Article

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Impact of Higenamine on the Hepatic Stellate Cells' Activation Stimulated by TGF-β1 *in Vitro*

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Objective: The current research aimed to explore the impact of higenamine on the hepatic stellate cells (HCC) activation Stimulated by transforming growth factor-β (TGF-β1) *in vitro*.

Methods: This is a prospective study, we investigated the impact of higenamine on the hepatic stellate cells (HSCs) activation stimulated by TGF-β1 *in vitro* and Liver fibrosis (LF) stimulated by tetrachloromethane (CCl_4) *in vivo*. Cell Counting Kit-8 (CCK-8) was adopted for detecting the proliferation of LX-2 cells, HSC stain. Reactive Oxygen Species (ROS) production was determined using fluoroprobe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). The expression levels of ROS-producing enzymes (NOX_2 and NOX_4), as well as Smad2, p-Smad3, p-Smad2, and Smad3 were quantified via western blot (WB). The mRNA/protein expression of extracellular matrix (ECM) proteins (collagen I (Col I) and α-smooth muscle actin (α-SMA)) was detected via Quantitative Real-time Polymerase Chain Reaction (RT-qPCR) and WB. Haematoxylin and eosin (H&E) dyeing was conducted using liver tissues to examine histopathological damage and fibrosis. The serum levels of fibrosis biochemical markers including hyaluronic acid (HA), PC-III, as well as Col IV were tested by Enzyme-Linked Immunosorbent Assay (ELISA).

Results: Higenamine suppressed the proliferation and ROS production in TGF-β1-intervened LX-2 cells. The increased levels of NOX2/NOX4 and NOX activity in TGF-β1-intervened LX-2 cells were reduced by higenamine. Higenamine inhibited the mRNA/protein expression of α-SMA and Col I in TGF-β1-intervened LX-2 cells. Furthermore, the TGF-β1-intevened phosphorylation of Smad3 and Smad2 was attenuated by higenamine.

Conclusion: To sum up, these findings showed that higenamine prevented HSCs' activation via the TGF-β1/Smad pathway. Higenamine also attenuated CCl_4-caused hepatic damage and fibrosis *in vivo*. Thus, higenamine is one possible therapeutic agent for LF prevention.

Keywords: Liver fibrosis; HSCs; higenamine; TGF-β1/Smad pathway; CCl_4

Introduction

Liver fibrosis (LF) is one wound-healing reaction generated against various factors, such as non-alcoholic steatohepatitis, non-alcoholic fatty liver disease, viral hepatitis, and alcohol consumption [1]. In the progression process of LF, the architecture of the liver is disrupted by a fibrous scar, which triggers deregulation of liver function, hepatocyte loss, and ultimately results in liver failure [2]. Reportedly, progressive LF facilitates the development of hepatocellular carcinoma (HCC) and cirrhosis. Therefore, LF is a severe health problem that triggers over 1 million deaths each year [3].

Different cell types and mediators participate in the progression of LF. Among these, hepatic stellate cells (HSCs), as resident mesenchymal cells, retain characteristics of resident fibroblasts and pericytes [4]. In fibrogenic liver, quiescent HSCs transdifferentiate into proliferative/migratory/contractile myofibroblasts, presenting secretory and pro-fibrogenic transcriptional performance, which is known as "HSCs activation" [5]. In addition, HSC are myofibroblast precursors in the liver, primarily responsible for collagen production and maintenance of hepatic extracellular matrix levels; transforming growth factor-β (TGF-β1) is a major driver of hepatic stellate cell activation and Liver fibrosis [6]. Thus, preventing the transdifferentiation or activation of HSCs is one of the major methods to prevent Liver fibrogenesis.

Higenamine is an alkaloid based on plant that is initially isolated from Aconitum, which has been used as local and traditional medicines for the treatment of many diseases like collapse, painful joints, oedema, syncope, and bronchial asthma [7]. According to studies over the last four decades, higenamine possesses many kinds of pharmacological properties such as anti-apoptotic, anti-thrombotic, anti-oxidative, immunomodulatory and anti-inflammatory influences [8]. Moreover, higenamine was found to improve cardiac and renal fibrosis in cardiorenal syndrome rats. Whereas, the role of higenamine in LF is still under exploration [9].

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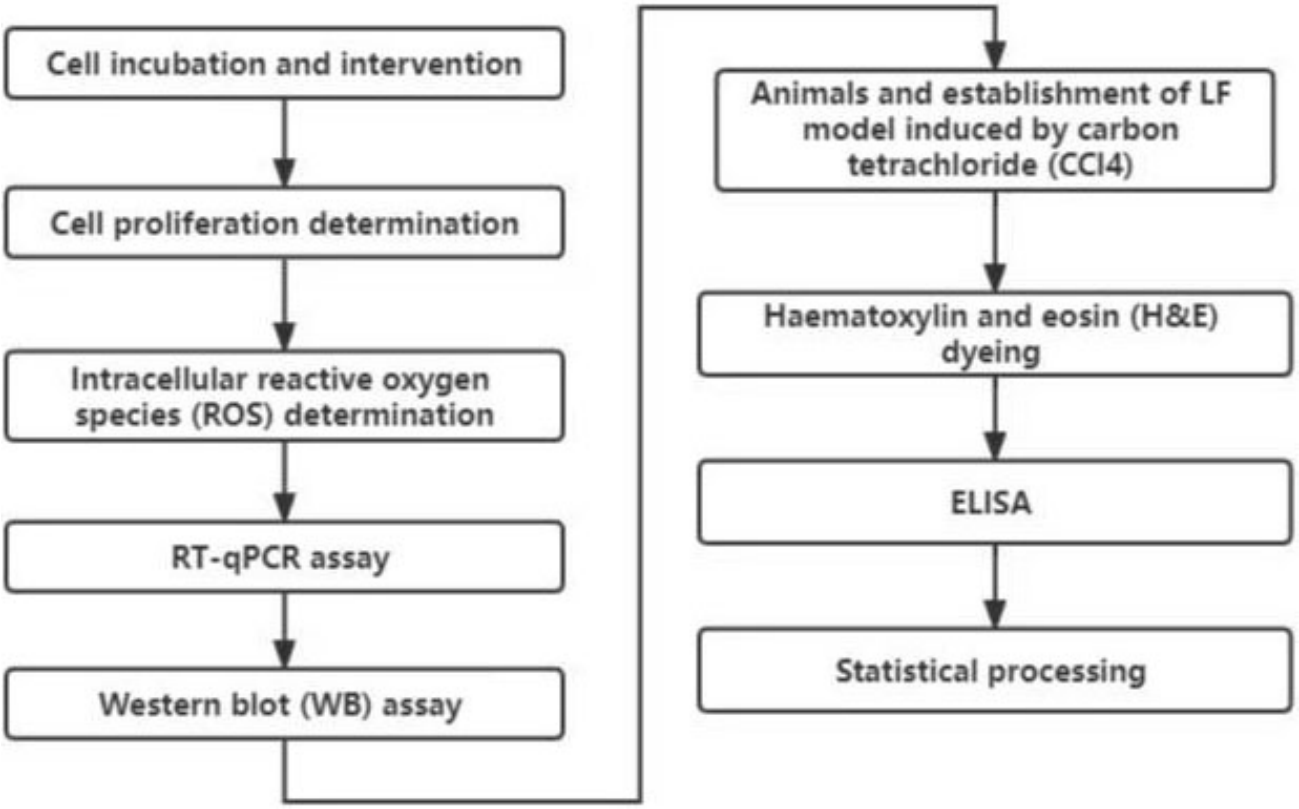

Fig. 1. Study flowchart.

This study probed into the effect of higenamine on TGF-β1-intervened activation of HSCs and uncovered that higenamine prevented the TGF-β1-stimulated activation of HSCs via TGF-β1/Smad pathway.

Materials and Methods

Cell Counting Kit-8 (CCK-8) was adopted for detecting the proliferation of LX-2 cells, HSC stain. Reactive Oxygen Species (ROS) production was determined using fluoroprobe H_2DCF-DA. The expression levels of ROS-producing enzymes (NOX2 and NOX4), as well as Smad2, p-Smad3, p-Smad2, and Smad3 were quantified via western blot (WB). The mRNA/protein expression of extracellular matrix (ECM) proteins (collagen I (Col I) and α-smooth muscle actin (α-SMA)) was detected via Quantitative Real-time Polymerase Chain Reaction (RT-qPCR) and WB. The study flowchart is shown in Fig. 1.

Cell Incubation and Intervention

The immortalised human HSCs, LX-2 cell strain (Merck KGaA, Darmstadt, Germany) was used in this study. Before incubation, the LX-2 cell line was tested for mycoplasma contamination and identified by STR analysis. The cells were treated by incubation (37 °C, 5% CO_2) in high-glucose DMEM containing 2% heat-inactivated FBS and 1% streptomycin/penicillin (Lonza, Basel, Switzerland) under humidified atmosphere. For HSCs' induction, LX-2 cells were given treatment through 10 ng/mL recombinant human TGF-β1 (MN 55413, R&D Systems, Minneapolis, MN, USA). Cells in the higenamine treatment groups were given pretreatment (12 h) by higenamine (5, 10 and 20 μM), followed by TGF-β1 stimulation.

Cell Proliferation Determination

The LX-2 cells' proliferation was studied via CCK-8. In brief, the cells were transferred to 96-well plates (5×10^3 cells/well), followed by 24-h incubation under conditions without serum. After 24-h incubation, every well was given 10 μL CCK-8 solution for 1 h incubation additionally. The absorbance (450 nm) was measured via one ELx800 microplate reader (Bio-Tek, Winooski, VT, USA).

Intracellular Reactive Oxygen Species (ROS) Determination

For measuring the ROS generation, LX-2 cells were intervened by 1 h incubation (37 °C) through 20 μM H_2DCF-DA (Sigma-Aldrich, St. Louis, MO, USA) away from light. After three times of washing via PBS, LX-2 cells were treated by 5-min centrifugation (14,000 *g*). Subsequently, the LX-2 cells were resuspended with ice-cold PBS, followed by the measurement of fluorescence intensity via one fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA; excitation wavelength: 485 nm; emission wavelength: 535 nm).

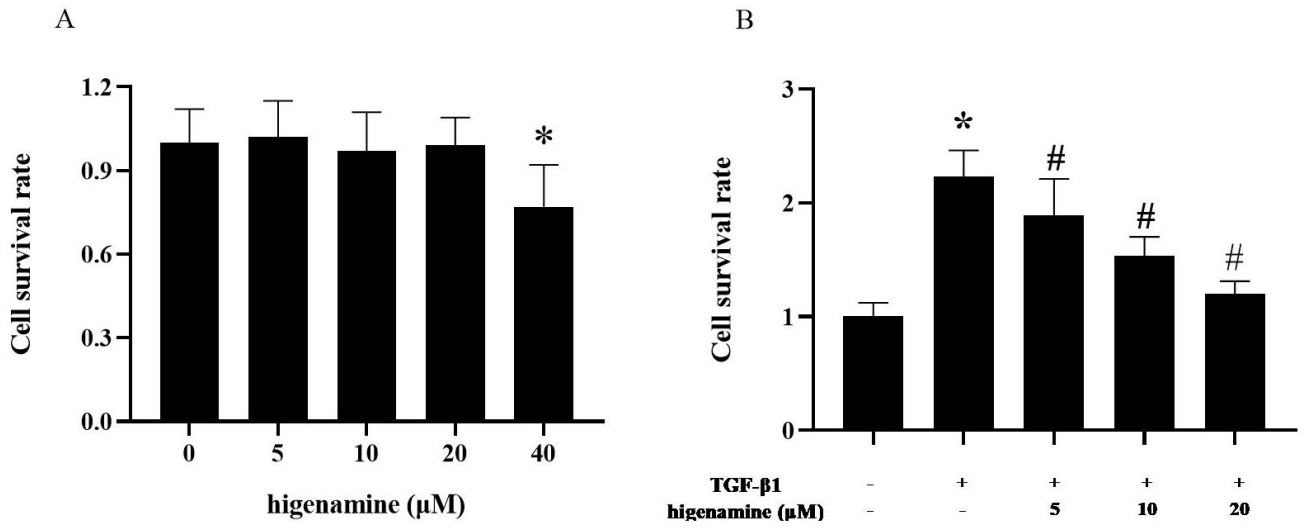

Fig. 2. Impact of higenamine on the proliferation of LX-2 cells. (A) LX-2 cells were given 24-h treatment via various concentrations of higenamine (0, 5, 10, 20 and 40 μM), and cell proliferation was measured using Cell Counting Kit-8 (CCK-8). (B) LX-2 cells were given 12-h pretreatment with higenamine (5, 10 and 20 μM), followed by transforming growth factor-β (TGF-β1) stimulation (10 ng/mL). CCK-8 assay was conducted for detecting the proliferation of LX-2 cells. *$p < 0.05$ vs. normal control group; $^{\#}p < 0.05$ vs. TGF-β1 stimulation; n = 10 for each group.

Detection of NADPH Oxidase (NOX) Activity

NOX activity was measured as previously described method. Briefly, LX-2 cells were acquired and the cellular lysates were obtained via lysis buffer. Homogenate (20 μL) was instantly placed in 180 μL phosphate buffer (pH 7.0) with 0.5 mg/mL salmon DNA, 0.02 mM DHE, as well as 0.2 mM NADPH. Finally, the Et fluorescence was determined via one fluorescence microplate reader (excitation wavelength: 480 nm; emission wavelength: 610 nm; Bio-Tek) during 30 min.

RT-qPCR Assay

LX-2 cells' total RNA was acquired via the RNeasy Mini Kit (QIAGEN, Hilden, Germany) under recommended protocols of the manufacturer, and then the cDNA was produced through reverse transcription via Super M-MLV Reverse Transcriptase (Bio-Tek). The mRNA expression of targeted genes was quantified through RT-qPCR via TaqMan Gene Expression Assays (Applied Biosystems, Foster, CA, USA). The levels were all treated through normalization to those of β-actin. α-smooth muscle actin (α-SMA) F 5′-GGG CAT CCA CGA AAC CAC CT-3′, R 5′-GAG CCG CCG ATC CAG ACA GA-3′; collagen I (Col I) F 5′-TCC TGC CGA TGT CGC TAT CC-3′, R 5′-TCG TGC AGC CAT CCA CAA GC-3′; β-actin F 5′-GGA GAT TAC TGC CCT GGC TCC TAG C-3′, R 5′-GGC CGG ACT CAT CGT ACT CCT GCT T-3′.

Western Blot (WB) Assay

Total proteins of LX-2 cells were acquired via a Whole Protein Extraction kit (Millipore, Temecula, CA, USA). Protein level was calculated via the BCA kit (Pierce Biotechnology, Rockford, IL, USA). Then samples with 40 μg protein were isolated via 12% SDS-PAGE, followed by electro-transferring to one PVDF membrane (Millipore). After immersion in 5% defatted milk, the membranes were treated by incubation (4 °C) overnight with specific I antibodies (Abcam, Cambridge, MA, USA) as follows: anti-α-SMA (1:1000), anti-Smad3 (1:500), anti-NOX2 (1:1000), anti-p-Smad3 (1:500), anti-Col I (1:1000), anti-NOX4 (1:1000), anti-p-Smad2 (1:500), anti-Smad2 (1:500), as well as anti-β-actin (1:5000). The membrane was subsequently treated by 1 h incubation (37 °C) with HRP-goat anti-rabbit IgG (Abcam) (diluted in 1:5000). The protein bands were treated by 1-min visualization (indoor temperature) by ECL detection reagent (NJ 08854, GE Healthcare, Piscataway, NJ, USA). Bio-Rad Image Lab Software (10219, Hercules, CA, USA) was used to analyze the bands.

Statistical Processing

The statistical processing was conducted via the Student *t*-test/one-way analysis of variance to compare the inter-group difference/multi-group difference. $p < 0.05$ denotes a notable difference. The transformed data were tested for normality using Shapiro-Wilk, and all log-transformed variables were normally distributed. Analyses were done via 6.0 GraphPad Prism (CA 92108, GraphPad Prism, San Diego, CA, USA).

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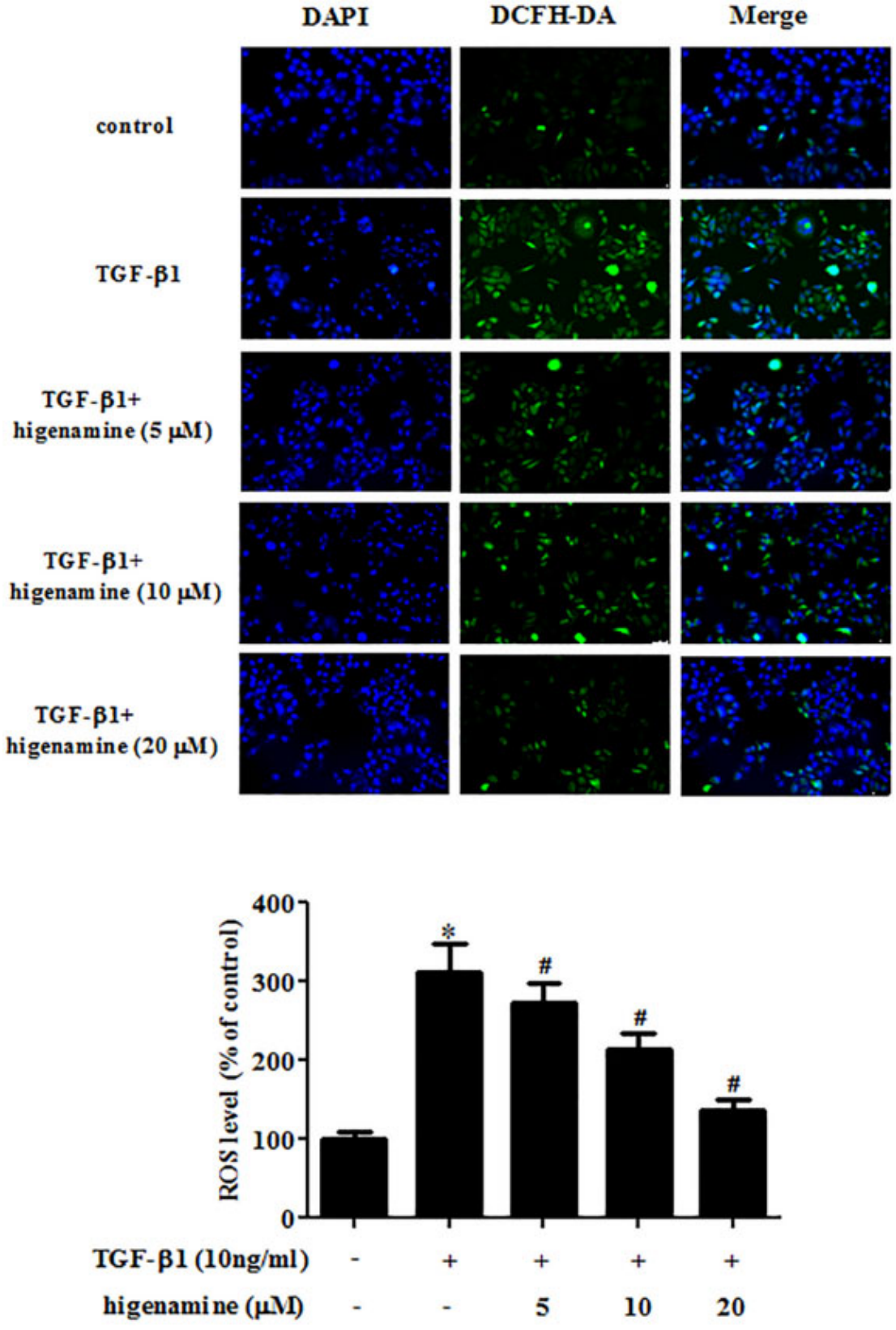

Fig. 3. Impact of higenamine on Reactive Oxygen Species (ROS) production in LX-2 cells. LX-2 cells in TGF-β1 stimulation group were given treatment via 10 ng/mL recombinant human TGF-β1. LX-2 cells in higenamine treatment groups were given 12-h pretreatment with higenamine (5, 10 and 20 μM), followed by TGF-β1 stimulation. ROS production was determined using fluoroprobe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). *$p < 0.05$ vs. normal control group; $^{\#}p < 0.05$ vs. TGF-β1 stimulation; n = 10 for each group.

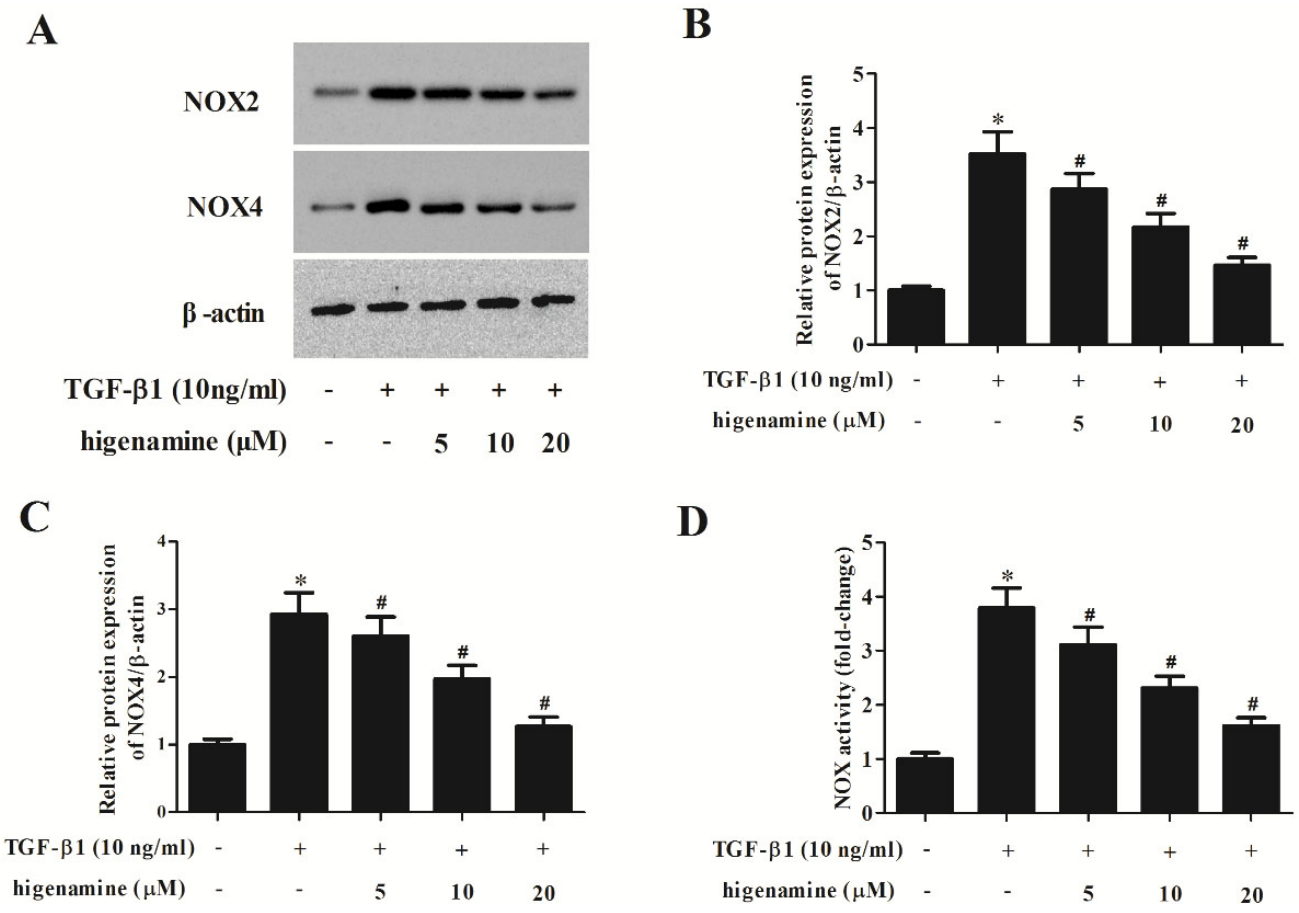

Fig. 4. Impact of higenamine on NOX2/4 expression and NOX activity in LX-2 cells. (A) The NOX2 and NOX4 expression was determined via western blot (WB). (B,C) Quantification analyses of NOX2 and NOX4. (D) NOX activity was also determined. *$p <$ 0.05 vs. normal control group; $^{\#}p < 0.05$ vs. TGF-β1 stimulation; n = 10 for each group.

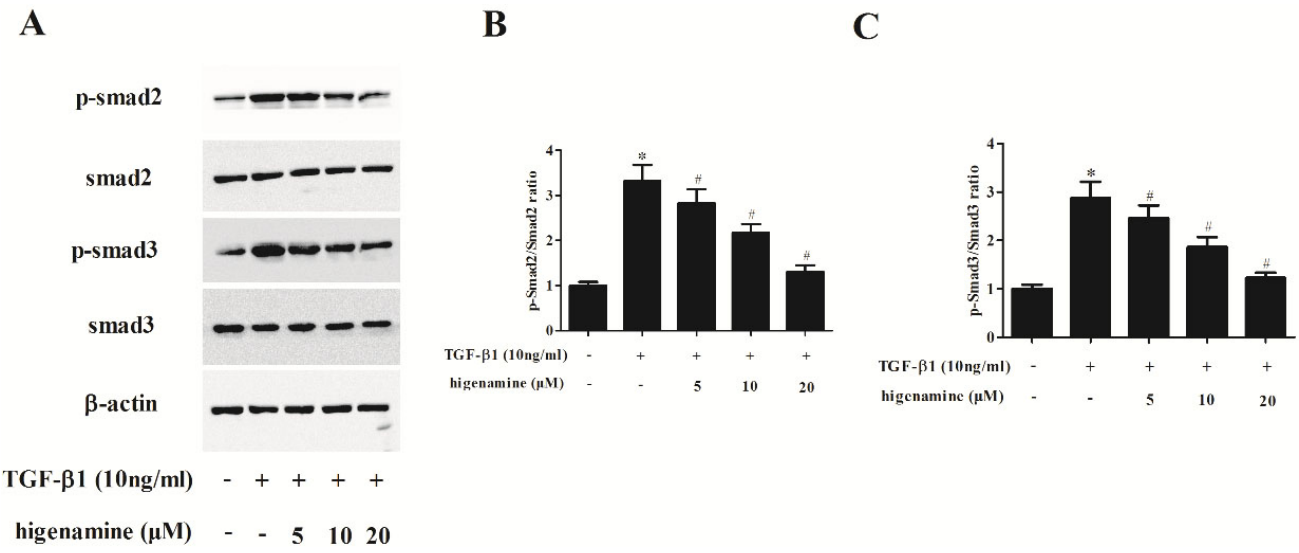

Fig. 5. Impact of higenamine on extracellular matrix (ECM) protein expression in LX-2 cells. (A,B) The α-smooth muscle actin (α-SMA) and collagen I (Col I) mRNA levels were determined via Quantitative Real-time Polymerase Chain Reaction (RT-qPCR). (C) The α-SMA and Col I protein levels were determined via WB. Quantification analyses of α-SMA and Col I. *$p <$ 0.05 vs. normal control group; $^{\#}p < 0.05$ vs. TGF-β1 stimulation; n = 10 for each group.

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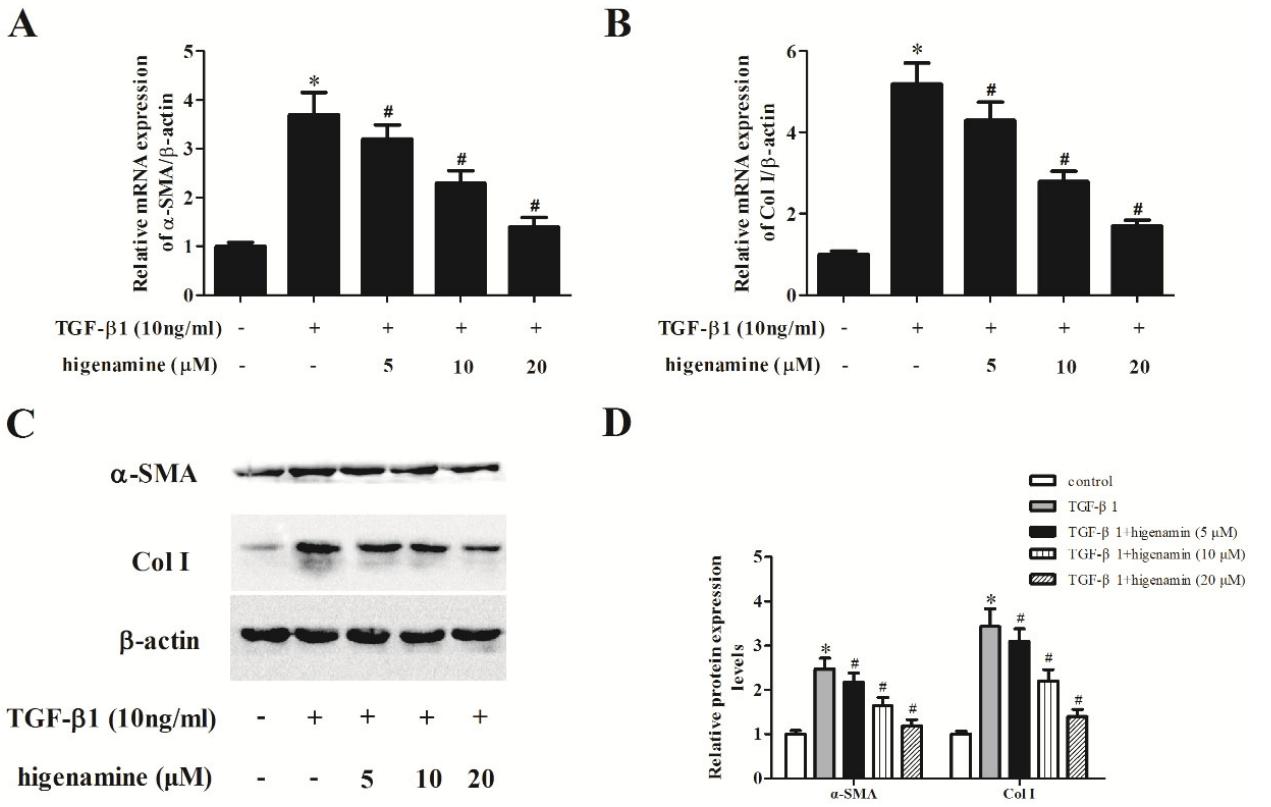

Fig. 6. Impact of higenamine on the TGF-β1/Smad pathway in LX-2 cells. (A) The expression of Smad2, p-Smad2, Smad3 and p-Smad3 was determined via WB. (B) The p-Smad2/Smad2 ratio. (C,D) The p-Smad3/Smad3 ratio. *$p < 0.05$ vs. normal control group; $^{\#}p < 0.05$ vs. TGF-β1 stimulation; n = 10 for each group.

Results

Higenamine Suppresses the Proliferation of TGF-β1-Intervened LX-2 Cells

Firstly, we determined the impact of higenamine on cell viability. As shown in Fig. 2A, administration of 40 μM higenamine greatly impacted cell viability, but the viability of LX-2 cells was not impacted by higenamine at concentrations of 5, 10 and 20 μM. Thus, higenamine (5–20 μM) was adopted in the later assays. According to the CCK-8 assay results, stimulation with TGF-β1 strongly enhanced the LX-2 cells' proliferation in contrast to the control cells. Whereas, the cell proliferation was dose-dependently decreased in LX-2 cells pretreated through higenamine in contrast to those stimulated by TGF-β1 (Fig. 2B).

Higenamine Inhibits ROS Production in TGF-β1-Intervened LX-2 Cells

Reportedly, ROS production takes a crucial part in the fibrosis [10]. Accordingly, we investigated the impact of higenamine on the ROS production in LX-2 cells in response to TGF-β1 stimulation. Fig. 3 presented that the production of ROS in LX-2 cells was markedly stimulated through TGF-β1. However, higenamine reduced the TGF-β1-treated ROS production in LX-2 cells in contrast to the TGF-β1-treated LX-2 cells.

Higenamine Inhibits the NOX Activity and NOX2/4 Expression in TGF-β1-Intervened LX-2 Cells

It is well-known that NOX is responsible for ROS production [11]. We further probed into the impact of higenamine on NOX2/4 expression and NOX activity. In TGF-β1-intervened LX-2 cells, the NOX2 and NOX4 expression greatly increased; however, higenamine treatment obviously mitigated NOX2 and NOX4 expression changes (Fig. 4A–C). Moreover, a significant increase in NOX activity was observed in TGF-β1-stimulated LX-2 cells. Pretreatment with higenamine gradually reversed the increased NOX activity as the increase in dosage (Fig. 4D).

Higenamine Inhibits Extracellular Matrix (ECM) Protein Expression in TGF-β1-Intervened LX-2 Cells

The potency of higenamine in preventing ECM protein expression was measured via RT-qPCR and WB. Results of RT-qPCR were displayed in Fig. 5A,B. The mRNA expression of α-SMA and Col I in TGF-β1-intervened LX-2 cells notably increased in contrast to those in control cells. Higenamine treatment greatly down regulated the α-SMA and Col I mRNA levels. WB analysis also proved that higenamine treatment attenuated the TGF-β1-stimulated protein levels of Col I andα-SMA (Fig. 5C).

Higenamine Inhibits LX-2 Cells' Activation through TGF-β1/Smad Pathway

As verified, TGF-β1/Smad pathway is strongly bound up with the fibrosis progression [12]. The changes of TGF-β1/Smad pathway' activation were detected by WB. As displayed in Fig. 6, the p-Smad2 and p-Smad3 levels dramatically increased in TGF-β1-intervend LX-2 cells. Treatment with higenamine greatly suppressed the TGF-β1-treated p-Smad2 and p-Smad3 levels.

Discussion

This study has proved that higenamine prevented TGF-β1-treated activation of the HSCs, as verified through decreased cell proliferation and ECM proteins expression. In addition, we found that the production of ROS was induced by TGF-β1 but prevented by higenamine treatment. Reportedly, several cellular events and extrahepatic factors induce HSCs activation directly during the fibrosis process [13]. TGF-β1 is the most extensively explored isoform in Liver fibrogenesis that synthetized as a latent precursor by various cells like endothelial cells, macrophages, as well as hepatocytes [14]. TGF-β1 promotes the fibrogenesis, proliferation, and contraction of HSCs. Our study used TGF-β1 to induce the activation of HSCs, thereby exploring the effect of higenamine on the activated HSCs. Targeting the TGF-β1/smad-dependent signalling pathway may be the pharmacological effect of higenamine in response to sustained CF activation and consequent fibrosis [15]. Because it is essential for the transformation of fibroblasts into myofibroblasts, when the TGF-β1 receptor is activated, Smad proteins (mainly Smad2/3) translocate into the nucleus and stimulate the transcription of profibrogenic genes. TGF-β1 is one critical mediator in tissue fibrosis (TF), which triggers tissue scarring primarily through activating its downstream Smad signaling that triggers overexpression of pro-fibrotic genes [16]. According to studies, TGF-β1/Smad pathway' dysregulation represents a crucial pathogenic mechanism in TF. Importantly, Smad2 and Smad3 are the two primary downstream regulators able to accelerate TGF-β1-mediated TF [17]. Our study demonstrated that higenamine prevented TGF-β1-induced expression of p-Smad2 as well as p-Smad3. The results indicated that higenamine might exert its anti-fibrotic role via TGF-β1/Smad pathway.

The results of this study have shown that higenamine inhibits ROS production in TGF-β1-intervened LX-2 cells. According to recent findings, disturbances in the formation and degradation of ROS take a critical part of LF. The production of the concomitant oxidative stress and ROS promotes HSCs activation and ECM production [18]. Excessive ROS gives rise to the activation and synthesis of many cytokines and growth factors via shared feed-forward and feedback mechanisms. The development of Liver fibrosis is often accompanied by an inflammatory response, and inflammation usually induces the activation of hepatic stellate cells, leading to further development of Liver fibrosis [19]. Activated hepatic stellate cells often accumulate in the damaged areas of the liver and further secrete inflammatory factors and chemokines. A vicious circle is formed between the two, in which inflammation leads to Liver fibrosis, which in turn promotes inflammation and leads to further deterioration of Liver fibrosis. In contrast, higenamine treatment prevents the production of ROS, which slows or inhibits liver damage and slows the process of fibrosis [20].

In this study, we confirmed that higenamine could inhibit the NOX activity and NOX2/4 expression in TGF-β1-intervened LX-2 cells. In the liver, NOX shows functional expression in the phagocytic and nonphagocytic forms [21]. Recent evidence indicates that NOX isoforms, like NOX1, NOX2 as well as NOX4, induce distinct profibrogenic actions in HSCs, the primary fibrogenic cell type in the liver. Besides, NOX-derived ROS facilitates hepatic fibrogenesis, thus, NOX2 and NOX4 as well as NOX activity were quantified. We found that higenamine also inhibited the expression of ROS-producing enzymes NOX2 and NOX4 [22]. These findings suggested that higenamine blocked the HSCs activation, indicating that higenamine might suppress LF.

Conclusion

This study establishes an effective monomer to delay the progression of Liver fibrosis based on the pathogenesis of the disease. It provides a reference value for the prevention and treatment of the disease. To sum up, this study highlighted an anti-fibrotic role of higenamine in LF with the involvement of TGF-β1/Smad pathway. Accordingly, higenamine is probably a therapeutic agent for the prevention of LF. Therefore, the TGF-β1/Smad pathway signaling mechanism also appears to be important in the process and progression of liver fiber development. Subsequently, we will use TGF-β1/Smad pathway as an entry point to further investigate the specific mechanism of action of norepinephrine *in vivo* and *in vitro*, thus providing a pharmacological basis for the prevention and treatment of cardiovascular diseases.

Availability of Data and Materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

JH, HX designed the research study. WL, PL performed the research. ML, FM conducted experiments, analyzed the data. All authors contributed to important editorial changes in the manuscript. All authors read and ap-

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proved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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