Luteoloside Ameliorates Palmitic Acid-Induced *in Vitro* Model of Non-alcoholic Fatty Liver Disease via Activating STAT3-Triggered Hepatocyte Regeneration

Abstract. Luteoloside (Lute), a bioactive natural ingredient, widely exists in nature and possesses hepatoprotective and hepatocyte proliferation-promoting properties. This study aimed to investigate whether Lute could counteract non-alcoholic fatty liver disease (NAFLD)-caused hepatocyte damage via its stimulation of hepatocyte regeneration efficacy and to explore the involved mechanism. LO2 cells and primary hepatocytes were used to examine the hepatocyte proliferation effects of Lute under physiological conditions and in the palmitic acid (PA)-induced *in vitro* model of NAFLD. STAT3 and cell cycle-related proteins (cyclin D1, c-myc and p21) were evaluated by Western blot. Under physiological conditions, LO2 cells and primary hepatocytes treated with various concentration of Lute for 12 and 24 h showed increased hepatocyte proliferation, especially with 20 μM treatment for 24 h. More notably, under the model conditions, co-incubation with 20 μM of Lute also markedly reversed PA-induced inhibition of cell proliferation and viability in primary hepatocytes. Mechanistically, Lute could activate STAT3 and subsequently increase cyclin D1 and c-myc expression, which positively regulates cell cycle progression, and decrease expression of p21, an inhibitor of cell cycle progression. Furthermore, Lute-induced hepatocyte proliferation-promoting efficacy was abolished by STAT3 inhibitor statistic. Collectively, Lute can alleviate PA-induced hepatocyte damage via activating STAT3-mediated hepatocyte regeneration.

Introduction

Non-alcoholic fatty liver disease (NAFLD), characterized by hepatic steatosis, has become one of the most common chronic liver diseases with 25% prevalence worldwide, and if unattended, it renders further hepatic injuries such as non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and even hepatocellular carcinoma (Younossi et al., 2016; Amirinejad et al., 2020). Currently, either drug therapy or lifestyle modifications for NAFLD management are focused on intervening “two hits” in NAFLD, namely, alleviating hepatic steatosis (the “first hit”), oxidative stress and inflammatory response (the “second hit”), and unfortunately both of them lack the anticipated outcomes (Zhang et al., 2015; Jin et al., 2017). Dowman et al. (2010) reported that the deficiency in hepatocyte regeneration may represent the “third hit” in NAFLD pathogenesis. However, few studies have investigated whether facilitating hepatocyte proliferation exhibits an anti-NAFLD efficacy.

The liver has a powerful regenerative capability, mediated by hepatocyte proliferation in response to liver injuries, and this is an indispensable process for successful treatment of some chronic liver diseases (Ezaki et al., 2009; Fausto et al., 2012). Multiple pathways and well-orchestrated stages are involved in the hepatocyte proliferation process, among which the STAT3 pathway plays a key role in the early stage of hepatocyte prolif-
Lonicera japonica is a medicinal plant that is known for its anti-nAFLD drugs. Potent agents for activating the STAT3 pathway include gao et al., 2012; Fu et al., 2019. Thus, the pursuit of which are responsible for the initiation of cell cycle gene expression (Taub, 2004). Lu et al. (2018) reported that Tmub1 suppressed liver regeneration by inactivating STAT3-mediated transcription of cell cycle genes such as cyclin D1 and c-myc, which are responsible for the initiation of cell cycle (Gao et al., 2012; Fu et al., 2019). Therefore, the present study aimed to explore whether Lute-boosted proliferation-promoting effect on LO2 cells (a human hepatocyte cell line) in a dependent manner (Zhao et al., 2019). However, whether Lute-mediated hepatic proliferation-promoting efficacy can rescue NAFLD is still unknown.

Therefore, in the present study, a palmitic acid (PA)-induced in vitro model of NAFLD was used to explore whether Lute-enhanced proliferation-promoting effect can counteract the “third hit” occurring in NAFLD and its underlying mechanism.

**Material and Methods**

**Reagents**

- Lute (HPLC ≥ 98%, BR, MW: 448.38, CAS: 5373-11-5), silymarin (Sil) (HPLC ≥ 98%, BR, MW: 482.44, CAS: 142797-34-0), sodium palmitate, and Oil Red O were bought from Sigma-Aldrich (St. Louis, MO). Stattic, a STAT3 inhibitor, was purchased from Apexbio (Shanghai, China). DMEM medium, phosphate-buffered saline (PBS), HBSS (Ca²⁺ and Mg²⁺ free), trypsin-EDTA, penicillin-streptomycin, ITS supplement, type I collagen, and dexamethasone were obtained from Gibco (Carlsbad, CA). Specific antibodies against p-STAT3, STAT3, c-myc, and p21 were from Cell Signaling Technology (Beverly, MA), and against β-actin from Santa Cruz Biotechnology (Santa Cruz, CA). Thiazolyl blue tetrazolium bromide (MTT) and 5-ethynyl-2'-deoxyuridine (EdU) Cell Proliferation Kit were provided as outlined above following the previously reported instructions.

**Cell culture and treatment**

LO2 cells, a human hepatocyte cell line, were bought from Shanghai Cell Bank of Chinese Academy of Sciences and primary hepatocytes were isolated from rats. LO2 cells and primary hepatocytes were respectively cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin, and William’s Medium E containing 5% FBS, ITS, 5 nM dexamethasone and 1% penicillin-streptomycin in a humidified incubator (Thermo Fisher Scientific, Waltham, MA) at 37 °C with 5% CO₂. PA stock solution (5 mM) was prepared per a previously reported protocol (Parra-Vargas et al., 2018). Under physiological conditions, namely, culture in normal medium without PA, LO2 cells and primary hepatocytes were co-incubated with Lute (5, 10, 20, 40, 80 μM) with or without stattic (5 mM) was prepared per a previously reported protocol. After the treatment as outlined above, 10 μl of MTT stock solution (5 mg/ml) was added into each well and incubated for another 4 h. Then, the supernatant was removed and the formazan crystals were dissolved using DMSO with gentle agitation for 15 min. Finally, absorbance was measured at the wavelength of 490 nm by a microplate reader (Thermo Fisher Scientific).

**Cell proliferation (EdU) assay**

LO2 cells and primary hepatocytes were seeded in 24-well plates at densities of 4 × 10⁵ cells/well and 3 × 10⁵ cells/well, respectively, and cultured overnight. After treatment, cell proliferation was detected using an EdU Cell Proliferation Kit following the manufacturer’s instructions.

**Oil Red O Staining**

Primary hepatocytes were seeded in 96-well plates at a density of 6 × 10⁶ cells/well and cultured overnight. Afterwards, the cells were co-treated with different concentrations of PA (62.5, 125, 250, and 500 μM) for 24 h. Then, lipid accumulation in primary hepatocytes was examined by Oil Red O staining according to the manufacturer’s protocol. Photographs were obtained with an inverted microscope (Carl Zeiss, Axiol Scope A1 pol, Jena, Germany) at 400× magnification.

**Western blot**

Western blot was performed after the cells were treated as outlined above following the previously reported method (Lee et al., 2019). Briefly, primary hepatocytes were lysed, and followed centrifugation at 12000 rpm for 15 min at 4 °C in a low-temperature/high speed centrifuge (Heraeus Megafuge 8R, Thermo Scientific). Then, the protein concentration was quantified with the BCA method and protein samples were prepared by boiling...
for 5 min with loading buffer. Equal amounts of proteins (40 μg) were subjected to 12% SDS-PAGE gel and then transferred to NC membranes, which were subsequently blocked by 5% BSA for 1 h at room temperature. After being rinsed with TBST, the NC membranes were sequentially incubated with the following primary antibodies: p-STAT3 (Abcam, Cambridge, UK, #ab267373, rabbit monoclonal), STAT3 (Abcam, #ab68153, rabbit monoclonal), cyclin D1 (CST, #55506, rabbit monoclonal), c-myc (CST, #18583, rabbit monoclonal), p21 (Abcam, #ab109520, rabbit monoclonal), and β-actin (Proteintech, #20536-1-AP, rabbit polyclonal) at 4°C overnight and HRP-conjugated secondary antibody (Proteintech, #SA00001-2, goat polyclonal) for 1 h at room temperature. Finally, the protein bands were detected with an enhanced chemiluminescence (eCL) kit and quantified using Image J (National Institutes of Health, Bethesda, MD).

Statistical analysis

Quantitative data are shown as the mean ± standard deviation (SD) and analysed by one-way analysis of variance (ANOVA) followed by examination of differences between pairs of means with Tukey’s multiple comparison test (SPSS 19.0). P values less than 0.05 were considered to be statistically significant.

Results

Lute promoted proliferation of LO2 cells and primary hepatocytes under the physiological conditions

To investigate the effect of Lute (Fig. 1) on cell viability and proliferation, LO2 cells and primary hepatocytes were co-cultured with various concentrations of Lute for 12 and 24 h. As illustrated in Fig. 2A and B, the cell viability of LO2 cells and primary hepatocytes was notably increased with 5 to 40 μM Lute treatment for 12 and 24 h (P < 0.05) and reached the maximum efficiency at 20 μM treatment for 24 h (P < 0.01), which was even better than the positive drug Sil (20 μM). Next, the cells were treated with 20 μM Lute for 24 h to further detect whether Lute exhibited a pro-proliferation effect on cells. As shown in Fig. 2C and D, compared to the control group, PA could remarkably decrease the cell viability and cell proliferation of primary hepatocytes (P < 0.01), while co-incubation with Lute or Sil for 24 h significantly reversed those alterations (P < 0.01), suggesting that Lute protected against PA-induced in vitro model of NAFLD mainly through promoting cell proliferation. Moreover, Lute showed robust effectivity in promoting cell proliferation in primary hepatocytes with EC50 equivalent to 6.82 μM.

Lute countered PA-induced inhibition of primary hepatocyte proliferation

We found that Lute could induce cell proliferation under physiological conditions. Next, we tested whether Lute could promote cell proliferation in a PA-induced in vitro model of NAFLD as well. First, we screened the best in vitro model conditions for NAFLD and found that 250 μM of PA incubation for 24 h could substantially increase lipid accumulation and reduce cell viability in primary hepatocytes (P < 0.01, Fig. 3A and B), suggesting successful establishment of an in vitro model of NAFLD. Then, we investigated Lute’s protective effects on cell viability and cell proliferation of primary hepatocytes under the above-screened model conditions. As presented in Fig. 3C and D, compared to the control group, PA could remarkably decrease the cell viability and cell proliferation of primary hepatocytes (P < 0.01), while co-incubation with Lute or Sil for 24 h significantly reversed those alterations (P < 0.01), suggesting that Lute protected against PA-induced in vitro model of NAFLD mainly through promoting cell proliferation. Moreover, Lute showed robust effectivity in promoting cell proliferation in primary hepatocytes with EC50 equivalent to 6.82 μM.

STAT3 mediated Lute-induced cell proliferation in primary hepatocytes

STAT3 plays a key role in the cell cycle through tuning its downstream proteins such as cyclin D1, c-myc and p21. We thus investigated the expression of STAT3 and its downstream proteins following Lute treatment to further elucidate its mechanism of promoting cell proliferation. The results showed that PA significantly inhibited p-STAT3 expression, which indicated inactivation of STAT3, and resultantly decreased cyclin D1 and c-myc expression and increased p21 expression. However, Lute
Fig. 2. Effects of Lute on LO2 cell and primary hepatocyte proliferation under physiological conditions

(A) Cell viability of LO2 cells following treatment with various concentrations of Lute (5, 10, 20, 40, and 80 μM) or Sil for 12 and 24 h. (B) Cell viability of primary hepatocytes following treatment with various concentrations of Lute (5, 10, 20, 40, and 80 μM) or Sil for 12 and 24 h. (C) Cell proliferation of LO2 cells following treatment with Lute (20 μM) and Sil (20 μM) for 24 h. (D) Cell proliferation of primary hepatocytes following treatment with Lute (20 μM) and Sil (20 μM) for 24 h. *P < 0.05, **P < 0.01 compared to the control group.
could dramatically reverse these changes in a dose-dependent manner (P < 0.01, Fig. 4A and B). Additionally, statin, an inhibitor of STAT3, abolished the promoting effect of Lute on cell proliferation (Fig. 5). Collectively, these results suggested that Lute promoted hepatocyte proliferation through triggering STAT3-regulated cell cycle-related proteins.

Discussion
NAFLD is a common chronic liver disease worldwide and can cause progressive hepatocellular injury. A number of previous studies focused on diminishing lipid accumulation, inflammation, or oxidative stress in the liver, yet ignored the potential curative effect of hepatocyte proliferation in NAFLD. Dowman et al. found that...
Fig. 4. Expression of cell cycle-related proteins following Lute (10 and 20 μM) treatment for 24 h in primary hepatocytes under the model conditions. (A) Protein expression of p-STAT3, STAT3, cyclin D1, c-myc, and p21 in primary hepatocytes. (B) Quantitative analysis of target proteins. *P < 0.05, **P < 0.01 compared to the control group.

Fig. 5. STAT3 activation mediated the pro-proliferation effects of Lute in primary hepatocytes. (A) Expression levels and quantitative analysis of p-STAT3 and STAT3 in primary hepatocytes after treatment with various concentrations of stattic (5, 10, 15, and 20 μM) for 24 h. (B) Cell viability of primary hepatocytes after co-culturing with Lute with or without stattic for 24 h. (C) Cell proliferation of primary hepatocytes following co-culturing with Lute with or without stattic for 24 h. *P < 0.01 compared to the control group; **P < 0.01 compared to the PA group; ***P < 0.01 compared to the Lute20 group.
inhibiting the replication of mature hepatocytes contributed to NAFLD progression and termed this phenomenon as the “third hit” in NAFLD (Downman et al., 2010). However, few studies have investigated the role of hepatocyte proliferation in NAFLD, and whether promoting hepatocyte proliferation can counteract NAFLD is still unknown. In this study, to the best of our knowledge, we were the first to demonstrate that Lute could promote hepatocyte proliferation and rescue cell viability of hepatocytes in a PA-induced in vitro model of NAFLD. Investigation of the mechanism revealed that activation of STAT3-regulated cell cycle-related proteins might contribute to the Lute’s favourable effects.

Under physiological conditions, Lute could facilitate hepatocyte proliferation in a dose-dependent manner within a certain concentration range, consistent with a previously reported work (Zhao et al., 2019). However, whether Lute can induce hepatocyte proliferation in NAFLD and its underlying mechanisms are undefined. STAT3 regulates expression of various genes in response to liver injuries and plays a crucial role in cell growth and proliferation (Moh et al., 2007). Cyclin D1 and c-myc are both downstream proteins of STAT3, which play key roles in the initiation of cell cycle (Kurinna and Barton, 2011). p21, a cyclin-dependent kinase inhibitor, negatively regulates cell cycle progression and blocks DNA synthesis, which is also modulated by STAT3 (Torbenson et al., 2002; Gartel and Radhakrishnan, 2005). In our study, Lute could increase hepatocyte proliferation under the in vitro NAFLD conditions and dose-dependently augment cell viability of hepatocytes. Further investigation found that Lute dose-dependently activated STAT3 and then up-regulated expression of cyclin D1 and c-myc and down-regulated p21 expression. Additionally, inhibition of STAT3 with stat3tumorigenesis in the liver. J. Hepatol. 43, 430-441.


