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Estrogen receptor-β-dependent effects of saikosaponin-d on the suppression of oxidative stress-induced rat hepatic stellate cell activation

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Abstract. Saikosaponin-d (SSd) is one of the major triterpenoid saponins derived from *Bupleurum falcatum* L., which has been reported to possess antifibrotic activity. At present, there is little information regarding the potential target of SSd in hepatic stellate cells (HSCs), which serve an important role in excessive extracellular matrix (ECM) deposition during the pathogenesis of hepatic fibrosis. Our recent study indicated that SSd may be considered a novel type of phytoestrogen with estrogen-like actions. Therefore, the present study aimed to investigate the effects of SSd on the proliferation and activation of HSCs, and the underlying mechanisms associated with estrogen receptors. In the present study, a rat HSC line (HSC-T6) was used and cultured with dimethyl sulfoxide, SSd, or estradiol (E_2; positive control), in the presence or absence of three estrogen receptor (ER) antagonists [ICI-182780, methylpiperidinopyrazole (MPP) or (R,R)-tetrahydrochrysene (THC)], for 24 h as pretreatment. Oxidative stress was induced by exposure to hydrogen peroxide for 4 h. Cell proliferation was assessed by MTT growth assay. Malondialdehyde (MDA), CuZn-superoxide dismutase (CuZn-SOD), tissue inhibitor of metalloproteinases-1 (TIMP-1), matrix metalloproteinase-1 (MMP-1), transforming growth factor-β1 (TGF-β1), hydroxyproline (Hyp) and collagen-1 (COL1) levels in cell culture supernatants were determined by ELISA. Reactive oxygen species (ROS) was detected by flow cytometry. Total and phosphorylated mitogen-activated protein kinases (MAPKs) and α-smooth muscle actin (α-SMA) were examined by western blot analysis. TGF-β1 mRNA expression was determined by RT-quantitative (q)PCR. SSd and E_2 were able to significantly suppress oxidative stress-induced proliferation and activation of HSC-T6 cells. Furthermore, SSd and E_2 were able to reduce ECM deposition, as demonstrated by the decrease in transforming growth factor-β1, hydroxyproline, collagen-1 and tissue inhibitor of metalloproteinases-1, and by the increase in matrix metalloproteinase-1. These results suggested that the possible molecular mechanism could involve downregulation of the reactive oxygen species/mitogen-activated protein kinases signaling pathway. Finally, the effects of SSd and E_2 could be blocked by co-incubation with ICI-182780 or THC, but not MPP, thus indicating that ERβ may be the potential target of SSd in HSC-T6 cells. In conclusion, these findings suggested that SSd may suppress oxidative stress-induced activation of HSCs, which relied on modulation of ERβ.

Introduction

Hepatic fibrosis is a reversible stage of numerous chronic liver diseases, which are associated with significant morbidity and mortality (1). Hepatic stellate cells (HSCs) are in close contact with hepatocytes and sinusoidal endothelial cells within the space of Disse, and are considered the primary cells that contribute to excessive extracellular matrix (ECM) deposition during the pathogenesis of hepatic fibrosis (2). Quiescent HSCs are characterized by a lack of proliferation, and vitamin A storage. In response to a fibrogenic stimulus, HSCs undergo an activation process, which includes loss of vitamin A stores, increased proliferation rate, increased ECM protein synthesis and transformation into α-smooth muscle actin (α-SMA)-positive

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Abbreviations: HSCs, hepatic stellate cells; ECM, extracellular matrix; ROS, reactive oxygen species; MAPKs, mitogen-activated protein kinases; SSd, saikosaponin-d; MDA, malondialdehyde; SOD, superoxide dismutase; TIMP-1, tissue inhibitor of metalloproteinases-1; MMP-1, matrix metalloproteinase-1; Hyp, hydroxyproline; COL1, collagen-1; TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin; ER, estrogen receptor

Key words: saikosaponin-d, estradiol, hepatic stellate cells, estrogen receptor, oxidative stress

myofibroblast-like cells (3,4). Therefore, HSCs are considered a target for the treatment of hepatic fibrosis (4). Oxidative stress (OS) results from the increased production of reactive oxygen species (ROS), and serves a crucial role in inducing HSC activation and fibrogenic potential (5). ROS are able to stimulate expression of the critical fibrosis-associated gene, transforming growth factor (TGF)-β1, via activating the mitogen-activated protein kinases (MAPKs) signaling pathway (6).

Gender has been identified as an independent risk factor for the progression from fibrosis to cirrhosis (7), which has a male:female ratio ranging between 2.3:1 and 2.6:1. It has previously been reported that estradiol (E_2) can attenuate dimethylnitrosamine (DMN)- or carbon tetrachloride (CCl_4)-induced liver fibrosis (7,8), and significantly inhibit HSC proliferation and transformation (9). Notably, E_2 suppresses hydrogen peroxide (H_2O_2)-induced activation of cultured rat HSCs via decreasing lipid peroxide levels (10). However, despite these benefits, the undesirable side effects of estrogen replacement therapy, including increased risk of breast and endometrial cancers, limit its clinical application; therefore, alternative drugs are required (11,12).

Traditional Chinese medicine has been used for thousands of years for the treatment of liver-related diseases. Bupleurum-containing herbal prescriptions, including sho-saiko-to (Xiao Chai Hu Tang) and Chaihu-Shugan-San, have been traditionally used in Asian countries to treat various liver diseases (13-15). Saikosaponin-d (SSd) is one of the major active pharmacological components extracted from *Bupleurum falcatum* L., which has been reported to alleviate CCl_4-induced hepatocyte injury by inhibiting lipid peroxidation (16). Furthermore, it exhibits suppressive effects on hepatic fibrosis in rats, which was induced by CCl_4 injections in combination with alcohol, high fat and low protein feeding, due to its protection against inflammatory hepatocyte injury (17). It has also been reported that SSd may inhibit proliferation and activation of HSC-T6 cells (18). Notably, our previous study demonstrated that SSd can induce estrogen response elements-luciferase activity in MCF-7 cells, thus suggesting that SSd exerts estrogen-like activity (19). However, whether SSd could suppress the activation of HSCs via the estrogen receptor (ER) signaling pathway, and which ER subtype is regulated by SSd in HSC-T6 cells, remains to be elucidated. Therefore, the present study aimed to investigate the effects of SSd on OS-induced activation of HSCs, as well as the underlying mechanisms associated with ERs.

Materials and methods

Materials. SSd (batch number: 110778-201409; purity, >95%) was purchased from National Institutes for Food and Drug Control (Beijing, China). SSd is quite stable at room temperature and retains its activity following exposure to organic solvents, including dimethyl sulfoxide (DMSO). ICI-182780, DMSO, bovine serum albumin, E_2 and phenol red-free Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Enhanced chemiluminescence (ECL) kit was purchased from EMD Millipore (Billerica, MA, USA). TRIzol® reagent was purchased from Invitrogen; Thermo Fisher Scientific, Inc. ReverTra Ace-α® reverse transcription (RT) kit and SYBR®-Green real-time polymerase chain reaction (PCR) master mix were purchased from Toyobo Life Science (Osaka, Japan). Fetal bovine serum (FBS) and charcoal-stripped FBS (sFBS) were obtained from Gibco; Thermo Fisher Scientific, Inc. The protein molecular weight marker was purchased from Pierce; Thermo Fisher Scientific, Inc. Total and phosphorylated MAPK primary antibodies [ERK (cat. no. 4695P), JNK (cat. no. 9258P), P38 (cat. no. 8690P), p-ERK (cat. no. 4370P), p-JNK (cat. no. 4668P), p-P38 (cat. no. 4511P)], β-actin antibody (cat. no. 4970S) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit-and goat anti-mouse antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). α-SMA primary antibody (cat. no. sc-32251) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). Rat malondialdehyde (MDA) ELISA test kit (cat. no. F16194), rat CuZn-superoxide dismutase (SOD) ELISA test kit (cat. no. F16742), rat hydroxyproline (Hyp) ELISA test kit (cat. no. F15649), rat collagen-1 (COL1) ELISA test kit (cat. no. F5730), rat TIMP-1 ELISA test kit (cat. no. F16930) and rat MMP-1 ELISA test kit (cat. no. F16160) were purchased from Westang Biological Science and Technology Co., Ltd. (Shanghai, China), and rat TGF-β1 ELISA test kit (cat. no. BMS623-3) was purchased from eBioscience;Thermo Fisher Scientific, Inc. Methylpiperidinopyrazole (MPP) dihydrochloride and (R,R)-tetrahydrochrysene (THC) were purchased from Tocris Bioscience (Bristol, UK). H_2O_2 solution was purchased from Tianjin Dongfang Chemical Co. (Tianjin, China). EDTA-free digestive juices were purchased from Invitrogen; Thermo Fisher Scientific, Inc.

Cell culture. Rat HSC-T6 cells (Cell Biological Research Institution of Chinese Academy of Sciences, Shanghai, China) were routinely cultured in DMEM supplemented with 5% FBS in an atmosphere containing 5% CO_2 at 37˚C. Cells were grown to 85% confluence and were then transferred to phenol red-free DMEM supplemented with 5% sFBS for 2 days, in order to minimize estrogenic activity of the serum. Cells were treated with SSd or E_2. SSd, E_2, ICI-182780, MPP and THC were all dissolved in DMSO. All were diluted in the medium immediately prior to use (final concentration of DMSO, <0.1%). DMSO (<0.1%) alone did not have any effect on the parameters measured.

Interaction with ICI-182780, MPP and THC. Some phytoestrogens, including genistein and daidzein, act as agonists and antagonists of ERs (20). In the present study, the effects of SSd alone, as well as its interaction with ICI-182780, MPP and THC, were examined. ICI-182780 is a pure ER antagonist; MPP is an antagonist specific to ERα; THC is an antagonist specific to ERβ. HSC-T6 cells were divided into 4 groups as follows: vehicle group (treated with DMSO at 37˚C for 24 h); ICI group (treated with 1 μM ICI-182780 at 37˚C for 24 h); MPP group (treated with 1 μM MPP at 37˚C for 24 h); THC group (treated with 1 μM THC at 37˚C for 24 h). Each group was divided into 4 subgroups as follows: control group (treated with DMSO at 37˚C for 24 h, then DMSO at 37˚C for 4 h); OS group (treated with DMSO at 37˚C for 24 h, then H_2O_2 at 37˚C for 4 h); SSd group (treated with 5 μM SSd at 37˚C for 24 h, then H_2O_2 at 37˚C for 4 h); E_2 group (treated with 1 μM E2 at 37˚C for 24 h, then H_2O_2 at 37˚C for 4 h). ER antagonist and drug treatment were administrated at the same time.

MTT growth assay. HSC-T6 cells were washed twice with PBS, counted and seeded into 96-well plates at a density of

0.8×10^4 cells/well. After 24 h, the cells completely attached to the wells. Cell proliferation was assessed after 24 h [cells in the OS groups were analyzed following 4 h induction with 0.2 mM H_2O_2, as previously described (21,22)]. Briefly, cells were incubated with 100 μl 0.5 mg/ml MTT solution for 4 h at 37˚C. The medium was then discarded and 200 μl DMSO was added for 24 h at room temperature. Absorbance was measured at 570 nm using an ELx800 universal microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell numbers were obtained as absorbance values. The results were expressed as proliferation of the treated cells relative to the control group.

Detection of MDA, CuZn-SOD, tissue inhibitor of metalloproteinases-1 (TIMP-1), matrix metalloproteinase-1 (MMP-1), TGF-β1, Hyp and COL1 levels in cell culture supernatants. HSC-T6 cells were seeded into 24-well plates (8×10^4 cells/well) and cultured in phenol red-free DMEM containing 5% sFBS. After 24 h, SSd and E_2 were added in the presence or absence of 1 μM ICI-182780, MPP or THC. After 24 h, cell culture supernatants were collected [cells in the OS groups were analyzed following 4 h induction with 0.2 mM H_2O_2, as previously described (21,22)] and stored at -20˚C. Subsequently, supernatants were analyzed using ELISA kits according to the manufacturer's protocols.

Detection of ROS in HSC-T6 cells. Intracellular ROS levels were measured by the conversion of non-fluorescent 2,7-dichlorofluorescein diacetate (DCFH-DA) into DCF. HSC-T6 cells were pretreated with SSd and E_2 in the presence or absence of ICI-182780, MPP or THC for 24 h. Cells in OS groups were stimulated with H_2O_2 for 4 h. Subsequently, cells were incubated in 10 μM DCFH-DA for 20 min at 37˚C. The cells were washed three times, harvested and resuspended in PBS. Fluorescence was detected using a BD FACSVerse™ flow cytometer (BD FACSuite™, version LSR 2).

Western blot analysis. HSC-T6 cells were seeded into 6-well plates and cultured in phenol red-free DMEM supplemented with 5% sFBS. Whole cell extracts were prepared using the M-PER lysis buffer (cat. no. 78501; Thermo Fisher Scientific, Inc.). Lysates were then centrifuged at 13,300 x g at 4˚C for 15 min to remove insoluble substances. Protein concentration was quantified using BCA assay according to the manufacturer's protocols. Total protein extracts (50 μg) from each sample were separated by 12% SDS-PAGE followed by electrotransfer onto polyvinylidene difluoride membranes (Immobilon-P; EMD Millipore). Membranes were blocked with 5% skimmed milk, and were incubated with rabbit polyclonal anti-rat extracellular signal-regulated kinase (ERK)/c-Jun N-terminal kinase (JNK)/p38 and phosphorylated (p)-ERK/p-JNK/p-p38 antibodies (1:1,000), mouse monoclonal anti-rat α-SMA (1:500) or mouse monoclonal anti-β-actin (1:2,000) diluted in 2% BSA at 4˚C overnight. Unbound primary antibodies were washed away using Tris-buffered saline containing 0.1% Tween-20. Immune complexes were probed using HRP-conjugated anti-mouse or anti-rabbit secondary antibodies diluted in 5% skimmed milk, which were incubated at room temperature for 2 h and were detected using an ECL western blot procedure (EMD Millipore, Billerica, MA, USA). Band density was semi-quantified following densitometric analysis of autoradiographs using a Bio-Rad GS-690 Scanner (Bio-Rad Laboratories, Inc.). Optical density values from the experimental groups were expressed as a mean percentage of control values, and differences were calculated by normalizing the density of each band to that of β-actin.

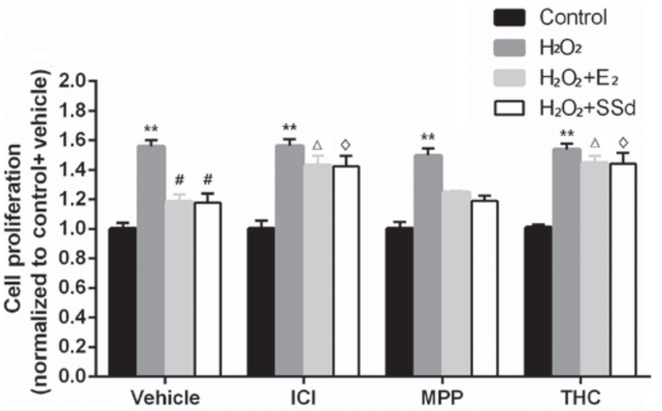

Figure 1. Effects of SSd and E_2 in the presence or absence of ER antagonists on oxidative stress-induced HSC proliferation. HSC-T6 cells were treated with DMSO, 1 μM E_2 or 5 μM SSd, in the presence or absence of 1 μM ICI-182780, MPP or THC for 24 h. Subsequently, oxidative stress was induced by 0.2 mM H_2O_2 for 4 h. Cell proliferation was detected using an MTT assay. Results are presented as the means ± SD, n=4. $^{**}P<0.01$ vs. the control group with the same ER antagonist treatment (ICI-182780, MPP, THC or DMSO); $^{\#}P<0.05$ vs. the H_2O_2 + DMSO group; $^{\triangle}P<0.05$ vs. the H_2O_2 + E_2 + DMSO group; $^{\diamond}P<0.05$ vs. the H_2O_2 + SSd + DMSO group. DMSO, dimethyl sulfoxide; E_2, estradiol; ER, estrogen receptor; H_2O_2, hydrogen peroxide; HSC, hepatic stellate cell; MPP; methylpiperidinopyrazole; SSd, saikosaponin-d; THC, (R,R)-tetrahydrochrysene.

RT-quantitative (q)PCR. HSC-T6 cells were seeded onto 6-well plates and cultured in phenol red-free DMEM containing 5% sFBS. After 24 h, SSd and E_2 were added, in the presence or absence of 1 μM ICI-182780, MPP or THC. Cells in the OS groups were analyzed after 4 h induction with 0.2 mM H_2O_2, as previously described (21,22). Total RNA was isolated using TRIzol® reagent, according to the manufacturer's protocol. Total RNA (3 μg) was used to generate cDNA in each sample using Superscript II reverse transcriptase with oligo(dT) primers (Toyobo Life Science, Osaka, Japan). qPCR was performed to quantify gene expression levels. Each qPCR reaction contained 2 μl diluted cDNA sample, 10 μl SYBR-Green PCR master mix, 0.5 μl 1 μM forward and reverse primers, and 7.5 μl ddH_2O. For detection of TGF-β1 and GAPDH (housekeeping gene) expression, the following primers were used: TGF-β1, forward 5'-TTG ACT TCC GCA AGG ACC TCG G-3', reverse 5'-GCG CCC GGG TTA TGC TGC TGG T-3'; and GAPDH, forward 5'-CCT CTA TGC CAA CAC AGT GC-3' and reverse 5'-GTA CTC CTG CTT GCT GAT CC-3' to yield 146 and 194 bp products within 35 PCR cycles, respectively. The amplification process was conducted as follows: Pre-denaturation at 95˚C for 3 min, denaturation at 95˚C for 45 sec, annealing at 50˚C for 45 sec and elongation at 72˚C for 45 sec, a final extension step at 72˚C for 10 min. qPCR was carried out using an ABI Prism 7900 HT Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The ratio of TGF-β1 to GAPDH was used to evaluate the significance of differences among the various groups. PCR results were quantified by using the $2^{-\Delta\Delta Cq}$ method (23).

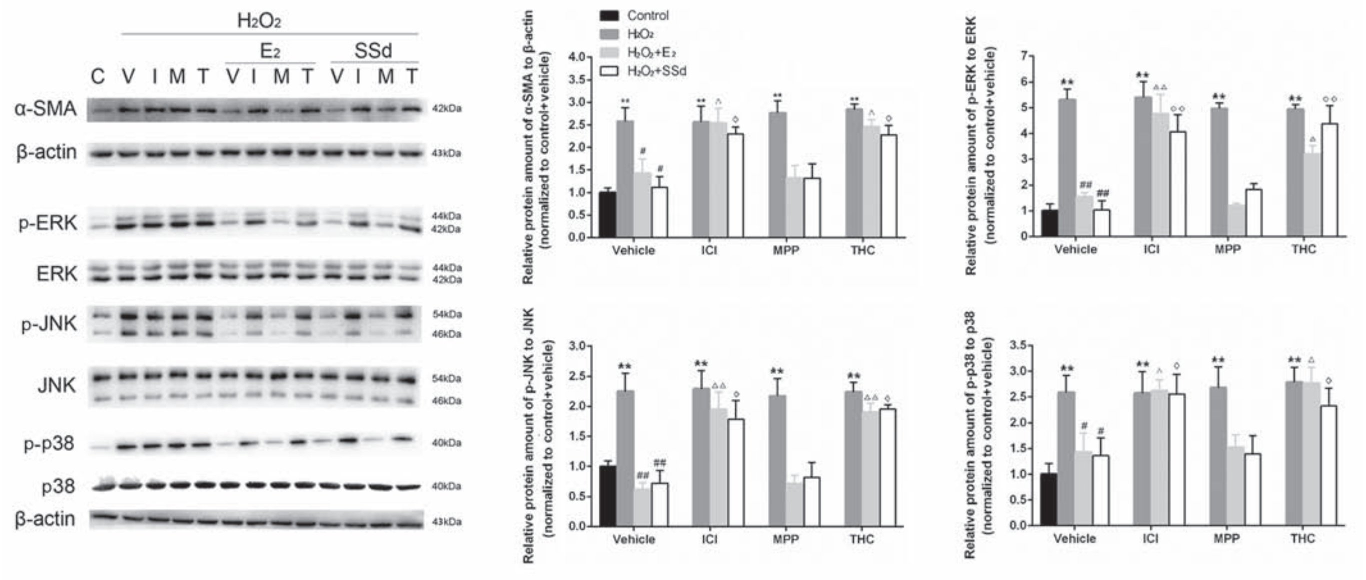

Figure 2. Effects of SSd and E_2 in the presence or absence of ER antagonists on oxidative stress-induced α-SMA, ERK1/2, JNK and p38 expression. HSC-T6 cells were treated with DMSO, 1 μM E_2 or 5 μM SSd, in the presence or absence of 1 μM ICI-182780, MPP or THC for 24 h. Subsequently, oxidative stress was induced by 0.2 mM H_2O_2 for 4 h. Expression levels of α-SMA, and total and p-ERK1/2, JNK and p38 were determined using western blot analysis. Levels of the constitutively expressed protein, β-actin, were comparable in all samples. Representative blotting images and relative semi-quantification of protein levels are presented. C, control; V, vehicle; I, ICI-182780; M, MPP; T, THC. Results are presented as the means ± standard deviation, n=4. $^{**}P<0.01$ vs. the control group with the same ER antagonist treatment (ICI-182780, MPP, THC or DMSO); $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. the H_2O_2 + DMSO group; $^{\triangle}P<0.05$ and $^{\triangle\triangle}P<0.01$ vs. the H_2O_2 + E_2 + DMSO group; $^{\Diamond}P<0.05$ and $^{\Diamond\Diamond}P<0.01$ vs. the H_2O_2 + SSd + DMSO group. α-SMA, α-smooth muscle actin; DMSO, dimethyl sulfoxide; E_2, estradiol; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; H_2O_2, hydrogen peroxide; HSC, hepatic stellate cell; JNK, c-Jun N-terminal kinase; MPP; methylpiperidinopyrazole; p-, phosphorylated; SSd, saikosaponin-d; THC, (R,R)-tetrahydrochrysene.

Statistical analysis. Results are presented as the means + standard deviation. Data were analyzed using an analysis of variance (one-way ANOVA; for multiple groups) with SPSS version 23.0. If ANOVA revealed an overall effect, intergroup differences were analyzed using Newman-Keuls test. Two-sided $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of SSd on the proliferation and activation of HSC-T6 cells in the presence or absence of ER antagonists. It has previously been reported that the protective effects of E_2 against hepatic fibrosis may be associated with its inhibition of HSC activation (7-9). As a phytoestrogen, SSd exerts similar effects (18). The results of an MTT assay indicated that SSd and E_2 significantly suppressed (n=4, $P<0.05$) the proliferation of HSC-T6 cells induced by H_2O_2 treatment (n=4, $P<0.01$) (Fig. 1). Western blot analysis indicated that the expression levels of α-SMA, a fibrotic marker that is highly expressed in activated HSCs, were reduced following incubation with SSd and E_2 (Fig. 2). Conversely, these effects were suppressed by ICI-182780 and THC (n=4, $P<0.05$), but not by MPP.

Effects of SSd on the synthesis and degradation of ECM in the presence or absence of ER antagonists. TGF-β1 is a key mediator that activates HSCs to produce ECM (1,24). In addition, MMP and TIMP are two important factors that regulate degradation of ECM (25). In the present study, SSd and E_2 were able to markedly decrease TGF-β1 (Fig. 3), Hyp, COL1 (Fig. 3A) and TIMP-1 expression (Fig. 4), and increase MMP-1 expression (Fig. 4), thus inhibiting H_2O_2-induced ECM formation. Conversely, the effects of SSd and E_2 were suppressed by ICI-182780 and THC (n=4, $P<0.05$), but not MPP.

Effects of SSd on H_2O_2-induced OS in the presence or absence of ER antagonists. OS is recognized as having a crucial role in HSC activation (5). Recent studies indicated that the liver-protective and antifibrotic effects of SSd may be attributed to its antioxidative capacity (26,27). Consequently, the antioxidative effects of SSd on H_2O_2-induced OS in HSC-T6 cells were explored. Flow cytometry revealed that the control group had low levels of ROS and that ER antagonists alone had no significant effect. Conversely, groups treated with H_2O_2 exhibited a significant increase in ROS (n=3, $P<0.01$), which could be reversed by SSd and E_2 (n=3, $P<0.01$) (Fig. 5A). MDA, which is an end product of lipid peroxidation, and the endogenous antioxidant CuZn-SOD, which indirectly reflects OS status, were detected by ELISA. The results indicated that increased MDA content induced by H_2O_2 (n=4, $P<0.01$) (Fig. 5B) was reduced by SSd and E_2 (n=4, $P<0.05$), whereas CuZn-SOD content (Fig. 5B) was increased by SSd and E_2 (n=4, $P<0.01$). However, the suppressive effects of SSd on OS could be inhibited by ICI-182780 and THC (n=4, $P<0.05$), but not MPP.

Effects of SSd on the MAPK signaling pathway in the presence or absence of ER antagonists. ROS can upregulate the expression of critical fibrosis-associated genes via activation of signal transduction pathways, including MAPKs (28,29). In the present study, the phosphorylation of three MAPK proteins (p-ERK, p-JNK and p-p38) was examined by western blot analysis, in order to provide further evidence regarding the antifibrotic effects of SSd. As shown in Fig. 2, the relative

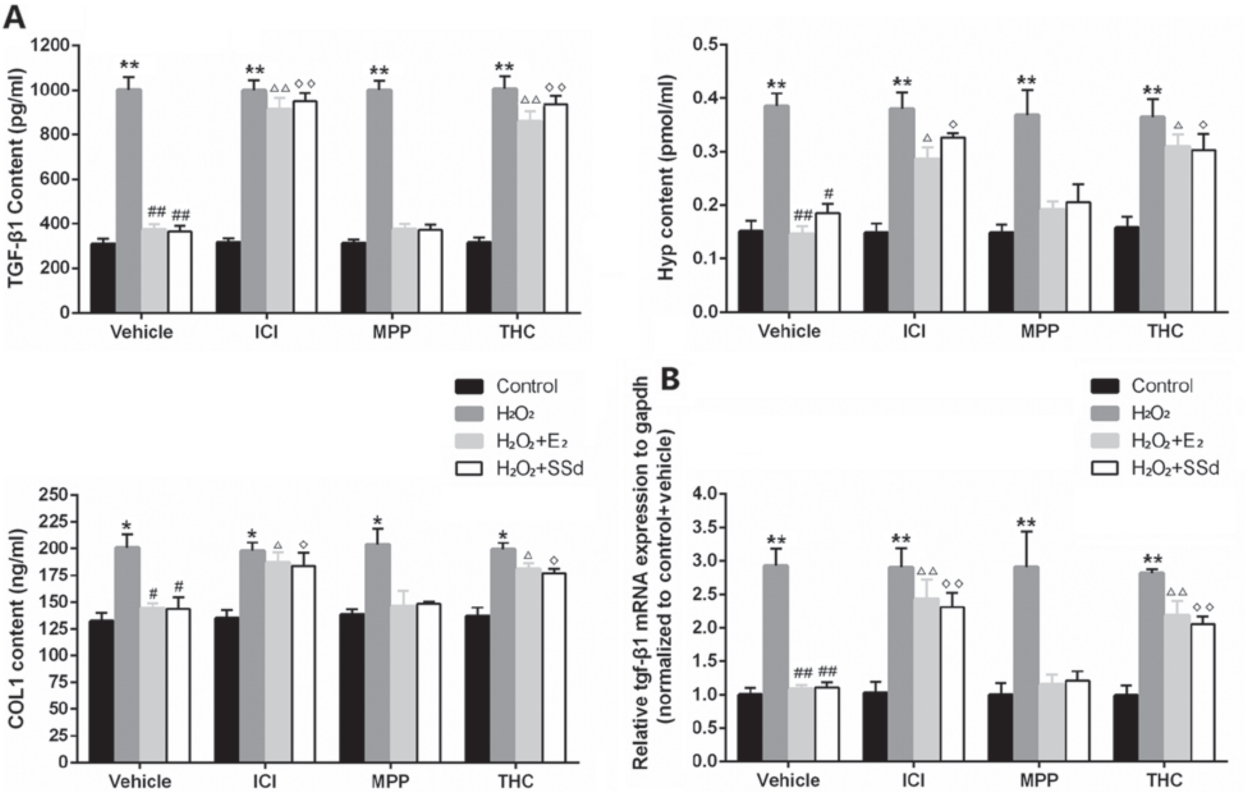

Figure 3. Effects of SSd and E_2 in the presence or absence of ER antagonists on oxidative stress-induced extracellular matrix synthesis. HSC-T6 cells were treated with DMSO, 1 μM E_2 or 5 μM SSd, in the presence or absence of 1 μM ICI-182780, MPP or THC for 24 h. Subsequently, oxidative stress was induced by 0.2 mM H_2O_2 for 4 h. (A) TGF-β1, Hyp, and COL1 contents in cell culture supernatants were detected by ELISA. (B) TGF-β1 mRNA expression was examined by quantitative polymerase chain reaction, with GAPDH mRNA used as an internal control. Results are presented as the means ± standard deviation, n=4. $^*P<0.05$ and $^{**}P<0.01$ vs. the control group with the same ER antagonist treatment (ICI-182780, MPP, THC or DMSO); $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. the H_2O_2 + DMSO group; $^{\triangle}P<0.05$ and $^{\triangle\triangle}P<0.01$ vs. the H_2O_2 + E_2 + DMSO group; $^{\diamond}P<0.05$ and $^{\diamond\diamond}P<0.01$ vs. the H_2O_2 + SSd + DMSO group. COL1, collagen-1; DMSO, dimethyl sulfoxide; E_2, estradiol; ER, estrogen receptor; H_2O_2, hydrogen peroxide; HSC, hepatic stellate cell; Hyp, hydroxyproline; MPP; methylpiperidinopyrazole; SSd, saikosaponin-d; TGF-β1, transforming growth factor-β1; THC, (R,R)-tetrahydrochrysene.

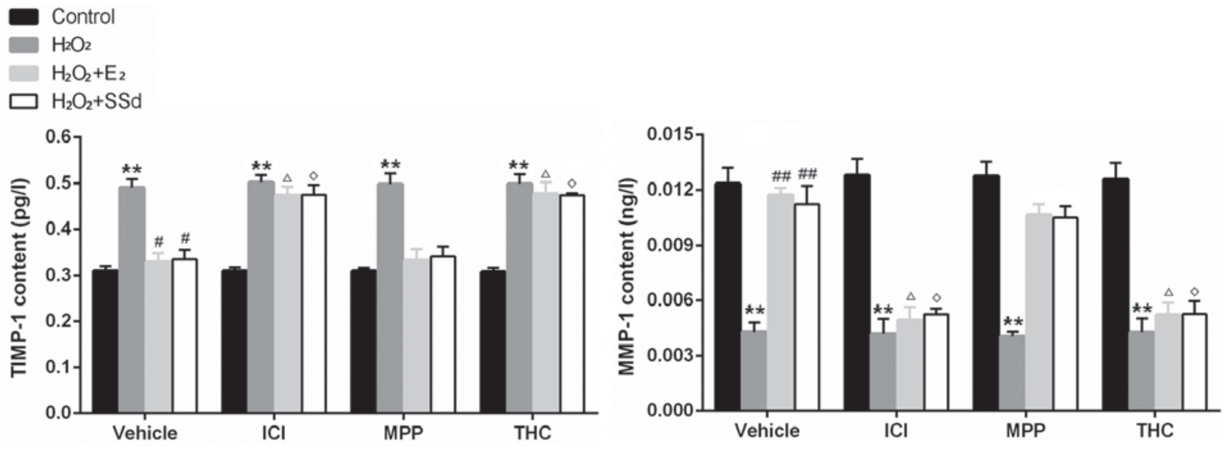

Figure 4. Effects of SSd and E_2 in the presence or absence of ER antagonists on oxidative stress-induced TIMP-1 and MMP-1 expression. HSC-T6 cells were treated with DMSO, 1 μM E_2 or 5 μM SSd, in the presence or absence of 1 μM ICI-182780, MPP or THC for 24 h. Subsequently, oxidative stress was induced by 0.2 mM H_2O_2 for 4 h. TIMP-1 and MMP-1 contents in cell culture supernatants were detected by ELISA. Results are presented as the means ± standard deviation, n=4. $^{**}P<0.01$ vs. the control group with the same ER antagonist treatment (ICI-182780, MPP, THC or DMSO); $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. the H_2O_2 + DMSO group; $^{\triangle}P<0.05$ vs. the H_2O_2 + E_2 + DMSO group; $^{\diamond}P<0.05$ vs. H_2O_2 + SSd + DMSO group. DMSO, dimethyl sulfoxide; E_2, estradiol; ER, estrogen receptor; H_2O_2, hydrogen peroxide; HSC, hepatic stellate cell; MMP-1, matrix metalloproteinase-1; MPP; methylpiperidinopyrazole; SSd, saikosaponin-d; THC, (R,R)-tetrahydrochrysene; TIMP-1, tissue inhibitor of metalloproteinases-1.

expression levels of p-ERK, p-JNK and p-p38 to their respective total proteins were increased following H_2O_2 treatment (n=4, $P<0.01$), whereas SSd and E_2 were able to significantly downregulate expression levels (n=4, $P<0.05$). Conversely, these effects were suppressed by coadministration with ICI-182780 and THC (n=4, $P<0.05$), but not MPP.

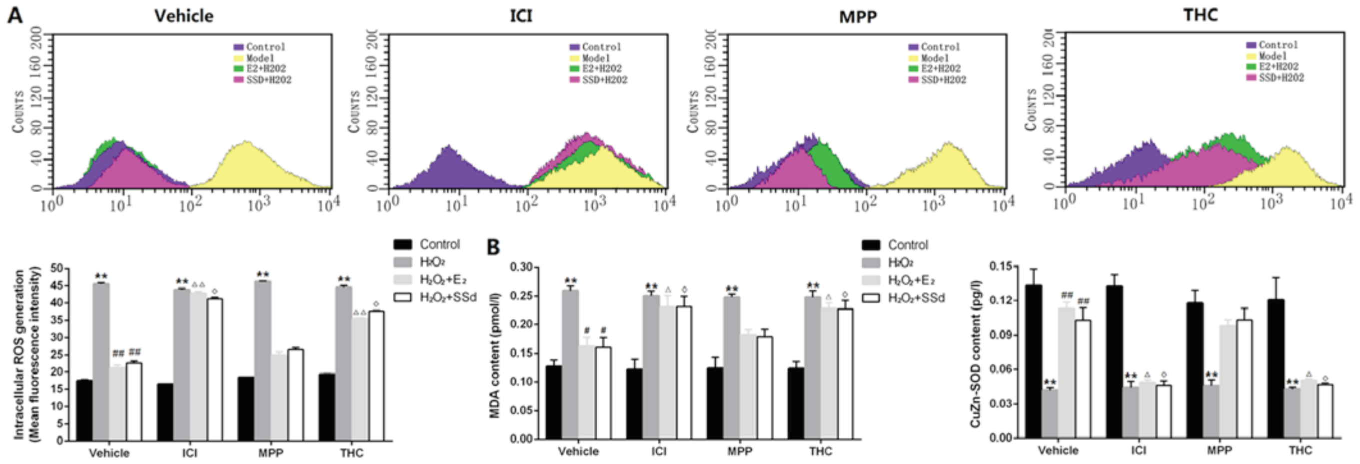

Figure 5. Effects of SSd and E_2 in the presence or absence of ER antagonists on oxidative stress-induced ROS generation, and MDA and CuZn-SOD activity. HSC-T6 cells were treated with DMSO, 1 μM E_2 or 5 μM SSd, in the presence or absence of 1 μM ICI-182780, MPP or THC for 24 h. Subsequently, oxidative stress was induced by 0.2 mM H_2O_2 for 4 h. (A) ROS generation was detected by flow cytometry. (B) MDA and SOD contents in cell culture supernatants were detected by ELISA. Representative flow cytometry images and quantification are presented. Results are presented as the means ± standard deviation, n=3 (A) and n=4 (B). $^{**}P<0.01$ vs. the control group with the same ER antagonist treatment (ICI-182780, MPP, THC or DMSO); $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. the H_2O_2 + DMSO group; $^{\triangle}P<0.05$ and $^{\triangle\triangle}P<0.01$ vs. the H_2O_2 + E_2 + DMSO group; $^{\Diamond}P<0.05$ vs. the H_2O_2 + SSd + DMSO group. CuZn-SOD, CuZn-superoxide dismutase; DMSO, dimethyl sulfoxide; E_2, estradiol; ER, estrogen receptor; H_2O_2, hydrogen peroxide; HSC, hepatic stellate cell; MDA, malondialdehyde; MPP; methylpiperidinopyrazole; ROS, reactive oxygen species; SSd, saikosaponin-d; THC, (R,R)-tetrahydrochrysene.

Discussion

Liver fibrosis and the final stage of liver fibrosis, cirrhosis, represent the final common pathway of virtually all chronic liver diseases (30-32). SSd has been reported to possess antifibrotic activity; however, there is currently little information regarding the effects of SSd on HSCs. Therefore, the present study aimed to investigate the effects of SSd on the proliferation and activation of HSCs, and the underlying mechanisms associated with ERs. The results strongly suggested that SSd may suppress OS-induced activation of HSCs in an ERβ-dependent manner. This finding may be of potential clinical interest, since previous studies have indicated that liver fibrosis is potentially reversible with the reduction of HSC activation (1,25).

Sex hormones may affect the development of hepatic fibrosis and cirrhosis (33). E_2 is an endogenous fibrosuppressant, which has been reported to attenuate liver fibrosis in DMN- or CCl_4-induced rat models (7,8). In addition, previous studies have demonstrated that the protective effects of E_2 against hepatic fibrosis may be associated with its ability to inhibit HSC activation (7-9). E_2 inhibits intracellular pathways and activation processes stimulated by H_2O_2 in cultured rat HSCs via its suppressive effect on lipid peroxidation (10). Previous studies have suggested that phytoestrogens, including isoflavones, resveratrol and genistein, may suppress the progression of liver fibrosis due to their weak estrogen-like activities (34-36). Our previous study demonstrated that SSd exerts estrogen-like actions via activation of the ER signaling pathway (19). The present study further verified that the suppressive effects of SSd on H_2O_2-induced activation of HSC-T6 cells could be reversed by coincubation with ER antagonists, thus confirming that the effects of SSd may be ER-dependent.

OS and ROS are predominant factors in HSC activation (37). Previous studies have reported that SSd possesses marked antioxidant activity (16,17,26,27). Consistently, the present study indicated that SSd could inhibit H_2O_2-induced OS in HSC-T6 cells, as evidenced by decreased ROS and MDA generation, and increased CuZn-SOD activity.

ROS can upregulate the expression of critical fibrosis-associated genes via activation of signal transduction pathways and transcription factors, including MAPKs, activator protein-1 and nuclear factor-κB (5). Suppression of ERK activation is associated with complete inhibition of HSC proliferation *in vitro* (38). In the liver, ERK1 is associated with TGF-β1-induced fibrotic signaling in HSCs (39), whereas ERK2 has a key role in hepatocyte survival (40). In addition, JNK inhibition not only prevents TGF-β-induced murine HSC activation and decreases TGF-β signaling in human HSCs *in vitro*, but also significantly reduces CCl_4-induced liver fibrosis *in vivo* (41). p38 MAPK is also associated with ECM synthesis and degradation. It has previously been reported that phosphorylation of p38 MAPK is augmented in activated HSCs, which is involved in TGF-β1-downregulated MMP-13 expression, as well as in upregulated COL1 expression (24,29). In the present study, OS increased the expression levels of p-ERK, p-JNK and p-p38, whereas SSd and E_2 could significantly downregulate these protein levels and hence inhibit activation of the MAPK pathway. These results are in agreement with the aforementioned involvement of the MAPK pathway. In addition, TGF-β1 is a key mediator that activates HSCs to produce ECM (1,24), whereas MMPs and TIMPs regulate ECM degradation (25). In the present study, SSd and E_2 were able to decrease the expression levels of TGF-β1, Hyp, COL1 and TIMP-1, and increase MMP-1 levels. These effects may induce positive remodeling of liver fibrosis.

The physiological effects of estrogens are mediated through two receptor subtypes, ERα and ERβ. It has been reported that only ERβ, not ERα, is expressed in primary cultured rat HSCs (42). However, our previous study detected both ERα and ERβ proteins by immunofluorescence and western blot analysis in the rat HSC line, HSC-T6 (43). This discrepancy regarding the presence of ERα may be due to the use of different HSC lines. Furthermore, the effects of genistein, a phytoestrogen, on OS and mitochondria may be due, at least in part, to increased ERβ presence, and may be due to upregulation of ERβ induced by genistein (44). It has also been demonstrated that 17β-E_2 protects ARPE-19 cells from OS via an ERβ-dependent mechanism (45). These results suggested that activation of ERβ may be associated with the reduction of ROS production. Conversely, ERα activation has been revealed to inhibit high-glucose-induced proliferation of vascular smooth muscle cells by downregulating ROS-mediated ERK activation, thus suggesting that selective activation of ERα is required for reducing OS (46). In the present study, the effects of SSd together with ICI-182780 (pure ER antagonist), MPP (ERα antagonist) or THC (ERβ antagonist) were explored, in order to determine which ER subtype contributes to the suppression of OS-induced HSC-T6 activation. The results revealed that the suppressive effects of SSd on H_2O_2-induced activation of HSC-T6 cells could be inhibited by coincubation with ICI-182780 or THC, but not MPP. These results strongly suggested that SSd suppresses OS-induced activation of HSCs, and this effect is largely dependent on modulation of ERβ.

In conclusion, the present study is the first, to the best of our knowledge, to suggest that the suppressive effects of SSd towards OS-induced HSC activation depend on ERβ activity, and may be at least partially attributed to inhibition of the ROS-MAPK signaling pathway. These results provided novel evidence regarding the target of SSd and may establish an experimental basis for the development of novel drugs for the treatment of hepatic fibrosis.

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References

1. Bataller R and Brenner DA: Liver fibrosis. J Clin Invest 115: 209-218, 2005.
2. Sánchez-Valle V, Chávez-Tapia NC, Uribe M and Méndez-Sánchez N: Role of oxidative stress and molecular changes in liver fibrosis: A review. Curr Med Chem 19: 4850-4860, 2012.
3. Li JT, Liao ZX, Ping J, Xu D and Wang H: Molecular mechanism of hepatic stellate cell activation and antifibrotic therapeutic strategies. J Gastroenterol 43: 419-428, 2008.
4. Schon HT, Bartneck M, Borkham-Kamphorst E, Nattermann J, Lammers T, Tacke F and Weiskirchen R: Pharmacological Intervention in Hepatic Stellate Cell Activation and Hepatic Fibrosis. Front Pharmacol 7: 33, 2016.
5. Ghatak S, Biswas A, Dhali GK, Chowdhury A, Boyer JL and Santra A: Oxidative stress and hepatic stellate cell activation are key events in arsenic induced liver fibrosis in mice. Toxicol Appl Pharmacol 251: 59-69, 2011.
6. Mormone E, George J and Nieto N: Molecular pathogenesis of hepatic fibrosis and current therapeutic approaches. Chem Biol Interact 193: 225-231, 2011.
7. Yasuda M, Shimizu I, Shiba M and Ito S: Suppressive effects of estradiol on dimethylnitrosamine-induced fibrosis of the liver in rats. Hepatology 29: 719-727, 1999.
8. Xu JW, Gong J, Chang XM, Luo JY, Dong L, Hao ZM, Jia A and Xu GP: Estrogen reduces CCl_4-induced liver fibrosis in rats. World J Gastroenterol 8: 883-887, 2002.
9. Shimizu I, Mizobuchi Y, Yasuda M, Shiba M, Ma YR, Horie T, Liu F and Ito S: Inhibitory effect of oestradiol on activation of rat hepatic stellate cells in vivo and in vitro. Gut 44: 127-136, 1999.
10. Itagaki T, Shimizu I, Cheng X, Yuan Y, Oshio A, Tamaki K, Fukuno H, Honda H, Okamura Y and Ito S: Opposing effects of oestradiol and progesterone on intracellular pathways and activation processes in the oxidative stress induced activation of cultured rat hepatic stellate cells. Gut 54: 1782-1789, 2005.
11. Cuzick J: Hormone replacement therapy and the risk of breast cancer. Eur J Cancer 44: 2344-2349, 2008.
12. Barnes PJ: Corticosteroids: The drugs to beat. Eur J Pharmacol 533: 2-14, 2006.
13. Lee JK, Kim JH and Shin HK: Therapeutic effects of the oriental herbal medicine Sho-saiko-to on liver cirrhosis and carcinoma. Hepatol Res 41: 825-837, 2011.
14. Deng G, Kurtz RC, Vickers A, Lau N, Yeung KS, Shia J and Cassileth B: A single arm phase II study of a Far-Eastern traditional herbal formulation (sho-sai-ko-to or xiao-chai-hu-tang) in chronic hepatitis C patients. J Ethnopharmacol 136: 83-87, 2011.
15. Qin F, Liu JY and Yuan JH: Chaihu-Shugan-San, an oriental herbal preparation, for the treatment of chronic gastritis: A meta-analysis of randomized controlled trials. J Ethnopharmacol 146: 433-439, 2013.
16. Fan J, Li X, Li P, Li N, Wang T, Shen H, Siow Y, Choy P and Gong Y: Saikosaponin-d attenuates the development of liver fibrosis by preventing hepatocyte injury. Biochem Cell Biol 85: 189-195, 2007.
17. Dang SS, Wang BF, Cheng YA, Song P, Liu ZG and Li ZF: Inhibitory effects of saikosaponin-d on CCl_4-induced hepatic fibrogenesis in rats. World J Gastroenterol 13: 557-563, 2007.
18. Chen MF, Huang CC, Liu PS, Chen CH and Shiu LY: Saikosaponin a and saikosaponin d inhibit proliferation and migratory activity of rat HSC-T6 cells. J Med Food 16: 793-800, 2013.
19. Wang P, Ren J, Tang J, Zhang D, Li B and Li Y: Estrogen-like activities of saikosaponin-d in vitro: A pilot study. Eur J Pharmacol 626: 159-165, 2010.
20. Richter DU, Mylonas I, Toth B, Scholz C, Briese V, Friese K and Jeschke U: Effects of phytoestrogens genistein and daidzein on progesterone and estrogen (estradiol) production of human term trophoblast cells in vitro. Gynecol Endocrinol 25: 32-38, 2009.
21. Gille JJ and Joenje H: Cell culture models for oxidative stress: Superoxide and hydrogen peroxide versus normobaric hyperoxia. Mutat Res 275: 405-414, 1992.
22. Kiyoshima T, Enoki N, Kobayashi I, Sakai T, Nagata K, Wada H, Fujiwara H, Ookuma Y and Sakai H: Oxidative stress caused by a low concentration of hydrogen peroxide induces senescence-like changes in mouse gingival fibroblasts. Int J Mol Med 30: 1007-1012, 2012.
23. Coates D, Zafar S and Milne T: Quantitative real-time gene profiling of human alveolar osteoblasts. Methods Mol Biol 1537: 447-459, 2017.
24. Lechuga CG, Hernández-Nazara ZH, Domínguez Rosales JA, Morris ER, Rincón AR, Rivas-Estilla AM, Esteban-Gamboa A and Rojkind M: TGF-beta1 modulates matrix metalloproteinase-13 expression in hepatic stellate cells by complex mechanisms involving p38MAPK, PI3-kinase, AKT, and p70S6k. Am J Physiol Gastrointest Liver Physiol 287: G974-G987, 2004.
25. Iredale JP: Hepatic stellate cell behavior during resolution of liver injury. Semin Liver Dis 21: 427-436, 2001.
26. Zhang BZ, Guo XT, Chen JW, Zhao Y, Cong X, Jiang ZL, Cao RF, Cui K, Gao SS and Tian WR: Saikosaponin-D attenuates heat stress-induced oxidative damage in LLC-PK1 cells by increasing the expression of anti-oxidant enzymes and HSP72. Am J Chin Med 42: 1261-1277, 2014.
27. Zhao L, Zhang H, Bao J, Liu J and Ji Z: Saikosaponin-d protects renal tubular epithelial cell against high glucose induced injury through modulation of SIRT3. Int J Clin Exp Med 8: 6472-6481, 2015.
28. Hong IH, Park SJ, Goo MJ, Lee HR, Park JK, Ki MR, Kim SH, Lee EM, Kim AY and Jeong KS: JNK1 and JNK2 regulate α-SMA in hepatic stellate cells during CCl_4-induced fibrosis in the rat liver. Pathol Int 63: 483-491, 2013.
29. Varela-Rey M, Montiel-Duarte C, Osés-Prieto JA, López-Zabalza MJ, Jaffrèzou JP, Rojkind M and Iraburu MJ: p38 MAPK mediates the regulation of alpha1(I) procollagen mRNA levels by TNF-alpha and TGF-beta in a cell line of rat hepatic stellate cells(1). FEBS Lett 528: 133-138, 2002.
30. Friedman SL: Liver fibrosis - from bench to bedside. J Hepatol 38 (Suppl 1): S38-S53, 2003.
31. Bhaskar ME: Management of cirrhosis and ascites. N Engl J Med 351: 300-301, author reply 300-301, 2004.

32. O'Beirne JP, Foxton MR and Heneghan MA: Management of cirrhosis and ascites. N Engl J Med 351: 300-301, author reply 300-301, 2004.
33. Xu JW, Gong J, Chang XM, Luo JY, Dong L, Jia A and Xu GP: Effects of estradiol on liver estrogen receptor-alpha and its mRNA expression in hepatic fibrosis in rats. World J Gastroenterol 10: 250-254, 2004.
34. Li JF, Chen BC, Lai DD, Jia ZR, Andersson R, Zhang B, Yao JG and Yu Z: Soy isoflavone delays the progression of thioacetamide-induced liver fibrosis in rats. Scand J Gastroenterol 46: 341-349, 2011.
35. Lee ES, Shin MO, Yoon S and Moon JO: Resveratrol inhibits dimethylnitrosamine-induced hepatic fibrosis in rats. Arch Pharm Res 33: 925-932, 2010.
36. Demiroren K, Dogan Y, Kocamaz H, Ozercan IH, Ilhan S, Ustundag B and Bahcecioglu IH: Protective effects of L-carnitine, N-acetylcysteine and genistein in an experimental model of liver fibrosis. Clin Res Hepatol Gastroenterol 38: 63-72, 2014.
37. Novo E, Busletta C, Bonzo LV, Povero D, Paternostro C, Mareschi K, Ferrero I, David E, Bertolani C, Caligiuri A, *et al*: Intracellular reactive oxygen species are required for directional migration of resident and bone marrow-derived hepatic pro-fibrogenic cells. J Hepatol 54: 964-974, 2011.
38. Marra F, Arrighi MC, Fazi M, Caligiuri A, Pinzani M, Romanelli RG, Efsen E, Laffi G and Gentilini P: Extracellular signal-regulated kinase activation differentially regulates platelet-derived growth factor's actions in hepatic stellate cells, and is induced by in vivo liver injury in the rat. Hepatology 30: 951-958, 1999.
39. Zhong W, Shen WF, Ning BF, Hu PF, Lin Y, Yue HY, Yin C, Hou JL, Chen YX, Zhang JP, *et al*: Inhibition of extracellular signal-regulated kinase 1 by adenovirus mediated small interfering RNA attenuates hepatic fibrosis in rats. Hepatology 50: 1524-1536, 2009.
40. Frémin C, Bessard A, Ezan F, Gailhouste L, Régeard M, Le Seyec J, Gilot D, Pagès G, Pouysségur J, Langouët S, *et al*: Multiple division cycles and long-term survival of hepatocytes are distinctly regulated by extracellular signal-regulated kinases ERK1 and ERK2. Hepatology 49: 930-939, 2009.
41. Kluwe J, Pradere JP, Gwak GY, Mencin A, De Minicis S, Osterreicher CH, Colmenero J, Bataller R and Schwabe RF: Modulation of hepatic fibrosis by c-Jun-N-terminal kinase inhibition. Gastroenterology 138: 347-359, 2010.
42. Zhou Y, Shimizu I, Lu G, Itonaga M, Okamura Y, Shono M, Honda H, Inoue S, Muramatsu M and Ito S: Hepatic stellate cells contain the functional estrogen receptor beta but not the estrogen receptor alpha in male and female rats. Biochem Biophys Res Commun 286: 1059-1065, 2001.
43. Li Y, Liu J, Lin L, Que R, Shen Y and Tao Z: Regulation of saikosaponin-d on the level of of ERα and ERβ protein in hepatic stellate cells. Trad Chin Drug Res Clin Pharmacol 27: 58-61, 2016 (In Chinese).
44. Nadal-Serrano M, Pons DG, Sastre-Serra J, Blanquer-Rosselló MM, Roca P and Oliver J: Genistein modulates oxidative stress in breast cancer cell lines according to ERα/ERβ ratio: Effects on mitochondrial functionality, sirtuins, uncoupling protein 2 and antioxidant enzymes. Int J Biochem Cell Biol 45: 2045-2051, 2013.
45. Giddabasappa A, Bauler M, Yepuru M, Chaum E, Dalton JT and Eswaraka J: 17-β estradiol protects ARPE-19 cells from oxidative stress through estrogen receptor-β. Invest Ophthalmol Vis Sci 51: 5278-5287, 2010.
46. Ortmann J, Veit M, Zingg S, Di Santo S, Traupe T, Yang Z, Völzmann J, Dubey RK, Christen S and Baumgartner I: Estrogen receptor-α but not -β or GPER inhibits high glucose-induced human VSMC proliferation: Potential role of ROS and ERK. J Clin Endocrinol Metab 96: 220-228, 2011.

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