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# Melatonin Upregulates the Activity of AMPK and Attenuates Lipid Accumulation in Alcohol-induced Rats

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## Abstract

**Aims:** Melatonin is supposed to be an effective hepatoprotective agent. The effects and mechanisms of melatonin on alcoholic fatty liver (AFL) have not been well explored. The aim of this study was to investigate the preventive and therapeutic effects of melatonin on alcohol-induced fatty liver rats.
**Methods:** The AFL rats were induced by intragastric infusion of alcohol plus a high-fat diet for 6 weeks, and melatonin (10, 20, 40 mg/kg) was administered by gastric perfusion. We also established fatty acid overload cell model in HepG2 cells to investigate the effect of melatonin on AMP-activated protein kinase (AMPK) activity.
**Results:** The results showed that melatonin (20 and 40 mg/kg) administration significantly reduced alcohol-induced hepatic steatosis with lowering activities of serum alanine aminotransferase, aspartate aminotransferase and levels of serum and hepatic triglyceride. The activity of superoxide dismutase was increased and the content of malondialdehyde was decreased in liver homogenates of rats treated with melatonin. Melatonin increased the phosphorylation of AMPK in the liver tissues of alcohol-induced rats as well. Additionally, *in vitro* studies showed that melatonin increased the expression of melatonin1A receptor (MT1R), whereas luzindole, a receptor antagonist of melatonin, had no effect on its expression. In addition, melatonin reduced the levels of adenosine 3′,5′-cyclic monophosphate (cAMP) and increased the phosphorylation of AMPK, and melatonin treatment could markedly reverse these effects.
**Conclusion:** In conclusion, melatonin could protect against liver injury caused by alcohol gastric perfusion. The effect may be related to alleviating lipid peroxidation and upregulating the activity of AMPK mediated by MT1R signaling pathway.

## INTRODUCTION

Alcoholic liver disease (ALD) is a main cause of morbidity and mortality all over the world (Gao and Bataller, 2011). Alcoholic fatty liver (AFL), the initial stage of the disease, is characterized by triglyceride (TG) accumulation in hepatocytes, which has been generally supposed to be a benign and reversible condition (Purohit *et al.*, 2009; Seth *et al.*,

2011). However, if alcohol abuse continues, hepatic steatosis can progress to the advanced stages of ALD, such as steatohepatitis, fibrosis, cirrhosis and even hepatocellular carcinoma, especially in the presence of co-factors including diabetes (Hassan, 2002), hepatitis virus infection (Gramenzi *et al.*, 2006; Gao and Bataller, 2011) and smoking (Kuper *et al.*, 2000; Marrero *et al.*, 2005). Therefore, understanding of the mechanisms by which chronic alcohol consumption disrupts lipid metabolism and subsequently accumulates excessive TG in hepatocytes is important and probably provides new insights into the prevention and treatment of ALD.

The mechanisms of ALD are complex and multifactorial. Many mechanisms are supposed to be involved in ALD, including oxidative stress, excess lipid synthesis and inflammation (Kanuri *et al.*, 2009). Therefore, treatment approaches that affect the antioxidant defense or regulate lipid metabolism may be beneficial in patients with ALD.

Melatonin, a lipophilic indoleamine derived from tryptophan, is secreted by the pineal gland. It exerts many physiological and biochemical functions via specific cell membrane and nuclear receptors, although many of its functions are receptor-independent, including interaction with cytosol proteins and scavenging of free radicals (Claustrat *et al.*, 2005; Galano *et al.*, 2011). In both *in vivo* and *in vitro* experiments, melatonin and its metabolites could directly scavenge a variety of free radicals and reactive oxygen intermediates (Tan *et al.*, 2007; Hardeland *et al.*, 2009). In addition, melatonin has been reported to reduce free radical levels via stimulating the activities of antioxidative enzymes (Rodriguez *et al.*, 2004; Pan *et al.*, 2006), including superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase. Several earlier studies showed that melatonin protects high-fat diet-induced nonalcoholic fatty liver disease (NAFLD) by attenuating oxidative stress and inhibiting inflammatory cytokine release (Pan *et al.*, 2006; Zaitone *et al.*, 2011; Hatzis *et al.*, 2013).

The AMP-activated protein kinase (AMPK) plays a key role in the control of carbohydrate and fat metabolism, acting as a metabolic master switch in response to the alterations in cellular energy charge. Once activated, AMPK leads to inhibition of lipogenesis and the stimulation of fatty acid oxidation (Viollet *et al.*, 2006; Lin *et al.*, 2007). The phosphorylation of AMPKα-$Thr^{172}$ is required for the activity of AMPK. Recent studies showed that AMPK activity can be attenuated in response to adenosine 3′,5′-cyclic monophosphate (cAMP)-elevating agents. As the study of Hurley *et al.* (2006) showed, the alteration of AMPK activity mediated by cAMP-elevating agents was the result of at least two limbs of regulation via modulation of multiple phosphorylation sites. In one limb, the phosphorylation of α-$Thr^{172}$ was reduced by inhibiting the calcium/calmodulin-dependent protein kinase (CaMKK), an AMPK kinase, mediated by cAMP/cAMP-dependent protein kinase (PKA). In the other limb, PKA, a downstream signal of PC12 cells, could directly phosphorylate AMPKα-$Ser^{485/491}$, whereas the phosphorylation of AMPKα-$Ser^{485/491}$ may decrease the accessibility of theα-$Thr^{172}$ site to its AMPK kinases. However, these researches were conducted with INS-1 cell or PC12 cells. The effect of cAMP on the activity of AMPK was not fully understood in fatty acid-overloaded HepG2 cells.

It is widely accepted that most physiological and biochemical functions of melatonin could be mediated by the G protein-coupled high-affinity membrane receptors MT1 (MT1R) and MT2 (Vanecek, 1998). The function of MT1R has been described in many tissues, including liver tissues (Naji *et al.*, 2004). The inhibition of the production of cAMP by a Gi/Go protein is one of the most important signaling pathways involving MT1 and MT2 receptors (Naji *et al.*, 2004; Ishii *et al.*, 2009). Whether melatonin can regulate AMPK activity mediated by Gi/cAMP pathway still remains unclear.

Although increasing evidence shows that melatonin exerts a protective role in NAFLD, its role in the pathogenesis of ALD remains obscure. The study of Hu *et al.* showed that melatonin protected against alcoholic liver injury by attenuating oxidative stress, inflammatory response and apoptosis, but its regulatory effects on lipid metabolism and its concrete mechanisms are still unknown (Hu *et al.*, 2009). The previous studies mainly focused on its antioxidant actions, whereas its specific mechanisms of regulating lipid metabolism are not fully understood. In this study, we explored the effects of melatonin on alcohol-induced AFL in rats. In the *in vitro* study, we mainly focused on the contribution of MT1R to the reducing lipid accumulation effect of melatonin, looking into its potential capacity to modulate cAMP levels and AMPK activation.

## MATERIALS AND METHODS

### Animals

Adult, male, Sprague–Dawley rats weighing 110–160 g were obtained from the Animal Department of Anhui Medical University. The animals were maintained on a 12 h light/12 h dark cycle under a regulated temperature (25 ± 1°C), and had free access to food and tap water. All animal studies were conducted with the approval of the Animal Care and Use Committee of Anhui Medical University, China (reference number: LLSC2013007).

### Experimental design and treatments

After acclimation for 1 week, rats were randomly divided into six groups: control group ($n = 8$) fed with regular diet, model group ($n = 8$) treated with alcohol for 6 weeks, melatonin groups (10, 20. 40 mg/kg; $n = 8$, respectively) treated with alcohol and different melatonin doses, positive drug group ($n = 8$) treated with alcohol and reduced glutathione (GSH, 180 mg/kg) for 6 weeks. All groups of rats were fed with the same fat-rich diet (89% common animal feeds + 1% cholesterol + 10% lard oil) except for control group. The rats of model group, melatonin groups and positive drug group received alcohol by gavage once a day, whereas normal group received equal amount of physiological saline. The daily doses and concentrations of alcohol were 5 ml/kg/day (35%, v/v) in the first 3 days and then increased progressively by 5% every 3 days up to a maximum of 5 ml/kg/day (55%, v/v). Melatonin (Sigma-Aldrich, St Louis, MO, USA) was gavaged at 4 pm every day with the doses of 10, 20, 40 mg/kg/day. In the positive drug group, GSH was injected intraperitoneally with the doses of 180 mg/kg. At the end of the experimental period, the final body weight of each animal was recorded. Then animals were anesthetized by injecting 10% chloral hydrate (3 ml/kg) intraperitoneally after fasting for 12 h and blood was collected by femoral artery puncture into tubes and centrifuged (1500 g, 10 min, 4°C). The serum was stored at −80°C till assayed as described below. The liver was rapidly dissected and washed with cold 0.9% saline (1:9, w:v). It was weighed and the liver index was calculated (liver weight/body weight × 100). Then one part of liver tissue was fixed in formalin for routine histological examination. The remaining livers were stored at −80°C if required.

### Histological examination of liver

Liver tissue sections were fixed in 4% formaldehyde saline and embedded in paraffin. Hematoxylin and eosin staining was performed for conventional morphological evaluation. Two experienced histologists made histological assessments under light microscope independently (Olympus, Olympus LX70, Japan).

### Analysis of liver function and serum lipids

Serum activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and levels of low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and TG were measured by spectrophotometry using commercially available kits (Nanjing Jiancheng Institute of Biotechnology, China).

### Hepatic triglyceride content determination

For hepatic TG determination, 100 mg of liver tissue was homogenized in 0.9% saline (1:9, w:v), and then TG was extracted with methanol:chloroform (1:2) (Pan *et al.*, 2006). The content of hepatic total TG was detected by enzyme-coupling colorimetric methods with commercial kits.

### Liver MDA content and SOD activity assaying

Livers were weighed and homogenized with 0.9% saline (1:9, w:v). Homogenates were centrifuged (1000 g, 10 min, 4°C) and the supernatant was collected. The content of MDA was detected by thiobarbituric acid (TBA) method and the activity of SOD was detected by the hydroxylamine method with commercially available kits (Nanjing Jiancheng Institute of Biotechnology, China).

### Cell culture

HepG2 cells purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China), were maintained in DMEM (Hyclone Co., Logan, UT, USA) containing 10% fetal calf serum (FBS, Hyclone Co.), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich, St Louis, MO)) at 37°C with 5% $CO_2$. To induce fatty acid overloading (Orlicky *et al.*, 2011), HepG2 cells at 70% confluence were exposed to a mixture of oleic acid and absolute alcohol (oleic acid: 100 μM, Sigma-Aldrich; absolute alcohol: 87 mM) for 48 h, with media being exchanged for fresh media after 24 h.

### Oil red O staining and cellular TG content determination

To investigate cellular neutral lipid droplet accumulation, HepG2 cells were stained by the oil red O (ORO) method (Hwang *et al.*, 2005). HepG2 cells were seeded into 24-well cell culture plates. After oleic acid and absolute alcohol treatments, different concentrations of melatonin ($10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$ and $10^{-5}$ M) or luzindole (5 μM) were added to the medium for 48 h. After the treatment, cells were washed three times with iced-cold PBS and fixed with 10% paraformaldehyde for 30 min. After fixation, cells were washed and stained with ORO and Harris hematoxylin. Fat droplets in HepG2 cells were stained red. To quantify ORO content levels, we added isopropanol to each sample shaken at room temperature for 5 min after ORO staining. Samples were read spectrophotometrically at 510 nm. The content of cellular TG was detected using a commercially available kit.

### Cyclic AMP immunoassay

For determination of the intracellular (cAMP) levels, cells were seeded into 24-well cell culture plates ($1 \times 10^5$ cells per well). After oleic acid and absolute alcohol treatments, different concentrations of melatonin ($10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$ and $10^{-5}$ M) or luzindole (5 μM) were added to the medium for 48 h. After the treatment, the medium was removed and 500 μl of 0.1 M HCl was added to the plate, incubating cells for 20 min at room temperature (Carbajo-Pescador *et al.*, 2011). Then, cells were scraped off with a cell scraper, transferred to a centrifuge tube and centrifuged for 10 min. The supernatant was collected, and intracellular cAMP was determined using a cAMP-specific ELISA kit (Yeyuan, Shanghai, China) according to the manufacturer's instructions.

### Western blot analysis

Total protein collected from snap-frozen liver or cultured cells was subjected to western blot analysis for phospho- or total forms of AMPK, MT1R (Bioss, China) and β-actin (Zhongshan Jinqiao, China). Protein concentration of each supernatant was quantified using a BCA kit (Biyuntian, China). Lysates were separated electrophoretically by 10% SDS–PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA), blocked in 5% nonfat milk dissolved in TBS containing 0.05% Tween (TBST) for 2 h and then incubated in the following primary antibodies: phospho- or total forms of AMPK, MT1R and β-actin. The membranes were then washed with TBST, incubated with secondary antibody, which was horseradish peroxidase conjugated to either goat anti-mouse IgG or anti-rabbit IgG (Zhongshan Jinqiao, China). The protein bands were detected by an enhanced chemiluminescence (ECL; Thermo Scientific) reaction and exposed to Image Quant LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Densitometry analysis of specific bands was performed by the Image J 14.0 software (National Institutes of Health, Bethesda, MD, USA).

### Statistical analysis

Dates are expressed as mean ± SD. Quantitative data for multiple comparisons between experimental groups were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's test. Values of $P < 0.05$ were considered as statistical significant.

## RESULTS

### Effects of melatonin on liver index

As given in Table 1, the weight gain was significantly lower in the model group than that in the control group, whereas treatment with melatonin and GSH showed no significant difference compared with the model group. Liver index in the model group was significantly higher than that in the control group, but was reduced in melatonin and GSH groups (Table 1).

### Effects of melatonin on pathological changes

No histological abnormalities were observed in the liver tissues of control group. The hepatic parenchyma manifested normal hepatocytes arranged around the central veins (Fig. 1A). All rats in alcohol-lavaged group had varying degrees of hepatic steatosis (Fig. 1B), which were significantly improved by melatonin or GSH administration.

**Table 1.** Effects of melatonin on body weight, wet liver weight and liver index alcohol-induced rats

| Groups | Body weight (g) | Wet liver weight (g) | Liver index (/100 g) |
|---|---|---|---|
| Control | 271 ± 33.3 | 7.42 ± 0.92 | 2.74 ± 0.16 |
| Model | 193 ± 38.1## | 6.36 ± 1.61 | 3.27 ± 0.31## |
| MEL (mg/kg) | | | |
| 10 | 200 ± 29.5 | 5.67 ± 0.85 | 2.85 ± 0.15* |
| 20 | 192 ± 33.7 | 5.57 ± 0.81 | 2.94 ± 0.42* |
| 40 | 197 ± 23.5 | 5.73 ± 0.73 | 2.91 ± 0.19* |
| GSH (mg/kg) | | | |
| 180 | 212 ± 50.2 | 6.35 ± 1.29 | 2.97 ± 0.32* |

Results are mean ± SD of eight rats.
MEL, melatonin.
*$P < 0.05$ *vs.* model group.

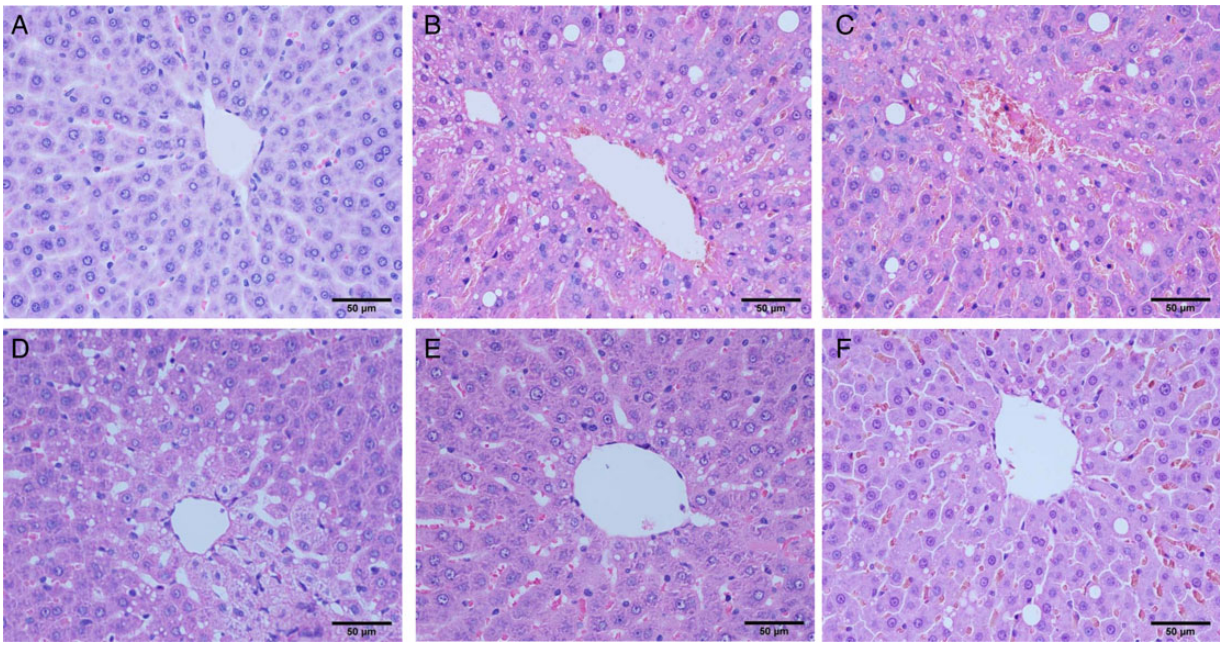



**Fig. 1.** Histology of the liver sections stained with hematoxylin and eosin (H&E, original magnification, ×200; MEL, melatonin). (**A**) Normal group. (**B**) Model group. (**C**) MEL group (10 mg/kg). (**D**) MEL group (20 mg/kg). (**E**) MEL group (40 mg/kg). (**F**) GSH group (180 mg/kg).

**Table 2.** Effects of melatonin on serum liver enzyme activities (AST and ALT), HDL-C, LDL-C, TG and hepatic TG level in alcohol-induced rats (MEL, melatonin)

| | Serum ALT (U/l) | Serum AST (U/l) | Serum TG (mg/dl) | Serum HDL-C (mg/dl) | Serum LDL-C (mg/dl) | Hepatic TG (mg/g) |
|---|---|---|---|---|---|---|
| Control | 36.26 ± 1.16 | 35.20 ± 3.72 | 58.05 ± 6.65 | 41.67 ± 9.00 | 29.74 ± 8.30 | 4.85 ± 1.03 |
| Model | 49.74 ± 3.03$^{\#\#}$ | 51.88 ± 2.54$^{\#\#}$ | 100.45 ± 12.72$^{\#\#}$ | 23.53 ± 3.22$^{\#\#}$ | 50.43 ± 5.61$^{\#\#}$ | 7.02 ± 0.57$^{\#\#}$ |
| MEL (mg/kg) | | | | | | |
| 10 | 47.46 ± 9.77 | 48.36 ± 3.67 | 88.21 ± 19.91 | 30.39 ± 6.35 | 44.82 ± 7.07 | 5.99 ± 0.63** |
| 20 | 39.40 ± 4.39** | 41.49 ± 2.34** | 90.02 ± 15.97 | 28.92 ± 6.55 | 39.22 ± 6.26* | 5.66 ± 0.53** |
| 40 | 37.09 ± 2.37** | 42.08 ± 3.31** | 70.75 ± 18.77** | 31.37 ± 3.03* | 37.93 ± 6.26** | 5.68 ± 0.54** |
| GSH (mg/kg) | | | | | | |
| 180 | 37.09 ± 2.37** | 47.26 ± 2.14* | 78.00 ± 11.34* | 35.78 ± 2.21** | 35.78 ± 6.42* | 5.24 ± 0.73** |

Results are mean ± SD of eight rats.
$^{\#\#}P < 0.01$ *vs.* control group, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* model group.

## Effects of melatonin on liver function, serum lipids and hepatic TG content

The results presented in Table 2 showed that alcohol administration caused a significant increase in the activities of AST and ALT and levels of serum LDL-C and both serum and hepatic TG. Treatment with melatonin and GSH showed a greater decrease in serum activities of ALT and AST (melatonin: 20, 40 mg/kg) and the levels of serum LDL-C (melatonin: 40 mg/kg), serum TG (melatonin: 40 mg/kg) and hepatic TG (melatonin: 10, 20, 40 mg/kg) than in the model group. The levels of HDL-C significantly decreased in the model group, melatonin (40 mg/kg) and GSH (180 mg/kg) treatment significantly reversed these values (Table 2).

## Effects of melatonin on MDA content and SOD activity in liver homogenates

Alcohol gavage led to a significant increase in the level of MDA and a marked decrease in the activities of SOD compared with control group. Melatonin (10, 20 and 40 mg/kg) and GSH (180 mg/kg) treatment significantly reversed these values (Fig. 2).

## Effects of melatonin on the phosphorylation of AMPK in liver tissue

AMPK is supposed to be a key player in regulating cellular energy balance, and AMPK phosphorylation levels at threonine 172 are currently thought as a marker of AMPK activity (Lin *et al.*, 2007). Therefore, we determined the phosphorylation of AMPK at threonine 172 in alcohol-induced rats. Results showed that the phosphorylated AMPK was inhibited in alcohol-induced rats compared with control group. Melatonin and GSH administration significantly alleviated the inhibitory effect (Fig. 3).

## Effects of melatonin on cellular lipid accumulation in HepG2 cells

To evaluate the inhibitory effects of melatonin on cellular lipid accumulation, HepG2 cells were exposed to a mixture of oleic acid and absolute alcohol and co-treated with different concentrations of melatonin, and fat decrease levels were detected by ORO staining after 48 h. As shown in Fig. 4A, in untreated control of HepG2 cells, ORO staining showed almost absence of intracellular lipid

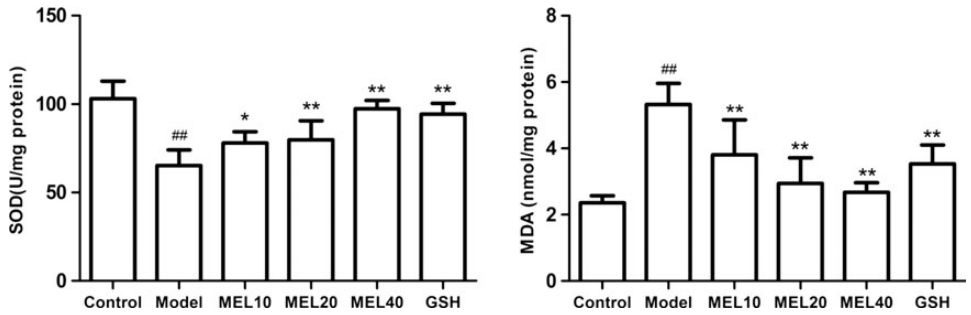



**Fig. 2.** Effects of melatonin on the activities of SOD and the content of MDA in liver homogenates of different groups. MEL, melatonin. Results are mean ± SD of eight rats. $^{\#\#}P<0.01$ *vs.* control group, $^{*}P<0.05$, $^{**}P<0.01$ *vs.* model group.

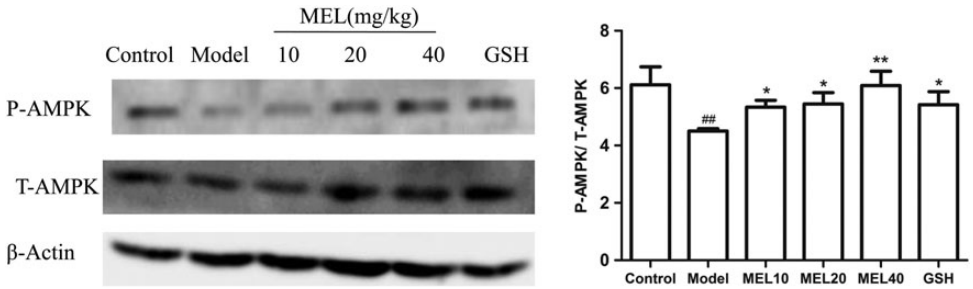



**Fig. 3.** Effects of melatonin on AMPK phosphorylation in alcohol-induced rats (MEL, melatonin). The phosphorylate AMPK (P-AMPK) and total form of AMPK (T-AMPK) protein levels were detected by western blot analysis. Results are mean ± SD of eight rats. $^{\#\#}P<0.01$ *vs.* normal group, $^{*}P<0.05$, $^{**}P<0.01$ *vs.* model group.

(Fig. 4A). After treatment with oleic acid and absolute alcohol, lipid droplets were significantly accumulated in the cytoplasm of HepG2 cells. Figure. 4B is the quantification of cellular lipid content. As shown in the figure, the intracellular lipid content could be reduced significantly by treatment with melatonin ($10^{-8}$, $10^{-7}$, $10^{-6}$ and $10^{-5}$ M). These results were further confirmed by the quantification of intracellular triglyceride content. As shown in Fig. 4, melatonin ($10^{-8}$, $10^{-7}$, $10^{-6}$ and $10^{-5}$ M) also showed a significant inhibitory effect on triglyceride accumulation in HepG2 cells, and reached its maximum effects at $10^{-6}$ M. Therefore, the concentration of melatonin ($10^{-6}$ M) was chosen as the candidate for the following experiments.

### Effects of melatonin on the phosphorylation of AMPK in HepG2 cells

The phosphorylation of AMPK cells was investigated by western blot in oleic acid and absolute alcohol-induced HepG2 cells. As shown in Fig. 5, melatonin administration remarkably enhanced the phosphorylation of AMPK compared with oleic acid and absolute alcohol-induced group. However, luzindole treatment significantly suppressed its effects.

### Effects of melatonin on MT1R protein expression and cAMP level in HepG2 cells

To evaluate the effects of melatonin on cAMP levels in oleic acid and absolute alcohol-induced HepG2 cells, cells were treated with different concentrations of melatonin. Using a cAMP-specific ELISA kit, we observed that melatonin ($10^{-7}$, $10^{-6}$ and $10^{-5}$ M) significantly decreased cAMP level, and reached its maximum at $10^{-6}$ M. However, the level of cAMP significantly increased in group treated with $10^{-6}$ M melatonin plus 5 μM luzindole (Fig. 6).

For an explanation of these effects, additional Western blot test of MT1R was performed to examine whether the effects were receptor-mediated. As illustrated in Fig. 7, MT1R significantly reduced in oleic acid and absolute alcohol-induced group compared with control group. Melatonin and luzindole treatment markedly enhanced its expression. These results suggested that the enhanced effects of melatonin on the phosphorylation of AMPK may be mediated by MT1R.

## DISCUSSION

There is increasing evidence that high-fat diet plays an important role in the progression of ALD (French *et al.*, 1986; Yuan *et al.*, 2006; Zhang *et al.*, 2013). French *et al.* (1986) found that high-fat diet was essential for the induction of fibrosis by alcohol in the rat. They also certified that dietary lipids were a key source of the lipids accumulating in the livers of ethanol-fed animals. Therefore, in our study, we used high-fat diet plus alcohol gavage to establish animal model of alcoholic liver injury. Our results demonstrated that chronic alcohol feeding caused hepatic steatosis as evidenced by the increase of liver index, accumulation of hepatic TG, elevation of serum ALT and AST and morphologic changes (small lipid droplets and mild inflammation in hepatocytes) in the liver. This method is a simple experimental model that mimics key aspects of ALD in humans, and could be useful for exploring the effects of melatonin on hepatic steatosis.

Melatonin is a well-known powerful endogenous antioxidant. Novel studies showed that melatonin ameliorated nonalcoholic fatty liver in experimental animals induced by high-fat diet (Pan *et al.*, 2006; Zaitone *et al.*, 2011; Hatzis *et al.*, 2013) or lipopolysaccharide (LPS) (Chen *et al.*, 2011). The role of melatonin in alleviating hepatic steatosis relates mainly to its antioxidant properties. However, our study found that melatonin administration significantly protected

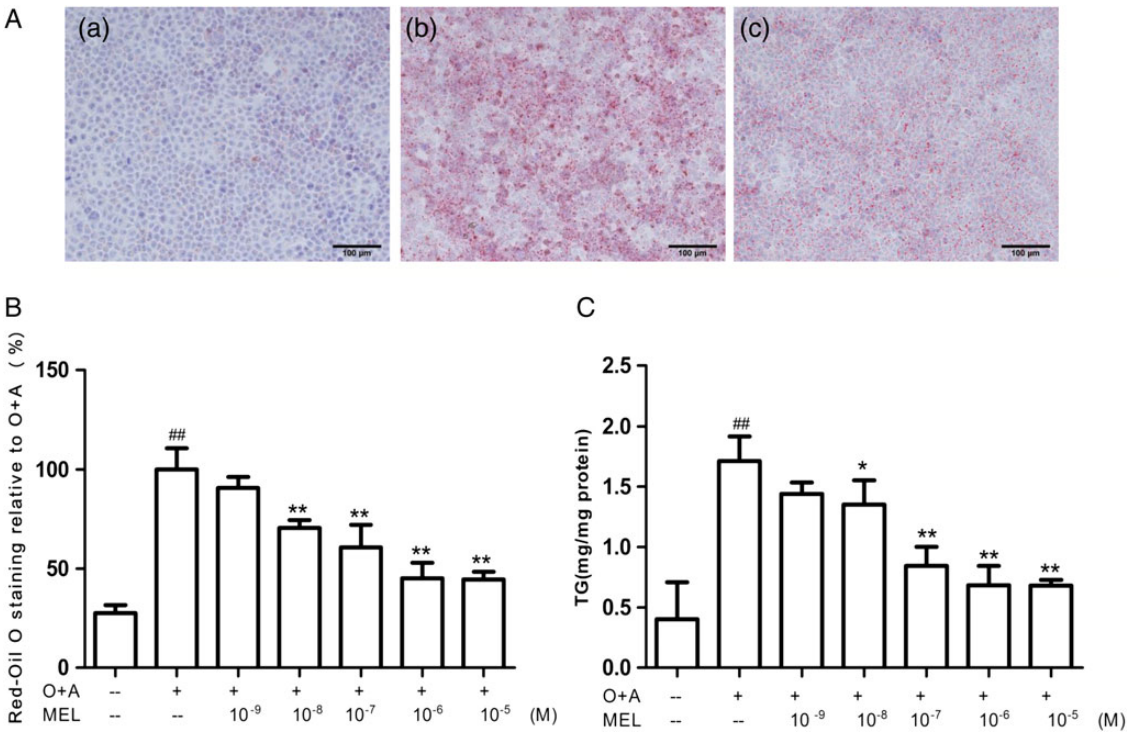



**Fig. 4.** Effects of melatonin on OA plus alcohol-induced cytoplasmic lipid droplet accumulation and TG content in HepG2 cells (MEL, melatonin). Cells are co-treated with OA (100 μM) and alcohol (87 mM) for 48 h in the presence of different concentrations of melatonin or not. (**A**) Representative photomicrographs of oil red O staining (Original magnification, ×400): (a) control; (b) Model; (c) melatonin ($10^{-6}$ M). (**B**) Quantification of fat drops stained by oil red O. (**C**) Cellular TG content. Data were expressed as mean ± SD. $^{\#\#}P < 0.01$ *vs.* control group, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* O + A group.

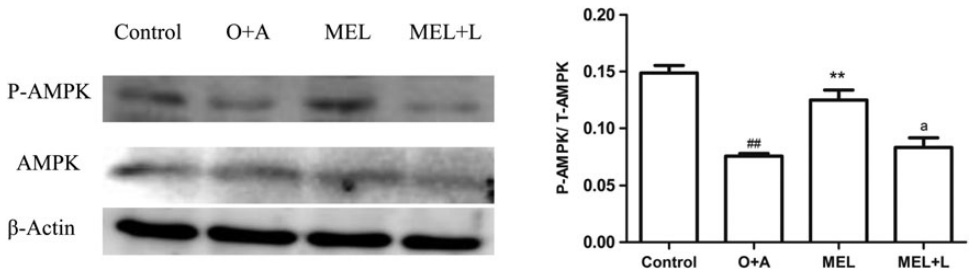



**Fig. 5.** Effects of melatonin on the phosphorylation of AMPK in HepG2 cells (MEL, melatonin; L, luzindole; O + A, OA plus alcohol). Cells were co-exposed to OA (100 μM), alcohol (87 mM) and melatonin ($10^{-6}$ M) for 48 h. The phosphorylate AMPK (P-AMPK), total form of AMPK (T-AMPK) protein levels were measured by western blot analysis. Data were expressed as mean ± SD. $^{\#\#}P < 0.01$ *vs.* normal group, $^{**}P < 0.01$ *vs.* O + A group, $^{a}P < 0.01$ *vs.* melatonin group.

rats against alcohol-induced fatty liver by decreasing elevated serum ALT and AST activities, reducing lipid levels in the serum and liver tissue and lightening hepatic lipid accumulation. Melatonin could decrease the liver index of rats as well, although it had no effects on its weight. These effects of melatonin were probably due to the fact that it could reduce hepatic cell edema and decrease lipid drop accumulation in the liver.

It is well known that oxidative stress plays an important role in the pathogenesis of alcohol-induced liver injury (Arteel, 2003). Oxidative stress results from an imbalance in the production of reactive oxygen species (ROS) and the ability of cells to scavenge them. Alcohol metabolism is an important source of ROS in alcohol-induced fatty liver disease (Arteel, 2003; Tsedensodnom *et al.*, 2013). The overproduction of ROS initiates lipid peroxidation within the cells, which results in the production of aldehyde byproducts such as MDA and *trans*-4-hydroxy-2-nonenal. Antioxidant enzymes, especially SOD and GSH-Px, reduce the levels of free radicals and neutralize the emerging ROS, which are important in preventing lipid peroxidation and maintaining the structure and function of biological membranes. Therefore, we investigated the protective effects of melatonin against alcohol-induced fatty liver by measuring MDA content and SOD activities in the liver. The results demonstrated that melatonin (10, 20 or 40 mg/kg) markedly decreased the levels of MDA and increased the activities of SOD and GSH-Px in the liver of rats induced by alcohol. These results suggested that the protective effects of melatonin on AFL may be partly mediated by its antioxidant actions.

Emerging evidence suggests that melatonin possesses hypolipidemic effects (Baydas *et al.*, 2002; Subramanian *et al.*, 2007; Butun *et al.*, 2013). It reduced plasma and hepatic cholesterol levels as well as hepatic triglyceride levels in hypercholesterolemic mice (Sener *et al.*,

2004). Melatonin also decreased serum or hepatic triglyceride levels in high-fat diet-induced rat (Marrero *et al.*, 2005; Pan *et al.*, 2006; Chen *et al.*, 2011; Zaitone *et al.*, 2011). In the study, we find that high dose of melatonin can reduce both serum and hepatic triglycerides in alcohol-induced rats.

In addition, as shown in pathological pictures, melatonin significantly decreased lipid droplets in hepatic parenchyma and improved hepatic steatosis. The effects of melatonin on decreasing lipid accumulation probably related to its regulating fat metabolism function.

It is well known that AMPK is an enzyme-sensing cellular energy balance that regulates the downstream signaling pathways toward an energy-conserving state. Phosphorylation of AMPKα-Thr$^{172}$ is required for the activity of AMPK. Therefore, we investigated the effects of melatonin on AMPK phosphorylation in alcohol-induced rats. Our result showed that melatonin significantly increased the activity of AMPK, suggesting its hypolipidemic effects probably related to AMPK activation.

To further investigate the mechanisms of the hypolipidemic effects of melatonin, we conducted the *in vitro* studies. Several stimulants, including alcohol (Grunnet *et al.*, 1985), OA (Lin *et al.*, 2007) and tetracycline (Amacher and Martin, 1997), can induce steatosis in cultured hepatocytes. Studies (Okamoto *et al.*, 2002; Lin *et al.*, 2007; Cui *et al.*, 2010) showed that treatment with OA in HepG2 cells can induce morphological similarities to steatotic hepatocytes. Orlicky *et al.* (2011) induced a cytoplasmic lipid droplet accumulation cell model incubating with OA and alcohol in HEK293 cells. These cellular fatty liver models are morphologically mimicking hepatic steatosis. On the basis of the previous studies, we induced the free acid overloading model by exposing HepG2 cells to a mixture of oleic acid and absolute alcohol. The ORO staining results suggested that we successfully reproduced the cell model of fatty acid overloading.

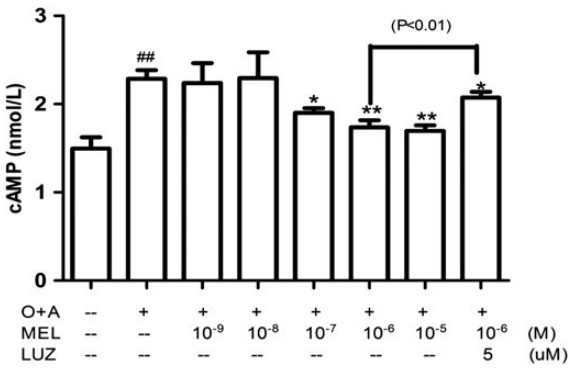
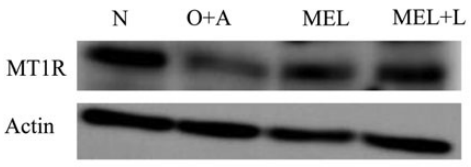



**Fig. 6.** Effect of melatonin and luzindole treatment on cAMP levels in HepG2 cells (MEL, melatonin; LUZ, luzindole; O + A, OA plus alcohol). Cells are co-treated with OA (100 μM) and alcohol (87 mM) for 48 h in the presence of different concentrations of melatonin with luzindole or not. Concentration of cAMP was measured using the cAMP immunoassay kit. Data were expressed as mean ± SD. $^{\#\#}P < 0.01$ *vs.* normal group, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* O + A group.

In addition to its receptor-independent activities, some of the physiological functions of melatonin are mediated by its interaction with two membrane receptors, MT1and MT2 receptor, which belong to the 7-transmembrane G protein-coupled receptor family (Ishii *et al.*, 2009). It is widely accepted that melatonin regulates cell functions by affecting several second messengers via its specific membrane receptors (Stumpf *et al.*, 2009; Wang *et al.*, 2014). Therefore, inhibition of the production of cAMP by coupling to Gi protein is one of the most important signaling pathways involving melatonin receptors (Ishii *et al.*, 2009; Stumpf *et al.*, 2009). Recently, some studies showed that the hypolipidemic effect of melatonin may be mediated by its specific MT1 and MT2 receptors (Brydon *et al.*, 2001; Ishii *et al.*, 2009). Therefore, in *in vitro* studies, we further investigated whether the effects of reducing TG content of melatonin are mediated by its specific MT1R, as MT2R is absent in HepG2 cells (Carbajo-Pescador *et al.*, 2011). We found that melatonin treatment significantly decreased the elevation of TG content in HepG2 cells induced by a mixture of oleic acid and absolute alcohol.

cAMP signaling systems are supposed to regulate many metabolic pathways, such as carbohydrate, lipid and protein metabolic pathways. Previous studies showed that there is a remarkable intersection between the cAMP and AMPK signaling pathways (Hurley *et al.*, 2006). Hurley *et al.* found that cAMP signaling significantly inhibited the activation of AMPK in INS-1 cell line, and the inhibitory effects may result from the inhibition of the activity of AMPK kinases (Hurley *et al.*, 2006). These finding are consistent with our results. In our studies, we found that melatonin treatment significantly decreased the elevation of cAMP level and reduced the inhibition of AMPK activity induced by a mixture of oleic acid and absolute alcohol, whereas luzindole administration markedly reversed its effects. These results suggested that hypolipidemic effects of melatonin may be mediated by MT1R/cAMP/AMPK pathways. However, some studies also showed that activation of PKA, a downstream signal of cAMP, causes a decrease in AMPK activity (Hutchinson *et al.*, 2005; Kimball *et al.*, 2004). These inconsistent results may result from variations in factors such as cell lines, methodology or disease model.

In our study, we found that melatonin unregulated the activity of AMPK in both *in vivo* and *in vitro* experiments. While the study of Hu *et al.* suggested that melatonin had no effects on hepatic expression of lipogenic genes in alcohol-induced rats, and they supposed that



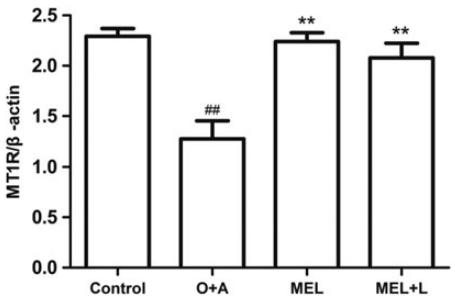




**Fig. 7.** Effects of melatonin on the level of MT1R protein expression in HepG2 cells (MEL, melatonin; L, luzindole; O + A, OA plus alcohol). Cells were co-exposed to OA (100 μM), alcohol (87 mM) and melatonin ($10^{-6}$ M) for 48 h. The MT1R protein levels were measured by western blot analysis. Data were expressed as mean ± SD. $^{\#\#}P < 0.01$ *vs.* normal group, $^{**}P < 0.01$ *vs.* O + A group.

melatonin may not directly affect lipid metabolism in hepatic cells, the different results may be due to the fact that the level of gene expression sometimes does not reflect the level protein expression and the activation of protein. Our study showed that the level of total AMPK had no significant difference among groups, but melatonin increased the level of phosphorylated AMPK in alcohol-induced rats. Then in *in vitro* study, we found that melatonin increased MT1R protein expression, decreased cAMP level and elevated the phosphorylation of AMPK, and luzindole treatment markedly reversed the latter two effects. Therefore, we assumed that melatonin upregulated the activity of AMPK probably mediated by MT1R signaling pathways.

In summary, melatonin treatment significantly improved the pathological abnormalities and decreased ALT, AST and TG in serum as well as TG in the livers of rats given alcohol gavage. Hepatic MDA level was decreased whereas SOD activities were increased in the rats induced by alcohol. In addition, hepatic AMPK activity was markedly inhibited by melatonin treatment in alcohol-induced rats. What is more, we provide evidence that melatonin probably plays a key role in reducing HepG2 cellular lipid accumulation by increasing AMPK phosphorylation via its MT1R. These findings suggest that melatonin exerts protective effects against AFL in rats induced by alcohol probably through two aspects of actions, including its antioxidant actions and the actions mediated by its receptors.

## FUNDING

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## CONFLICT OF INTEREST STATEMENT

None declared.

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