of JS treatment against H_2O_2-induced inflammation and apoptosis in HIG-82 synovial cells were investigated. To set up an in vitro inflammation model, H_2O_2 was utilized, which is a well-known stimulus to induce inflammation and oxidative stress in various cells [22]. Besides, we also determined the optimum concentration of JS and H_2O_2 treatment to avoid any cytotoxic effects on HIG-82 synoviocytes. We tested a range of JS treatments (0–1883 μg/mL) and found that JS did not exert any significant changes in cell viability below 188 μg/mL; however, cytotoxicity and a decrease in cell viability were observed at 188–1883 μg/mL concentration. Based on our previous studies [23, 24] and current results, a lower dose (70–100 μg/mL) was selected for further experimental treatments. H_2O_2 at 100 μM was used as a stimulus for the induction of inflammation based on our previous studies [25]. The MTT results showed that treatment of HIG-82 cells with H_2O_2 leads to a significant decline in cell viability. Interestingly, posttreatment with JS not only improved the cell viability but significantly enhanced HIG-82 cell viability under H_2O_2 treatment. Next, to ascertain the role of JS in inflammatory conditions, we determined the expressions of inflammation-associated markers MMP-13, ADAMTS-2, -8, and -17 in H_2O_2-treated HIG-82 cells. As shown by western blot, the protein expressions of ECM-degrading proteases MMP-13, ADAMTS-2, -8, and -17 were markedly elevated in H_2O_2-treated HIG-82 cells. However, treatment with JS significantly reverted those expressions in a dose-dependent manner (70–100 μg/mL). In addition, DCFDA staining showed that H_2O_2-induced intracellular ROS was also abated by treatment with JS. These observations were suggestive of an anti-inflammatory and antioxidant effect of JS in H_2O_2-treated HIG-82 synoviocytes. These results corroborated the previous studies that showed a protective role of JS treatment against pathological inflammation and oxidative stresses in various cell types [23, 24]. Further, we tested the protective efficacy of JS against multiple inflammation inducing agents (H_2O_2, MIA, LPS, and TNF-α) in HIG-82 cells. Treatment with MIA, LPS, TNF-α, and H_2O_2 resulted in increased expressions of

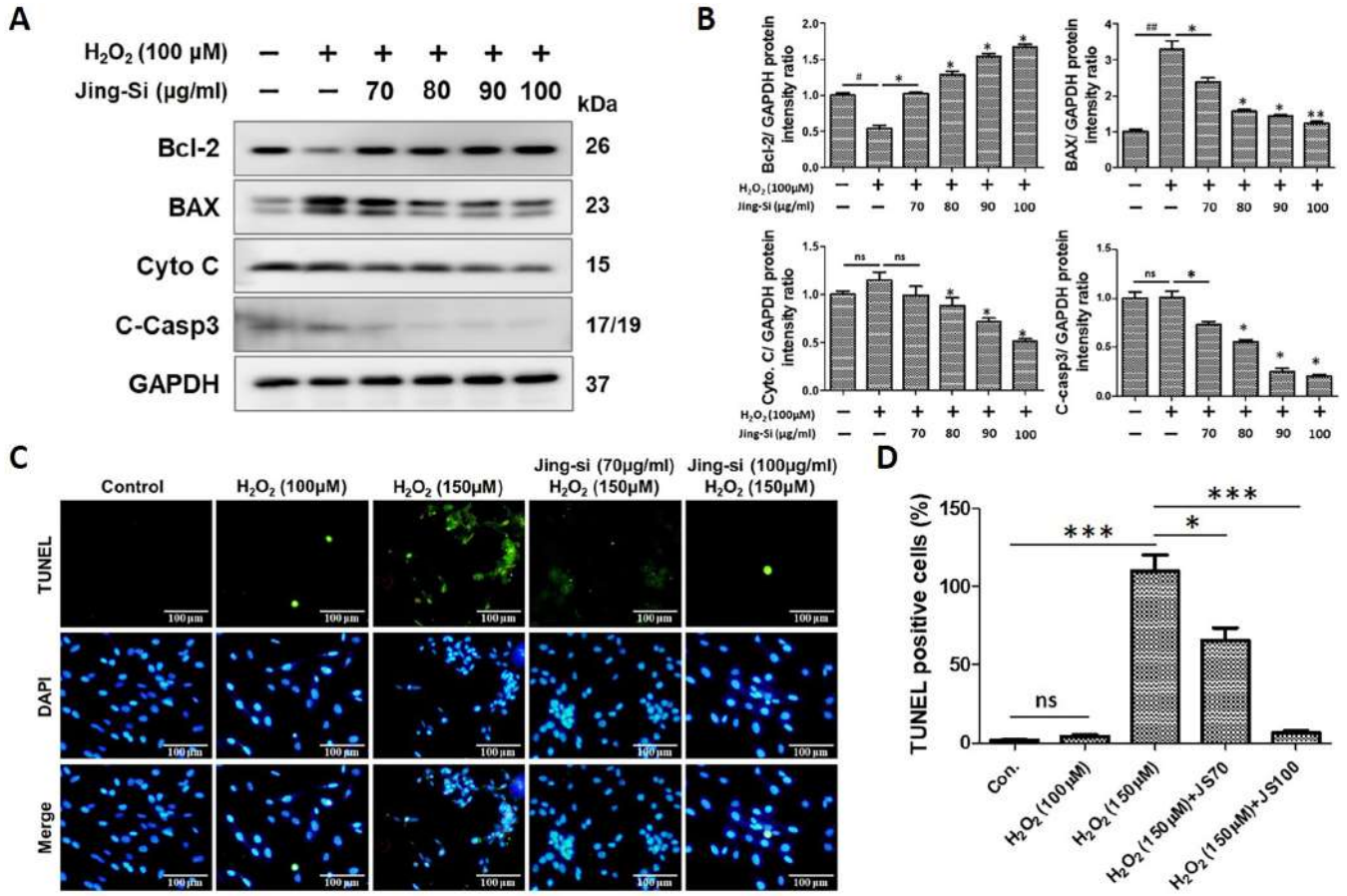

FIGURE 4 | JS treatment decreases mitochondrial-associated apoptosis markers under H_2O_2-induced inflammation in HIG-82 cells. (A) Western blotting displaying the protein expression levels of Bcl-2, Bax, Cytochrome C, and cleaved Caspase-3 in HIG-82 cells. HIG-82 cells were pretreated with H_2O_2 (100 μM) for 24 h, followed by treatment with JS (70–100 μg/mL) for another 24 h. (B) Quantification of protein expressions relative to GAPDH is shown in graphs (ns: not significant, $*p<0.05$, $**p<0.01$; $\#p<0.05$, $\#\#p<0.01$). (C) TUNEL assay showing the apoptosis in HIG-82 cells. The cells were pretreated with H_2O_2 (100 or 150 μM) for 24 h, followed by treatment with JS (70 or 100 μg/mL) for another 24 h. (D) The graph represents percentage of apoptotic TUNEL-positive cells (green) (nuclei: blue) as compared to controls (ns: not significant, $*p<0.05$, $***p<0.001$).

MMP-13, ADAMTS-2, Cytochrome C, and cleaved Caspase 3, whereas, aggrecan, Collagen II and Bcl-2 were decreased, in HIG-82 cells. However, above effects were conspicuously reversed by JS treatment. Moreover, there was a dose-dependent (70–100 μg/mL of JS) suppression of Bax, Cytochrome C, and cleaved Caspase 3, and increased expression of Bcl-2 under H_2O_2 treatment in HIG-82 cells. The results confirmed that posttreatment with JS significantly repressed the inflammation associated apoptosis induction by H_2O_2 in HIG-82 synoviocytes. Furthermore, the results from subcellular protein fractionation showed that Bax localization was enhanced in mitochondrial protein portions, whereas the cytoplasm of H_2O_2-treated HIG-82 cells contained higher Cytochrome C expression. Remarkably, both the mitochondrial localization of Bax and the Cytochrome C release into cytoplasm were decreased by JS in H_2O_2-treated HIG-82 cells.

Both extrinsic (receptor-mediated) and intrinsic (mitochondria-mediated) apoptotic mechanisms are implicated in cell death processes [26]. Usually, H_2O_2-induced oxidative stress sets off the mitochondria-mediated apoptotic cascade [27]. In current investigation, we found that in H_2O_2-incited HIG-82 cells, the expression of key apoptotic markers linked to the mitochondria-mediated apoptotic pathway was upregulated, whereas survival factors were downregulated. These results were consistent with our hypothesis, and interestingly, these patterns were reversed by JS treatment in HIG-82 synoviocytes. The inflammation and ROS, both are interconnected phenomenon and work as a double-edged sword in cell protection and apoptosis. The inflammatory and apoptotic processes in RA have been widely investigated in H_2O_2-triggered RA fibroblast-like human synovial cell models, which implicated the involvement of MAPK and NF-κB pathways [28]. In this study, H_2O_2 exposure triggered inflammation and apoptosis in HIG-82 synoviocytes, which are hallmarks of RA. The results exhibited an amplified level of proteases linked to cartilage degradation, a common manifestation of RA. The results displayed that JS treatment, not only inhibits inflammation but also apoptosis induced by H_2O_2 in HIG-82 cells. JS treatment led to the downregulation of several inflammation associated cartilage degrading proteases, including MMP 13, ADAMTS-2, ADAMTS-8, and ADAMTS-17, as well as the crucial proapoptotic proteins Bax, Cytochrome C, and cleaved Caspase-3. Previous studies have suggested that NLRP3 inflammasome, which is a multiprotein complex, plays a critical role in modulating inflammatory signaling and the immune system. The activation of NLRP3

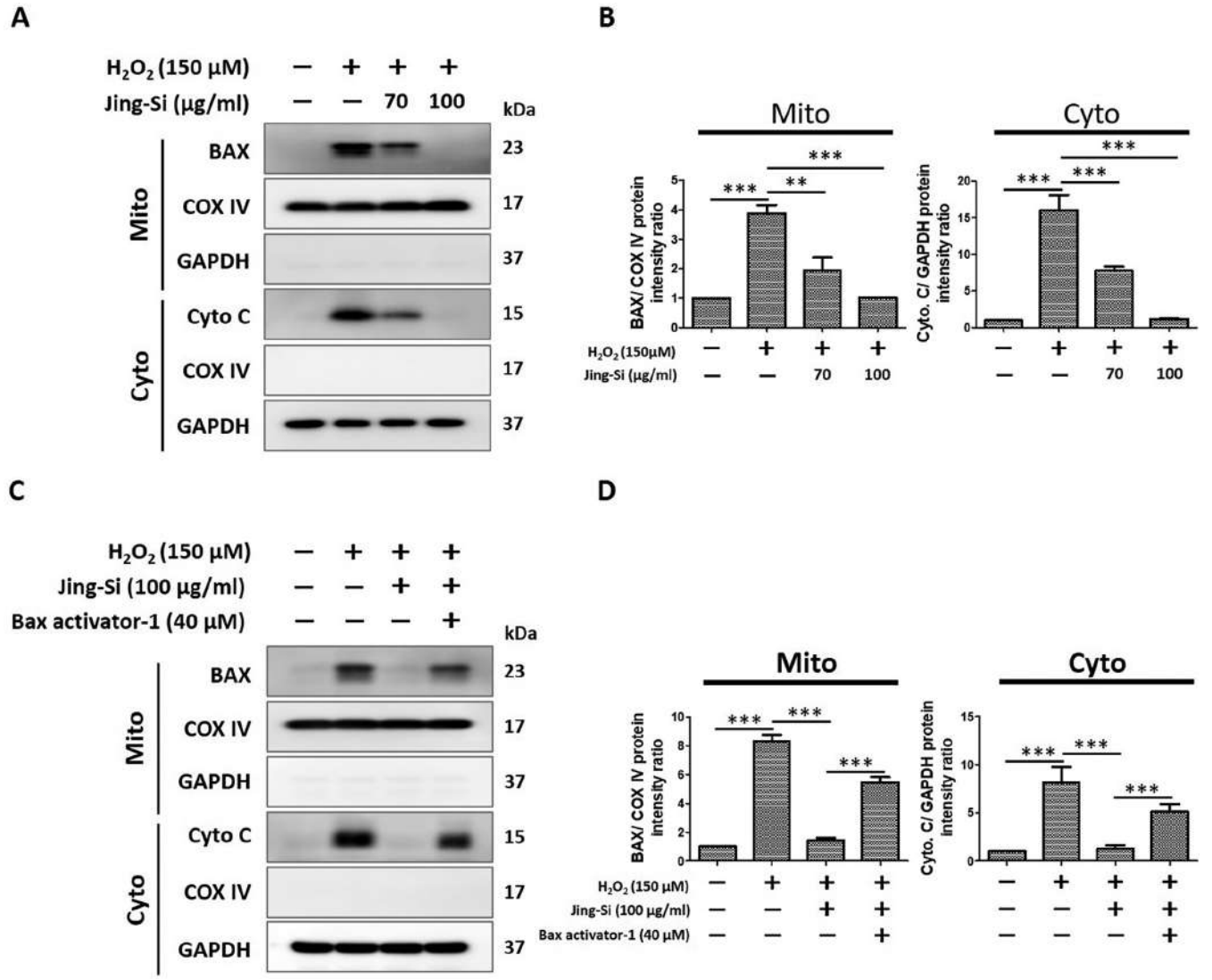

FIGURE 5 | JS rescues inflammation-associated intrinsic apoptosis in HIG-82 synovial cells by regulating mitochondrial localization of Bax and Cytochrome C release. (A) Western blotting showing the expression of mitochondrial apoptosis markers Bax and Cytochrome C in HIG-82 cells. The cells were pretreated with H_2O_2 (150 μM) for 24 h, followed by treatment with JS (70 or 100 μg/mL) for another 24 h. The mitochondrial and cytosolic protein fractions were extracted and subjected to western blotting examination. (B) The graph represents quantification of Bax (relative to COX IV) in mitochondrial protein fraction and Cytochrome C (relative to GAPDH) in cytosolic protein fraction. (**$p < 0.01$, ***$p < 0.001$). (C) HIG-82 cells were pretreated with H_2O_2 (150 μM) for 24 h, followed by incubation with JS (70 or 100 μg/mL) for 24 h. Next, the cells were rinsed with PBS and incubated with Bax activator-1 (40 μM) for 24 h. The cytosolic and mitochondrial protein fractions were separated and western blotting was performed. (D) The quantification of Bax in mitochondrial protein fraction, while Cytochrome C in cytosolic protein fraction is presented in adjacent graph. (***p < 0.001).

inflammasome triggers the release of pro-inflammatory cytokines IL-1β and IL-18, finally culminating into pyroptosis [29].

Rheumatic diseases resulting from the unnecessary activation of inflammation is mediated via NLRP3 inflammasome which promotes the induction of pro-inflammatory cytokines and ROS [30]. The ROS production incited by activation of the NLRP3 inflammasome leads to further enhancement of inflammatory processes and a vicious cycle of ROS and inflammation continues which keeps aggravating the affected tissue damage [1, 31]. This study showed that, following JS treatment, H_2O_2 instigated inflammatory response was markedly diminished, signifying that the anti-inflammatory mechanism of JS chiefly involves the suppression of inflammation via inhibition of NLRP3 inflammasome [23]. The destruction of the ECM is strongly affected by MMPs. In the milieu of RA, MMPs produced from synovial fibroblasts contribute to cartilage obliteration in the affected joints [32]. In this study, MMP-13 expression was increased in H_2O_2-instigated HIG-82 cells, but this effect was alleviated subsequent to JS treatment. Higher levels of MMP-13 are known to aggravate the tissue destruction in RA [32]. MMPs and ADAMTSs are well recognized to play substantial roles in breakdown of ECM type II collagen and aggrecan, and are thus potential targets for development of new arthritis therapies [33]. In patients with RA, increased expression of ADAMTS is reported to enhance apoptosis of synovial cells [33, 34]. In current study, there was an increased expression of ADAMTS-17, ADAMTS-2, and ADAMTS-8 in H_2O_2-stimulated

HIG-82 synoviocytes. Interestingly, in line with previous results, their expressions were ameliorated following JS treatment. Cellular apoptotic pathways could be triggered by both mitochondria-mediated as well as receptor-mediated signaling mechanisms [35]. In this study, the key apoptotic indicators linked with the mitochondria-mediated apoptotic pathway, including Bax, Cytochrome C, and cleaved Caspase-3 were prominently enhanced, whereas survival marker Bcl-2 was decreased in H_2O_2-stimulated HIG-82 cells. These findings were in concordance with our previous results, and notably, these effects were reverted upon JS treatment. Our findings determined that JS suppresses the H_2O_2-induced inflammation and associated apoptosis, as well as inhibits the proteases involved in matrix degradation in the pathogenesis of RA (Figure 6). Nevertheless, we justify for further investigations to corroborate the above findings and to find the deeper associations between JS elicited mechanisms in inflammatory diseases.

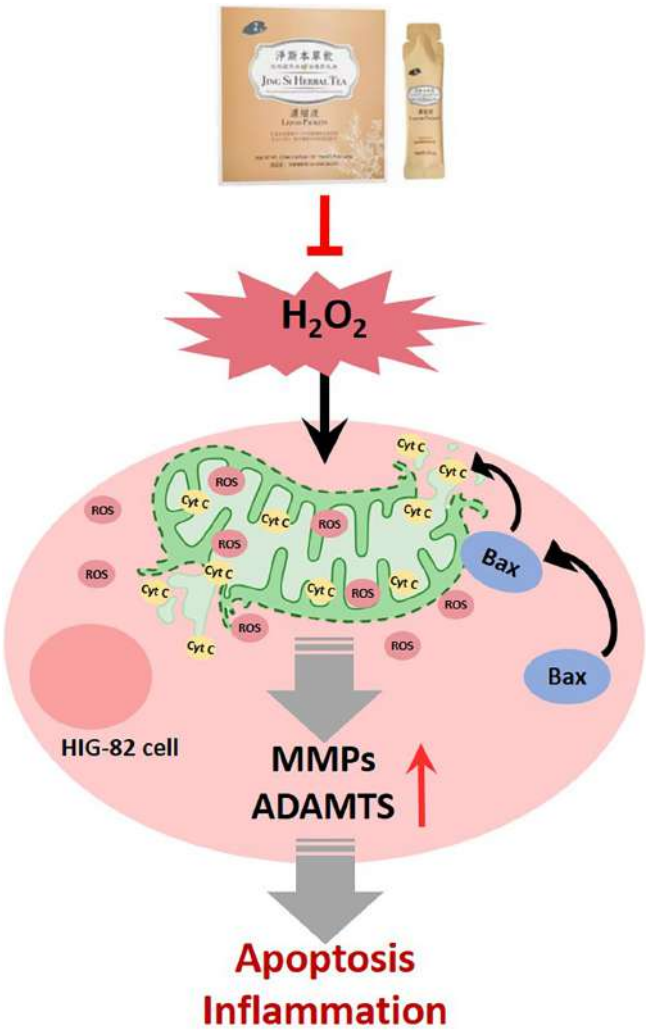

FIGURE 6 | Graphic summary. This work highlights JS's remarkable capacity to effectively reverse inflammation and apoptosis in H_2O_2-stimulated HIG-82 synoviocytes. JS treatment suppressed inflammation and apoptosis through decreased production of enzymes linked to the destruction of the ECM (MMPs and ADAMTs) and reduced ROS, mitochondrial localization of Bax, and Cytochrome C release. These results corroborate JS's therapeutic potential in ameliorating H_2O_2-triggered synovial cell inflammation and apoptosis. ADAMTS, A Disintegrin-like and Metalloproteinases with Thrombospondin Motifs; ECM, Extracellular matrix; MMP, matrix metallopeptidase; ROS, reactive oxygen species.

5 | Conclusion

This study reveals the noteworthy ability of JS to efficiently reverse inflammation and apoptosis in H_2O_2- stimulated HIG-82 synoviocytes. H_2O_2-triggered inflammation leads to ECM destruction, whereas JS administration represses cellular inflammation and apoptosis induced by H_2O_2 exposure in HIG-82 cells. This was accomplished by the reduced expression of enzymes associated with ECM degradation and increased expression of survival markers by JS treatment. These findings accentuate the therapeutic potential of JS in attenuating H_2O_2-triggered inflammation and apoptosis in synovial cells.

Author Contributions

Tsung-Jung Ho and **Chih-Yang Huang:** conceptualization. **Shih-Wen Kao**, **Yu-Chun Chang**, and **Feng-Huei Lin:** performed experiments. **Tai-Lung Huang** and **Tung-Sheng Chen:** analysis and validation, **Wei-Wen Kuo** and **Chih-Yang Huang:** funding acquisition and resources. **Shinn-Zong Lin**, **Kuan-Ho Lin**, **Wei-Wen Kuo**, **Tsung-Jung Ho**, and **Chih-Yang Huang:** interpretation, drafting, and revision of the manuscript. All authors have read and authorized the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data supporting the findings of current study are available within the manuscript and would be available from the corresponding author upon reasonable request.

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