doi.org/10.36721/PJPS.2024.37.1.REG.033-041.1

Salvianolate ameliorates inflammation and oxidative stress in high-glucose induced HK-2 cells by blocking TGF-β/Smad signal pathway

Qianyu Fu, Longhua Zhou, Yuqin Fan, Fenfen Liu, Yuanyuan Fan, Xin Zhang, Li Wang and Lan Cheng*
Jiangxi Provicial People's Hospital, The First Affiliated Hospital of Nanchang Medical College

Abstract: The objective of this research is to assess how salvianolate impacts inflammation and oxidative stress in a laboratory setting, as well as to investigate the underlying mechanisms. HK-2 cells were subjected to different treatments, including normal glucose, mannitol, high glucose and high glucose plus salvianolate. Cell proliferation, death, MDA levels, IL-1β, IL-6, TNF-α, MCP-1 concentrations, ROS levels, MMP, MPTP, and ATP levels were assessed using various kits. The protein expressions of NOX4, TGF-β1, P-Smad2, P-Smad3, Smad4 and Smad7 were ascertained through western blot analysis. Our results indicated salvianolate could reduce the release of IL-1β, IL-6, TNF-α, as well as MCP-1, alleviate the levels of oxidative stress markers NOX4 and MDA, and improve mitochondrial function by increasing MMP and ATP levels while reducing ROS and MPTP opening. Furthermore, salvianolate inhibited the TGF-β1/Smad2, Smad3 signaling pathway, suppressed Smad4 expression, and increased Smad7 expression. Salvianolate seems to mitigate inflammation and oxidative stress through a variety of mechanisms. These discoveries offer valuable understanding into the possible mechanisms by which salvianolate may be employed in the treatment of diabetic nephropathy.

Keywords: Salvianolate, inflammation, oxidative stress, HK-2 cell, TGF-β/Smad signal pathway.

INTRODUCTION

Chronic kidney disease (CKD) poses a significant public health concern among individuals diagnosed with type 2 diabetes mellitus (T2DM). The elevated occurrence of CKD has been brought about by the high prevalence of T2DM. According to the data released by Liu *et al*. (2010), the prevalence of diabetic kidney disease (DKD) in China is 10.7%, which has emerged as a significant factor adding to the disease burden in China. DKD typically begins and advances with inflammation and oxidative stress, triggering harm to renal cells and consequent deterioration in function (Rapa *et al.*, 2019). The current treatment for DKD includes lifestyle modifications, controlling hypertension and diabetes and reducing proteinuria and inflammation. Advanced-stage patients can also consider options such as hemodialysis and kidney transplantation. However, these measures cannot reverse the loss of kidney function (Breyer and Susztak, 2016). Certain medications may cause adverse reactions, such as hypotension and electrolyte imbalances. Hemodialysis and kidney transplantation are expensive and may be difficult to access in certain regions.

Derived from the roots of the Salvia miltiorrhiza plant, salvianolate boasts a lengthy history of traditional medicinal usage and is renowned for its favorable safety record and well-tolerated nature. It is widely recognized for its cardiovascular protective effects and its ability to improve symptoms of ischemic stroke (Luan *et al.*, 2020; Ren *et al.*, 2019). Due to the fact that patients suffering from DKD frequently face an elevated risk of cardiovascular diseases and ischemic strokes, there are currently limited medications available for simultaneously treating the diabetes-related complications of the heart, brain and kidneys. Therefore, there is interest in whether salvianolate has similar effects of cardiovascular and cerebrovascular protection in DKD. Researches on the treatment of DKD with salvianolate is still in its early stages and has certain limitations and controversies. The size and quality of the study samples are limited. Furthermore, there are variations in the results among different studies. Further research is necessary to validate findings, gain mechanistic insights, understand clinical implications and develop targeted therapeutic interventions.

Conventional inflammatory indicators like IL-1β, IL-6, as well as TNF-α hold significant roles in the inflammatory response and have undergone extensive research. However, our research introduces a new perspective and innovation by examining the expression of MCP-1. MCP-1 performs a unique function in the context of the inflammatory response and kidney damage. The changes in its expression levels may serve as important indicators for assessing the severity of diabetic nephropathy and predicting patient prognosis (Tesch, 2008). In recent studies, Ma *et al.* (2021), Du *et al.* (2021) and Liu *et al.* (2022) have all identified the decrease in MCP-1 levels in kidney tissue or cells as one of the indicators of improvement in the inflammatory response of diabetic kidney disease. By evaluating the levels of MCP-1. Our

*Corresponding author: e-mail: 471907791@qq.com

research offers a more encompassing evaluation of inflammation's role in diabetic nephropathy. The notion of "tubulocentric" early diabetic nephropathy underscores the pivotal role of renal tubules in the onset as well as advancement of diabetic kidney disease, encompassing inflammation and oxidative stress within these tubules (Vallon and Komers, 2011). HK-2 cells exhibit characteristics and functions associated with renal tubules. If our study confirms that salvianolate inhibits inflammation and oxidative stress in HK-2 cells in vitro, It will provide a theoretical basis for the therapeutic indication of salvianolate in diabetic nephropathy and facilitate further animal and clinical trials.

To substantiate our hypothesis, cell proliferation and death were evaluated using the cell proliferation ELISA, BrdU kit, and LDH cytotoxicity assay kit. The measurement of MDA levels was conducted with the MDA assay kit for lipid peroxidation, while the concentrations of IL-1β, IL-6, TNF-α, as well as MCP-1 were quantified through the utilization of their corresponding ELISA kits. ROS levels were determined utilizing the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate in conjunction with the ROS assay kit. MMP was evaluated using the fluorescent probe JC-1 in conjunction with the MMP assay kit. MPTP was detected employing the MPTP fluorescence assay kit. ATP levels were gauged with the ATP assay kit. The protein expressions of NOX4, TGF-β1, P-Smad2, P-Smad3, Smad4 and Smad7 were ascertained through western blot analysis.

MATERIALS AND METHODS

Cell culture and treatment

HK-2 cells (ATCC, Manassas, USA, catalog#: HTX2165) was inoculated into 96-well tissue culture plates and cultured in K-SFM (Sigma-Aldrich, St. Louis, USA, catalog#: 131-500A) which was supplemented with 10% FBS (Gibco, Carlsbad, USA, catalog#: 10099141C), 100 U/mL penicillin and 100U/mL streptomycin (Gibco, Carlsbad, USA, catalog#: 15140122). The cells were maintained in a humidified chamber at 37°C with 5% CO2. Cell quantification in each well was performed using a hemacytometer (Reichert, Buffalo, NY).

To establish the experimental setup, cells were allowed to reach a confluency of 70% to 80%. Subsequently, they were subjected to a 24-hour incubation period in serum-free medium, aiming to arrest and synchronize cell growth. A medium with a glucose (Sigma-Aldrich, St. Louis, USA, catalog#: G8270) concentration of 5.5mM was utilized as the control group referred to as normal-glucose (NG). As an osmotic control, the medium was composed of 5.5mM glucose along with 24.5mM mannitol (Sigma-Aldrich, St. Louis, USA, catalog#: M9546), referred to as the osmotic (MA) control. Finally, a medium containing 30mM glucose was employed to represent the high glucose (HG) group (Li *et al.*, 2022).

Cell proliferation assay and cytotoxicity assay

Cells were pretreated with different concentrations of salvianolate (Shanghai Green Valley Pharmaceutical Co., Ltd., Shanghai, China, catalog#: 16110121), including 0uM, 0.1uM, 1.0uM, 10uM, 50uM, and 100uM, for 24 hours followed by stimulated with 30mM glucose for 48 hours. The normal control group comprised cells stimulated with 5.5mM glucose, whereas the osmotic control group consisted of cells stimulated with 24.5mM mannitol in addition to 5.5mM glucose. Then cell proliferation assay was performed. Cells were exposed to different concentrations of salvianolate (0uM, 0.1uM, 1.0uM, 10uM, 50uM and 100uM) for a duration of 24 hours prior to conducting cell cytotoxicity analysis. The in vitro assessment of HK-2 cell proliferation following treatment with salvianolate utilized the Cell Proliferation ELISA, BrdU kit (Roche, Basel, Switzerland, catalog#: 11647229001) as per the manufacturer's guidelines. The measurement of light emissions was conducted using a Multi-Mode Micro plate Reader (Epoch 2; BioTek, Vermont, USA) equipped with a 450nm absorbance filter to determine BrdU incorporation. The impact of salvianolate on HK-2 cell cytotoxicity was evaluated with the LDH Cytotoxicity Assay Kit (Roche, Basel, Switzerland, catalog#: 11644793001) in accordance with the manufacturer's guidelines and absorbance measurements were conducted at 490 nm using the Multi-Mode Microplate Reader. These experiments were performed in triplicate and reiterated in three distinct test runs (Jiang *et al.*, 2021).

Quantification of the IL-1β, IL-6, TNF-αand MCP-1

HK-2 cells were subjected to a 24-hour treatment regimen, either with or without 10μM salvianolate, followed by a subsequent 48-hour exposure to 30mM glucose stimulation. Thereafter, the supernatant was collected to measure the levels of IL-1β (Beyotime, Nanjing, China, catalog#: PI305), IL-6 (Beyotime, Nanjing, China, catalog#: PI330), TNF-α (Beyotime, Nanjing, China, catalog#: PT518) and MCP-1 (Abcam, Cambridge, UK, catalog#: ab203359) using their respective ELISA kits in compliance with the manufacturer's provided directions. The absorbance wavelength used for detecting IL-1β, IL-6, TNF-α and MCP-1 is 450 nm using a Multi-Mode Microplate Reader and their concentrations were calculated according to the standard curve (Yun *et al.*, 2022).

Measurement of MDA

Cell treatment same as above. MDA activity in cell supernatants was conducted with the MDA assay kit for lipid peroxidation (Beyotime, Nanjing, China, catalog#: S0131S) according to instructions from manufacturer. Use a Multi-Mode Microplate Reader to measure the

absorbance at a wavelength of 532 nm and calculate the concentration of MDA based on the standard curve (Lu *et al.*, 2021).

ROS, MMP, MPTP and ATP levels analysis

ROS levels were determined utilizing the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate in conjunction with the ROS assay kit. (Beyotime, Nanjing, China, catalog#: S0033S) following the manufacturer's instructions. Fluorescence intensity was evaluated using a Multi-Mode Micro plate Reader. The excitation and emission wavelengths were set at 485 nm and 528 nm, respectively. ROS levels were determined based on fluorescent intensity normalized per nanogram of protein. MMP was evaluated using the fluorescent probe JC-1 in conjunction with the MMP assay kit (Solarbio, Beijing, China, catalog#: M8650). In a nutshell, isolated mitochondria were combined with the JC-1 staining working solution. Following thorough mixing, a Multi-Mode Micro plate Reader was employed to measure the fluorescence intensity of both mitochondrial JC-1 monomers (green fluorescence; excitation wavelength 490 nm, emission wavelength 530nm) and aggregates (red fluorescence; excitation wavelength 525nm, emission wavelength 590nm). MMP was computed by determining the fluorescence ratio of red to green per milligram of protein. MPTP opening was detected using the MPTP Fluorescence Assay Kit (Beyotime, Nanjing, China, catalog#: C2009S) following the provided instructions. Using a Multi-Mode Microplate Reader, fluorescence intensity was measured and the excitation and emission wavelengths were configured at 494 and 517nm, respectively. MPTP was calculated based on relative fluorescence intensity per milligram of protein. ATP levels were determined with the ATP assay kit (Abcam, Cambridge, UK, catalog#: ab83355), following the manufacturer's protocols. Fluorescence intensity measurements were conducted utilizing a Multi-Mode Micro plate Reader, employing an excitation wavelength of 535 nm and an emission wavelength of 587 nm. Finally, ATP levels were computed based on a standard curve. All data were presented as fold changes compared to the control group (Li *et al.*, 2020).

Western blotting

The cells were collected after treatment and RIPA cell lysate (Solarbio, Beijing, China, catalog#: R0010) was added. The protein concentration was detected by a BCA protein detection kit (Solarbio, Beijing, China, catalog#: PC0020). The protein sample was heated and denatured in a boiling water bath (HI1210, Leica, Baden-Württemberg, Germany). Then, prepare PAGE gel as follow: the lower separating gel was prepared using a 12% gel, while the upper stacking gel was prepared using a 5% gel. Place the prepared PAGE gel into the electrophoresis tank (Mini protein 3 cell, BIO-RAD, California, USA) and add an appropriate amount of electrophoresis buffer (Glycine 14.4g, Tris base 3.0g, Methanol 200ml, and make up to 1L with ddH2O). The denatured protein sample of 20μg was added to the loading hole for PAGE gel electrophoresis. The stacking gel was run at 80V for 40 minutes, followed by the separating gel at 120V for 50 minutes. After the protein was separated, it was transferred wet to the PVDF membrane (Millipore, Darmstadt, Germany, catalog#: IPVH00010) for electro transfer at 90V for 50minutes. The transformed membrane was blocked with 5% skimmed milk overnight at room temperature. The primary antibodies including TGF-β1 (Bioswam, Shanghai, China, catalog#: PAB39276); p-Smad2 (Abcam, Cambridge, UK, catalog#: ab188334), Smad2 (Bioswam, Shanghai, China, catalog#: PAB35421), p-Smad3(Abcam, Cambridge, UK, catalog#: ab52903), Smad3 (Bioswam, Shanghai, China, catalog#: PAB44700), Smad4 (Bioswam, Shanghai, China, catalog#: PAB43811), Smad7 (Bioswam, Shanghai, China, catalog#: PAB40077) ; NOX4 (Bioswam, Shanghai, China, catalog#: PAB30655) and GAPDH (Bioswam, Shanghai, China, catalog#: PAB36269) were diluted (1:1000) and incubated for 1 hour at room temperature, and then the HRP-conjugated corresponding secondary antibody (Bioswam, Shanghai, China, catalog#: SAB43714) was diluted (1:20000) and incubated for 1hour at room temperature. The immunoreactive proteins were visualized with the ECL kit (Millipore, Darmstadt, Germany, catalog#: WBKLS0500) and the band densities were quantified through TANON GIS software (Tanon, Shanghai, China) using a fully automated chemiluminescence analyzer (Tanon-5200, Tanon, Shanghai, China), Analysis on each protein sample was repeated 3 times (Li *et al.*, 2022).

STATISTICAL ANALYSIS

Quantitative data were presented as the mean ± standard deviation (SD). Statistical analysis was conducted using SPSS 26.0 software for Windows (SPSS Inc., Chicago, USA). Group differences were assessed through one-way ANOVA, with statistical significance denoted by a P-value of less than 0.05.

RESULTS

Normal-glucose (NG) medium contained a glucose concentration of 5.5 mM as the negative control, mannitol (MA) medium consisted of 5.5mM glucose plus 24.5mM mannitol as osmotic control and high glucose (HG) medium contained 30mM glucose as the positive control. Cells were pre-exposed to various concentrations of salvianolate, including 0.1μM, 1.0μM, 10μM, 50μM and 100μM, for a 24-hour period. Subsequently, they were stimulated with 30 mM glucose for 48 hours. The treated cells were labeled as HG+S (0.1μM), HG+S (1.0μM), HG+S (10μM), HG+S (50μM) and HG+S (100μM), respectively. The results shown in fig. 1A indicate a

marked increase in HK-2 cell proliferation when they were cultured under high glucose (HG) conditions, in contrast to normal glucose (NG) conditions. However, the mannitol (MA) group did not exhibit significant proliferation. Salvianolate at concentrations of 1.0uM, 10uM, 50uM and 100uM significantly inhibited cell proliferation under high glucose induction as shown in fig. 1A with statistically significant differences.

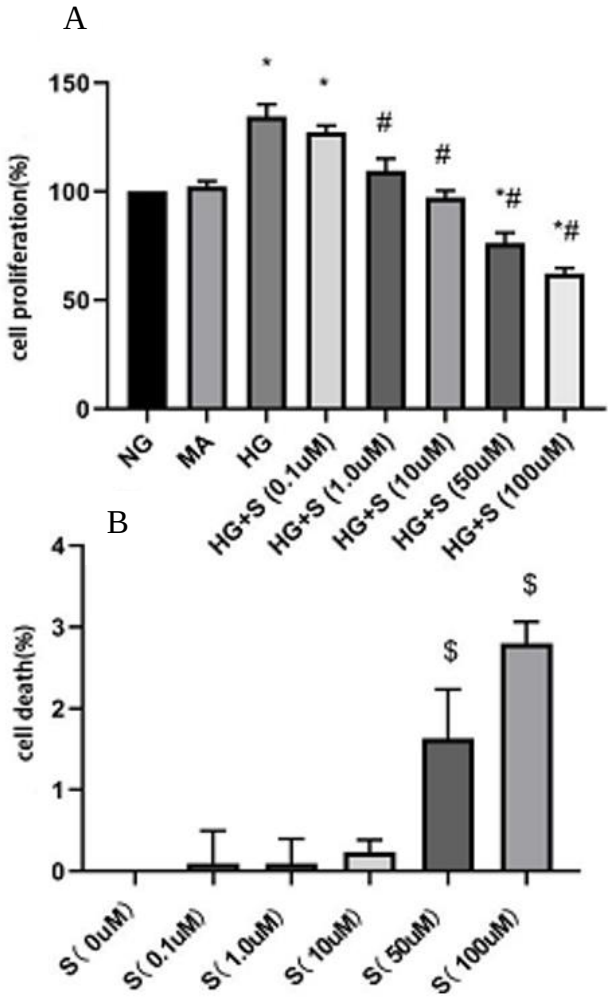

Fig. 1: The effects of salvianolate at different concentrations on the proliferation and death of HK-2 cells.

Salvianolate dose-dependently inhibited the proliferation of HK-2 cells prompted by high glucose.(A) . The impact of salvianolate on cell death in HK-2 cells at varying concentrations.(B). Data were presented as fold of the control group. *P<0.05 vs. NG group, #P<0.05 vs. HG group. $P<0.05 vs. 0uM salvianolate.

The cells were exposed to different concentrations of salvianolate (0uM, 0.1uM, 1.0uM, 10uM, 50uM, and 100uM) and a medium containing 5.5mmol/L glucose for a duration of 24 hours prior to conducting cell cytotoxicity analysis and these cells were labeled as S (0uM), S (0.1uM), S (1.0uM), S (10uM), S (50uM) and S (100uM), respectively. The effects of salvianolate at concentrations of 0.1uM, 1.0uM and 10uM did not show a statistically significant difference in comparison to 0uM on cell death. However, compared to 0uM salvianolate, the proportion of cell death increased significantly after 50uM and 100uM salvianolate treatment, as shown in fig. 1B with statistically significant differences. This suggests that salvianolate's inhibitory effect on cell proliferation is independent of its cytotoxicity. We chose a concentration of 10uM for the next experiment because it had a stronger inhibitory effect on cell proliferation and a lesser cytotoxicity.

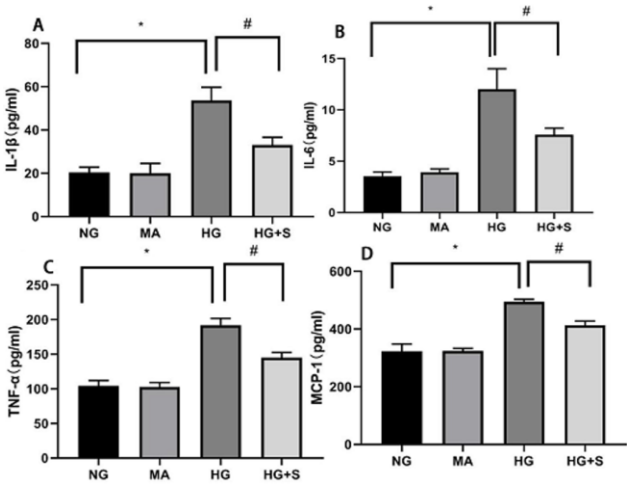

Fig. 2: The impact of salvianolate on the secretion of IL-1 β , IL-6, TNF- α , as well as MCP-1 in HK-2 cells.

The secretions of IL-1 β (A), IL-6 (B), TNF- α (C), as well as MCP-1(D) in HK-2 cells were measured after exposure to 30mmol/L glucose or mannitol, with or without a 1μmol/L dosage of salvianolate. A noteworthy distinction was evident amid the NG group and the HG group (*P<0.05) and similarly, a fundamental differentiation was perceived in the midst of the HG group and the HG+S group (#P<0.05).

Cells were exposed to a culture medium embracing 5.5 mM glucose, which was labeled as the NG group. Another set of cells received treatment with a culture medium comprising 5.5mM glucose plus 24.5mM mannitol, identified as the MA group. Additionally, cells were cultured in a medium encompassing only 30mM glucose which was referred to as the HG group. Cells were pretreated with 10uM salvianolate for 24 hours, followed by stimulation with 30mM glucose for 48 hours, and labeled as the HG+S group. The secretions of IL-1β, IL-6, TNF-α, as well as MCP-1 in the supernatant of HK-2 cells elevated when incubated in high glucose, in contrast to cells treated with normal physiological glucose levels. This increase was not due to osmotic stress, as the use of mannitol did not evoke an increase in the levels of these inflammatory cytokines. Treatment of HK-2 cells

with 10umol/L salvianolate instigated a significant decrease in the secretions of IL-1β, IL-6, TNF-α, as well as MCP-1 in comparison to untreated cells. (see fig. 2A, B, C and D).

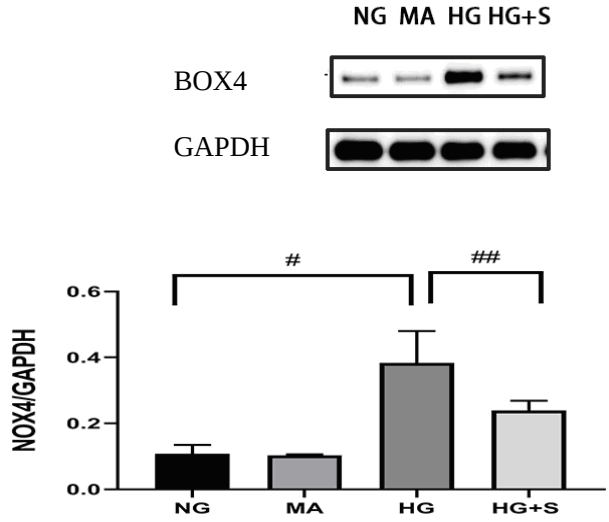

Fig. 3: The impact of salvianolate on the secretion of NOX4 protein in HK-2 cells.

Western blot and the corresponding quantitative data for NOX4 expression in HK-2 cells treated with 30 mmol/L glucose or mannitol, with or without 10μmol/L salvianolate treatment. GAPDH served as the loading control. (##P<0.05 in contrast to HG group; #P<0.05 in contrast to NG group).

The findings indicated an intensified NOX4 protein secretion in HK-2 cells when cultivated in elevated glucose conditions as opposed to standard glucose conditions. However, treatment of the HK-2 cells containing 10umol/L salvianolate triggered a significant decrease of NOX4 protein secretion when compared to the cells without salvianolate treatment (as shown in fig. 3).

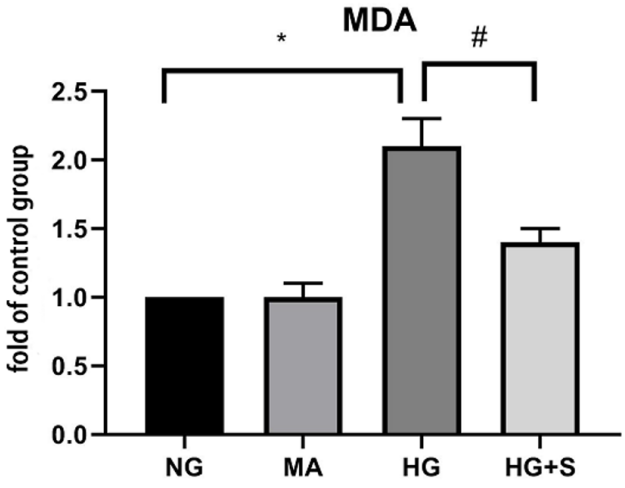

Fig. 4: The impact of salvianolate on the levels of MDA in HK-2 cells.

MDA levels were assessed in HK-2 cells subjected to treatment with 30mmol/L glucose or mannitol, with or without 10umol/L salvianolate treatment. Data were presented as fold of the control group. *P<0.05 compared to NG group. #P<0.05 compared to HG group.

Diabetic nephropathy involves increased oxidative stress activity as an paramount mechanism in its development. Our results showed that, when exposed to high glucose stimulation, a marked increase occurred in the level of MDA, which is a marker of oxidative stress, in HK-2 cells. However, a noticeable reduction in the MDA level prompted by high glucose was effectively achieved in HK-2 cells with the use of 10uM salvianolate. (see fig. 4).

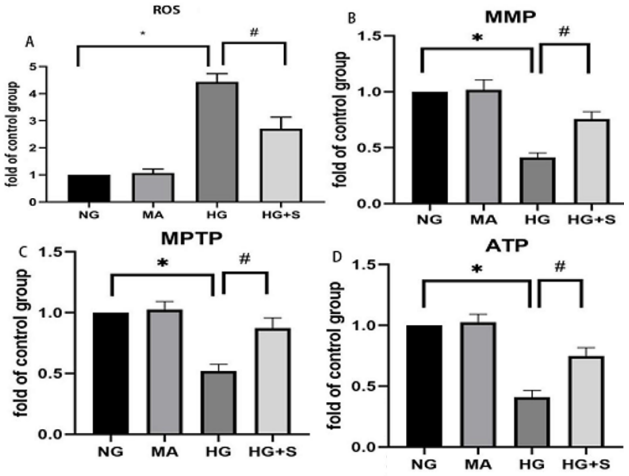

Fig. 5: The impact of salvianolate on the levels of mitochondrial ROS, MMP, MPTP opening and ATP in HK-2 cells.

Mitochondrial ROS levels (A), MMP (B), MPTP opening (C) and ATP (D) were quantified in HK-2 cells subjected to treatment with 30mmol/L glucose or mannitol, with or without 10 μ mol/L salvianolate. The results were presented as fold changes relative to the control group. (*P<0.05 in contrast to the NG group; #P<0.05 in contrast to the HG group).

MPTP expression was visualized using MPTP fluorescence assay, where stronger fluorescence indicated reduced MPTP opening. Cells from the high glucose-treated HK-2 group exhibited elevated ROS (fig. 5A) and MPTP (fig. 5C) levels compared to the normal glucose group. Notably, 10μM salvianolate effectively mitigated the heightened ROS and MPTP levels impelled by high glucose in HK-2 cells. Conversely, the high glucose group displayed diminished MMP and ATP levels in HK-2 cells contrasted to the normal glucose group (fig. 5B, D). Importantly, 10μM salvianolate significantly augmented the restoration of MMP and ATP levels in cells from the high glucose-treated HK-2 group.

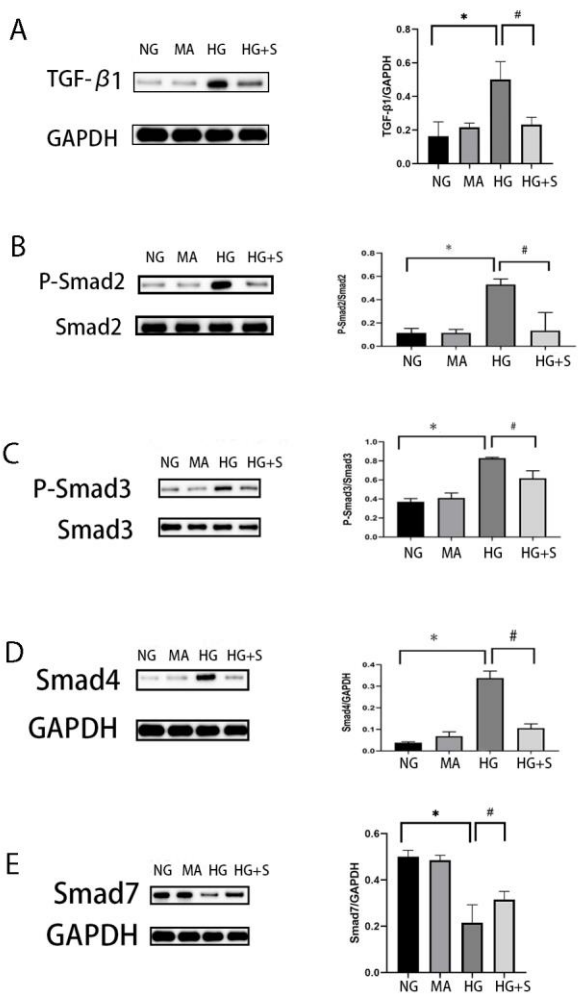

Fig. 6: The impact of salvianolate on the protein expressions of TGF- β 1, P-Smad2, P-Smad3, Smad4 as well as Smad7 in HK-2 cells.

Western blot and the corresponding quantitative data for protein expressions of TGF-β1 (A), P-Smad2 (B), P-Smad3 (C), Smad4 (D) as well as Smad7 (E) in HK-2 cells treated with 30mmol/L glucose or mannitol with or without 10umol/L salvianolate treatment. #P<0.05 versus HG group; *P<0.05 versus NG group.

The protein secretion of TGF-β1 showed an increase in HK-2 cells subjected to high glucose incubation, as opposed to cells maintained under normal physiological glucose levels. Stimulation of HK-2 cells with 10μmol/L salvianolate triggered a notable decrease in the protein secretion of TGF-β1 compared to cells without salvianolate treatment (see fig. 6A). The protein expressions of P-Smad2, P-Smad3 and Smad4 enhanced in HK-2 cells incubated in high glucose in contrast to cells cultured in a normal physiological level of glucose. Meanwhile, Smad7 decreased under the same conditions. Stimulation of HK-2 cells with 10μmol/L salvianolate elicited a prominent decrease in the protein secretions of P-Smad2, P-Smad3, and Smad4, and a material increase in the protein secretion of Smad7 compared to cells without salvianolate treatment (see fig. 6B, C, D, E).

DISCUSSION

A high-glucose environment can induce proliferation of HK-2 cells, possibly due to increased energy supply and stimulation of insulin-like growth factor release. We utilized HK-2 cells, a cell line with characteristics of renal tubular epithelial cells, as a research model for diabetic nephropathy. Cells were exposed to high glucose concentrations to mimic hyperglycemia in diabetic patients. We observed that high glucose treatment induced inflammatory responses, such as increased synthesis of inflammatory cytokines like TNF-α and IL-6. We also detected abnormalities in cellular energy metabolism, such as impaired mitochondrial function and heightened oxidative stress. These observations and experimental results provide preliminary evidence that a high-glucose environment mimics the pathological features and cellular responses associated with diabetic nephropathy. However, comprehensive validation of the high-glucose model for diabetic nephropathy would require further studies, including animal models to assess parameters like changes in glomerular filtration rate and increased protein excretion in urine. Further research is warranted.

In the progression of DKD, inflammation and oxidative stress play pivotal roles. Individuals suffering from DKD often demonstrate elevated plasma levels of pro-inflammatory cytokines, incorporating interleukins IL-1 and IL-6, TNF-α, as well as MCP-1 (Pérez-Morales *et al.*, 2019). MCP-1 can be found in renal endothelial, mesangial and monocytic cells, with its expression being regulated by TNF-α and IL-1 (Deshmane *et al.*, 2009) and it is further encouraged by oxidative stress in the context of diabetes (Singh *et al.*, 2021). Nadkarni *et al* discussed the relationship between MCP-1 and diabetic nephropathy. They reported that a strong association was found between an elevated urinary MCP-1 level and a decreased eGFR in diabetic patients, suggesting that the levels of excreted MCP-1 might serve as a valuable marker for predicting the occurrence, progression, and outlook of diabetic kidney disease (Nadkarni *et al.*, 2016). Salvianolate demonstrates anti-inflammatory properties by modulating diverse inflammatory mediators and signaling pathways. One of the key mechanisms by which it alleviates inflammation is through its antioxidant activity. Inflammation frequently correlates with the evolution of ROS and oxidative stress. Salvianolate's antioxidant properties can help neutralize ROS and reduce oxidative stress, thereby attenuating inflammation. By reducing oxidative stress, salvianolate can alleviate inflammation and its detrimental effects on tissues and organs, including the kidneys (Liang *et al.*, 2021). Within the realm of diabetes, chronic inflammation is intricately

linked to the progression of complications, notably DKD. Elevated blood glucose levels and other metabolic irregularities give rise to the generation of pro-inflammatory cytokines as well as the initiation of inflammatory pathways. This persistent, low-level inflammation can have a hand in tissue damage, hindered renal function and the onset of diabetic nephropathy (Duran-Salgado and Rubio-Guerra, 2014). Salvianolate shows potential in alleviating inflammation in diabetes and its complications. It can inhibit the secretions of pro-inflammatory cytokines, for example, IL-1β, TNF-α, as well as IL-6, which are engaged in inflammation associated with DKD (Hou *et al.*, 2017). Additionally, studies have found that salvianolate can modulate several signaling pathways participating in inflammation, like nuclear factor-kappa B (NF-κB) (Oh *et al.*, 2011) as well as mitogen-activated protein kinase (MAPK) pathways (Qi *et al.*, 2017). The pathways regulate the expression of genes contributing to inflammation. By inhibiting these pathways, salvianolate helps to dampen the inflammatory response and alleviate the progression of DKD. There is a considerable amount of research on salvianolate and inflammation. However, most of these studies focus on cardiovascular and cerebrovascular diseases. Our study, on the other hand, is one of the few that investigates the interaction between salvianolate and inflammation in diabetic nephropathy. Although our research represents a small step in this field, these preliminary findings provide valuable insights for further research and clinical practice, aiming to explore new avenues for the treatment and management of DKD.

DKD has been linked to oxidative stress. The protein NOX4 is a pivotal contributor to oxidative stress (Sedeek *et al.*, 2010). NOX4 is upregulated in the kidneys of diabetic animals. The increase in oxidative stress resulting from elevated NOX4 expression can contribute to the development and progression of DKD (Sedeek *et al.*, 2010). Research has shown that NOX4 influences overseeing multiple cellular processes, including angiogenesis, inflammation, and fibrosis, which are closely connected with diabetic nephropathy (Yang *et al.*, 2018). Moreover, recent findings indicate that suppressing NOX4 activity can mitigate oxidative stress and enhance renal function in diabetic animal models. Consequently, targeting NOX4 may present a promising approach for managing this condition (Lee *et al.*, 2020). This study has demonstrated that salvianolate can effectively suppress NOX4 activity in cells from the kidney cultured in high glucose conditions. These findings offer an additional theoretical underpinning for employing salvianolate in the treatment of DKD.

ROS levels, the change in MMP, the opening of the MPTP, The level of ATP in the mitochondria, and the level of MDA have demonstrated a strong connection to the development and advancement of DKD (Ahmad *et al.*, 2021; Chang *et al.*, 2005; Jha *et al.*, 2016; Lindblom *et al.*, 2020; Mise *et al.*, 2020). Elevated ROS and MDA levels can contribute to oxidative stress. MMP changes and MPTP opening can result in mitochondrial malfunction, which has likewise been linked to the progression of DKD. Decreased ATP levels in the mitochondria may further facilitate oxidative stress. In our study, salvianolate has been shown to improve mitochondrial function, as measured by changes in ROS levels, MMP, MPTP opening and ATP levels in renal cells cultured in high glucose. Furthermore, it has been demonstrated that salvianolate can diminish MDA levels in kidney cells cultivated in elevated glucose conditions.

The TGF-β/Smad signaling pathway is a multifaceted cellular signaling system that holds significance in numerous physiological and pathological processes, notably including the advancement of DKD (Lan, 2012). In the context of DKD, the initiation of the TGF-β/Smad pathway can give rise to the generation of diverse cytokines and growth factors. These molecules, in turn, can incite oxidative stress and inflammation, contributing to extracellular matrix degradation and the advancement of DKD. Conversely, oxidative stress and inflammation can also trigger the TGF-β/Smad pathway, creating a detrimental cycle that intensifies the disease progression. Lu *et al* (Lu *et al.*, 2019) conducted a study on a rat model of renal fibrosis and found that suppressing the TGF-β and downstream Smad2, Smad3, and Smad4 signaling pathways with salvianolic acid effectively reduced the levels of inflammatory factors and improved kidney function. Similarly, Ai *et al* (Ai *et al.*, 2015) discovered that GQ5, a small-molecule phenol compound, produced impressive renal protective effects by inhibiting the p-Smad3 induced by TGF-β and blocking the entry of Smad2, Smad3, and Smad4 into the nucleus. Jiang *et al* (Jiang *et al.*, 2021) also found that inhibiting the TGF-β/Smad pathway reduced oxidative stress and inflammation in DKD and decreased proteinuria. We also believe that inhibiting the TGF-β/Smad pathway is an effective method for preventing and treating DKD by reducing oxidative stress and inflammation. Therefore, salvianolate can inhibit the excessive onset of TGF-β/Smad pathway induced by high glucose, thus preventing and treating DKD.

CONCLUSION

Based on the study results, the mechanism by which salvianolate improves DKD can be summarized as follows: Inhibition of Inflammatory Response: The study results demonstrate that salvianolate can reduce the secretions of IL-1β, IL-6, TNF-α, as well as MCP-1. This indicates its inhibitory effect on alleviating the inflammatory response induced by DKD.

Mitigation of Oxidative Stress: Salvianolate reduces oxidative stress levels by decreasing oxidative stress markers, namely, NOX4 and MDA. This implies that it

can reduce the production of ROS and lipid peroxidation, both of which contribute to kidney damage in DKD.

Improvement of Mitochondrial Function: Salvianolate enhances mitochondrial function by increasing MMP and ATP levels while reducing ROS production and preventing the opening of MPTP. This suggests that it contributes to improving the energy-generating capacity of kidney cells and preserving mitochondrial integrity.

Inhibition of TGF-β1/Smad Pathway: Salvianolate inhibits the TGF-β1/Smad2 and Smad3 signaling pathway, suppresses Smad4 expression and increases Smad7 expression. This indicates its potential to modulate the TGF-β1/Smad pathway associated with fibrosis and inflammation in DKD.

In summary, salvianolate appears to ameliorate high-glucose-induced inflammation and oxidative stress in renal cells through multiple mechanisms. These mechanisms encompass inflammation inhibition, oxidative stress relief, enhancement of mitochondrial function, and modulation of the TGF-β1/Smad pathway. These discoveries offer valuable perspectives into the potential mechanisms through which salvianolate may treat DKD.

ACKNOWLEDGEMENT

This work was supported by grants from the Science and Technology Project of Jiangxi Province Administration of Traditional Chinese Medicines (Grant No. 2021B665). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

Ahmad AA, Draves SO and Rosca M (2021). Mitochondria in diabetic kidney disease. *Cells*, **10**(11): 2945-2975

Ai J, Nie J, He J, Guo Q, Li M, Lei Y, Liu Y, Zhou Z, Zhu F, Liang M, Cheng Y and Hou FF (2015). GQ5 hinders renal fibrosis in obstructive nephropathy by selectively inhibiting TGF-β-induced Smad3 phosphorylation. *J. Am. Soc. Nephrol.*, **26**(8): 1827-1838.

Breyer MD and Susztak K (2016). The next generation of therapeutics for chronic kidney disease. *Nat. Rev. Drug Discov.*, **15**(8): 568-588.

Chang JM, Kuo MC, Kuo HT, Chiu YW and Chen HC (2005). Increased glomerular and extracellular malondialdehyde levels in patients and rats with diabetic nephropathy. *J. Lab. Clin. Med.*, **146**(4): 210-215.

Deshmane SL, Kremlev S, Amini S and Sawaya BE (2009) Monocyte chemoattractant protein-1 (MCP-1): An overview. *J. Interferon Cytokine Res.*, **29**(6): 313-326.

Du Q, Fu YX, Shu AM, Lv X, Chen YP, Gao YY, Chen J, Wang W, Lv GH, Lu JF and Xu HQ (2021). Loganin alleviates macrophage infiltration and activation by inhibiting the MCP-1/CCR2 axis in diabetic nephropathy. *Life Sci.*, **272**: 118808.

Duran-Salgado MB, and Rubio-Guerra AF. (2014). Diabetic nephropathy and inflammation. *World J. Diabetes*, **5**(3): 393-398.

Hou B, Qiang G, Zhao Y, Yang X, Chen X, Yan Y, Wang X, Liu C, Zhang L and Du G (2017). Salvianolic acid a protects against diabetic nephropathy through ameliorating glomerular endothelial dysfunction via inhibiting AGE-RAGE signaling. *Cell Physiol. Biochem.*, **44**(6): 2378-2394.

Jha JC, Banal C, Chow BS, Cooper ME and Jandeleit-Dahm K (2016). Diabetes and kidney disease: Role of oxidative stress. *Antioxid. Redox. Signal.*, **25**(12): 657-684.

Jiang Y, Xie F, Lv X, Wang S, Liao X, Yu Y, Dai Q, Zhang Y, Meng J, Hu G, Peng Z and Tao L (2021). Mefunidone ameliorates diabetic kidney disease in STZ and db/db mice. *FASEB J.*, **35**(1): e21198.

Lan HY (2012). Transforming growth factor-β/Smad signalling in diabetic nephropathy. *Clin. Exp. Pharmacol. Physiol.*, **39**(8): 731-738.

Lee SR, An EJ, Kim J and Bae YS (2020). Function of NADPH oxidases in diabetic nephropathy and development of nox inhibitors. *Biomol. Ther.* (Seoul). **28**(1): 25-33.

Li Q, Liang X, Yang Y, Zeng X, Zhong X and Huang C (2020). Panax notoginseng saponins ameliorate cisplatin-induced mitochondrial injury via the HIF-1α/mitochondria/ROS pathway. *FEBS Open Bio*., **10**(1): 118-126.

Li Y, Deng X, Zhuang W, Li Y, Xue H, Lv X and Zhu S (2022). Tanshinone IIA down-regulates -transforming growth factor beta 1 to relieve renal tubular epithelial cell inflammation and pyroptosis caused by high glucose. *Bioengineered*, **13**(5): 12224-12236.

Liang Y, Liu H, Fang Y, Lin P, Lu Z, Zhang P, Jiao X, Teng J, Ding X and Dai Y (2021). Salvianolate ameliorates oxidative stress and podocyte injury through modulation of NOX4 activity in db/db mice. *J. Cell Mol. Med.*, **25**(2): 1012-1023.

Lindblom R, Higgins GC, Nguyen TV, Arnstein M, Henstridge DC, Granata C, Snelson M, Thallas-Bonke V, Cooper ME, Forbes JM and Coughlan MT (2020). Delineating a role for the mitochondrial permeability transition pore in diabetic kidney disease by targeting cyclophilin D. *Clin. Sci.*, (Lond). **134**(2): 239-259.

Liu XQ, Jiang L, Li YY, Huang YB, Hu XR, Zhu W, Wang X, Wu YG, Meng XM and Qi XM (2022). Wogonin protects glomerular podocytes by targeting Bcl-2-mediated autophagy and apoptosis in diabetic kidney disease. *Acta Pharmacol. Sin.*, **43**(1): 96-110.

Liu Z, Fu C, Wang W and Xu B (2010). Prevalence of

chronic complications of type 2 diabetes mellitus in outpatients - a cross-sectional hospital based survey in urban China. *Health Qual. Life Outcomes,* **8**(1): 62-70.

Lu H, Sun X, Jia M, Sun F, Zhu J, Chen X, Chen K and Jiang K (2021). Rosiglitazone suppresses renal crystal deposition by ameliorating tubular injury resulted from oxidative stress and inflammatory response via promoting the Nrf2/HO-1 pathway and shifting macrophage polarization. *Oxid. Med. Cell Longev.*, 5527137.

Lu XY, Zou W, Dong YQ, Zhou D, Tong XL, Dong ZL, Liang GY, Tang L, and Liu MH (2019) Anti-renal fibrosis effect of asperulosidic acid via TGF-β1/smad2/smad3 and NF-κB signaling pathways in a rat model of unilateral ureteral obstruction. *Phytomedicine*, **53**: 274-285.

Luan P, Xu J, Ding X, Cui Q, Jiang L, Xu Y, Zhu Y, Li R, Lin G, Tian P and Zhang J (2020). Neuroprotective effect of salvianolate on cerebral ischaemia-reperfusion injury in rats by inhibiting the Caspase-3 signal pathway. *Eur. J. Pharmacol.,* **872**: 172944.

Ma L, Wu F, Shao Q, Chen G, Xu L and Lu F (2021). Baicalin alleviates oxidative stress and inflammation in diabetic nephropathy via Nrf2 and MAPK signaling pathway. *Drug Des. Devel. Ther.*, **15**: 3207-3221.

Mise K, Galvan DL and Danesh FR (2020). Shaping up mitochondria in diabetic nephropathy. *Kidney360*. **1**(9): 982-992.

Nadkarni GN, Rao V, Ismail-Beigi F, Fonseca VA, Shah SV, Simonson MS, Cantley L, Devarajan P, Parikh CR and Coca SG (2016). Association of urinary biomarkers of inflammation, injury, and fibrosis with renal function decline: The ACCORD trial. *Clin. J. Am. Soc. Nephrol.,* **11**(8): 1343-1352.

Oh KS, Oh BK, Mun J, Seo HW and Lee BH (2011). Salvianolic acid A suppress lipopolysaccharide-induced NF-κB signaling pathway by targeting IKKβ. *Int. Immunopharmacol.,* **11**(11): 1901-1906.

Pérez-Morales RE, Del Pino MD, Valdivielso JM, Ortiz A, Mora-Fernández C and Navarro-González JF (2019). Inflammation in diabetic kidney disease. *Nephron*, **143**(1): 12-16.

Qi JY, Yu J, Huang DH, Guo LH, Wang L, Huang X, Huang HD, Zhou M, Zhang MZ and Wu J (2017). Salvianolate reduces murine myocardial ischemia and reperfusion injury via ERK1/2 signaling pathways *in vivo*. *Chin. J. Integr. Med.*, **23**(1): 40-47.

Rapa SF, Di Iorio BR, Campiglia P, Heidland A and Marzocco S (2019). Inflammation and oxidative stress in chronic kidney disease-potential therapeutic role of minerals, vitamins and plant-derived metabolites. *Int. J. Mol. Sci.*, **21**(1): 263-288.

Ren J, Fu L, Nile SH, Zhang J and Kai G (2019). Salvia miltiorrhiza in Treating Cardiovascular Diseases: A Review on Its Pharmacological and Clinical Applications. Front Pharmacol. 10: 753-767.

Sedeek M, Callera G, Montezano A, Gutsol A, Heitz F, Szyndralewiez C, Page P, Kennedy CR, Burns KD, Touyz RM and Hébert RL (2010). Critical role of Nox4-based NADPH oxidase in glucose-induced oxidative stress in the kidney: implications in type 2 diabetic nephropathy. *Am. J. Physiol. Renal Physiol.*, **299**(6): F1348-1358.

Singh S, Anshita D and Ravichandiran V (2021). MCP-1: Function, regulation, and involvement in disease. *Int. Immunopharmacol.,* **101**(Pt B): 107598.

Tesch GH (2008). MCP-1/CCL2: a new diagnostic marker and therapeutic target for progressive renal injury in diabetic nephropathy. *Am. J. Physiol. Renal Physiol.*, **294**(4): F697-701.

Vallon V and Komers R (2011). Pathophysiology of the diabetic kidney. *Compr. Physiol.*, **1**(3): 1175-1232.

Yang Q, Wu FR, Wang JN, Gao L, Jiang L, Li HD, Ma Q, Liu XQ, Wei B, Zhou L, Wen J, Ma TT, Li J and Meng XM (2018). Nox4 in renal diseases: An update. *Free Radic. Biol. Med.,* **124**: 466-472.

Yun J, Ren J, Liu Y, Dai L, Song L, Ma X, Luo S and Song Y (2022). MicroRNA (miR)-590-3p alleviates high-glucose induced renal tubular epithelial cell damage by targeting C-X3-C motif chemokine ligand 1 (CX3CL1) in diabetic nephropathy. *Bioengineered*, **13**(1): 634-644.