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Increased hepatic mitophagy by acute alcohol feeding ameliorates liver injury in rats: involvement of the hypoxia-inducible factor-1α pathway

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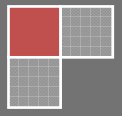
ABSTRACT

Mitophagy plays key roles in the process of acute alcoholic liver injury. Many studies show that HIF-1α may exert direct or indirect effects on liver injury. However, whether HIF-1α can interfere with liver mitophagy remains elusive. The rats were randomly divided into four groups: (1) control group(C); (2) 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) group (CY); (3) five-day alcohol intake group (CA); and (4) five-day-alcohol intake plus YC-1 group (CAY). The rats were investigated to determine the following: BNIP3, HIF-1α, LC3II, Beclin1 mRNA and protein expressions; mitochondrial ROS production; mitochondrial TBARS level; aconitase and ATP synthase activities; mitochondrial inner membrane potential; the number of mtDNA and mitochondrial respiration functions in liver tissue; and serum ALT and AST. The results showed that acute alcohol intake caused significant increased HIF1-α, BNIP3, LC3II and Beclin1 levels and decreased mtDNA copy number. Meanwhile, mitochondrial oxidative injury increased with decreased respiratory function. Added HIF1-α inhibitor resulted in significantly lower HIF1-α, BNIP3, LC3II and Beclin1 expression and increased mtDNA copy number compared to the single acute alcohol intake. However, mitochondrial oxidative injury further increased with further decreased respiratory function. It showed that acute alcohol consumption induced mitophagy may involve the HIF-1a pathway, which can ameliorate liver injury. However, it was not enough to completely clear the damaged mitochondria, resulting in acute alcoholic liver injury in rats.

KEYWORDS

Acute alcoholic liver injury; Hypoxia-inducible factor-1α; mitochondrial oxidative injury; Mitophagy; Rats.



INTRODUCTION

Alcohol induced damage is a disease with a wide clinical spectrum,including fatty liver, hepatic fibrosis, hepatocirrhosis and cancer[1]. Massive drinking can cause mitochondrial lipid, protein, and DNA oxidatived, production of reactive oxgen specises (ROS)[2-5]. Alcohol-induced ROS generation results in mitochondrial damage, which further induces mitochondrial dysfunction[6].

Acute alcohol intake can cause an enhanced accumulation of dysfunctional mitochondria[2]. Previous studies showed that increased autophagy can alleviate or prevent alcoholic liver injury[7-10]. Ding et al.[7,8,11] reported that an increased number of autophagosomes are found in ethanol-treated hepatocytes by electron microscopy. Ethanol-induced autophagy seems to target damaged mitochondria and accumulated lipid droplets selectively but not long-lived proteins. This process is called mitophagy[12]. Mitophagy induced by acute ethanol treatment possibly functions in attenuating ethanol-induced liver injury because damaged mitochondria is a well-known key change in the liver after alcohol binge drinking[8].

However, the change in mitophagy during acute alcoholic liver injury formation remains unclear. Mitophagy is activated by fasting, nutrient deprivation, hypoxia, and so on[12,13]. Zhang et al. showed that hypoxia results in mitophagy increased, and that this process requires hypoxia-dependent factor-1-dependent expression of BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3)[14]. Some studies demonstrated that chronic and acute alcohol administrated caused liver hypoxia[15]. Other studies have postulated an increase in hypoxia-inducible factor-1α (HIF-1α) mRNA as a mechanism of ethanol-induced liver injury[16]. Considering these studies, we hypothesized that hypoxia induced by acute ethanol consumption upregulates HIF-1α expression, which subsequently enhances mitophagy in the livers of rats. The stimulation of mitophagy may be considered as a stress-protective mechanism during acute alcoholic liver injury.

3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1), a small molecule inhibitor of HIF-1α expression, can inhibit HIF-1α protein expression but not inhibit HIF-1α mRNA expression[17]. In this study, we used YC-1 as an inhibitor of HIF-1α expression to study the relationship between HIF-1α and mitophagy in liver after acute alcoholic liver injury in vivo.

EXPERIMENTAL

Animals and treatments

Forty-eight male Sprague-Dawley rats (age: 6 -8weeks; body weight: 176.31 ± 4.65 g) were randomly divided into four groups: (1) control group (C; n = 12), (2) YC-1 group (CY; n = 12), (3) five-day alcohol intake group (CA; n = 12), and (4) five-day alcohol intake plus YC-1 group (CAY; n = 12). YC-1 was administered intraperitoneally (10 mg/kg, once a day for 5 d) parallel to alcohol treatment. Alcohol (5 g/kg body weight) was diluted in water[18] and applied to the rats by gastric intubation using an appropriate stainless steel needle, whereas the control rats received the same volume water for 5 days. All experiments were approved by the Institutional Review Board of the Shandong University of Technology under the guidelines of the Chinese Academy of Sciences.

Preparation of liver mitochondria

The mitochondrial preparation method was slightly modified from Bo,et al[19] and Kessova, I.G, et al[20]. Mitochondrial protein content was quantified according to Lowry et al report[21].

Measurements of mitochondrial thiobarbituric acid-reactive substances (TBARS) content and serum ALT and AST

According to N.Jiang et al report[22], mitochondrial TBARS content was determined in liver as a marker of lipid peroxidation. Serum ALT and AST were measured using a commercial kit (Jiancheng Co., NJ). The process was manipulated according to the manufacturer's instruction.

Determination of liver mitochondrial ROS generation, mitochondrial potential, ATP synthase activity, aconitase activity, and mitochondrial respiration functions

Liver mitochondrial ROS generation was determined by dichlorofluoroscein (DCF) as a probe according to our previous study[19] and report[6]. The inner mitochondrial membrane potential ($\Delta\psi$) was measured using Rhodamine 123 (Sigma) according to our previous report[19]. ATP synthase activity was determined by using BioOrbit 20/20^n luminometer (Turku, Finland) and expressed as nmol/sec per mg protein[19]. Aconitase activity was monitored for 3–5 min by determining the absorbance at 340nm in freshly prepared 50 mM Tris-HCl buffer in a quartz cuvette. Mitochondrial respiratory function was determined using a Clark-type oxygen electrode (YSI, USA) according to our previous study[19].

Measurements of the number of mtDNA copy

According to the previous study[23], the mtDNA copy number was measured by real-time PCR technique.

Determination of BNIP3, HIF-1α, microtubule-associated protein 1 light chain 3(LC3II), and Beclin 1 mRNA expression

The expressions of BNIP3, HIF-1α, LC3II, and Beclin1 were determined in the liver tissue by real-time quantitative reverse-transcription PCR (RT-PCR). RT-PCR was performed in a fluorescence temperature cycler (ABI Co., USA). Fluorescence signal was plotted against the cycle number of the samples and the external standards.

Measurements of BNIP3, HIF-1α, LC3II, and Beclin 1 protein expression

Frozen liver tissue was extracted in SDS loading buffer, and heated at 100 °C for 5 min. The extracted materials were separated on a 15% SDS-PAGE gel. The proteins were transferred to a polyvinylidene difluoride membrane, and immunological detection was performed using polyclonal antibodies against BNIP3, HIF-1α, LC3II, and Beclin 1 (Abcam) at 1:1000 dilution. Anti-rabbit antibodies were used as secondary antibodies. Detection was achieved using an enhanced chemiluminescence (ECL) detection system.

Data analyses

Data were analyzed by using two-way ANOVA. When a main effect was significantly detected, then the Bonferroni post hoc test was used to compare differences between diverse groups. The SPSS(Inc., version 13.0) was used for all analyses. The significance level was set at $p<0.05$.

RESULTS

Acute alcohol consumption induced liver injury

After 5 d of alcohol feeding, lipid peroxidation, as assayed by the formation of TBARS in the liver mitochondria, was increased by 59.77% ($p < 0.01$) (Figure 1A); blood ALT and AST were increased by 80.35% ($p < 0.01$) and 51.36% ($p < 0.01$), respectively(Figure 1C and D); mitochondrial ROS production increased by more than twofold ($p < 0.01$) (Figure 1B); and aconitase activity decreased by 48.29% ($p < 0.01$)(Figure 2A). As shown in Figure 2B and C, liver ATP synthase activity and mitochondrial $\Delta\psi$ were transiently decreased ($p < 0.05$) in CA rats. For the assessment of liver mitochondrial respiration functions in the CA group, state 4 respiration rate was 61.27% higher ($p < 0.01$), state 3 respiration rate and RCR were 35.96% ($p < 0.01$) and 60.15% ($p < 0.01$) lower, respectively, than the control group (TABLE 1).

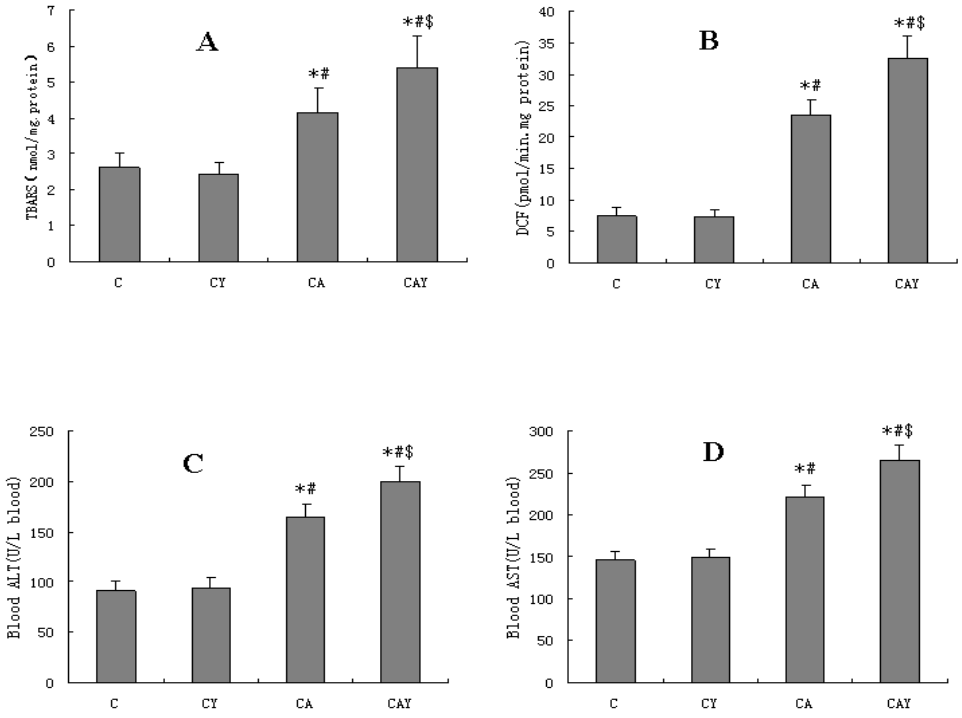


Figure 1 A. : TBARS levels, DCF formation, blood ALT and AST in different groups; Note:*p < 0.01 vs. C, #p < 0.01 vs. CY, $p < 0.05 vs. CA.

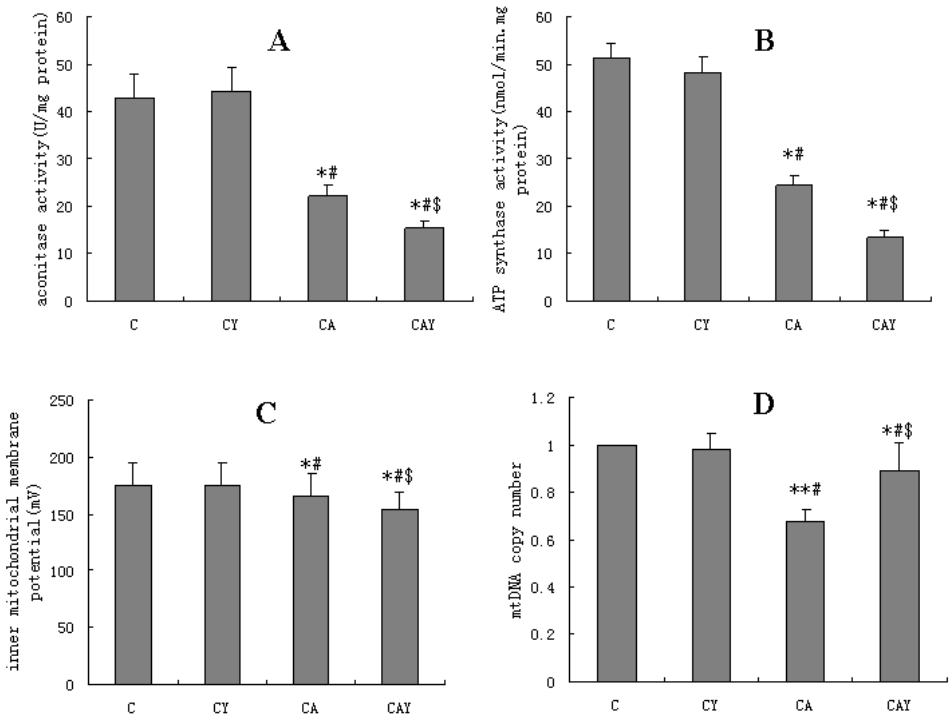


Figure 2 A : Aconitase activity, ATP synthase activity, inner mitochondrial membrance potential and number of mtDNA copy in different groups (*p < 0.05, **p < 0.01 vs. C; $^{\#}$p < 0.05 vs. CY, $^{\$}$p < 0.05 vs. CA).

TABLE 1 : Mitochondrial respiration functions in rat liver.

Group	State 3(nmol O_2/min·mg protein)	State 4(nmol O_2/min·mg protein)	RCR
C	25.14±2.14	9.45±0.72	2.66±0.34
CY	24.22±2.09	10.09±0.87	2.41±0.32
CA	16.10±1.65$^{*\#}$	15.24±1.35$^{*\#}$	1.06±0.20$^{*\#}$
CAY	14.22±1.06$^{*\#\$}$	17.45±1.66$^{*\#\$}$	0.82±0.18$^{*\#\$}$

Data are means ± SEM (*n* = 12). RCR, respiratory control ratio. *p < 0.01 vs. C; $^{\#}$p < 0.01 vs. CY; $^{\$}$p < 0.05 vs. CA.

Acute alcohol consumption enhanced liver mitophagy by increasing HIF-1α expression

The mRNA and protein levels in CA group were obtained and compared with the control group: BNIP3, 188% ($p < 0.05$; Figure 3B) and 131% ($p < 0.05$; Figure 4B); LC3II, 88.1% ($p < 0.01$; Figure 3C) and 56% ($p < 0.01$; Figure 4C); and Beclin1 74.5% ($p < 0.01$; Figure 3D) and 64% ($p < 0.01$; Figure 4D). mtDNA copy number was also significantly lower in the CA group than in the control group(Figure 2D). To prove our hypothesis that acute alcohol consumption-induced mitophagy was related to an increased HIF-1α expression, we examined HIF-1α expression. The results showed that BNIP3, LC3II, and Beclin 1 expressions were consistent with HIF-1α expression (Figure 3 and Figure 4). YC-1, did not alter HIF-1α mRNA significantly ($p > 0.05$; Figure 3A; C rats vs. CY rats, and CA rats vs. CAY rats). However, HIF-1α protein content decreased significantly ($p < 0.05$; Figure 4A; CA group vs. CAY group). At the same time, the inhibited HIF-1α protein expression decreased the mRNA and protein expressions of BNIP3, LC3II, and Beclin1, compared to CA group (Figure 3 B, C and D ; Figure 4 B, C and D). The number of mtDNA copy was significantly increased ($p < 0.05$; Figure 2D).

Enhanced liver mitophagy ameliorated acute alcohol-induced liver injury

BNIP3, LC3II, and Beclin1 mRNAs and proteins were reduced when the rats were administered with HIF-1α inhibitor in the CAY group, however, the number of mtDNA increased, compared to CA group. At the same time, mitochondrial TBARS, serum ALT and AST, mitochondrial ROS production, and state 4 respiration rate were significantly increased, but aconitase and ATP synthase activities, RCR, state 3 respiration rate, and $\Delta\psi$ were markedly decreased (TABLE 1 ; Figure 1,2,3 and 4).

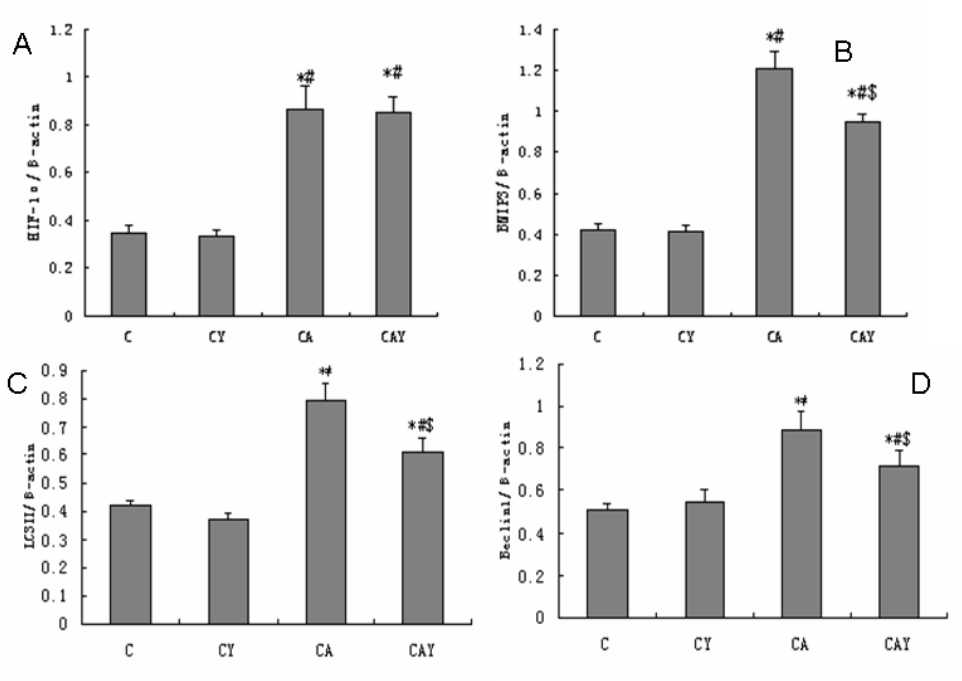


Figure 3A : HIF-1*α*, BNIP3, LC3 II and Beclin1 mRNA relative abundance in the liver cells in different groups; Note: *p < 0.01 vs. C, #p < 0.01 vs. CY, $p < 0.05 vs. CA.

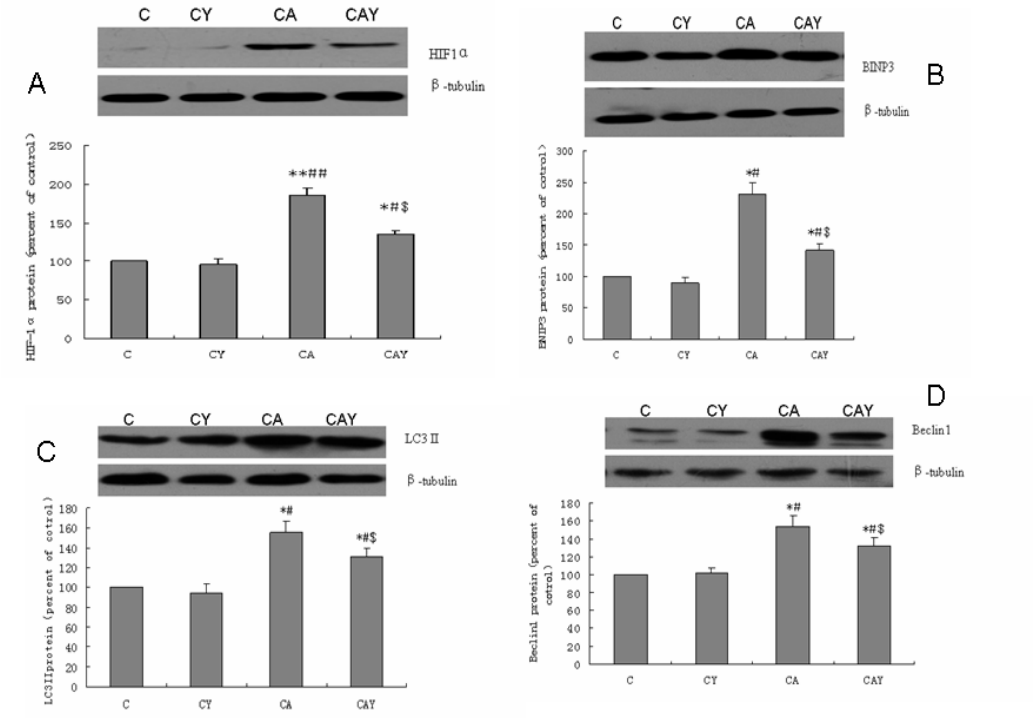


Figure 4 A. HIF-1α protein content in the liver cells in different groups (*p < 0.05, **p < 0.01 vs. C, #p < 0.05, ##p < 0.01 vs. CY, $p < 0.05 vs. CA); B. BNIP3 protein content in the liver cells in different groups; C. LC3 II protein content in the liver cells in different groups; D. Beclin 1 protein content in the liver cells in different groups; Note: Figure B. C. D: *p < 0.01 vs. C, #p < 0.01 vs. CY, $p < 0.01 vs. CA.

DISCUSSION

Acute and chronic ethanol treatments caused an increased ROS production; peroxidation of lipid, protein, and DNA as well as mitochondrial dysfunction was induced[4]. Previous investigations showed that the impairment of mitochondrial function results from ethanol treatment that induces ROS production and causes cell toxicity[24]. In our present study, serum

ALT and AST, mitochondrial TBARS, mitochondrial ROS production, and state 4 respiration rate were increased after acute alcohol consumption. By contrast, aconitase activity, $\Delta\psi$, ATP synthase activity, state 3 respiration rate, and RCR were markedly reduced. These results strongly suggested that the five-day alcohol consumption induced serious liver injury in rats.

Although acute alcoholic liver injury poses risks, the pathogenesis remains unclear. Previous studies showed that the early manifestations of alcohol liver injury include mitochondrial dysfunction, mitochondrial morphology change[25], and damaged mitochondria accumulation[6,26,27]. Therefore, the timely removal of damaged mitochondria is one of the important approaches to prevent acute alcoholic liver injury[7]. Clearance of damaged mitochondria is associated with mitophagy[28]. Our study found that the number of mtDNA copy decreased significantly, but BNIP3, LC3II, and Beclin1 expressions increased after acute alcohol intake. This result indicated that mitophagy occurred in the liver cell during acute alcoholic liver injury. Ding et al.[11] and Eid et al.[29] also found that alcohol intake induces mitophagy. Increased mitophagy in hepatocytes after alcohol intake corresponds to ethanol toxicity and can represent a possible protective mechanism by stimulating mitophagy[29]. The inhibition of autophagy promotes binge ethanol-induced hepatotoxicity, steatosis, and oxidant stress[30].

Although mitophagy can control mitochondrial quality, the mechanism remains unclear. Considering that acute alcohol-induced liver hypoxia and the relationship between mitophagy and HIF-1α, we suggested that mitophagy enhancement was associated with HIF-1α expression in acute alcoholic liver damage. Our results strongly supported this hypothesis by showing that HIF-1α protein expression in liver cells increased markedly after acute alcohol intake, causing an increase in mitophagy-related proteins BNIP3, LC3II, and Beclin1, but a significant decrease in the number of mtDNA copy was found. To test our hypothesis, we injected YC-1 to the experimental rats and found that the protein content of the rat liver HIF-1α was significantly reduced compared with the control rats. BNIP3, LC3II, and Beclin 1 mRNA and protein expressions also decreased markedly, but the number of mtDNA copy was increased significantly. These results clearly demonstrated that mitophagy induced by acute alcoholic liver injury was associated with increased HIF-1α expression in rats.

Previous studies suggested that mitophagy can remove damaged mitochondria to maintain proper functioning of the mitochondria[31]. Although mitophagy was enhanced by acute alcohol consumption in this study, mitochondrial ROS generation, TBARS content, serum ALT and AST, and mitochondrial state 4 respiration rate increased, but aconitase activity, mitochondrial membrane potential, ATP synthase activity, mitochondrial state 3 respiration rate, and RCR decreased. It seemed that mitophagy enhancement was conflict with the decreased mitochondrial function and increased liver injury. However, we think this result is not paradoxical. Although mitophagy enhancement induced by acute alcoholic liver injury removes a part of the damaged mitochondria, binge drinking results in a large number of damaged mitochondria accumulated. The ability of mitophagy to clear damaged mitochondria was limited, when beyond its limit, a large number of damaged mitochondria accumulation was inevitable. Therefore, mitophagy induced by acute alcohol consumption can not completely prevent liver injury. However, a protective mechanism by stimulating mitophagy was observed. The degree of alcohol-induced liver injury is determined by a balance between mitophagy and damaged mitochondria accumulation. If mitophagy can not clear the damaged mitochondria in time, damaged mitochondria continue to accumulate, resulting in liver injury.

CONCLUSION

It showed that acute alcohol consumption induced mitophagy may involve the HIF-1a pathway, which can ameliorate liver injury. However, it was not enough to completely clear the damaged mitochondria, resulting in acute alcoholic liver injury in rats.

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REFERENCES

[1] M.R.Lucey, P.Mathurin, T.R.Morgan; *The New England journal of medicine*. **360**, 2758-69 **(2009)**.
[2] A.Dey, A.I.Cederbaum; *Hepatology (Baltimore, Md.)*. **43**, S63-74 **(2006)**.
[3] A.Mansouri, C.Demeilliers, S.Amsellem, D.Pessayre, B.Fromenty; *The Journal of pharmacology and experimental therapeutics*. **298**, 737-43 **(2001)**.
[4] S.K.Mantena, A.L.King, K.K.Andringa; *World journal of gastroenterology : WJG*. **13**, 4967-73 **(2007)**.
[5] A.Venkatraman, A.Landar, A.J.Davis; *The Journal of biological chemistry*. **279**, 22092-101 **(2004)**.
[6] D.Gyamfi, H.E.Everitt, I.Tewfik, D.L.Clemens, V.B.Patel; *Free radical biology & medicine*. **53**, 2131-45 **(2012)**.
[7] W.X.Ding, M.Li, X.Chen, et al; *Gastroenterology*. **139**, 1740-52 **(2010)**.
[8] W.X.Ding, S.Manley, H.M.Ni; *Experimental biology and medicine (Maywood, N.J.)*. **236**, 546-56 **(2011)**.
[9] H.Dong, M.J.Czaja; *Trends in endocrinology and metabolism: TEM*. **22**, 234-40 **(2011)**.
[10] R.Singh, S.Kaushik, Y.Wang; *Nature*. **458**, 1131-5 **(2009)**.
[11] W.X.Ding, M.Li, X.M.Yin; *Autophagy*. **7**, 248-9 **(2011)**.
[12] R.J.Youle, D.P.Narendra; *Nature reviews. Molecular cell biology*. **12**, 9-14 **(2011)**.

[13] W.X.Ding, X.M.Yin; *Biological chemistry*. **393**, 547-64 **(2012)**.
[14] H.Zhang, M.Bosch-Marce, L.A.Shimoda; *The Journal of biological chemistry*. **283**, 10892-903 **(2008)**.
[15] S.W.French; *Hepatology research : the official journal of the Japan Society of Hepatology*. **29**, 69-74 **(2004)**.
[16] B.Nath, I.Levin, T.Csak, et al; *Hepatology (Baltimore, Md.)*. **53**, 1526-37 **(2011)**.
[17] E.J.Yeo, Y.S.Chun, Y.S.Cho; *Journal of the National Cancer Institute*. **95**, 516-25 **(2003)**.
[18] A.Mansouri, C.Demeilliers, S.Amsellem, D.Pessayre, B.Fromenty; *The Journal of pharmacology and experimental therapeutics*. **298**, 737-43 **(2001)**.
[19] H.Bo, N.Jiang, G.Ma, et al; *Free radical biology & medicine*. **44**, 1373-81 **(2008)**.
[20] I.G.Kessova, A.I.Cederbaum; *Free radical biology & medicine*. **42**, 1470-80 **(2007)**.
[21] O.H.Lowry, N.J.Rosebrough, A.L.Farr, R.J.Randall; *The Journal of biological chemistry*. **193**, 265-75 **(1951)**.
[22] N.Jiang, G.Zhang, H.Bo; *Free radical biology & medicine*. **46**, 138-45 **(2009)**.
[23] D.M.Medeiros; *Methods (San Diego, Calif.)*. **46**, 288-94 **(2008)**.
[24] S.M.Bailey, C.C.Cunningham; *Free radical biology & medicine*. **32**, 11-6 **(2002)**.
[25] J.H.Lefkowitch; *Clinics in liver disease*. **9**, 37-53 **(2005)**.
[26] S.Das, N.Hajnoczky, A.N.Antony; *Pflugers Archiv : European journal of physiology*. **464**, 101-9 **(2012)**.
[27] E.D.Degli, J.Hamelin, N.Bosselut; *Biochem Res Int*. **2012**, 387626 **(2012)**.
[28] C.Luo, Y.Li, H.Wang; *Journal of cellular biochemistry*. **114**, 212-9 **(2013)**.
[29] N.Eid, Y.Ito, K.Maemura, Y.Otsuki; *Journal of molecular histology*. **44**, 311-26 **(2013)**.
[30] D.Wu, X.Wang, R.Zhou, L.Yang, A.I.Cederbaum; *Free radical biology & medicine*. **53**, 1346-57 **(2012)**.
[31] Y.Kurihara, T.Kanki, Y.Aoki; *The Journal of biological chemistry*. **287**, 3265-72 **(2012)**.