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Full Length Article

Regulation of Bcl-2 and the NF-kB Signaling Pathway by Succinyl Rotundic Acid in Livers of Rats with Alcoholic Hepatitis

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Abstract

In this study, the protective effects of succinyl rotundic acids on alcoholic hepatitis in irradiated rats as well as the effects of Bcl-2-Bax-caspase-3 and the NF-kB signal pathways were studied. SD rats were divided into four groups randomly: normal; model; and succinyl rotundic acid low-, middle-, and high-dose groups. Distilled water, 60% ethanol and 60% ethanol +SRA, respectively, were given for 30 days. ELISA was used to measure serum levels of LDH, AST, ALT, NOS, NO, MDA, GSH and TG. Western blotting was used to measure protein levels of Bcl-2, Bax, caspase-3, NF-kB p 65, IKBA, HO-1, Nrf2 and CYP2E1. Compared with the model group, LDH, AST, ALT, NOS, NO, MDA and TG levels were lower in serum of low-, middle-, and high-dose groups ($P < 0.01$, $P < 0.05$ and $P < 0.05$ in all); GSH content was greater in serum of low-, middle- and high-dose groups ($P < 0.05$). Levels of Bcl-2, HO-1, and Nrf2 were greater ($P < 0.01$ in all); those of Bax, caspase-3, NF-kB p65, IKBA, and CYP2E1 were lower ($P < 0.01$ and $P < 0.001$ in all). These findings suggest that succinyl rotundic acid reduces inflammatory reactions by reducing levels of NOS and NO, regulating levels of Bcl-2, Bax, caspase-3, NF-kB, and anti-oxidative stress pathways, and has an antagonistic effect on alcoholic liver injury. The agent has potential to treat clinical alcoholic liver disease.

Keywords: Hepatitis rats; Protective effect; Serum levels; Western blotting

Introduction

Alcoholic liver disease (ALD) is caused by long-term heavy drinking (Li *et al.* 2011). It usually manifests as fatty liver in the initial stage and develops into alcoholic hepatitis, cirrhosis and hepatic fibrosis. There is a lack of large-scale epidemiological survey data on alcoholic liver disease in China (Wei *et al.* 2015; Ciardullo *et al.* 2020).

The first-line therapy for severe alcoholic hepatitis is corticosteroids; however, the evidence for its effectiveness in reducing mortality remains unclear. Pentoxifylline is an alternative therapy (Maryconi and Mitchell 2014). Succinyl rotundic acid has pharmacological effects including antioxidation, anti-inflammation, scavenging oxygen free radicals, and others. It treats liver diseases such as acute liver injury, nonalcoholic fatty liver, liver fibrosis, liver cancer, and others. In view of these problems, our research group designed succinyl rotundic acid (Li *et al.* 2017) to treat alcoholic liver disease.

This study established a chronic ethanol-induced liver injury model in rats, studied its hepatoprotective effects, explored the relevant mechanisms, and measured liver function and liver tissue oxidation injury indexes to evaluate the therapeutic effects of succinyl rotundic acid (Hsu *et al.* 2015) on alcoholic liver disease.

Materials and Methods

Materials sample

Succinyl rotundic acid (SRA) was obtained by chemical modification of RA. RA was heated in the presence of pyridine and reacted with succinic anhydride. Cooling to room temperature, after the completion of an action in ice water, dilute hydrochloric acid was added to adjust the pH to 4–. After filtration, the filter residue was washed with water until it was colorless. The final SRA product was obtained by recrystallizing. The purity of SRA was 95.70% by HPLC.

Sprague Dawley (SD) rats, male, weights 180–220 g were used. After adaptive feeding, 60 SD rats were divided into normal, model, succinyl rotundic acid low-dose (10 μmol/L), middle-dose (20 μmol/L), and high-dose (40 μmol/L) groups according to body weight. In the normal

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group, the rats were given equal volume distilled water. Except for the normal group, rats in each group were given 10 mL/kg of 60% ethanol solution orally every day. After 6 h, the drug group was given the corresponding drug orally according to the designed dose (2.0 mL/kg·d) for 30 days. The model group was not given drugs. After the final administration, the rats were fasted for 16 h without withholding drinking water, weighed, and blood and liver tissues were taken for each index test.

LDH, AST, ALT, NOS and NO measurement

Serum was separated and levels of lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine aminotransferase (ALT) were determined using an automatic blood biochemical analyzer. The activities of nitric oxide synthase (NOS) and nitric oxide (NO) were determined by a chemical chromogenic method according to the kit instructions.

MDA, GSH and TG measurement

Liver tissue was homogenized according to kit instructions. The levels of malondialdehyde (MDA), glutathione (GSH) and triglyceride (TG) in liver tissue were measured according to the kit instructions.

Western blotting

Liver tissue homogenates was prepared using RIPA to obtain protein samples. A BCA protein concentration determination kit was used to measure protein concentrations. After SDS-PAGE electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes and washed in TBST. Membranes were incubated with antibodies to Bcl-2 (1:700), Bax (1:2000), caspase-3 (1:700), NF-kB p 65 (1:2000), IKBA (1:2000), HO-1 (1:2 000), Nrf2 (1:2000), CYP2E1 (1:2000), and β-actin (1:2000) overnight at 2–8ºC; then added goat anti-rabbit secondary antibody (1:2000). Detection, exposure, development and fixation involved ultra-sensitive enhanced chemiluminescence (ECL) reagents. Gel-Pro-Analyzer software (Media Cybernetics Inc.) was used to measure the optical density.

Statistical analysis

The data was expressed as M ± S. Use Bonferroni post–hoc test for multiple comparisons and one-way ANOVA was used for inter-group comparison. Use GraphPad Prism 5.0 software to process data and numbers.

Results

Body weight

The initial body mass in each group of rats showed no significant differences before intragastric administration. After 30 days of continuous gavage, the body weights in the normal group were higher than those of other groups, however there without significance (Table 1 and Fig. 1A).

Content of LDH, AST, ALT, NOS and NO in liver tissue

LDH, AST, NOS and NO levels in the model group were significantly greater differences ($P < 0.01$, $P < 0.05$, $P < 0.001$, and $P < 0.001$, respectively) than those of the the normal group. Serum LDH levels in the low-, middle-, and high-dose groups were significantly lower than those of the model group ($P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively, Fig. 1B); AST content in serum was significantly lower in the three groups ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively, Fig. 1C). Serum ALT levels in the low-, middle-, and high-dose groups were significantly lower ($P < 0.05$, $P < 0.05$ and $P < 0.01$, respectively, Fig. 2A). Serum levels of NOS and NO were significantly lower in the middle- and high-dose groups ($P < 0.05$ and $P < 0.01$, respectively, Fig. 2B–C and Table 2).

Changes of MDA, GSH and TG in liver tissue

Levels of MDA and TG in the model group were significantly higher, while levels of GSH were significantly lower ($P < 0.001$, $P < 0.05$, and $P < 0.01$, respectively) than those of the normal group. MDA levels in liver tissue were significantly lower in the middle-, and high-dose groups ($P < 0.05$ and $P < 0.01$, respectively Fig. 3A), GSH levels in liver tissue were significantly higher in the high-dose group ($P < 0.05$, Fig. 3B), TG levels in liver tissue were significantly lower in the high-dose group ($P < 0.05$, Fig. 3C and Table 3) than in the model group.

Western blotting

Liver levels of Bcl-2 in the model group were significantly lower ($P < 0.001$), and levels of Bax and caspase-3 were significantly greater ($P < 0.001$ in all) than in the normal group. Levels of Bcl-2 in liver in the high-dose group were significantly greater ($P < 0.01$), and levels of Bax and caspase-3 in the high- and middle-dose groups were significantly lower ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 4A–B) than in the model group.

Western blotting revealed that levels of CYP2E1 in liver tissue in the model group were significantly greater than those of the normal group ($P < 0.001$). The high-, middle-, and low-dose groups showed significantly lower levels of CYP2E1 ($P < 0.001$, $P < 0.01$, and $P < 0.05$ respectively) (Fig. 4C–D) than those of the model group. These findings suggest that succinyl rotundic acid inhibits hepatic levels of CYP2E1 in rats with alcoholic hepatitis, thereby promoting the rapid decomposition of ethanol and reducing ethanol-induced damage to the liver.

Hepatic levels of Nrf2 in the model group were significantly lower than those of the normal group ($P <$

Table 1: Body weights and body weight increase of rats in various groups (x ± s)

Group	n	Body weight		Weight increase
		Before experiment	After experiment	
Blank control	12	193.56 ± 4.78	258.31 ± 5.96	64.75 ± 11.25
Model control	10	195.24 ± 5.26	225.15 ± 7.82	29.91 ± 10.32
Low dose	10	196.23 ± 4.32	245.89 ± 6.15	49.66 ± 9.68
Middle dose	11	195.34 ± 3.18	244.12 ± 5.93	48.78 ± 10.48
High dose	11	196.54 ± 5.69	245.79 ± 6.48	49.25 ± 11.24

Table 2: Levels of serum LDH, AST, ALT, NOS, NO in alcohol liver injury of rats (x ± s)

Groups	LDH (U/L)	AST (U/L)	ALT (U/L)	NOS (U/mL)	NO (μmol/L)
Blank control	1015.26 ± 105.48	105.46 ± 17.28	32.78 ± 5.29	19.56 ± 3.48	20.47 ± 3.47
Model control	1534.15 ± 114.25**	157.68 ± 24.31*	45.37 ± 4.89	34.75 ± 2.09***	35.28 ± 2.08***
Low dose	1089.24 ± 123.14$^{\&\&}$	101.57 ± 12.36$^{\&}$	31.36 ± 5.19$^{\&}$	28.47 ± 2.58$^{\#}$	30.09 ± 1.48$^{\#\#}$
Middle dose	947.26 ± 108.97$^{\&\&\&}$	93.27 ± 15.68$^{\&\&}$	29.48 ± 3.48$^{\&}$	25.67 ± 2.08$^{\&}$	28.32 ± 2.05$^{\#\&}$
High dose	908.46 ± 98.56$^{\&\&\&}$	87.68 ± 17.29$^{\&\&}$	26.18 ± 5.17$^{\&\&}$	22.34 ± 2.81$^{\&\&}$	25.79 ± 2.36$^{\&\&}$

Note: *Blank control *vs*. Model control; $^{\#}$Blank control *vs*. dose groups; $^{\&}$Model control *vs*. dose groups

Table 3: Levels of hepatic MDA, GSH, TG in alcohol liver injury of rats (x ± s)

Groups	MDA (nmol/mg)	GSH (mmol/mg)	TG (mmol/g)
Blank control	0.62 ± 0.11	21.15 ± 2.45	0.31 ± 0.02
Model control	1.42 ± 0.15***	11.34 ± 1.78**	0.48 ± 0.08*
Low dose	1.24 ± 0.23$^{\#\#}$	13.52 ± 1.63$^{\#}$	0.44 ± 0.05
Middle dose	1.01 ± 0.10$^{\&}$	15.69 ± 2.78	0.38 ± 0.06
High dose	0.85 ± 0.09$^{\&\&}$	18.45 ± 2.05$^{\&}$	0.33 ± 0.04$^{\&}$

*Blank control *vs*. Model control; $^{\#}$Blank control *vs*. dose groups; $^{\&}$Model control *vs*. dose groups

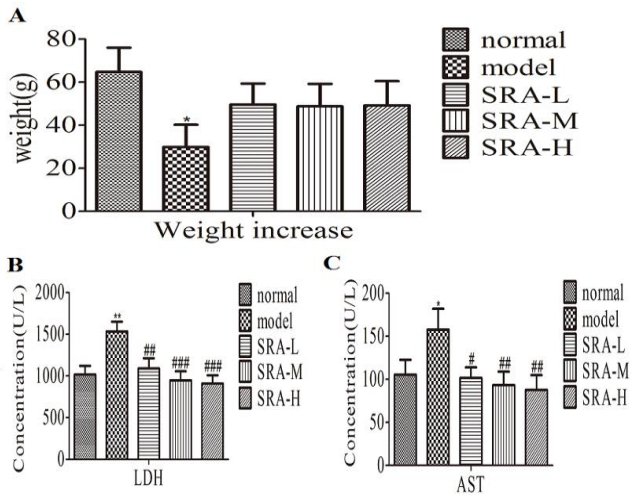

Fig. 1: Body weight changes of SRA on SD rats and effects of SRA on LDH and AST levels in serum. Values are expressed as means ± SEM. Compared with normal group: $^{*}P < 0.05$, $^{**}P < 0.01$; compared with model group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$

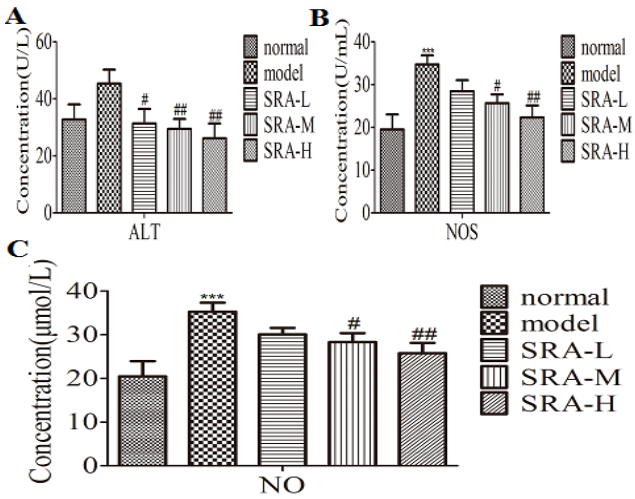

Fig. 2: Effects of SRA on ALT, NOS and NO levels in serum. Values are expressed as means ± SEM. Compared with normal

0.001). The high-dose group showed significantly higher Nrf2 levels ($P < 0.01$) (Fig 4C–D) than those of the model group. These findings suggest that succinyl rotundic acid increases levels of Nrf2 in liver tissue of rats with alcoholic liver injury, increasing the amount of nuclear transfer, further activating the expression of downstream antioxidant proteins, and enhancing the oxidation defense system so as to protect the liver.

Levels of HO-1 in liver tissue in the model group were significantly lower ($P < 0.001$) than those of the normal group. The high- and middle-dose groups showed significantly greater levels of HO-1 protein ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 4C–D) than those of the model group. These findings suggest that succinyl rotundic acid enhances anti-oxidation and anti-apoptosis effects, improving cell survival in liver tissue, and enhancing the

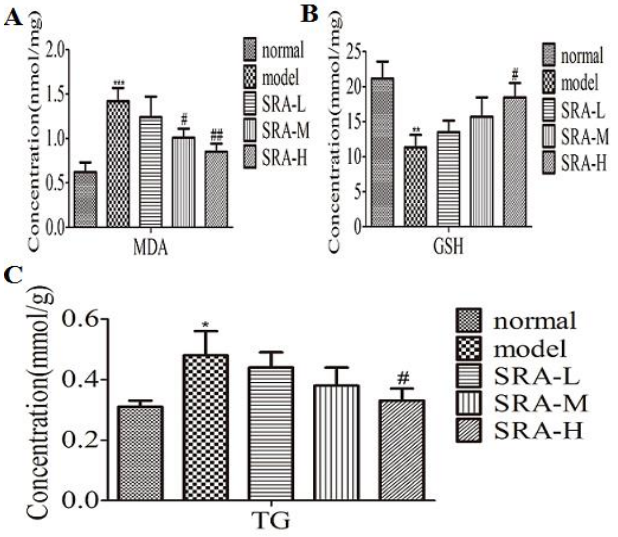

Fig. 3: Effects of SRA on MDA, GSH and TG levels in serum. Values are expressed as means ± SEM. Compared with normal group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; compared with model group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$

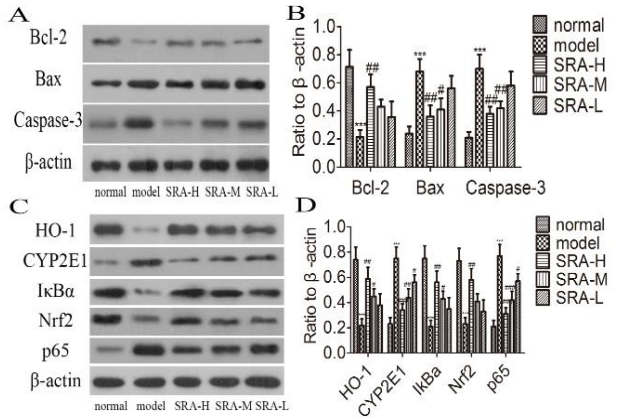

Fig. 4: The effect of SRA on NF-κB and Bcl-2 signaling pathway in rat. Values are expressed as means ± SEM. Compared with normal group: $^{***}P < 0.001$; compared with model group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$

oxidation defense system by up-regulating the expression of antioxidant protein HO-1 in livers of rats with alcoholic hepatitis.

Levels of IKBA in the model group were significantly lower ($P < 0.001$) than those of the normal group. The high- and middle-dose group showed significantly greater levels of IKBA than those of the normal group ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 4C–D). These findings suggest that succinyl rotundic acid inhibits the degradation of IKBA protein in the livers of rats with alcoholic liver injury and reduces the release of NF-KB, thereby inhibiting inflammation.

Levels NF-kB p 65 protein were significantly lower than those of the normal group ($P < 0.001$). Compared with the model group, the high-, middle-, and low-dose groups showed significantly lower levels of NF-kB p 65 ($P < 0.001$, $P < 0.001$, and $P < 0.05$, respectively) (Fig. 4C–D). These findings suggest that succinyl rotundic acid inhibits levels of NF-kB p 65 protein in liver tissue of rats with alcoholic liver injury, reducing the release of inflammatory factors, and thereby inhibiting inflammation.

Discussion

In this study, rats in the model group showed significant liver injury after long-term administration of ethanol. Levels of serum LDH, AST, and NOS significantly increased (Baghdasaryan *et al.* 2019; Sehgal *et al.* 2020; Wu *et al.* 2020; Zou *et al.* 2020), suggesting abnormal liver function and liver cell injury. MDA levels in liver tissue increased significantly and those of GSH decreased, suggesting lipid peroxidation damage in liver tissue. The increase of TG levels in liver tissue suggests fatty degeneration of liver tissue. Succinyl rotundic acid reduced serum levels of LDH, AST, NOS, and NO (Zhang *et al.* 2002; Tang *et al.* 2009; He *et al.* 2021), increasing GSH activity, and reducing levels of MDA and TG (Jiao *et al.* 2020; Noto *et al.* 2020), suggesting that it ameliorates alcoholic liver injury and reduces lipid peroxidation damage and steatosis. Levels of Bcl-2 in liver decreased while levels of Bax and caspase-3 increased (Raisova *et al.* 2001; Klemm *et al.* 2008; Mao *et al.* 2008). Succinyl rotundic acid inhibited expression of CYP2E1 protein in liver of rats with alcoholic hepatitis, thereby promoting the rapid decomposition of ethanol and reducing ethanol-induced liver damage. By up-regulating the expression of Nrf2 protein in liver tissue with alcoholic liver injury and increasing amounts of nuclear transfer, the expression of downstream antioxidant proteins are further activated and the oxidation defense system (Zhang *et al.* 2000; Hong *et al.* 2016) is activated so as to achieve liver protection.

By up-regulating the expression of antioxidant protein HO-1 in the liver of rats with alcoholic hepatitis, anti-oxidative and anti-apoptotic capabilities are enhanced, cell survival in liver tissues is improved, and the oxidation defense system is enhanced. SRA inhibited the degradation of IKBA protein in liver tissue of rats with alcoholic liver injury and reduced the release of NF-KB p65 (Chen *et al.* 2011; Huang *et al.* 2017), thereby inhibiting inflammation. SRA inhibited levels of NF-kB p 65 in alcoholic liver injury tissue and reduced the release of inflammatory factors, thereby inhibiting inflammation.

Conclusion

Succinyl rotundic acid reduces inflammatory reactions by reducing the levels of NOS and NO, regulating levels of Bcl-2, Bax, and Caspase-3, and regulating NF-kB and anti-oxidative stress pathways. It has an antagonistic effect on alcoholic liver injury and has potential for treating clinical alcoholic liver disease.

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Author Contributions

Yajie Zhang: Overall instructor; Yufang He, Fang Xia, Xu Wang: Responsible for the experiment and operation; Lijing Li and Minlun Nan: Experimental operation support.

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