

Original Article

Mesangial cells can be affected by elevated concentration of free fatty acids in the cell proliferation, cell cycle and apoptosis

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Abstract: Elevated serum levels of free fatty acids (FFAs) are correlated with the deterioration of renal function in patients with chronic kidney disease (CKD). However, it was still unclear whether there would be and what were the effects of elevated FFAs on mesangial cells. A cell line of rat mesangial cells (HBZY-1) was treated with different concentrations of linoleic acids (LA) (0.25, 0.5, 1.0 mmol/L). Non-treated cells served as controls. Intracellular lipid deposition, cell proliferation activity, cell cycle, and apoptosis of mesangial cells were respectively assessed by Oil Red O staining, MTT, and flow cytometry at 24, 48, and 72 hours after stimulation. One-way ANOVA was used to do statistical analysis and $P < 0.05$ considered as significant. Compared with controls, the cells treated with 0.5 and 1.0 mmol/L LA were significantly decreased in cell proliferation activity, but increased in the count of intracellular lipid droplets, percentage of G0/G1 cell cycle arrest, and apoptotic rate ($P < 0.05$ or $P < 0.01$). Elevated concentrations of FFAs (≥ 0.5 mmol/L LA) may bring adverse effects on mesangial cells, including inhibition of cell proliferation activity and induction of cell cycle arrest and apoptosis.

Keywords: Mesangial cells, free fatty acids, cell proliferation, cell cycle, apoptosis

Introduction

Mesangial cells are specialized smooth muscle cells around tiny blood vessels in the kidney accounting for 30%~40% of intrinsic glomerular cell totals and help regulate the filtration process of blood while providing support for the glomerular structure [1]. Their abnormal proliferation, apoptosis and phenotype transdifferentiation are closely related to glomerular sclerosis and renal disease progression [2].

Free fatty acids (FFAs) are important energy-generating nutrients for the body [3]. But once the concentration of FFAs exceeds the normal physiological range, it may bring harm to cells and tissues or organs. Reportedly, a high concentration of fats or lipids in the blood is an independent risk factor for chronic kidney disease (CKD) [4]. Furthermore, elevated serum levels of FFAs is a concomitant feature of CKD

and positively correlated with the deterioration of renal function in patients with CKD [5].

Linoleic acid (LA) is a polyunsaturated omega-6 fatty acid and abundant in many nuts, fatty seeds and their derived vegetable oils. It is an essential fatty acid that must be consumed for proper health because it is one of the lipids of cell membranes and it can not be synthesized from other food components [6, 7]. It has been reported that 0.2 mmol/L oleic acid (monounsaturated omega-9 fatty acid) can induce a myofibroblast phenotype transdifferentiation in mesangial cells [8]. This study tested the hypothesis that elevated concentrations of LA could also induce pathological changes of mesangial cells.

The serum level of FFAs observed in healthy controls ranges from 0.258 to 0.495 (mean 0.388) mmol/L, and the level in the patients

with coronary artery disease is from 0.299 to 0.625 (mean 0.45) mmol/L [9]. Other studies reported that the average level in the patients with type-2 diabetes and hyperlipidemia were respectively 0.6268 ± 0.2971 mmol/L [10] and 0.9415 ± 0.0551 mmol/L [11]. From these, the serum concentration of FFAs alter in the range of from 0.25 to 1.0 mmol/L were determined. Therefore, three different concentrations of LA, 0.25, 0.5 and 1.0 mmol/L were used to investigate the effects of LA on mesangial cells in the aspects of intracellular lipid deposition, cell proliferation, cell cycle, and cell apoptosis.

Materials and methods

Reagents

The main reagents used in this study included: DMEM/F12 (1:1) (Hyclone, USA); fetal bovine serum (FBS) (Hyclone, USA); linoleic acids (LA) (MP, USA); 1% FFAs-free bovine serum albumin (BSA) (MP, USA); propidium iodide (PI) (MP, USA); MTT (MP, USA); RNase (MP, USA); Annexin V-FITC Apoptosis Detection kit (MP, USA); Binding buffer (MP, USA); Oil Red O (MP, USA); DMSO (Sigma, USA); paraformaldehyde (Sigma, USA).

Cell line & cell culture

A cell line of rat mesangial cells (HBZY-1) purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) was maintained in growth media (DMEM with 10% FBS, 2% penicillin/streptomycin, 5.5 mmol/L glucose) and then put into an incubator for culturing (37 Celsius, 5% CO₂, 100% humidity). When the cells grew and covered about 80% the bottom, they were in the log phase. After digestion with 0.25% trypsin, the cells were collected, suspended, diluted to a concentration of 2.5×10^4 cells/ml, and then switched to 24-well (800 ul per well or 2×10^4 cells per well) or 96-well culture plate (200 ul per well or 5×10^3 cells per well). All cells were plated in the wells at least 24 hours before treatment.

Cell stimulation

As FFAs are fat-soluble and *in vivo* are transported via the blood in the form of free fatty acid-albumin complex, FFA-free bovine serum albumin was selected as the carrier of FFAs and we used three different concentrations of LA

(0.25, 0.5, 1 mmol/L) to simulate the effects of high FFAs on mesangial cells *in vivo*. To induce LA overloading, the mesangial cells were exposed to a mixture of the three different concentrations of LA in media containing 1% FFAs-free BSA. The cells that were treated with the media only containing 10% FBS or 1% FFAs-free BSA served as controls.

Detection of intracellular lipid deposition

Then, 24 h after treatment, the cells were rinsed twice with PBS and followed by 30 minute fixation (4% paraformaldehyde) and 10 minute staining using 0.2% Oil Red O staining solution (0.5 g Oil Red O powder dissolved in 60% ethanol). Then the Oil Red O staining solution was discarded with pipette. The cells were rinsed twice with 60% isopropyl alcohol and stained again with hematoxylin dye for 10 minutes. Finally, the cells were washed with PBS and observed under an inverted microscope. The number of lipid droplets was counted in 5 high power fields.

MTT cell proliferation assay

At 24, 48, and 72 hours after the treatment, the culture media was discarded and the cells were rinsed once with PBS. 200 ul 0.5% MTT solution was added to each well. After 4 hour incubation, the supernatant was pipetted out and then 150 ul DMSO added to. The 96-well culture plate was shaken by a swing bed for 10 minutes and then put on microplate reader (Sigma, USA) for measuring absorbance value at 490 nm wavelength.

Cell cycle analysis

The cells were collected 48 h after treatment, centrifuged (1000 rpm, 5 minutes) and washed once with PBS. After fixation in 70% pre-cooling ethanol (1 ml) for 1~2 h at 4 Celsius, the cells were re-suspended with PBS, treated with RNase (50 mg/mL), and re-suspended in PBS containing PI (0.05 mg/mL in 3.8 mol/L sodium citrate) at room temperature for 30 minutes. Then, the stained cells were analyzed using flow cytometry (FACS Caliber, Becton-Dickinson, San Jose, CA) according to the manufacturer's instructions and the data stored as list-mode files. DNA cell cycle histograms were analyzed and modeled using ModFit and WinList software (Verity Software House, Topsham, ME, USA). Twenty thousand cells were analyzed in triplicate for each sample.

Effects of FFAs on mesangial cells

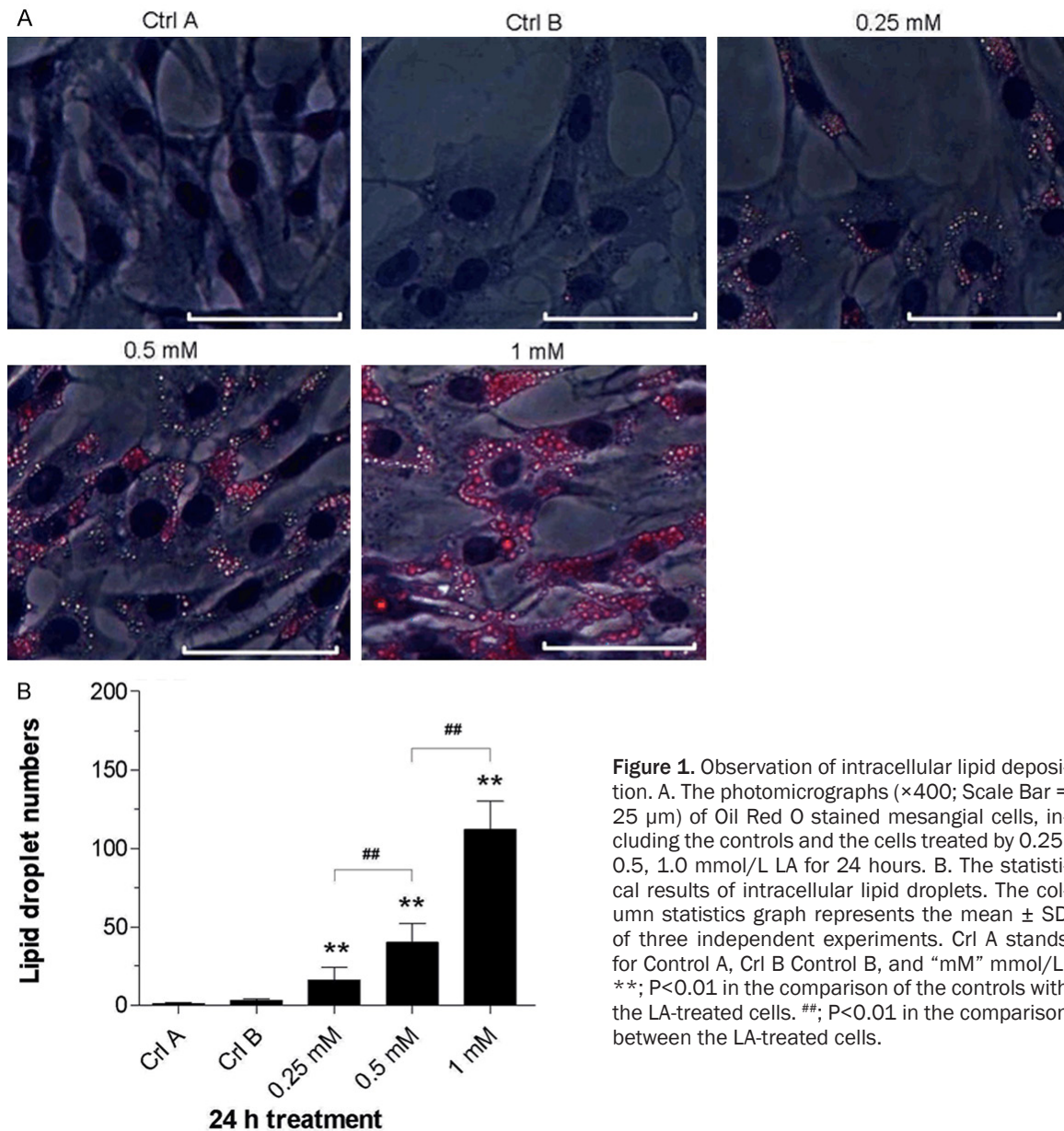


Figure 1. Observation of intracellular lipid deposition. A. The photomicrographs ($\times 400$; Scale Bar = 25 μm) of Oil Red O stained mesangial cells, including the controls and the cells treated by 0.25, 0.5, 1.0 mmol/L LA for 24 hours. B. The statistical results of intracellular lipid droplets. The column statistics graph represents the mean \pm SD of three independent experiments. Ctrl A stands for Control A, Ctrl B Control B, and "mM" mmol/L. **, $P < 0.01$ in the comparison of the controls with the LA-treated cells. ##, $P < 0.01$ in the comparison between the LA-treated cells.

Cell apoptosis assay

Cell apoptosis was determined using an Annexin V-FITC Apoptosis Detection kit according to the manufacturer's protocol [12]. Then 48 hours after treatment, the cell culture solution that contained suspended apoptotic or necrotic cells was collected in a centrifuge tube. Furthermore, the cells were washed twice with 37 Celsius preheated PBS and then digested with 1 ml 37 Celsius preheated 0.25% trypsin for 2 minutes. Subsequently the collected culture solution was pipetted to the cells and made them mixed well. After centrifugation (1000 rpm, 5 minutes), the supernatant was

discarded and the cells were collected again and re-suspended in 500 μL 1:4 deionized-water diluted binding buffer and adjusted to a concentration of $1 \times 10^6/\text{mL}$. 100 μL of cell suspension was pipetted into 5 ml FACS tubes, added 5 μL Annexin V-FITC, followed by mixing, incubation at room temperature in the dark for 15 minutes, adding 10 μL PI solution (120 $\mu\text{L}/\text{mL}$), mixing gently and 5 minutes standing on ice bath in the dark. Then, the apoptotic cells were analyzed using flow cytometry. Annexin V-FITC and PI emissions were detected in the FL 1 and FL 2 channels. Early apoptotic cells were expressed as percentage of cells positive for annexin V and negative for PI.

Effects of FFAs on mesangial cells

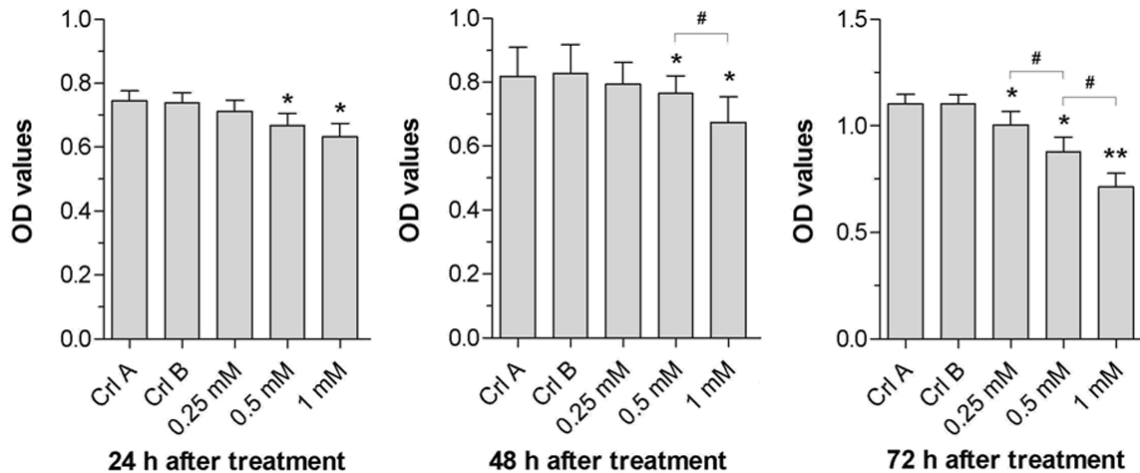


Figure 2. Cell proliferation. The column statistics graph represents the mean \pm SD of absorbance OD value of three independent experiments. **, $P < 0.01$ and *, $P < 0.05$ in the comparison of the controls with the LA-treated cells. #, $P < 0.05$ in the comparison between the LA-treated cells.

Statistical analysis

All data are expressed as mean \pm SD and processed with the statistics software package SPSS 13.0. The data of each group, including intracellular lipid droplets, absorbance OD value of MTT colorimetric assay, percentage of cell cycle phase and apoptotic rate, were first tested by the Levene's test for homogeneity of variance, and then the group differences were analyzed with LSD test in one-way ANOVA if homogeneity of variance or with Dunnett T3 test if heterogeneity of variance. $P < 0.05$ would normally be considered significant.

Results

Intracellular lipid deposition

The cells of two controls had little distribution of red-dyed lipid droplets in the cytoplasm and morphologically they were normal. In the cytoplasm of cells treated with 0.25, 0.5, and 1.0 mmol/L LA, the red dyed lipid droplets could be clearly seen and their counts and size were significantly increased with the concentration rise ($P < 0.01$). Additionally, the 0.5 and 1.0 mmol/L LA-treated cells looked plumper in cytoplasm and larger in nucleus and part of them could be seen membrane damage and nucleus offsets (Figure 1A, 1B).

Cell proliferation

The cells' absorbance value (OD value) was measured at 490 nm wavelength in the MTT colorimetric assay 24, 48, and 72 hours after treatment respectively. There was no signifi-

cant difference between the two controls ($P > 0.05$). Compared with the controls, the cells treated with 0.5 and 1.0 mmol/L LA were significantly decreased in the OD value at 24, 48, 72 hours after treatment ($P < 0.01$ or $P < 0.05$), indicating that elevated concentration of LA may inhibit mesangial cell proliferation (Figure 2).

Cell cycle

Cell cycle was determined 48 hours after treatment. Compared to the controls, the percentage of cells in G0/G1 phase was significantly higher in the cells treated with 0.5 and 1.0 mmol/L LA ($P < 0.05$). Additionally, the percentage of cells in G0/G1 phase went up gradually with the increase of concentration of LA. The percentage of cells in S phase was decreased significantly in the cells treated with 1.0 mmol/L LA ($P < 0.05$). There was no significant difference in the percentage of mesangial cells in G2/M phase between the LA-treated cells and controls (Figure 3A, 3B).

Cell apoptosis

Cell apoptosis was determined 48 hours after treatment. Compared to the controls, the apoptotic rate of cells treated with 0.5 and 1.0 mmol/L LA were significantly increased ($P < 0.01$). The higher the concentration, the higher the apoptotic rate. The difference in the apoptotic rate between 0.5 and 1.0 mmol/L LA treated cells was also significant ($P < 0.01$) (Figure 4), meaning that elevated concentra-

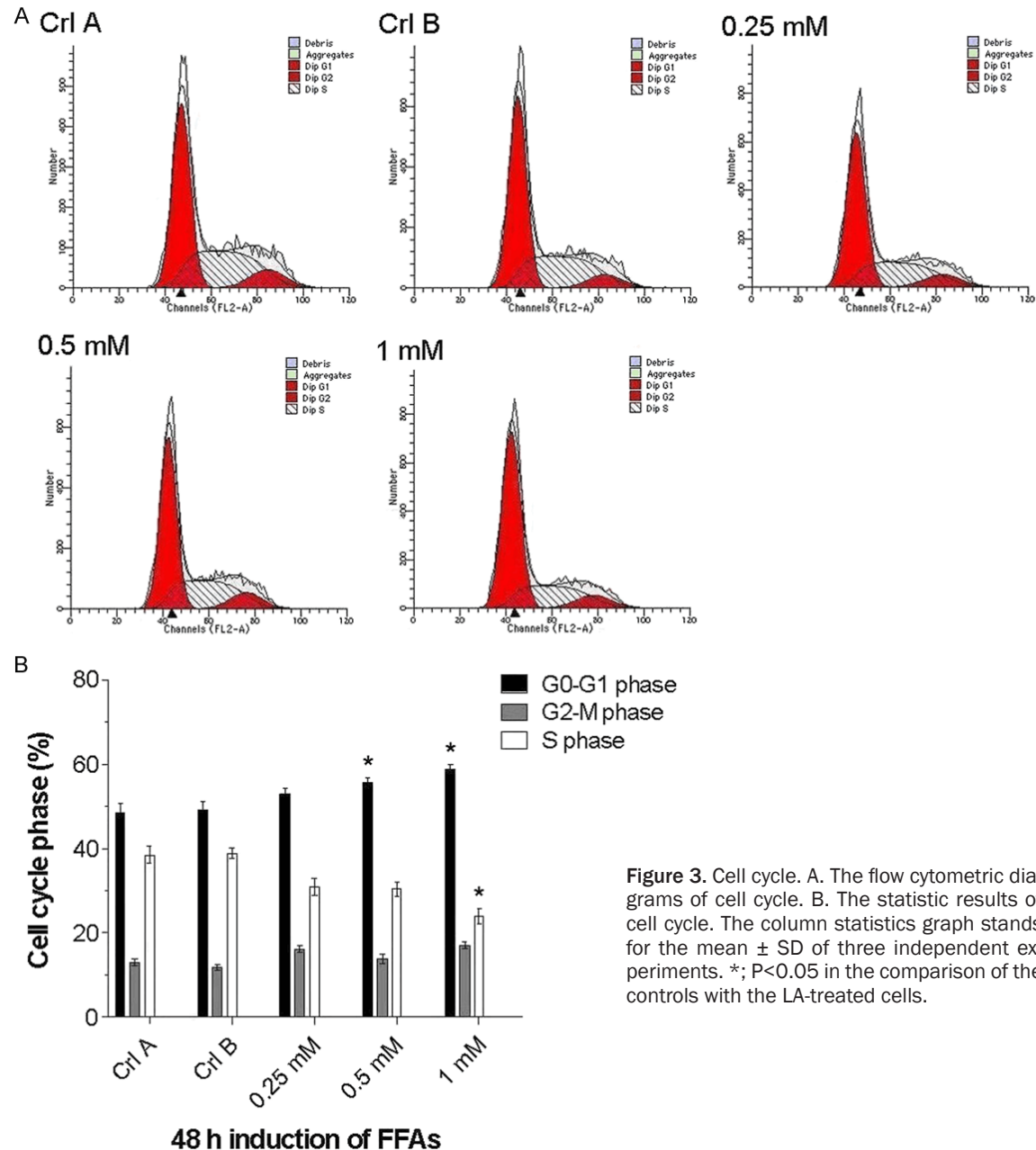


Figure 3. Cell cycle. A. The flow cytometric diagrams of cell cycle. B. The statistic results of cell cycle. The column statistics graph stands for the mean \pm SD of three independent experiments. *; $P < 0.05$ in the comparison of the controls with the LA-treated cells.

tions of LA might induce apoptosis of mesangial cells.

Discussion

Fat stored in adipose tissue is transported to other tissues in the form of FFAs hydrolyzed from triglycerides and combined with albumin. By participating in and adjusting intracellular lipid metabolism, FFAs provide energy for the cells of vital tissues or organs, such as skeletal muscle, heart, liver, and kidney [3]. It has been demonstrated that high concentrations of FFAs are associated with insulin resistance [13],

type 2 diabetes [14], fatty liver disease [15], hypertension [16], atherosclerosis [17], myocardial dysfunction [18], and chronic kidney disease (CKD). High-fat diet may cause macrophage infiltration and foam cell formation in rats, leading to glomerulosclerosis [19]. Hypercholesterolemia may accelerate the rate of progression of kidney disease [20]. Therefore it is of great importance to investigate the adverse effects of elevated concentrations of LA on mesangial cells.

Previous clinical studies proved that the normal level of serum FFAs ranged from 0.258 to

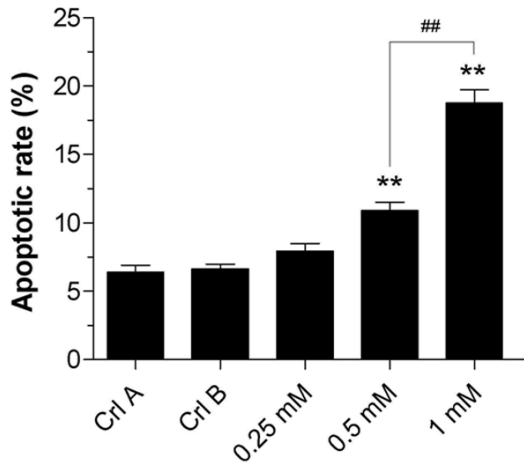


Figure 4. Apoptotic rate. The column statistics graph stands for the mean \pm SD of apoptotic rate of three independent experiments. **: $P < 0.01$ in the comparison of the controls with the LA-treated cells. **: $P < 0.01$ in the comparison between the LA-treated cells.

0.495 (mean 0.388) mmol/L, and the serum level of FFAs in the patients with type-2 diabetes and hyperlipidemia was often elevated over 0.4 mmol/L [9-11]. The findings of the current study confirmed that when the concentration of LA was over 0.4 mmol/L, it did bring adverse effects on the mesangial cells.

First, Oil Red O Staining was observed as the concentration of LA ≥ 0.5 mmol/L, the LA lipid droplets were deposited in a large quantity in the mesangial cells. This is likely a reflection of the lipotoxicity, a metabolic syndrome that results from the accumulation of lipid intermediates in non-adipose tissue [21]. Next, proliferation activity of mesangial cells, cell cycle, and apoptotic rate were determined. The concentration of LA ≥ 0.5 mmol/L, the inhibition of proliferation activity of mesangial cells, the cell cycle arrest in G0/G1 phase, and the cell apoptotic rate increase was observed.

Previous studies *in vitro* demonstrated that elevated concentrations of FFAs can inhibit vascular endothelial cell proliferation [22], can induce hepatic cell [23] and tubular epithelial cell apoptosis [24], and can lead to severe renal tubular interstitial damage [25]. Other previous studies proved that high concentrations of FFAs can injure cell membranes to make the membrane permeability increased, which induces membrane permeation of lipids and eventually

leads to pathological reactions of cell morphology and function [26, 27]. The findings of this study are consistent with these previous studies.

High concentrations of FFAs can lead to mitochondrial dysfunction and endoplasmic reticulum stress [28, 29]. Is it by mitochondrial dysfunction or endoplasmic reticulum stress for the elevated concentration of LA to impact cell proliferation of mesangial cells or to induce cell cycle arrest and apoptosis? Or is it by other unknown ways? These questions are still unsettled and need more studies.

Based on the findings of this study, it can be concluded that elevated concentrations of FFAs may bring adverse effects on mesangial cells, including inhibiting cell proliferation activity, inducing cell cycle arrested in G0/G1 phase and inducing cell apoptosis. In spite of based on the experiments *in vitro*, the findings are still of a certain clinical significance. It is to call attention to the potential risk of chronic renal injury incurred by the serum concentration of FFAs ≥ 0.5 mmol/L.

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Disclosure of conflict of interest

None.

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