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Protective effect of ligustrazine on accelerated anti-glomerular basement membrane antibody nephritis in rats is based on its antioxidant properties

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Abstract

Ligustrazine has a renoprotective effect against nephritis. In this study, we further characterized the renoprotective properties of ligustrazine in an experimental model using accelerated anti-glomerular basement membrane antibody (AGBM-Ab).

Ligustrazine was given i.p. once daily at 50, 100 mg/kg for 15 days after singly giving i.v. of rabbit anti-rat glomerular basement membrane serum, and showed dose-dependent inhibition the elevation of urinary protein, serum creatinine and blood urea nitrogen as well as the development of glomerular histological changes. Ligustrazine (50 mg/kg) had no affect on glutathione (GSH) content, glutathione peroxidase and catalase activities, but decreased the malondialdehyde (MDA) content and increased superoxide dismutase (SOD) activity in nephritis induced by AGBM-Ab. Ligustrazine (100 mg/kg) significantly decreased MDA content while significantly increased GSH content and SOD, glutathione peroxidase, catalase activities of kidney tissues in the rats treated with AGBM-Ab alone.

In conclusion, our results show that ligustrazine has protective activity against accelerated AGBM-Ab nephritis, and its renoprotective effect may be due to its antioxidant properties and inhibition reactive oxygen species (ROS).

Keywords: Ligustrazine; Nephritis; Antioxidants; Effect; (Rat)

1. Introduction

Anti-glomerular basement membrane antibody (AGBM-Ab) nephritis has been generated in rabbits (Fujimoto et al., 1964), rats (Hammer and Dixon, 1963) and mice (Park et al., 1998) by the administration of heterologous antibody against glomerular basement membrane. AGBM-Ab nephritis is a well-established experimental model of human glomerular immune injury resulting in glomerulonephritis (Hammer and Dixon, 1963; Unanue and Dixon, 1967).

After administration of AGBM-Ab, the nephron filtration rate falls precipitously, both as a consequence of reductions of the glomerular ultrafiltration coefficient and as a result of renal vasoconstriction producing reductions in renal plasma flow. Antibodies directed to the antigens on the glomerular basement membrane produce a rapid inflammatory reaction characterized by accumulation of polymorphonuclear leukocytes and later by transition to a mononuclear cell response (Allison et al., 1990). The rapidity of the glomerular capillary inflammatory reaction is related to the site of the antigen/antibody reaction, the endocapillary luminal surface, where the endothelium is fenestrated, allowing easy access of the antibody to its antigenic site (Yingwei et al., 2006).

Traditional Chinese medicine is the natural therapeutic agent used under the guidance of the theory of traditional Chinese medical sciences and has been used to treat human diseases in China for centuries. People are becoming increasingly interested in traditional Chinese medicines because of their low toxicity and good therapeutic performance. Ligustrazine (tetramethypyrazine) (Fig. 1A), a bioactive component contained in Chuanxiong (*Ligusticum chuanxiong* Hort), is widely applied

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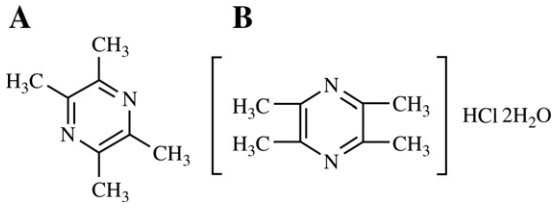

Fig. 1. Chemical structure: A: Ligustrazine, B: Ligustrazine hydrochloride.

in the treatment of vascular diseases in China, e.g. myocardial and cerebral infarction (Li et al., 2006). It has been reported that ligustrazine can increase coronary blood flow and reduce myocardial ischemia in animal study (Dai and Bache, 1985). It has been reported that ligustrazine-mediated vascular relaxation in which both Ca^{2+}-dependent and ATP-dependent KB^{+} channels have been involved (Tsai et al., 2002). It blocks calcium channels, reduces the bioactivity of platelets and platelet aggregation, and inhibits free radicals (Zou et al., 2001). In addition, ligustrazine has been demonstrated to play a protective role in ischemia-reperfusion kidney injury in rats (Sun et al., 2002; Feng et al., 2004). Ligustrazine used to treat patients with proliferative glomerulonephritis showed signs of reduced progression of their disease (Huang and Zhan, 1998). The mechanism of ligustrazine may be due to its scavenging effect on superoxides (Liu et al., 2002).

This study was designed to elucidate the renoprotective effect of ligustrazine on accelerated AGBM-Ab nephritis in rats.

2. Materials and methods

2.1. Chemicals

Ligustrazine hydrochloride (Fig. 1B) was purchased from Weifang Fine Chemical Co. Ltd. (Shanghai, China). Normal rabbit IgG was purchased from Beijing Zhongshan Biotechnology Co. Ltd (Beijing, China). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2. Animals

The animals used in this study were approved by the Animal Care Committee of Wuhan University. Male Sprague–Dawley rats and New Zealand white (NZW) rabbits were purchased from Wuhan University Laboratory Animal Center (Wuhan, China).

2.3. Preparation of AGBM-Ab

Rat glomerular basement membrane was prepared using the previous method (Krakower and Greenspon, 1951). The preparation of AGBM-Ab was described previously (Wada et al., 1996). Briefly, five NZW rabbits were immunized with glomerular basement membrane antigen (5 mg, s.c.) in complete Freund's adjuvant, at five times with 1 week intervals. Antisera from the five animals were collected after the final injection of antigen on day 7th. These sera were pooled together and used to induce AGBM-Ab nephritis throughout this study.

2.4. Induction of accelerated AGBM-Ab nephritis in rats

Rats were immunized with normal rabbit IgG (5 mg, s.c.) in 0.5 ml of complete Freund's adjuvant. Five days later, accelerated AGBM-Ab nephritis was induced by the injection of a nephritogenic dose (10 ml/kg, i.v.) of rabbit anti-rat glomerular basement membrane serum through a tail vein (Hattori et al., 1994; Boyce and Holdsworth, 1985).

2.5. Experimental procedures

In this investigation, 40 healthy adult male Sprague–Dawley rats (8-week-old weighing 180±20 g) were used. The animals were housed in the metabolic cages at room temperature (20–24 °C) and regular light cycle(12 light/12 dark) with feed and water *ad libitum*. Rats were divided into four groups of ten animals. Control rats were singly injected with normal rabbit serum (10 ml/kg, i.v.) through tail vein which were followed by the injection of 0.9% NaCl (5 ml/kg, i.p.) for 15 days. Rats in nephritis model group were singly injected with rabbit anti-rat glomerular basement membrane serum (10 ml/kg, i.v.) which were followed by the treatment of 0.9% NaCl (5 ml/kg, i.p.) for 15 days. Rats in ligustrazine hydrochloride group were injected with rabbit anti-rat glomerular basement membrane serum (10 ml/kg, i.v.) and then injected with ligustrazine hydrochloride (50 or 100 mg/kg, i.p.) for 15 days, respectively.

The urine samples were collected at 1, 3, 5, 7, 10 and 15 days respectively after administration of ligustrazine hydrochloride.

Table 1
Effects of ligustrazine on urinary protein levels in accelerated AGBM-Ab nephritis

Group	Urinary protein (mg/24 h urine)					
	1	3	5	7	10	15 (days)
Control	1.8±1.1	1.6±0.7	1.4±0.7	2.4±1.2	2.7±0.6	2.3±1.3
Model	1.7±1.6	19.0±17.0[a]	45±14.0[a]	59.0±18.0[a]	87±13.0[a]	124.6±14.7[a]
Model+ligustrazine (50 mg/kg)	1.7±1.1	15.4±12.0	38±11.0	50.0±18.0	67±13.0[b]	104.2±10.7[b]
Model+ligustrazine (100 mg/kg)	1.6±0.6	2.1±0.6[c]	3.9±1.0[c]	4.0±1.0[c]	3.3±0.9[c]	3.5±0.8[c]

Note: Model (accelerated AGBM-Ab nephritis).
Mean±S.D. (n=10).
[a] $P<0.01$ vs. control group.
[b] $P<0.05$.
[c] $P<0.01$ vs. model group.

Table 2
Effects of ligustrazine on blood urea nitrogen and serum creatinine contents in accelerated AGBM-Ab nephritis

Group	Blood urea nitrogen (mM/L)	Serum creatinine (μM/L)
Control	5.8±0.6	40.8±7.6
Model	18.7±0.4 [a]	110.8±15.0 [a]
Model+ligustrazine (50 mg/kg)	14.6±0.4 [b]	100.2±11.0 [b]
Model+ligustrazine (100 mg/kg)	6.2±0.5 [c]	45.3±8.6 [c]

Mean±S.D. (n=10).
[a] P<0.01 vs. control group.
[b] P<0.05.
[c] P<0.01 vs. model group.

All animals were anesthetized with 45 mg/kg sodium pentobarbital and sacrificed at 4 h after the last administration. Blood samples were collected to measure serum creatinine and blood urea nitrogen. The samples were centrifuged at 200 ×g for 5 min at 4 °C. Kidneys were removed rapidly, excised and sectioned for histological analysis. Other kidney tissues were homogenized in Tris-HCl buffer (0.05 mol/L Tris-HCl, 1.15% KCl, pH7.4) using a Polytron homogeniser. The homogenate was centrifuged at 18,000 ×g (4 °C) for 30 min, and the supernatant was utilized for biochemical analysis.

2.6. Biochemical assays

Urinary protein content was measured according to the sulfosalicylic acid colorimetric method and expressed as mg/24 h urine (Salant and Cybulsky, 1988). Serum creatinine and blood urea nitrogen concentrations were measured using an autoanalyzer (Beckman Instruments, Fullerton, CA, USA). The concentrations of malondialdehyde (MDA) were determined by the reaction with thiobarbituric acid (Ohkawa et al., 1979). Superoxide dismutase (SOD) activity determination is based on the inhibition of pyrogallol autooxidation (Misra and Fridovich, 1972). Glutathione (GSH) level was measured colorimetrically as protein-free sulfhydryl content using 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (Beutler et al., 1963). Glutathione peroxidase was measured by the enzymatic method (Flohe and Gunzler, 1984). Catalase activity determination method was determinated by the rate constant of the H_2O_2 decomposition rate at 240 nm (Aebi, 1984). Total protein content was determined using Lowry method (Lowry et al., 1951).

2.7. Histopathological examinations

Kidneys were fixed with 10% neutral buffered formaldehyde for 2 days, then dehydrated and embedded in paraffin. Paraffin sections were made at 3 μm and stained with haematoxylin/eosin (HE) and periodic acid-Schiff (PAS) for microscopic study.

Twenty-five glomeruli per section were observed under a light microscope (PAS stain) to evaluate each of the histopathological parameters, the extent of crescent formation, adhesion and fibrinoid necrosis was scored as 1 (mild), 2 (moderate) and 3 (severe). The number of the glomeruli corresponding to each score is given as n_1, n_2 and n_3. A crescent formation index (CI), an adhesion index (AI) and a fibrinoid necrosis index (FI) were calculated from the following formula (Nagao et al., 1998).

$$\text{CI, AI and FI} = 1 \times n_1 + 2 \times n_2 + 3 \times n_3.$$

The index of glomerular lesions (IGL) was calculated to evaluate the extent of glomerular lesions as follows:

$$\text{IGL} = \frac{(3 \times \text{CI}) + (2 \times \text{AI}) + (1 \times \text{FI})}{(3+2+1) \times 25}$$

2.8. Statistical analysis

Results were expressed as mean±S.D.; the differences between groups were analyzed by one-way analysis of variance and the Student–Neumann–Keuls' t-test. Statistical significance was defined as P<0.05.

3. Results

3.1. Effects of ligustrazine on urinary protein levels in accelerated AGBM-Ab nephritis

As shown in Table 1, a single dose of AGBM-Ab caused a significant increase (P<0.01) in urinary protein 3, 5, 7, 10, 15 days after treatment. Co-administration with ligustrazine (100 mg/kg) significantly reduced the levels of urinary protein at all time points examined (P<0.01), while ligustrazine (50 mg/kg) decreased urinary protein levels after AGBM-Ab treatment on days 10 and 15 (P<0.05).

Table 3
Effects of ligustrazine on kidneys MDA, GSH levels and SOD, glutathione peroxidase, catalase activities in accelerated AGBM-Ab nephritis

Group	MDA	SOD	GSH	Glutathione peroxidase	Catalase
	nM/mg protein	U/mg protein	nM/mg protein	μM/min/mg protein	mM/min/mg protein
Control	1.10±0.03	74.2±1.7	19.2±1.6	126±29	43.0±6.0
Model	2.64±0.08 [a]	34.0±3.3 [a]	5.9±1.4 [a]	51±11 [a]	34.4±7.7 [b]
Model+ligustrazine (50 mg/kg)	1.86±0.04 [c]	42.8±3.2 [c]	6.6±0.6	48±14	33.8±7.4
Model+ligustrazine (100 mg/kg)	1.52±0.06 [d]	69.4±4.0 [d]	18.4±1.8 [d]	114±39 [d]	67.8±4.6 [d]

Mean±S.D. (n=10).
[a] P<0.01 vs. control group.
[b] P<0.05.
[c] P<0.05.
[d] P<0.01 vs. model group.

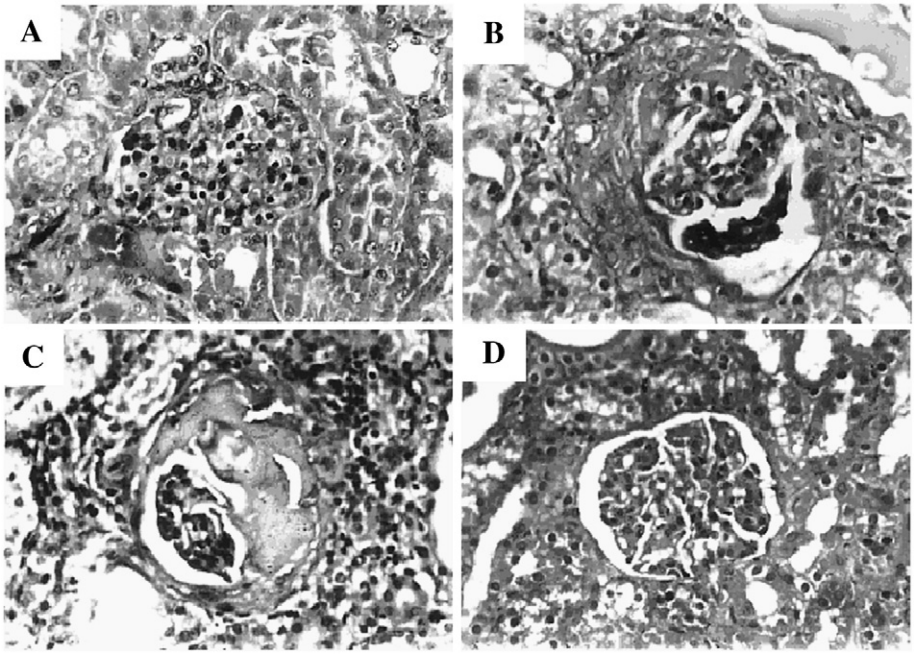

Fig. 2. Light microscopy of renal tissue from rats (HE stain, *x*400). (A) Control group: normal glomerular structure. (B) Nephritis model group: glomerular cell proliferation, glomerular hypertrophy, protein exudation in capsule, crescent formation. (C) Nephritis model+ligustrazine (50 mg/kg) group: glomerular pathological changes slightly improve. (D) Nephritis model+ligustrazine (100 mg/kg) group: glomerular pathological changes obvious improve and glomerular injury recovery normal.

3.2. Effects of ligustrazine on serum creatinine and blood urea nitrogen contents in accelerated AGBM-Ab nephritis

Table 2 indicates that rats treated with AGBM-Ab alone showed significant elevation in both serum creatinine and blood urea nitrogen levels in comparison with the control group ($P<0.01$). Ligustrazine (50, 100 mg/kg) significantly decreased the levels of serum creatinine and blood urea nitrogen induced by AGBM-Ab with dose-dependence ($P<0.05$, $P<0.01$ respectively).

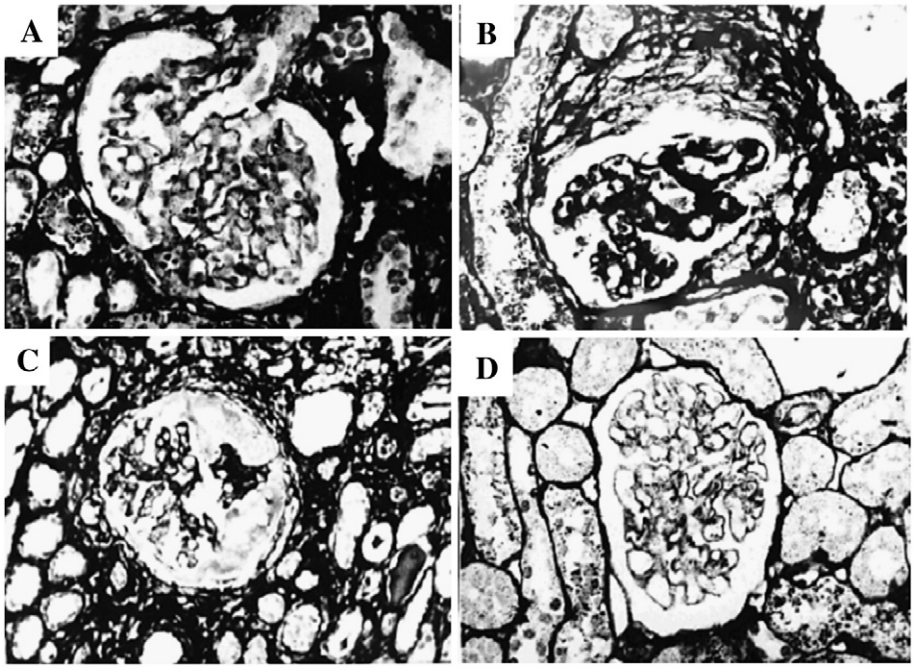

Fig. 3. Light microscopy of renal tissue from rats (PAS stain, *x*400). (A) Control group: normal glomerular structure. (B) Nephritis model group: glomerular cell proliferation, thickening of glomerular basement membrane, occlusion of glomerular loops, crescent formation. (C) Nephritis model+ligustrazine (50 mg/kg) group: glomerular pathological changes slightly improve. (D) Nephritis model+ligustrazine (100 mg/kg) group: glomerular pathological changes obvious improve and glomerular injury recovery normal.

Table 4
Effects of ligustrazine on Index of glomerular lesions in accelerated AGBM-Ab nephritis

Group	Index of glomerular lesions
Model	3.12±0.43
Model+ligustrazine (50 mg/kg)	2.38±0.35[a]
Model+ligustrazine (100 mg/kg)	0.52±0.08[b]

Mean±S.D. (n=10).
[a] P<0.05.
[b] P<0.01 vs. model group.

3.3. Effects of ligustrazine on kidneys MDA, GSH levels and SOD, glutathione peroxidase, catalase activities in accelerated AGBM-Ab nephritis

The activities of SOD, glutathione peroxidase and catalase as well as GSH content were lower while MDA was higher in the AGBM-Ab nephritis group than those in the control (P<0.05, Table 3). Ligustrazine (50 mg/kg) had no affect on the activities of glutathione peroxidase and catalase as well as GSH content, while it decreased MDA content and increased SOD activity when compared with the model group (P<0.05). However, the large dose of ligustrazine (100 mg/kg) significantly decreased MDA content and increased GSH content and the activities of SOD, glutathione peroxidase and catalase when compared with rats treated with AGBM-Ab alone (P<0.01).

3.4. Effects of ligustrazine on kidneys histology in accelerated AGBM-Ab nephritis

Kidneys in control showed normal glomerular structure (Figs. 2A and 3A). In comparison, AGBM-Ab serum treatment caused evident morphological alterations, indicating by thickening of glomerular basement membrane, glomerular proliferation, hypertrophy, protein exudation in capsule, occlusion of glomerular loops and crescent formation (Figs. 2B and 3B; Table 4). Glomerular pathological damages were slightly reversed in rats treated with ligustrazine of 50 mg/kg (Figs. 2C and 3C; Table 4). Ligustrazine of 100 mg/kg significantly ameliorated glomerular pathological changes induced by AGBM-Ab serum, and glomerular injury recovery normal (Figs. 2D and 3D; Table 4).

4. Discussion

Anti-glomerular basement membrane antibody (AGBM-Ab) mediated nephritis is characterized by the binding of autoantibodies to glomerular basement membrane, leading to rapidly progressive glomerulonephritis that often results in irreversible loss of renal function and glomerular sclerosis. The nephrotoxic potential of AGBM-Ab has been demonstrated in animal experiments (Zhao and Ming-Hui, 2005). After injection of AGBM-Ab serum, transient infiltration of polymorphonuclear leukocytes in glomeruli, thickening of glomerular basement membrane, and swelling of endothelial cells were observed (first phase). These findings are similar to the acute phase of human glomerulonephritis. The second phase began 5 to 7 days after injection, the glomeruli showed remarkable cellularity with crescent formation and urinary protein was increased. Persistant mesangial cell proliferation and glomerular basement membrane thickening, glomerular inflammation was exacerbated glomerulosclerosis, which may be similar to chronic glomerulonephritis in humans. Our findings are consistent with previous reports that the first phase and the second phase noted changes in urinary protein and glomerular histopathological damages after injection of AGBM-Ab serum. In previous report, ligustrazine has been used to postpone or halt the progress of renal failure and proliferative glomerulonephritis in Chinese patients (Huang and Zhan, 1998; Tang, 2003). In this study, the effect has been demonstrated in laboratory animals. Ligustrazine significantly inhibited urinary protein excretion, serum creatinine and blood urea nitrogen levels as well as histological alterations which include thickening of glomerular basement membrane, glomerular proliferation, hypertrophy, and crescent formation in accelerated AGBM-Ab nephritis rats.

Advances in our understanding of glomerulonephritis have come from both clinical and research studies. New animal models of human glomerulonephritis, human and mouse genetic studies, and molecular and immunological tools have helped to identify new inflammatory mediators and regulatory molecules, resulting in a better understanding of the pathogenesis of human glomerulonephritis and novel therapeutic strategies (Jolanta et al., 2007). Growth factors (Pierina et al., 2004), cytokines (Koryakova et al., 2006) and extracellular matrix (Wang et al., 2005) have been involved in glomerulonephritis, though the overall mechanism remains to be clarified. Generation of reactive oxygen species (ROS) from circulating inflammatory cells and from local resident cells seems to play a role in the tissue damage associated with the acute inflammatory response. Production of ROS has been known to occur with exposure to AGBM-Ab in the rat model. Acute inflammation of glomeruli and rapid progression of chronic glomerulonephritis can be induced by AGBM-Ab injection, suggesting ROS generation may play a critical role in the progression of glomerular lesions (Gunther et al., 2006; Narsipur et al., 2003). It is well known that the attack by free radicals can be protected by many biologic antioxidant and scavenging systems. For example, the damage of cell constituents by ROS including the superoxide radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) can be prevented via various enzymatic and non-enzymatic mechanisms. In this enzymatic defense mechanism, SOD, glutathione peroxidase, and catalase play principle roles. On the other hand, many non-enzymatic free radical scavengers such as vitamins C and GSH exist in tissues and plasma. The antioxidants (enzymatic and non-enzymatic antioxidants) are the barriers (both endogenous and exogenous) against free radicals' attacks (Bergamini and Seghieri, 2006). SOD enzyme catalyzes the dismutation of $O_2^{\cdot}$ to H_2O_2 and molecular oxygen (O_2), while glutathione peroxidase and catalase catalyze the degradation of H_2O_2 to O_2 and water (Dobashi et al., 2000). In this study, we found that GSH content, the activities of SOD, glutathione peroxidase and catalase in kidneys significantly decreased in accelerated AGBM-Ab nephritis, which indicated that the oxygen-derived free radicals

can be increased, and the capability in scavenging free radicals are decreased after the injection of AGBM-Ab serum. Free radicals lead to lipid peroxidation, especially for multivalence unsaturated fatty acids on glomerular basement membrane, and the damage of glomerular basement membrane, which would further proceed to the destruction of the organ's construction and function. The production of MDA parallels lipid peroxidation, but lipid peroxidation may induce the generation of MDA. MDA contents in kidneys were significantly increased in accelerated AGBM-Ab nephritis in our experiment. If ROS are critical to the inflammatory process, then administration of antioxidants may ameliorate this process of tissue damage. In this experiment study, we found that in rat treated with ligustrazine (100 mg/kg), GSH contents and the activities of SOD, glutathione peroxidase and catalase in kidneys were significantly increased, while MDA contents in kidneys were significantly decreased in accelerated AGBM-Ab nephritis. Generation of ROS was reduced by ligustrazine.

In conclusion, the AGBM-Ab successfully induces nephritis by inducing the formation of ROS, while ligustrazine (50, 100 mg/kg) can act as a protection with dose-dependence due to its antioxidant effect. The high levels of urinary protein, serum creatinine and blood urea nitrogen as well as kidneys histological alterations can be improved by ligustrazine in accelerated AGBM-Ab nephritis in rats. This study may provide experimental evidences that ligustrazine can be used as a therapeutic drug in accelerated AGBM-Ab nephritis.

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