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Rapamycin attenuates palmitate-induced lipid aggregation by up-regulating sirt-1 signaling in AML12 hepatocytes

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Rapamycin (Rap), a specific inhibitor of the mTOR signaling, has been shown to affect lipid metabolism *in vitro* and *in vivo*. Sirt-1, an NAD^+ dependent deacetylase, regulates a variety of cellular processes, including aging, lifespan extension and glucose and lipid metabolism. Herein, we applied a cellular steatosis model to investigate whether rapamycin's role in lipid metabolism is sirt 1-associated. Cells were exposed to palmitate stimulation for 48 h with or without rapamycin treatment. Lipid droplets in AML12 cells were observed by oil red O staining, and the intracellular lipid content was measured. We found that rapamycin treatment, at a relatively low concentration, significantly attenuated lipid aggregation, whereas knockdown of sirt-1 by siRNA abrogated rapamycin's effect on ameliorating lipid accumulation. Moreover, rapamycin exposure increased the expression levels of sirt-1 and AMPK, and enhanced sirt-1 deacetylase activity in steatotic AML12 hepatocytes. This is the first report demonstrating that rapamycin ameliorates lipid accumulation through upregulating sirt-1 signaling supporting the hypothesis that rapamycin may positively influence sirt-1 signaling in maintaining metabolic homeostasis.

1. Introduction

Mammalian target of rapamycin (mTOR), a highly conserved serine-thereonine kinase, integrates a variety of biological signals of cell growth and metabolism. Rapamycin, the classical inhibitor of mTOR signaling, has therefore been widely used as immunosuppressor and anticancer agent due to its strong anti-proliferative capacity (Wang et al. 2015). Rapamycin exposure influences glucose and lipid metabolism both *in vitro* and *in vivo*. In diabetic *Psammomys obesus,* rapamycin treatment resulted in a robust increase of serum lipids (Fraenkel et al. 2008). Chronic rapamycin treatment reduced adiposity and fat cell number which was associated with a coordinated downregulation of genes involved in both lipid uptake and output in rats (Houde et al. 2010). Furthermore, in different mammalian cells, rapamycin treatment was verified to stimulate lipolysis and inhibit *de novo* lipogenesis (Chakrabarti et al. 2010). In accordance with previous studies, we found that rapamycin given to diabetic mice at a dose of 2 mg/kg exacerbated metabolic defects including hyperglycemia and dyslipidemia (Wang et al. 2015).

Sirt-1, an NAD^+ dependent deacetylase, regulates central metabolic functions such as lipogenesis, protein synthesis, gluconeogenesis and bile acid (BA) homeostasis through deacetylation (García-Rodríguez et al. 2014). Increased sirt-1 deacetylase activity attenuated lipid accumulation in human HepG2 hepatocytes exposed to high glucose (Hou et al. 2008), and lack of sirt-1 leads to liver steatosis in the sirt1+/- mice (Xu et al. 2010). In differentiated fat cells, upregulation of sirt-1 triggered lipolysis and loss of fat, which were thought to be the underlying mechanism connecting calorie restriction to life extension in mammals (Picard et al. 2004). In a high-fat diet-induced mice model, sirt-1 protected against metabolic damage due to induction of antioxidant proteins such as MnSOD and Nrf1, or lower activation of proinflammatory cytokines (Pfluger et al. 2008). Recent studies further unveiled the interesting role of sirt-1 in brown adipose tissue degeneration process (Xu et al. 2016). Plenty of studies have investigated the mechanism through which rapamycin treatment affects lipid metabolism, including inhibiting lipid storage by promoting lipolysis (Chakrabarti et al. 2010) and downregulating the expression of genes involved in lipid homeostasis such as perilipin, sterol regulatory element-binding protein 1 (SREBP1) and lipin 1 (Pereira et al. 2013), acetyl-coenzyme A carboxylase I and mitochondrial glycerol phosphate acyltransferase (Brown et al. 2007). However, whether rapamycin's influence on lipid metabolism is *via* sirt-1 signaling has not been fully characterized yet. In this work, we demonstrated that rapamycin treatment significantly ameliorated lipid accumulation, increased the protein expression levels of sirt-1 and AMPK, and furthermore, enhanced sirt-1 deacetylase activity in a palmitate-induced cellular steatosis model.

2. Investigations and results

2.1. *Rapamycin significantly enhanced cell viability*

Palmitate stimulation significantly decreased cell viability (approximately 70% to that of normal AML12 cells). As shown in Fig. 1, cells responded dose-dependently to rapamycin treatment, and we found that at a relatively lower concentration, rapamycin treatment (50 nM) significantly increased cell viability (0.780±0.023 *vs.* 0.708±0.048, $p<0.05$) when compared with the PA group. Therefore, this concentration (50 nM) is selected for later studies.

2.2. *Rapamycin significantly ameliorated lipid accumulation in AML12 cells*

As shown in Fig. 2A and 2B, Oil red O staining assay and intracellular TG content detection both demonstrated that palmitate (450 μM) induced a profound increase of TG accumulation compared to normal cells (0.12±0.007 mmol/gprot for PA group *vs.* 0.02±0.004 mmol/gprot for AML12 group, $p<0.01$), and knockdown of sirt-1

with siRNA further exacerbated lipid aggregation (0.29±0.006 mmol/gprot for Sirt-1 siRNA+PA group *vs.* 0.16±0.014 mmol/gprot for NC siRNA+PA group, p<0.05), suggesting the pivotal role sirt-1 played in maintaining lipid homeostasis. Rapamycin challenge decreased nearly four fifth of TG content compared to NC siRNA+PA group (0.03±0.004 mmol/gprot for NC siRNA+PA+Rap group *vs.* 0.16±0.014 mmol/gprot for NC siRNA+PA group, p<0.05), whereas knockdown of sirt-1 significantly abrogated this effect (0.13±0.003 mmol/gprot for sirt-1 siRNA+PA+Rap group *vs.* 0.03±0.004 mmol/gprot for NC siRNA+PA+Rap group, p<0.01), and furthermore, rapamycin merely decreased approximately half of the TG content (0.13±0.003 mmol/gprot for sirt-1 siRNA+PA+Rap group *vs.* 0.29±0.006 mmol/gprot for sirt-1 siRNA+PA group, p<0.01) when compared with sirt-1 siRNA+PA group. All these indicated that rapamycin ameliorated lipid accumulation through sirt-1 signaling. A similar tendency existed in intracellular FFA evaluation, as exhibited in Fig. 2C.

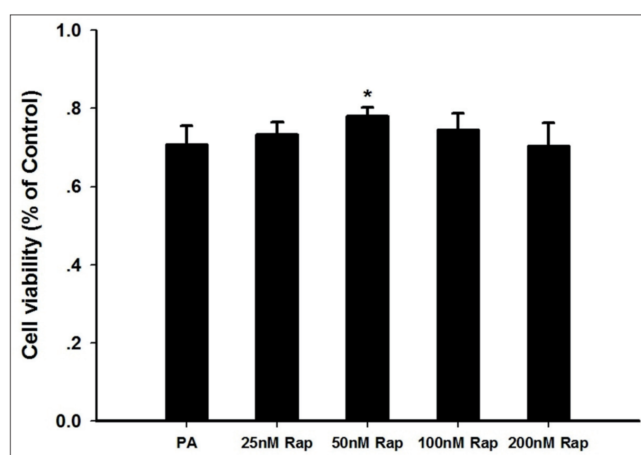

Fig. 1: MTT assay indicated that PA induced significantly decreased cell viabiligy of the cells, whereas rapamycin treatment (50nM) significantly increased cell viability of palmitate-exposed steatotic cells. *p<0.05 vs. PA-treated group.

2.3. *Rapamycin significantly increased sirt-1 and AMPK expression in AML12 cells*

We performed WB to test the hypothesis that rapamycin treatment increased sirt-1 expression. As indicated in Fig. 3A, 3B and 3D, exposure to PA led to a significant decrease of sirt-1 and AMPK protein expression levels. Rapamycin treatment significantly upregulated sirt-1 and AMPK expression levels when compared to the PA group (p<0.05). Interestingly, rapamycin was capable of increasing sirt-1 and AMPK expression significantly without the presence of PA stimulation. We further completed WB analyses in sirt-1 siRNA-treated groups, and consistent results were obtained. As exhibited in Fig. 4A, 4B and 4D, sirt-1 siRNA transfection induced significant downregulation of sirt-1 and p-AMPKα/AMPKα expression levels (p<0.01), and rapamycin exposure significantly enhanced sirt-1 and p-AMPKα/AMPKα expression levels when compared with sirt-1 siRNA group and NC siRNA group (p<0.01).

2.4. *Rapamycin significantly increased sirt-1 activity in AML12 cells*

As exhibited in Fig. 5, PA exposure significantly decreased sirt-1 deacetylase activity (p<0.01), whereas rapamycin treatment enhanced sirt-1 activity significantly (p<0.05).

3. Discussion

Data regarding the influence of mTOR pathway modulation on sirt-1 signaling are sparse. Our present research is the first report on rapamycin's influence on sirt-1 signaling in a palmitate-induced steatotic cell model. We found that rapamycin treatment induced significant attenuation of lipid accumulation and upregulation of sirt-1 and AMPK expression levels in addition to enhanced sirt-1 activity in the steatotic AML12 hepatocytes.

It has been shown that palmitate induced activation of mammalian target of rapamycin complex 1 and insulin resistance in hepatocytes (Hatanaka et al. 2014; Mordier et al. 2007). Consistent with previous investigations, we found that palmitate-treated cells exhibited profound lipid aggregation and significant increase in p-S6K1/S6K1 ratio (S6K1 is known as a down-stream signal of mTOR signaling cascade), indicating significant activation of mTOR signaling. Furthermore, sirt-1 and AMPK expression levels were significantly decreased in PA-exposed cells, which was in accordance with previous research (Kwon et al. 2015; Tong et al. 2015).

Rapamycin has been verified to affect metabolic homeostasis in *in vitro* and *in vivo* models. Previous studies demonstrated that rapamycin treatment effectively prevented the onset of type 1 diabetes (Baeder et al. 1992; Jiang et al. 2009). However, some investigations have paradoxically shown that rapamycin inhibits pancreatic β cell proliferation, reduces glucose-stimulated insulin release (Barlow et al. 2012) and induces insulin resistance by upregulating hepatic gluconeogenesis (Houde et al. 2010). As for lipid metabolism, rapamycin exposure was demonstrated to promote lipolysis, to inhibit lipogenesis and to prevent fat storage in cellular models (Brown et al. 2007; Houde et al. 2010), whereas in animal models, rapamycin treatment led to reduced size of both intraabdominal and subcutaneous fat pads, dyslipidemia and weight loss (Chakrabarti et al. 2008; Fraenkel et al. 2008; Ray et al. 2014; Wang et al. 2015). In the present paper, rapamycin treatment at a concentration of 50 nM significantly increased the cell viability of the PA-exposed steatotic cells. Therefore, we hypothesized that rapamycin treatment at a concentration of 50 nM was capable of exerting beneficial effects on the viability and overall condition of the cells, and we selected this concentration (50 nM) for later studies regarding rapamycin's role in lipid homeostasis. Consistent with our hypothesis, rapamycin treatment at this concentration significantly ameliorated intracelluar lipid accumulation (TG and FFA contents), whereas knockdown of sirt-1 abrogated this effect, indicating that rapamycin influenced lipid homeostasis *via* sirt-1 signaling.

Sirt-1, the important and "famous" NAD^+ dependent deacetylase, has been widely studied due to its vital role in controlling vital biological signals including lifespan extension, metabolic homeostasis, *etc*. It has been reported that sirt-1 may act to promote growth and survival of neurons in the central nervous system *via* its negative modulation of mTOR signaling (Guo et al. 2011) and sirt-1 activator (SRT1720) improves the follicle reserve and prolongs the ovarian lifespan of diet-induced obesity in female mice *via* activating SIRT1 and suppressing mTOR signaling (Zhou et al. 2014), suggesting that sirt-1 may negatively modulate mTOR signaling. On the contrary, mTOR pathway modulation influenced mTOR-dependent phosphorylation of sirt-1 at serine47, resulting in the inhibition of the deacetylase activity of sirt-1 (Back et al. 2011). Therefore, we hypothesized that inhibition of the mTOR pathway may positively influence sirt-1 signaling. Our present data supported our previous hypothesis that rapamycin, a classical inhibitor of the mTOR signaling, significantly upregulates sirt-1 expression levels and enhances sirt-1 deacetylase activity in palmitate-induced steatotic hepatocytes.

AMPK, the downstream signal of sirt-1 signaling, is believed to exert beneficial effects on maintaining lipid homeostasis *via* the sirt-1-AMPK pathway (Choi et al. 2015; Yang et al. 2014). Lots of natural components including berberine (Kim et al. 2009) and theaflavins (Lin el al. 2007) have also been reported to attenuate lipid accumulation through activating AMPK signaling. In our present research, rapamycin treatment not only increased sirt-1 expression level but also enhanced the expression level of AMPK, indicating that rapamycin ameliorated lipid accumulation through the sirt1-AMPK pathway in the palmitate-exposed steatotic hepatocytes.

In conclusion, rapamycin treatment ameliorated lipid aggregation in palmitate-induced steatotic hepatocytes. The protective action of rapamycin against palmitate-induced steatosis in AML12 cells appears to be mediated through the upregulation of sirt1-AMPK signaling.

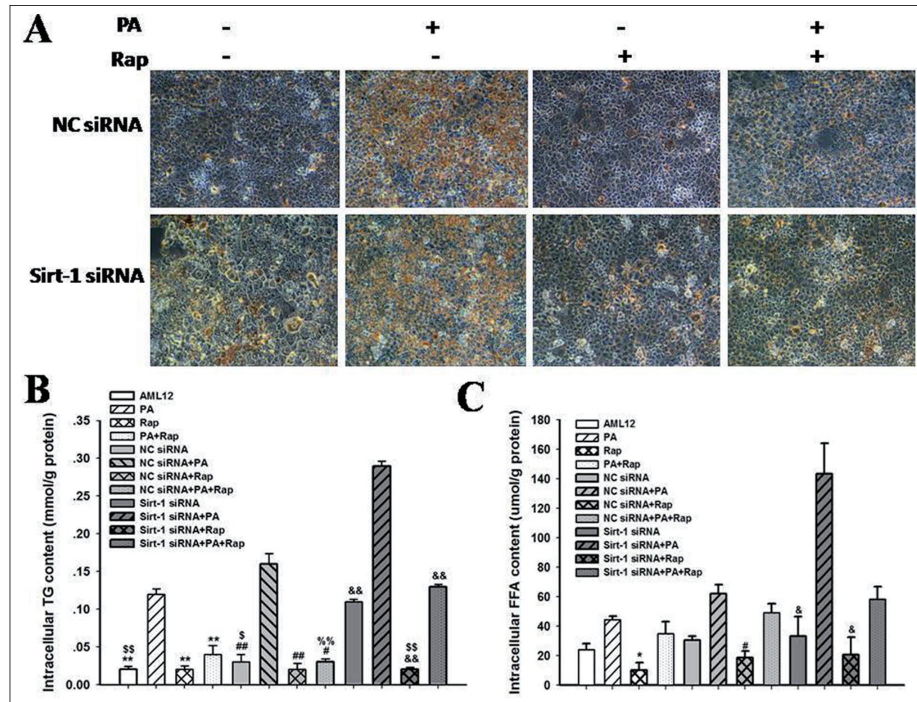

Fig. 2: Oil red O assay (A) and intracellular lipid contents detection (B and C) demonstrated that both PA treatment (450μM) and knockdown of sirt-1 signicantly induced lipid aggregation in AML12 hepatocytes. Rapamycin treatment significantly ameliorated lipid accumulation both in NC siRNA and sirt-1 siRNA-treated hepatocytes (A). Correspondingly, rapamycin treatment significantly decreased intracellular TG content in PA-treated cell (B). Similar tendency was obtained in intracellular FFA evaluation (C). * $p<0.05$, ** $p<0.01$ vs. PA group; # $p<0.05$, ## $p<0.01$ vs.NC siRNA+PA; % $p<0.05$, %% $p<0.01$ vs. Sirt-1 siRNA+PA+Rap group; & $p<0.05$, && $p<0.01$ vs. Sirt-1 siRNA+PA group; $p<0.05$, $p<0.01$ vs. Sirt-1 siRNA group.

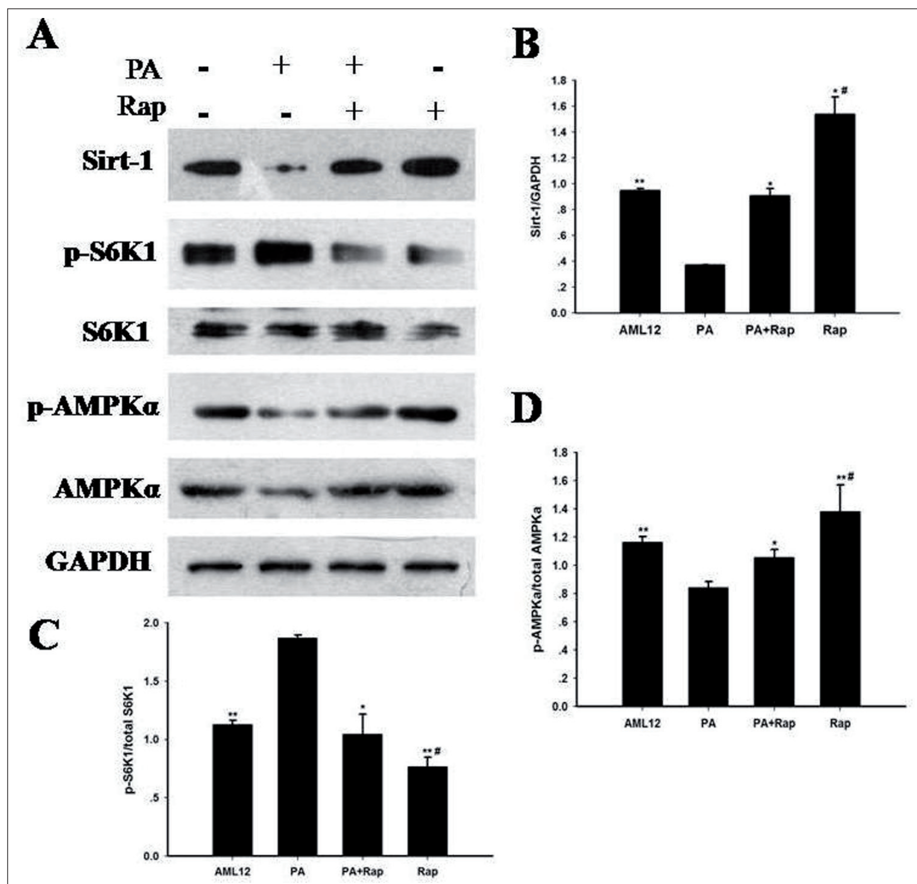

Fig. 3: WB assay demonstrated that PA stimulation induced significantly decreased protein expression levels of sirt-1 and p-AMPK/AMPK, whereas rapamycin treatment significantly increased the protein expression levels of sirt-1 and p-AMPK/AMPK in the PA-treated steatotic cells (A, B and D). Furthermore, when normal AML12 cells were treated with rapamycin, the protein expression levels of sirt-1 and p-AMPK/AMPK were also significantly up-regulated. As rapamycin was a specific mTOR signaling inhibitor, rapamycin treatment significantly down-regulated the p-S6K1/S6K1 ratio (C). * $p<0.05$, ** $p<0.01$ vs. PA-treated group; # $p<0.05$ vs. normal AML12 group.

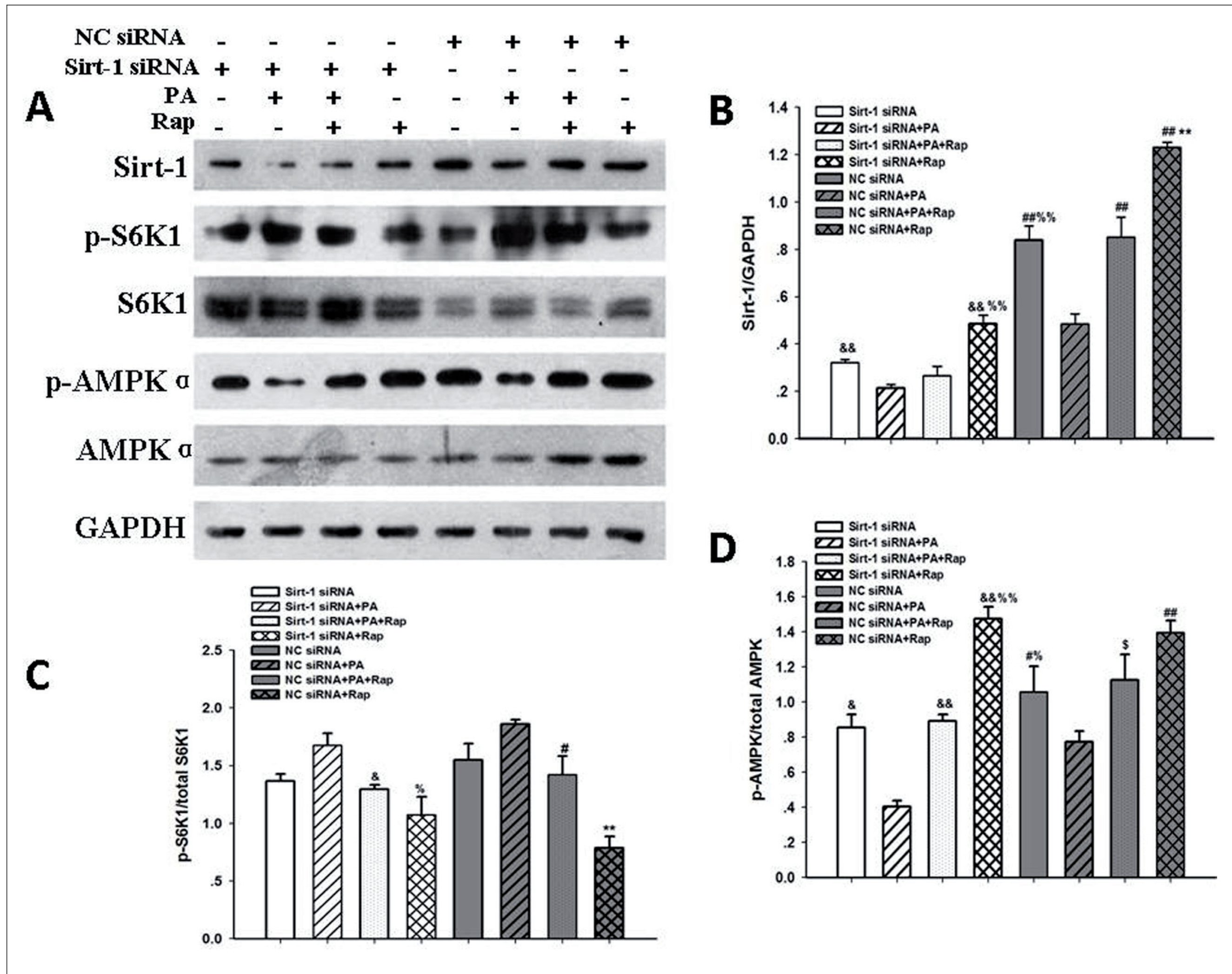

Fig. 4: WB assay demonstrated that knockdown of sirt-1 led to significantly decreased expression level of sirt-1 (A and B). Rapamycin treatment significantly increased the protein expression levels of sirt-1 and p-AMPK/AMPK both in NC siRNA and sirt-1 siRNA-treated hepatocytes (A , B and D). Correspondingly, rapamycin treatment significantly down-regulated p-S6K1/S6K1 ration, indicating the block of mTOR signaling (C). * p<0.05, ** p<0.01 vs. NC siRNA group; # p<0.05, ## p<0.01 vs.NC siRNA+PA; % p<0.05, %% p<0.01 vs. Sirt-1 siRNA group; & p<005, && p<001 vs. Sirt-1 siRNA+PA group.

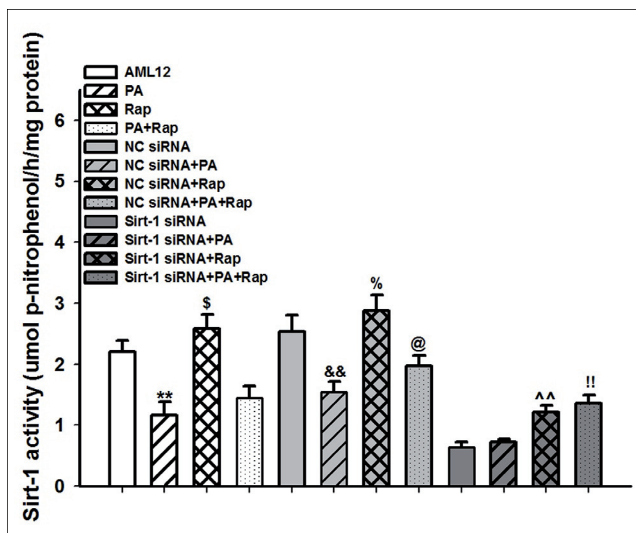

Fig. 5: PA exposure significantly decreased sirt-1 deacetylase activity, whereas rapamycin treatment significantly increased sirt-1 deacetylase activity. ** p<0.01 vs. AML12 group; $ p<0.05 vs. AML12 group; && p<0.05 vs. NC siRNA group; % p<0.05 vs. NC siRNA group; @ p<0.05 vs. NC siRNA+PA group; ^^ p<0.01 vs. Sirt-1 siRNA group; !! p<0.01 vs. Sirt-1 siRNA+PA group.

4. Experimental

4.1. Chemicals and reagents

Palmitate, dexamethasone, and bovine serum albumin (BSA) were purchased from Sigma. Culture mixture containing insulin, transferrin and selenium was obtained from Invitrogen. Primary antibodies against sirt-1, p-AMPKα, AMPKα, S6K1, p-S6K1 (Thr 389) were all from Cell Signaling Technology (Beverly, MA, USA).

4.2. Cell culture

The mouse AML12 liver cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in DMEM/F12 (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum (Hyclone), 1% antibiotics, 5 mg/L insulin, 5 mg/L transferrin, 5 µg/L selenium acid and 40 ng/ml dexamethasone in a 37 °C incubator with 5 % CO_2. Cells were subcultured beyond 80% confluence.

4.3. Preparation of palmitate-BSA complex

Palmitic acid was dissolved at 70 °C in 0.1 M NaOH to obtain a 100 mM stock solution, and a 5% (w/v) solution of fatty-acid-free BSA was prepared in serum-free DMEM/F12 medium. The two solutions were suitably combined to prepare BSA-conjugated palmitate. The mixture was conveniently further diluted in DMEM/F12 to reach the desired final concentrations.

4.4. MTT assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. AML12 cells were plated into 96-well until they reached approximately 60% confluency, and stimulated with a culture medium

containing paltimate (450 μM) for 48 h with or without different concentrations of rapamycin (25 nM, 50 nM, 100 nM, 200 nM). At the end of the exposure, MTT (20 μL, 5 mg/mL) solution was added to each well and the plates were incubated at 37 °C for additional 4 h. The medium was then removed, and 100 μL DMSO was applied to each well to dissolve the purple formazan crystals. Absorbance was measured at 492 nm, and results were standardized using vehicle group values.

4.5. Oil red staining

Intracellular lipid accumulation was evaluated by staining of neutral fats and cholesterol esters with Oil Red O. Cells were fixed with 4% formalin and stained with freshly diluted Oil Red O solution (0.5%) for 20 min. After washing, Oil Red O was extracted by isopropanol and its optical density was monitored spectrophotometrically at 492 nm.

4.6. Sirt-1 siRNA transfection

AML12 cells were plated in antibiotic-free medium at least 24 h before transfection and transiently transfected with either control scrambled siRNA (NC siRNA) or sirt1-specific siRNA(sirt-1 siRNA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The siRNA that provided the most effective inhibition was used for the experiments.

4.7. TG and FFA assay

To determine intracellular triglyceride (TG) and free fatty acid (FFA) content, cells were seeded in 6-well plates. Intracellular TG and FFA content were evaluated after lysis of the cells, and determined by commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the protocal provided by manufacturer. Results were normalized by the protein content.

4.8. Sirt-1 activity assay

A commercial assay kit (Qiyi Bioengineering Inc., Shanghai, China) was used to measure the sirt-1 deacetylase activity according to the manufacturer's protocol. The absorbance was measured at 405 nm using a microplate reader.

4.9. Western Blotting

Cells were harvested and lysed in RIPA buffer containing 150 mM NaCl, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors. Total cell proteins were separated by 12% SDS–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane, and then hybridized with primary antibodies overnight at 4 °C. The proteins were washed in TBST (Tris-buffered saline containing 0.1% Tween 20) and detected using horseradish peroxidase-conjugated secondary antibodies. After washing, the optical density values of bands were measured with Image J software.

4.10. Statistical analysis

Data are shown as means±SE. One-way analysis of variance (ANOVA) was used to test the homogeneity for variance and LSD test was applied to test the significance of differences between multiple groups. A P value of less than 0.05 was considered significant. The final results represented the average data of three independent tests.

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Conflict of interest: None declared.

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