

Influence of oleic acid in different solvent media on BRL 3A cell growth and viability

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Oleic acid (OA) is widely used in pathology studies of hepatocellular lipid deposition. Identifying the effects of different solvents on OA-induced liver lipid deposition would be beneficial for studies on hepatocytes. We treated BRL 3A cells with OA dissolved in different solvents. After 12 h incubation, cell viability was assessed using MTT assays. Reactive oxygen species (ROS), triglyceride (TG) and total cholesterol (TC) counts, and the expression level of glucose regulated protein (GRP78), sterol regulatory element binding protein (SREBP-1C) and fatty acid synthase (FAS) were analyzed. Water, PBS and DMSO were disadvantageous to the dissolution of OA and did not cause an OA-induced response in hepatocytes. In the alcohol+OA-treated cells, the severe ER stress, oxidative stress and cellular fat deposition were significantly increased. BSA promoted cell growth and the cells treated with 1.2% BSA+OA showed a lower grade TG and endoplasmic reticulum stress compared with KOH+OA and alcohol+OA treatments. KOH had no significant influence on BRL 3A cells viability. When treated with OA dissolved in KOH, BRL 3A cells showed a typical hepatocyte damage. KOH was considered the suitable choice for an OA solvent for BRL 3A cells in hepatic lipodosis research.

Key words: oleic acid; non-alcoholic fatty liver disease; liver lipid deposition

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Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; FAS, fatty acid synthase; GRP78, glucose regulated protein 78; H₂O, distilled water; KOH, potassium hydroxide; OA, oleic acid; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SREBP-1C, sterol regulatory element binding protein 1c; TC, total cholesterol; TG, triglyceride

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the types of fatty liver which occurs when fat is deposited in the liver due to the causes other than excessive alcohol use. In most of the cases of NAFLD a metabolic syndrome is also present (Xu *et al.*, 2016a). A high-fat diet is the major pathogenesis factor as the most dietary fat sources contain abundant free fatty acids (FFAs). Liver and adipose tissue are the major organs of lipid metabolism and take part in modulating lipid oxidative capacity and energy demands (Xu *et al.*, 2015). A surplus of FFAs in non-adipose cells, especially hepatocytes, may activate deleterious pathways leading to a cell dysfunction (Xu *et al.*, 2016b). Furthermore, high levels of FFAs could contribute to mitochondrial dysfunction on the

level of production of reactive oxygen species and activation of endoplasmic reticulum stress-associated mechanisms (Perla *et al.*, 2017). Oleic acid (OA) is a unique unsaturated fatty acid which plays a key role in cellular activity, metabolism, and nuclear events (Imai *et al.*, 2003). It is synthesized *de novo* from palmitic acid or dietary palmitic acid by palmitoyl-CoA elongation, or from stearic acid by stearoyl-CoA desaturation (Cinti *et al.*, 1992). OA is widely used in pathological studies of hepatic lipodosis. In *in vitro* studies, the various solvents for dissolving OA are used (Cairns *et al.*, 2017, Li *et al.*, 2015, Weng *et al.*, 2017), but the effect of these solvents on hepatocytes are unclear, thus, identifying the effects of OA in different solvents on hepatocytes would be beneficial. In this study, OA was dissolved in distilled water (H₂O), potassium hydroxide (KOH), alcohol, phosphate-buffered saline (PBS), bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) to evaluate the effect of different solvents on OA-induced hepatic lipodosis, viability, oxidative stress and endoplasmic reticulum stress.

MATERIALS AND METHODS

Cell cultures and treatment. The BRL3A rat liver cell line was purchased from Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (GIBCO, Life Technologies, USA) containing 10% FBS (GIBCO, Life Technologies) with 1% Penicillin-Streptomycin Solution (Solarbio Life Sciences, Beijing, China) in a humidified incubator at 37°C with 5% CO₂. Stock solutions of 158.5 μl OA were prepared in 10 ml of 0.1 mM KOH (Liu *et al.*, 2014, Wang *et al.*, 2015), 0.1 mM PBS, alcohol (absolute ethyl ethanol; Lagrutta *et al.*, 2017, Chen *et al.*, 2014, Liao *et al.*, 2014), BSA (0.4%, 0.8%, 1.2%) (Moravcova *et al.*, 2015, Seo *et al.*, 2014) and DMSO (0.1%, 0.4%, 0.8%) (Rogue *et al.*, 2014, Zhang *et al.*, 2004), respectively, and diluted in DMEM culture medium to a final concentration of OA at 1.2 mM. About 5×10⁴ BRL 3A cells per well were seeded on the six-well culture plates and treated with different solvents and OA in different solvents. The cells were harvested after 12 hours.

Assessment of BRL 3A cell viability. BRL 3A cells were seeded in 96-well culture plates at about 3000 cells per well. To determine cell viability, cells were treated for 6, 12, 24 or 48 h. A final concentration of 5 mg/ml methylthiazolyldiphenyl-tetrazolium bromide (MTT) was added for the last 4 h of the incubation time. Then the medium was removed carefully and added to 150 μl DMSO for 0.5 h and which was next used for cell viability analysis using spectrophotometer at 492 nm. Four independent experiments were performed for each group.

Table 1. Primer sets used in PCR

Gene	Forward (5'-3')	Reverse (5'-3')
Tbp	ACTCCTGCCACACCAGCC	GGTCAAGTTTACAGCCAAGATTCA
Grp-78	AACCAGATGAGGCTGTAGCATA	CACAGTGTCTCTCGGAATCAGTT
Srebp-1c	GACGACGGAGCCATGGATT	GGGAAGTCACTGTCTTGGTTGTT
Fas	CTATTGTGGACGGAGGTATC	TGCTGTAGCCCAGAAGAG

TG and TC concentration determination. After the incubation, the cells were lysed by brief sonication in 2% Triton X-100 in an ice bath. The lysates were centrifuged at 10000×g at 4°C for 10 min. The supernatants were collected for TG and TC analysis using commercial enzymatic kits (Pulilai Biotechnology, Beijing, China). Four independent experiments were performed for each group.

Measurement of ROS production. After collecting cells according to ROS kit (Applygen, Beijing, China) instructions, cells were treated with 10 μM DCFH-DA in PBS for 40 min at 37°C and photographed using a fluorescence microscope for ROS analysis.

Lipid synthesis activity and endoplasmic reticulum stress determination. Expression of sterol regulatory element binding protein (SREBP-1C) and fatty acid synthase (FAS) were used for determination of lipid synthesis activity of the cells, and expression of glucose regulated protein (GRP78) was used for endoplasmic reticulum stress determination. Total RNA was extracted using TRIZOL reagent (Invitrogen,

USA). RNA was reverse transcribed into cDNA using high-capacity cDNA reverse transcription kits (Invitrogen, Switzerland) according to the manufacturer's instructions. mRNA levels for GRP78, SREBP-1C and FAS were analyzed using Applied Biosystems 7300 real-time polymerase chain reaction (PCR) system and SYBR Premix Ex TaqI (TaKaRa, Dalian, China). Primers were designed using Primer Express software from Applied Biosystems and synthesized (Table 1).

Statistical analysis. Data are provided as means ± S.E.M. All data were tested for significance using unpaired Student t-test or ANOVA. Only results with $p < 0.05$ were considered statistically significant.

RESULTS

Cell viability assays

For a 12 h stimulation, the surviving fractions of the 0.8% BSA+OA, 1.2% BSA and the 1.2% BSA+OA treated cells were significantly higher than in the control groups ($p < 0.05$, Table 2). Also for a 24 h stimulation, the survival rates of the 0.8% BSA group, the 0.8% BSA+OA group, the 1.2% BSA group and the 1.2% BSA+OA group, were significantly higher than in the control groups ($p < 0.05$). On

Table 2. Cell viability assays in BRL 3A cells with OA in different solvents

	6 h		12 h		24 h	
	OD Count	Fraction Surviving	OD Count	Fraction Surviving	OD Count	Fraction Surviving
Control	0.673±0.02	100%	0.697±0.015	100%	0.655±0.001	100%
H ₂ O	0.653±0.003	96.93%	0.663±0.038	95.17%	0.626±0.02	95.57%
H ₂ O+OA	0.622±0.021	98.33%	0.680±0.016	97.56%	0.727±0.014	101.00%
KOH	0.673±0.04	99.90%	0.695±0.007	99.71%	0.639±0.03	97.61%
KOH+OA	0.696±0.043	103.32%	0.712±0.008	102.15%	0.709±0.091	108.35%
Alcohol	0.607±0.03	90.10%	0.644±0.005	92.35%	0.541±0.02*	82.59%
Alcohol+OA	0.556±0.012	92.57%	0.671±0.030	96.27%	0.530±0.022*	89.96%
PBS	0.644±0.007	95.59%	0.650±0.007	93.26%	0.617±0.005	94.20%
PBS+OA	0.697±0.018	103.47%	0.641±0.053	91.97%	0.607±0.004	92.63%
0.4% BSA	0.611±0.01	95.69%	0.728±0.051	104.50%	0.668±0.03	101.99%
0.4% BSA+OA	0.701±0.023	104.06%	0.745±0.063	106.89%	0.731±0.013	111.61%
0.8% BSA	0.651±0.03	96.68%	0.782±0.017	112.15%	0.759±0.01*	115.94%
0.8% BSA+OA	0.764±0.018	113.51%	0.795±0.026*	114.06%	0.776±0.023*	118.58%
1.2% BSA	0.756±0.02	112.33%	0.869±0.063*	124.73%	0.997±0.08*	152.29%
1.2% BSA+OA	0.690±0.041	102.48%	0.829±0.012*	118.94%	0.791±0.018*	120.88%
0.1% DMSO	0.632±0.01	93.91%	0.674±0.002	96.75%	0.625±0.07	95.42%
0.1% DMSO+OA	0.657±0.015	97.52%	0.632±0.108	94.70%	0.527±0.047	95.45%
0.4% DMSO	0.638±0.02	94.75%	0.686±0.043	98.37%	0.636±0.01	97.20%
0.4% DMSO+OA	0.612±0.077	90.89%	0.782±0.039	90.67%	0.627±0.032	95.82%
0.8% DMSO	0.595±0.01	88.32%	0.604±0.045	86.66%	0.517±0.03*	78.93%
0.8% DMSO+OA	0.488±0.018*	82.48%	0.604±0.038	86.66%	0.517±0.014*	78.92%

Fraction Surviving: OD of treated samples/OD of control samples*100. Statistical significance: * $p < 0.05$ vs. control at the same time point; ** $p < 0.01$ vs. control at the same time point.

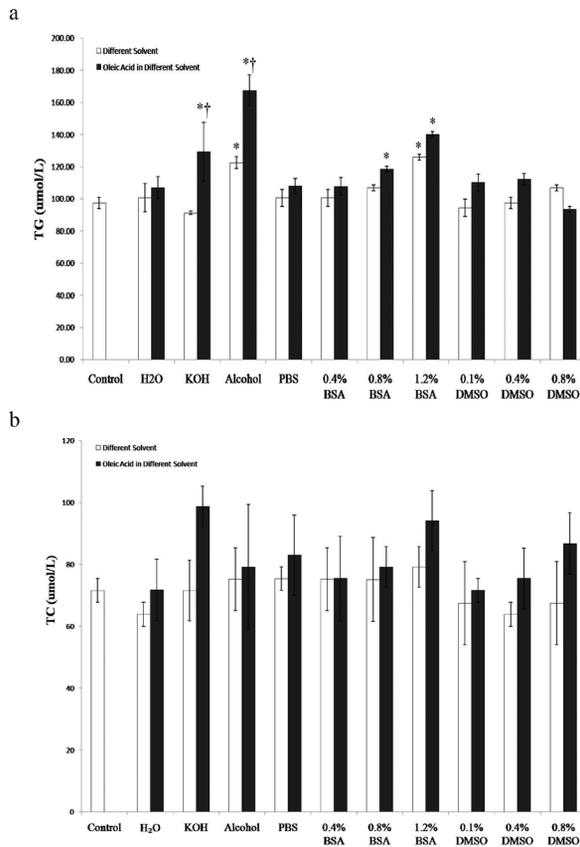


Figure 1. TG and TC counts in BRL 3A cells treated with OA in different solvents or solvents only

TG and TC counts in BRL 3A cell which treated with solvent or OA in different solvents for 12 h. (a) BRL 3A cells were seeded in six-well culture plates and treated with OA in different solvents or the solvents only for 12 h. The supernatants were collected for TG analysis using commercial enzymatic kits and a microplate multifunction reader. (b) BRL 3A cells were seeded in six-well culture plates and treated with OA in different solvents or the solvents only for 12 h. The supernatants were collected for TC analysis using commercial enzymatic kits and a microplate multifunction reader. *indicates a significant difference ($p < 0.05$) between solvent and control groups; † indicates a significant difference ($p < 0.05$) between solvent+OA and corresponding solvent group; neither *, nor †, indicates no significant difference ($p > 0.05$).

the other hand, the surviving fractions in the alcohol group, alcohol+OA group, 0.8% DMSO group and the 0.8% DMSO+OA group were significantly lower than in the control groups ($p < 0.05$) after 24 hour stimulation.

Effect of OA in different solvents on BRL-3A induction of TG and TC counts

TG counts for the alcohol, 1.2% BSA, KOH+OA, alcohol+OA, 0.8% BSA+OA and 1.2% BSA+OA groups were significantly higher than in the control group ($p < 0.05$) (Fig. 1). TG count for the KOH+OA group, alcohol+OA group and 1.2% BSA+OA group were significantly higher than in the corresponding solvents group. No significant difference was seen in TC counts among different solvent groups and normal control groups (Fig. 1).

Intracellular ROS

As showed in Fig. 2, the observations were further corroborated by an increased ROS production

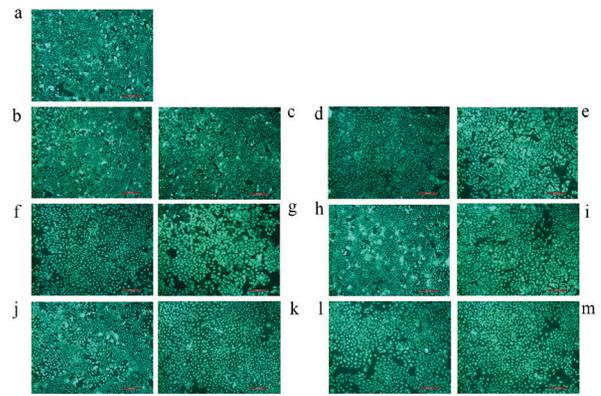


Figure 2. ROS analysis using a fluorescence microscope.

The intensity of fluorescence corresponds to the amount of ROS. The stronger the fluorescence intensity in the picture, the more ROS in the BRL 3A cells. The weaker the fluorescence intensity in the picture, the less ROS in the BRL 3A cells. Figures a, b, c, d, e, f, g, h, i, j, k, l and m correspond to control H₂O, H₂O+OA, KOH, KOH+OA, Alcohol, Alcohol+OA, PBS, PBS+OA, BSA, BSA+OA, DMSO and DMSO+OA groups, respectively.

in the alcohol and DMSO groups. ROS production in the KOH+OA and alcohol+OA groups was significantly increased compared to the corresponding solvent groups. ROS production in the PBS+OA, BSA+OA and DMSO+OA groups was increased compared to the corresponding solvent groups. No significant difference in ROS production was observed between H₂O+OA and H₂O groups.

Effect of OA in different solvents on GRP78, SREBP-1C and FAS mRNA in BRL 3A cells

GRP78 mRNA levels were significantly higher in the alcohol, KOH+OA, alcohol+OA and 1.2% BSA+OA groups compared to the control groups ($p < 0.05$, Fig. 3). GRP78 mRNA was significantly higher in the KOH+OA and alcohol+OA groups compared to corresponding solvent groups ($p < 0.05$). Other groups had higher GRP78 expression than the control groups, but the differences were not significant.

SREBP-1C mRNA levels were significantly lower in the alcohol, KOH+OA, alcohol+OA, 1.2% BSA+OA, 0.8% BSA+OA and 0.8% DMSO+OA groups compared to the control group ($p < 0.05$). SREBP-1C mRNA in the KOH+OA, 0.8% DMSO and 0.8% BSA groups was significantly lower than in the corresponding solvent groups ($p < 0.05$). FAS mRNA levels were significantly lower in the H₂O+OA, KOH+OA, alcohol+OA, 0.4% BSA, 0.8% DMSO and 0.8% DMSO+OA groups compared to the control group ($p < 0.05$). FAS mRNA levels in the KOH+OA groups were significantly lower than in the corresponding solvent groups ($p < 0.05$).

DISCUSSION

NAFLD becomes increasingly prevalent due to the worldwide obesity epidemic and currently affects about one billion people worldwide (Webster, 2017). Steatosis is the earliest and most common stage of NAFLD and is often referred to as the “first hit”. During this stage, FFAs accumulate in the cells, which is followed by the production of ROS and lipid peroxidation. It has been proposed that the lipotoxicity of FFAs increases the liver’s vulnerability to a “second hit” involving environmental and/or genetic factors, which ultimately can lead

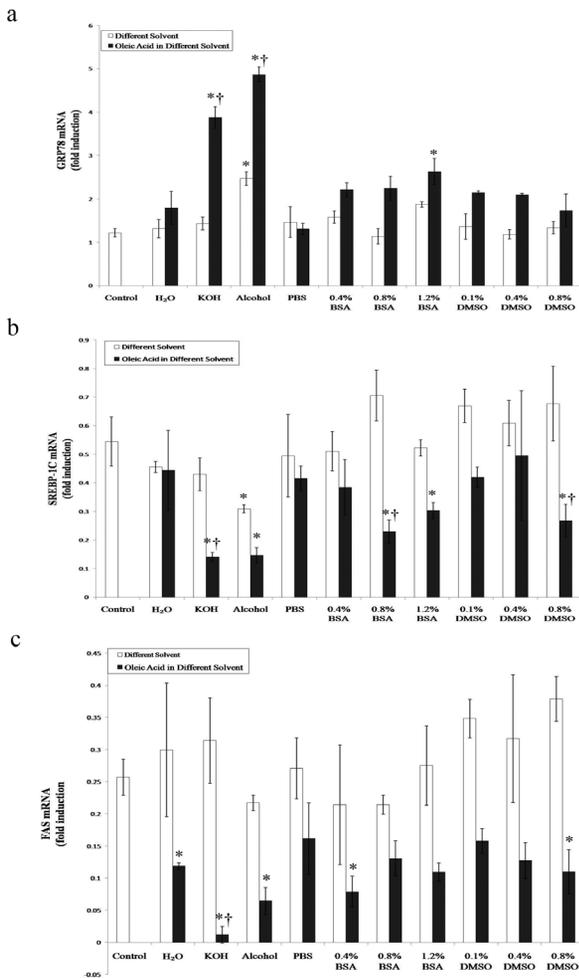


Figure 3. GRP78, SREBP-1C and FAS mRNA counts in BRL 3A cells

Figures **a**, **b** and **c** correspond to GRP78, SREBP-1C and FAS mRNA counts, respectively. *indicates a significant difference ($p < 0.05$) between solvent and control groups; † indicates a significant difference ($p < 0.05$) between solvent+OA and corresponding solvent groups; neither *nor †, indicates no significant difference ($p > 0.05$).

to end-stage liver disease (Periasamy *et al.*, 2014). Growing evidence suggests that endoplasmic reticulum (ER) stress may link saturated fatty acids to NAFLD (Mota *et al.*, 2016). ER stress causes the activation of the unfolded protein response (UPR) and the sterol regulatory element-binding protein (SREBP) pathway (Zhao & Ackerman 2006). Once activated, the UPR increases the expression of intraluminal ER chaperones, especially GRP78, in order to cope with the accumulation of unfolded or misfolded proteins. Hence GRP78 is a marker of endoplasmic reticulum stress (Moslehi *et al.*, 2017). In contrast, SREBP-1 acts as a transcription factor that regulates the genes that control the synthesis of fatty acids and the cellular uptake of lipoproteins (Zhang *et al.*, 2014). SREBP-1c regulates hepatic lipogenic gene transcription and insulin-induced lipogenesis, and its target genes include FAS, acetyl coenzyme A carboxylase (ACC), and low-density lipoprotein receptor (Liao *et al.*, 2010). SREBP-1c is involved in almost all hepatic fatty acid and TG synthesis, gene transcription and TG synthesis, gene transcription and transformation. FAS is a

fatty acid synthase. Both SREBP-1C and FAS are regulators of *de novo* fat synthesis (Zhou *et al.*, 2017).

In previous work (Muller *et al.*, 2010) have demonstrated that non-esterified fatty acid (NEFA) caused NAFLD-like changes within hepatocytes, including lipid accumulation, oxidative stress and cell death (Muller *et al.*, 2012). High concentrations of NEFAs can induce oxidative stress in hepatocytes by increasing the levels of ROS (Shi *et al.*, 2015; Song *et al.*, 2016; Du *et al.*, 2017). To study the pathogenesis mechanism of NAFLD *in vitro*, fatty acids were used to induce hepatic lipid deposition. But the effects of the use of the different solvents for fatty acids remained unclear. Alcohol has been used in many studies (Lagrutta *et al.*, 2017; Chen *et al.*, 2014; Liao *et al.*, 2014) as a solvent of oleic acid. Some experiments have proved that low concentration of DMSO (Rogue *et al.*, 2014; Zhang *et al.*, 2004) exerted little damage to the cells, so we chose 0.8% 0.4% 0.1% concentration of DMSO for our experimentation. KOH was chosen as the solvent for oleic acid because many NEFA models of non-alcoholic fatty liver were dissolved in KOH (Liu *et al.*, 2014; Wang *et al.*, 2015). For an overall evaluation of the influence of oleic acid in different solvent media on the cells, PBS and H₂O were also used as solvents in this research. In the present study, BRL 3A cells were treated with OA in different solvents to explore the influence of these solvents on hepatic lipid deposition. We screened the mild solvents of oleic acid to see the effects of different solvents on cell viability, toxicity and the degree of oxidative stress and hepatocyte lipid deposition. It was found that the OA dissolved in H₂O and PBS had no significant effect on hepatocytes' response, which did not differ from the control group. This was probably because H₂O and PBS are disadvantageous to the dissolution of OA. Alcohol is commonly used in the study of alcoholic fatty liver disease (Zhu *et al.*, 2014; Zhang *et al.*, 2014). OA dissolves in high concentration of alcohol, but high alcohol concentration is also toxic to the cells, resulting in oxidative and endoplasmic reticulum stress (Caires *et al.*, 2012). In this study, cells treated with alcohol+OA had significantly increased and severe ER stress, oxidative stress and hepatic fat deposition, compared to the other groups. However, as the alcohol also leads to liver injury, it was considered not appropriate as a solvent to the study of fatty acid in liver cell degeneration. DMSO is a universal solvent routinely used in experimental and biological disciplines. It is often used to solubilize drug molecules that are otherwise poorly soluble at concentrations of a 10% (v/v) range (Galvao *et al.*, 2014). In this study we observed that although when the concentration of DMSO is low, it has little effect on the cell, it is cytotoxic at higher DMSO concentration, causing oxidative stress and endoplasmic reticulum stress. The survival rates of the 1.2% BSA group and the 1.2% BSA+OA group were significantly higher than in the control groups. That was probably because BSA can promote cell growth as a nutrient. The cells treated with BSA showed a lower grade TG and endoplasmic reticulum stress compared with KOH+OA and Alcohol+OA groups. In addition, 1.2% BSA+OA treated cells showed a significantly increased fat deposition compared to the control and BSA groups. KOH was usually used as the solvent for fatty acids (Song *et al.*, 2016). In this study, KOH group had no significant influence on cell viability, lipid deposition and oxidative stress as compared the control group, while KOH+OA group exhibited a typical hepatocyte damage. In summa-

ry, we consider KOH to be the most suitable choice for an OA solvent to be used for BRL 3A cells.

Conflict of interest statement

The authors have declared that no competing interest exists.

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