Effects of Ling Zhi on Cardiac Heterotopic Transplantation

— Immunopharmacological Study (9)

ZHANG Luoxiu & ZHAO Lianhua

Abstract Half heart tissue from new born mice was grafted to the pinna of adult mouse ears in order to evaluate the influence of Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst) on the survival of grafts. Electrical activity was used to ascertain the viability of the grafted heart muscle. The results showed that Ling Zhi alone had no significant effect on the survival time of grafted heart. Only when LZ in combination with suboptimal dosage of cyclophosphamide had somewhat increase in graft survival time as compared to cyclosphosphamide alone. No obvious synergic activity could be detected when LZ combined with optimal dosage of cyclophosphamide. The mechanism of Ling Zhi in this study was discussed.

Key words Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst); Heterotopic transplantation of heart.

From the work reported before it has been found that LZ exhibited regulative activity on some aspects of immune function. It was demonstrated that LZ was capable of acting as a biological response modifiers. It diversely regulated lymphocytes proliferation, inhibited antibody reaction and also suppressed delayed type hypersensitive reaction (DTH), contactive dermatitis, Arthus reaction and rat passive cutaneous anaphylactic reaction (PCA). The purpose of this study was to investigate the effect of LZ alone or in combination with cyclophosphamide on heterotopic transplantation of the mouse heart muscle.

Materials and Methods

Animals

Kunming (KM) mice, male, 5 ~ 6 weeks old, were used as cardiac transplant recipients and new born mice (within 24 h after born) of the same hybrid strain were used as cardiac donors. All mice were supplied by the Animal Center, Shanghai Medical University.

Dept. of Pharmacology, School of Pharmacy, Shanghai Medical University, Shanghai, China

Reagents

LZ was prepared as before.

Cardiac transplantation technique [1 ~4]

This experiment was performed as described by Fulmer [1] and modified by Judd[4]. Recipient mice were anesthetized by ip. 2.5% pentobarbital sodium (0.5 mg / 10 g). An incision 3 ~ 5 mm in length was made with a scapel parallel to the body axis and 1 ~ 2 mm distal to the ear-skull junction. The incision penetrated only the epidermis and dermis, not the cartilage. A pouch 3 ~ 4 mm in diameter was then formed between the skin and cartilage by blunt dissection with small curved forceps.

The donor heart was excised and cut almost equally to 2 pieces with particular attention. The graft probably contained one atria and one ventricle. The pieces of heart transplanted were approximately 3 x 3 mm in size. All tissue was grafted promptly following removal from donor except for a period of one to three minutes in Hanks solution while the host ear was prepared. The grafts were inserted into the ear pouch. Gentle pressure with the side of the forceps was applied to the ear in an effort to remove any trapped air in order to facilitate direct adhesion of donor and recipient tissue.

Electrical activity record

Heart grafts in the ears of anesthetized mice were monitored with physiograph. Electrodes for recording the electrocardiogram of heart in situ were made from pin needles and were inserted through the ear tissue on opposite sides of the heart graft on a line parallel to the long axis of the body. The end point of graft survival was taken as the time of cessation of electrical activity. The results were expressed as mean ± SD. The statistical analysis were performed by Student's t test.

Results

Influence of LZ alone treatment

Recipients were treated with Ling Zhi 125 and 500 mg/kg po. qd. x 9 starting on the day of transplantation. The duration of survival of viable grafts in Ling Zhi treatment groups were 11.6 ± 3.0 and 12.5 ± 1.7 days respectively. No significant prolongation of grafts-survival was observed when compared with untreated control group which survival time was 12.5 ± 2.6 days (Fig. 9-1).

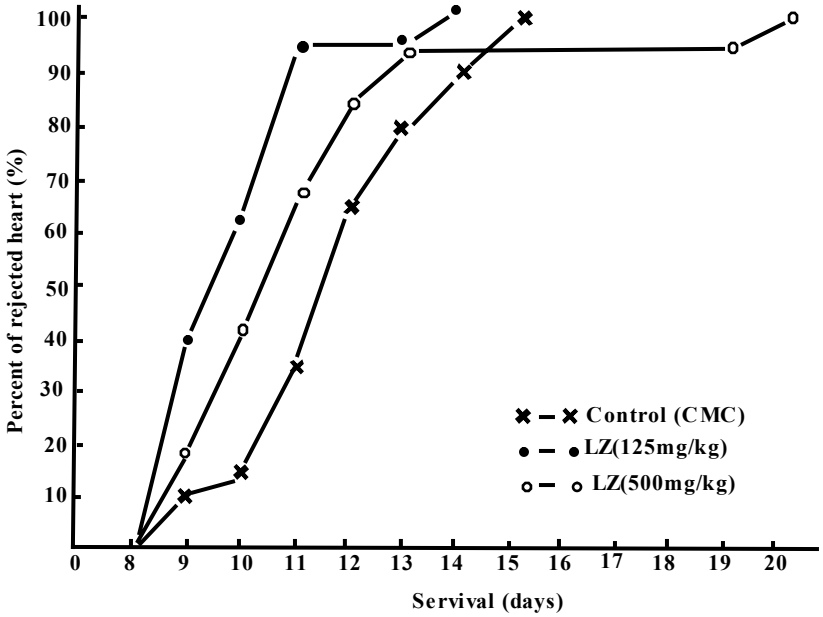

Fig. 9-1 Effect of LZ on time of implanted heart survival in mice
LZ: po. qd. X 9

Influence of treatment with Cyclophosphamide 10 mg/kg or 6 mg/kg alone and in combination with LZ

Treatment of cardiac transplanted mice with Cyclophosphamide (CYA) 10 mg/kg qd. x 9 starting on the day of transplantation resulted in a slight prolongation of graft survival time. The duration of survival time was 14.8 ± 2.2 days with significant increase when compared with control untreated group.

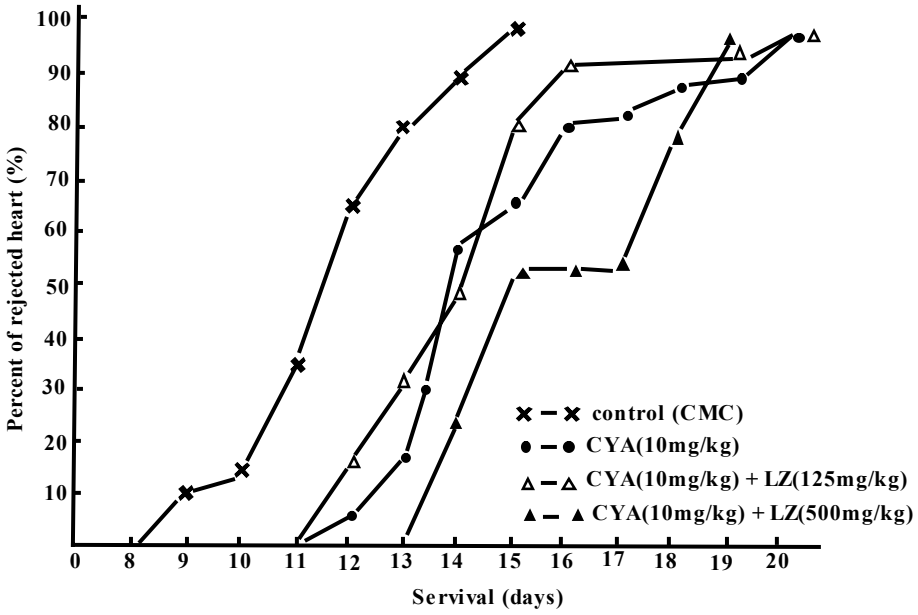

Fig. 9-2 Effect of LZ on time of implanted heart survival in mice
CYA: ip. qd. X 9; LZ: po. qd. X 9

LZ 125 and 500 mg/kg po. qd. x 9 in combination with CYA 10 mg/kg ip. qd. x 9 had no significant prologation in duration of survival time as compared with mice which received CYA 10 mg/kg alone group (Fig. 9-2).

Cardiac allografted mice which received combination therapy of CYA 6 mg/kg and LZ 125 or 500 mg/kg po. starting on the day of transplantation had somewhat increase in graft survival as compared with mice which received CYA 6 mg/kg ip. alone (Fig. 9-3).

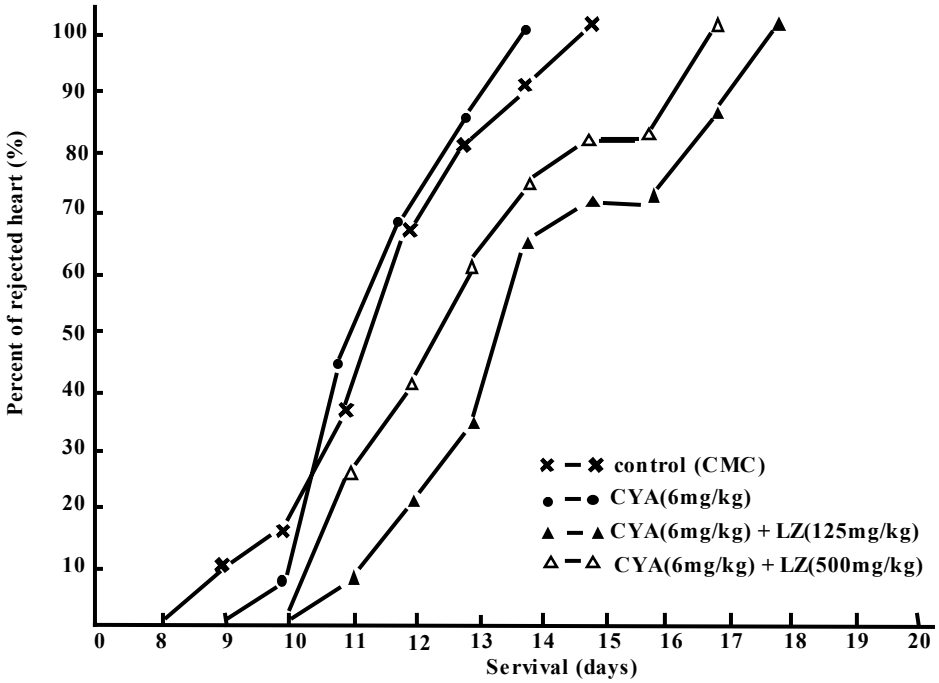

Fig. 9-3 Effect of LZ on time of implanted heart survival in mice
CYA: ip. qd. X 9; LZ: po. qd. X 9

Influence of treatment with Cyclophosphamide 15 mg/kg ip. q2d alone and in combination with LZ

Treatment of cardiac allografted mice with CYA 15 mg/kg ip. q2d alone. Starting on the day of transplantation no significant effect on graft survival time could be detected. It was 12.1 ± 2.0 days when compared with 11.4 ± 2.4 days of control untreated group.

However, when LZ 125, 250 mg/kg po. qd. x 9 in combination with CYA 15 mg/kg ip. q2d resulted in a slight increase in duration of survival time as compared with untreated control group. It was 14.2 ± 3.7 and 14.9 ± 4.5 days in LZ combination group. But had no significant effect when compared with the CYA 15 mg/kg ip. q2d alone group. Even increasing the dosage of LZ to 600 mg/kg no better result could be obtained (Fig. 9-4).

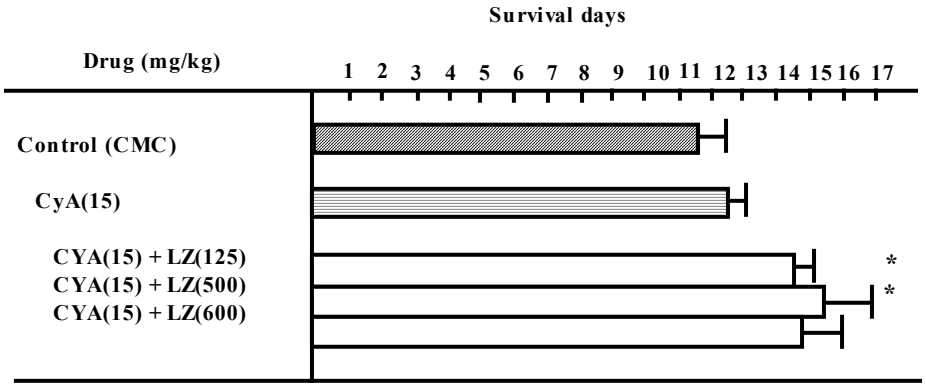

Fig. 9-4 Effect of LZ on time of implanted heart survival in mice compared with control, *P< 0.05; ± SE
CYA: ip. qod. X 5; LZ; po. qd. X 9

Discussion

Transplantation of cardiac tissue into the mouse ear has been demonstrated to be a relatively simple model to investigate the interaction between host and grafts. It is a more sensitive indicator of transplant rejection than the in vitro assays for cellular immunity. The case with which numerous new born heart allograft can be performed and the precision with which graft survival and function can be monitored by electrocardiographic evaluation suggests that the mouse heart transplant model is greatly superior to the skin allograft technique in measuring transplantation immunity [4 ~ 6]. Also it is a useful model to discover the effect of some immunosuppressive therapy on the survival of allografts [5].

In this study electrical activity of grafted heart tissue can be discovered in almost all of the recipience mice at 6 days after operation. The mean survival time of 20 mice of untreated control group was 12.5 ± 2.6 days. LZ alone did not show any superior effect on this model however when LZ in combination with suboptimal dosage of Cyclophosphamide (6 mg/kg qd. x 9 or 15 mg/kg qod x 5) would express slight increase in the duration of grafts survival time as compared mice which received suboptimal dosage of Cyclophosphamide alone. This effect of LZ was probably attributable to its regulative capacity on immune system, suppress the antibody response, DTH etc. Besides the anticoagulative and vascular dilating activity of LZ may also contribute to a more adequate blood supply to the graft. This is a primary study. Farther investigation is necessary.

Hepatoprotective Activity of Ling Zhi

— Immunopharmacological Study (10)

ZHANG Luoxiu WU Wengrong CAO Zhifang

Abstract The effect of hot water extract from Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst) on experimental liver injury induced by D-galactosamine combined with Endotoxin in mice were studied. Daily oral administration of 250 or 500 mg/kg LZ significantly decreased SGPT activity in D-galactosamine and Endotoxin intoxicated mice. It was also observed that LZ obviously protected tetrachloride produced cytotoxicity in primary cultured hepatocytes and reduced the release of lactate dehydrogenase (LDH) and glutamic pyrovic transaminase (GPT) from rat liver cells. There was a noticeable improvement in mice with liver injury induced by single oral administration of tetrachloride. These results suggest that LZ has a therapeutic effect on liver injury by improving liver functions.

Key words Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst); Liver injury; Tetrachloride; D-galactosamine; Endotoxin.

LZ is the dried fruit body of a fungus Ganoderma Lucidum, Fr. Karst and has been used in Chinese traditional medicine as a tonic and sedative drug for a long time. From the Chinese ancient pharmacopea "Ben Cao" it was recorded that LZ possessed the activity to reinforce and nourish the deficiencies of five parenchymatous viscera. In this study we specially observed the activity of the hot water extract of LZ on experimental pathological mode of liver injury.

It was reported in 1967 that D-galactosamine induced rat liver injury and the pathological change was similar to those of human hepatitis. However, there was s large inter and intra species variations with respect to the susceptibility of the animals. Mice were less susceptible to D-galactosamine than rats. It was also known that D-galactosamine induced hepatitis in mice was potentiated by administration of Endotoxin[(1)]. In order to study the protective activity of LZ on liver injury we were interested in this similar model. Besides, CCI_4 is a typical hepatic toxin. In this paper, we studied the effect of LZ on rat liver cells damaged by CCI_4 in vivo and in vitro.

Dept. of Pharmacology, School of Pharmacy, Shanghai Medical University, Shanghai, China

Materials and Methods

Animals

Kunming (KM) Mice, 21 ~ 26 g, Sprague Danley (SD) rats, 180 ~ 220 g, were supplied by the Animal Center, Shanghai Medical University.

Reagents

The hot water extract from LZ planted in Japan was provided by Wakan Shoyaku Botany Institute. LZ extract a brown powder was suspended in saline containing 0.2% carboxymethylcellulose sodium (CMC) for in vivo study and for in vitro study 10 mg of LZ was prepared in 10 ml NS then the tube put in 80°C water bath shaken for 2 h. After centrifugation the soluble part was named Part A. If it was prepared by three times rapid frozen and thawed then it was named as frozen thawed part (Part B) after centrifugation.

D-galactosamine was prepared by Chong Qing Medical University. Endotoxin 055B5 was provided by Second Millitary Medical University. Collagenase I type was purchased from Sigma Chemical Co. RPMI – 1640 medium was purchased from GIBCO Co. it contained 15 mmol/L Hepes, 100 U/ml Penicilline, 100 μg/ml Streptomycin and 10% NCS.

Mice liver injury induced by D-galactosamine combined with Endotoxin

Mice were randomly divided into 4 groups. The first group was normal untreated animals, the second group received D-galactosamine 500 mg/kg ip. 30 min later ip. endotoxin 100 μg/mouse. The third group received Endotoxin only and the fourth group received D-galactosamine only. 10 h after injection of toxic agent the serum was separated by centrifugation (3 000 rpm x 10 min) from blood. The activity of glutamic-oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) was assayed. Analysis of variance test followed by individual comparison by Student's t test was utilized to determine the significance of mean (± SD) values of various groups of animals.

Effect of LZ on D-galactosamine/Endotoxin induced liver injury

Mice were randomly divided into 3 groups. The first group received D-galactosamine 500 mg/kg ip. and Endotoxin 100 μg/mouse only as a control group. The second and third groups received 250 or 500 mg/kg LZ po. qd. x 2 respectively. 1h after last administration of LZ ip. D-galactosamine and Endotoxin. 10h later blood was removed from the mice to assay SGPT and SGOT activity.

The assay of IL-1 like substance in serum

The activity of IL-1 like substance of serum from the above experiment were assayed by means of thymus cells proliferation test. The thymus cells of BALB/c mice were used. After washing twice, the cells number was adjusted to 1 x 10^7 /ml contained Con A 2.5 μg/ml. 1 x 10^6 cells were seeded into each well of 96 well microplate. 0.1 ml of different dilution of serum was added in and incubated at 37°C 5% CO_2 for 72 h. 6h before termination of incubation each well was pulsed with 0.925 x 10^7Bq of ^{3}H-TdR were measured.

Effect of LZ on isolated rat hepatocytes intoxicated by CCI_4

SD rats (180 ~ 220 g) were used as liver donors. They were allowed food and water. Isolated hepatocytes were prepared by the method of Berry and Friend (1969) [(2)] with minor modifications. Under ether anesthesia, the portal vein was cannulated and the liver was perfused under a constant pressure. The flow rate was 9 ml/min with calcium – free physiological solution (96 mM NaCI, 1.4 mM KCI, 0.74 mM $MgSO_4$, 2.5 mM KH_2PO_4, 30 mM $NaHCO_3$ and 21.7 mM sodium gluconate) gased with 95% O_2/, 5% CO_2 (pH 7.4) and warmed at 37°C. Physiological solution contained 0.02% collagenase was continued to perfuse for a further 8 min. Then the third physiological solution contained Ca^{2+} and bovine albumin 1% without collagenase was perfused for another 4 min. The liver was then placed in a beaker containing buffer solution plus bovine serum albumin and Ca^{2+} the capsule was disrupted gently with a spatula. After gently shaking (40 cycles/min) the cell suspension was then filtered through nylon gauze. The cells suspension was centrifugated (500 rpm for 1 min) and washed twice. Finally cells resuspended in RPMI – 1640 medium with 10% NCS. Viability of the cells was estimated by the trypan blue test with an index of 90 ~ 92%. 1 ml of cells (5 x 10^6) were seeded in 24 well microplate. Each group had triplicate cultures.

LZ Part A or Part B was added to make a final concentration 0.01 ~ 10 mg/ml. 20 μl of CC I_4 was added at the same time. Incubation were carried out at 37°C 5% $CO_2$1 h. After centrifugation (3 000 rpm x 10 min) the supernatants were determined the release of lactate dehydrogenase and GPT from liver cells. The enzyme activity represented the total value of 100% lysis was obtained by lysing hepatocytes with 1% Triton X-100 for 1 h.

Effect of LZ on CCI_4 intoxicated mice in vivo

Mice were randomly divided into 4 groups. Group A control, group B ip. 0.01% CCI_4 0.18 ml/10g. Group C and D received LZ 250 or 500 mg/kg po. qd. x 2. 1 h after the second oral administration of LZ the same dosage of CCI_4 as above were given by intraperitoneal injection. 24 h later blood was removed and the serum were assayed for SGPT activity.

Results

D-galactosamine and Endotoxin induced liver injury in mice

The results showed the D-galactosamine alone resulted in a slight increase in GPT activity ($P < 0.05$). However, when D-galactosamine combined with Endotoxin 100 μg/mouse produced an apparent increase in SGPT level ($P < 0.01$). While this amount of Endotoxin alone did not produce significant liver injury (Tab. 10-1).

Tab. 10-1 Serum GPT and GOT activity in mice treated with D-galactosamine and Endotoxin

Group	Galn (mg/kg)	ET (μg/mouse)	n	SGPT (μg/ml ± SD)	SGOT (μg/ml ± SD)
A	—	—	5	92 ± 53	67 ± 25
B	500	100	5	454 ± 209**	106 ± 16*
C	500	—	5	157 ± 88*	74 ± 26*
D	—	100	5	59 ± 17**	48 ± 13*

B compared with A; C and compared with B. *P<0.05; **P<0.01

Effect of LZ on D-galactosamine/Endotoxin induced liver injury

Mice were randomly divided into 3 groups. The first group received D-galactosamine 500mg/kg ip. and Endotoxin 100 μg/mouse only as a control group. The second and third groups received 250 or 500 mg/kg of LZ po. qd. x 2 respectively. 1 h after last administration of LZ ip. D-galactosamine and Endotoxin, 10 h later blood was removed from mice. The activities of SGPT demonstrated that LZ decreased the level of SGPT in a dose dependent fashion ($P < 0.05 \sim 0.01$) (Tab. 10-2).

Tab. 10-2 Protective activity of LZ on D-galactosamine and endotoxin induced liver injury in vivo

Group	mg/kg	Galn/Endotoxin	n	SGPT (U/ml ± SD)	SGOT (U/ml ± SD)
Control	—	+	6	1 474 ± 548	238 ± 101
LZ	250	+	6	583 ± 733**	127 ± 44*
LZ	500	+	6	310 ± 182**	162 ± 161

Galn: 500 mg/kg; Endotoxin: 100 μg/mouse. *P<0.05; **P<0.01

Tab. 10-3 Effect of LZ on IL-1 like substance in serum of mice intoxicated by D-galactosamine and endotoxin

Group	N	Dilution of serum		
		1 : 40	1 : 20	1 : 10
Control	5	16 319 ± 6 261	5 828 ± 1 241	627 ± 158
Galn / ET	4	7 240 ± 4 843*	2 876 ± 2 439*	480 ± 393
LZ + G/E	5	11 351 ± 7 384	3 085 ± 1 746*	308 ± 88**

$\overline{X}$ ± SD; cpm / 1 x 10^6 cell
compare with control, *P<0.05; **P<0.01
Galn 500 mg/kg; Endotoxin 100 μg/mouse; LZ 500 mg/kg

The serum from each group of mice in above experiment was taken to study. It was found that the level of IL-1 like substance in intoxicated mice was lower than that of control group. LZ appeared to elevate the IL-1 level in serum. It suggested that LZ was able to regulate the immune function of liver injured mice (Tab. 10-3).

Protective activity of LZ against tetrachloride toxicity in isolated rat hepatocytes

From the results indicated in Tab. 10-4 it was shown that both part A and part B of LZ induced an apparent decrease in GPT and LDH level released from liver cells (P< 0.01).

Tab. 10-4 Effect of LZ on hepatocytes intoxicated by CCl_4 in vitro

Group	CCl_4	LZ	LDH		GPT	
	μl / well	Mg/ml	$\overline{X}$ ± SD	Releasing %	$\overline{X}$ ± SD	Releasing %
Control	—	—	6 800 ± 200	39.4	43 ± 4	23.6
CCl_4	20	—	14 483 ± 525	84.0	145 ± 5	79.7
Part A	20	0.01	10 225 ± 225**	59.3	85 ± 6**	46.7
	20	0.1	9 667 ± 289**	56.0	86 ± 6**	47.5
	20	1	9 417 ± 144**	54.6	85 ± 2**	46.9
	20	10	9 983 ± 475**	57.9	51 ± 5**	28.0
Part B	20	0.01	7 717 ± 375**	44.7	62 ± 3**	34.1
	20	0.1	9 667 ± 289**	56.0	88 ± 4**	48.1
	20	1	8 700 ± 260**	50.4	73 ± 6**	40.3
	20	10	10 900 ± 450**	63.2	48 ± 3**	26.4

Hepatocytes: 1 : 40; culture time: 1 h
Part A: soluble part; Part B: frozen-thawed part
N = 3; compared with CCl_4 group: **P<0.01

Effect of LZ on tetrachloride intoxicated mice

Mice were randomly divided into 4 groups. Group A control group B 0.01% CCI_4 0.18 ml/10 g ip. groups C and D received LZ 250 or 500 mg/kg po. qd. x 2. 1 h after the second oral administration of LZ the same dosage of CCI_4 as above were given by intraperitoneal injection. 24 h later blood was removed and the serum were assayed after convenient dilution.

It was found that LZ decreased the SGPT level significantly ($P < 0.05$) (Tab. 10-5).

Tab. 10-5 Protective activity of LZ on CCI_4 induced liver injury in vivo

Group	Dosage (mg/kg)	CCl_4 (ml / 10g)	n	GPT (U/ml ± SD)	GOT (U/ml ± SD)
Control	—	—	9	52 ± 7	164 ± 96
CCl_4	—	0.18	9	1 015 ± 0	1 426 ± 756
LZ	250	0.18	10	883 ± 235	2 192 ± 1 366
LZ	250	0.18	10	712 ± 396*	1 660 ± 930

*$P<0.05$, compare with CCl_4 group

Discussion

Although more than thousands of years ago the ancient Chinese medicinal work had already noticed the activity of LZ on liver. But the liver described in Ben Cao implicated a wider meaning. Our interest was to determine the protective activity of LZ on experimental pathological model of liver injury. From the results stated above LZ improved the liver injury produced by CCI_4, D-galactosamine combined with Endotoxin mice as well as the isolated primary cultured liver cells. These results suggest that LZ possesses hepatoprotective activity.

GPT and GOT are enzymes usually released from liver cells which injured by intoxication or infection. These enzymes represent the degenertion and necrosis of liver cells. Because a great amount of GOT enzyme exists within the cells of heart, kidney and skelector muscles therefore GPT is more specific to indicate liver injury.[4]

Lactic acid dehydrotenase (LDH) reflected the liver injury. LZ could decrease the level of LDH of rat liver cells intoxicated by CCI_4.

IL-1 is an important monocytrkine with wide biological activities relating its important role in immune and certain pathological events. The relationship between IL-1 and liver injury is not available. In this study it was found that the IL-1 like substance in serum of D-galactosamine and Endotoxin intoxicated mice was lower than that of normal mice. LZ which protects mice against liver injury would be able to elevate IL-1 like substance in serum. This further indicates that LZ regulates immune function.

Effects of Ling Zhi on Hemopoietic System in Mice

— Immunopharmacological Study (11)

JIA Yongfeng ZHOU Xianbiao MENG Hong ZHANG Luoxiu

Abstract The effect of Ling Zhi (LZ) on the hemopoietic function in normal mice and in mice with suppressed function induced by cyclophosphamide (CYA) were studied. LZ 250, 500 or 1 000 mg/kg po. qd. x 6 could enhance the bone marrow nucleated cells (BMNC) proliferation, increase the number of white blood cell (WBC) and the amount of hematoglobin (Hb) of peripheral blood. LZ also improved BMNC, WBC, Hb of mice treated with CYA (40 mg/kg). 250 mg/kg of LZ had the best therapeutic effectiveness. Our results indicated that LZ could stimulate the hemopoietic function of mice.

Key words Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst); White blood cell; Hematoglobin; Bone marrow nucleated cell.

Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst), a Chinese medicinal herb, has been used to protect or cure many kinds of diseases for two thousand years. The clinical data showed: LZ had affects on leukopenia and could improve blood depression caused by other disorders. However, there was no experimental data of LZ effecting on hemopoietic system.

Our purpose was to investigate the effect of LZ from Japan on hemopoietic system, filling the blank of basic pharmacological data on this aspect.

Materials and Methods

Animals

Kunming (KM) mice, male, 20 ± 2g, supplied by the Animal Center, Shanghai Medical University.

Dept. of Pharmacology, School of Pharmacy, Shanghai Medical University, Shanghai, China

Reagents

Blood from tail vein of mice was taken twice and its WBC was counted before experiment. The animals were chosen and randomly divided into 4 groups, including control group, CYA group and LZ groups with different dosages. Control group was oral administration of 0.2% CMC; CYA group was intrapertioneal injection of CYA 40 mg/kg.[4]

LZ hot water extract form Ganoderma Lucidum was provided by Wakan Shoyaku Bnotany Institute, Tokyo, Japan. LZ extract was grinded and dissolved in normal saline containing 0.2% CMC, shaken at 80°C water bath for 4 h, stored at 4°C.

Cyclophosphamide (No. 12 Pharmaceutical Factory of Shanghai). CYA was dissolved in sterile saline. Final concentration was 0.2%, stored at 4°C.

Reference heamatin hydrochloride (R.H.Hc) solution. 5.055mg R.H.Hc (Shanghai Research Institute of Biochemistry) were dissolved with 0.1 mol/L NaOH, total volume was 0.5L. The concentration of Hb was 250 g/L.

Animal model of blood depression

Kunming mice Cyclophosphamide 40 mg/kg ip. d0 ~ d3 and d6.

Standard curve of Hb[3]

The reference heamatin hydrochloride solution was diluted with 0.1 mol/L NaOH to a series of difference concentrations.

The absorption of these solutions was done with spectrophotometer Model 721, λ 395nm. The data showed in Tab. 11-1. Linear regression line was drawn according to the absorption and the amount of Hb. This regression line was called Hb standard curve.

$$\text{Hb(g/L)} = \frac{\text{concentration of H.HC}}{4} \times \frac{64\,458 \times 1\,000}{1\,000}$$

Tab. 11-1 Absorption of reference heamatin hydrochloride solution

		No. of tube									
		1	2	3	4	5	6	7	8	9	10
Volume of R. H. Hc (ml)		1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Volume of NzOH (m)		9.0	8.0	7.0	6.0	5.0	4.0	3.0	2.0	1.0	0.0
Hb (g/L)		25	50	75	100	125	150	175	200	225	250
Absorption	Y1	62	122	164	264	370	426	468	586	614	718
(x 1 000)	Y2	32	89	156	303	341	448	513	580	671	700
	$\bar{}$	47	105	160	283	355	437	491	583	642	709

Regression Line: $Y = 327.51X \pm 12.59$

Leukocyte count and Hb determination

20 μl blood from mice tail vein was mixed with 380 μl 0.1 mol/L HCI and took out 40 μl for cell count, another 100 μl mixture was added into 4.9 ml 0.1 mol/L NaOH, the absorption of those solutions was done using spectrophotometer Model 721 at λ 395nm and its concentration of Hb was read from the standard curve of Hb.

BMNC count [2, 5]

Mice were killed and a femur was taken. After getting rid of femural surface tissue, the bone marrow cavity was opened and washed with saline, then centrifuged 1 500 rpm 10 min. The pallet was collected and mixed with 2ml 3% HAc, this solution was further 10 times diluted with 3% Hac and take 50 μl for cells count.

Cell counts were expressed in number as the mean ± SD, these data were compared for statistical significance by Student's t test.

Results

Effect of LZ on the number of WBC in peripheral blood of normal mice

Leukocyte count level was within the normal range during the first week. On d10, WBC count of groups treated with LZ exceeded. The mice in group of 250 mg/kg LZ had significant difference with that in control group (Tab. 11-2).

Tab. 11-2 Effect of LZ on the number of WBC in peripheral blood of normal Mice

LZ (mg/kg)	Number of WBC (/mm^3)				
	d0	d2	d4	d6	d10
Control	10 744 ± 1 628	11 155 ± 2 068	10 510 ± 1 557	11 328 ± 1 923	11 962 ± 311
250	10 763 ± 994	12 223 ± 746	8 364 ± 1 242	12 490 ± 1 401	13 783 ± 1 637*
500	10 780 ± 1 086	12 000 ± 1 828	9 433 ± 3 027	13 000 ± 2 211	13 300 ± 1 517

LZ po. qd. x 6; $\overline{X}$ ± SD; n = 5; *P<0.05 compared with control

Enhance of BMNC proliferation by LZ in normal mice

The number of BMNC of LZ groups was significantly higher than that of control group on d8, and the action of LZ 250 mg/kg group was still existed on d10 (Tab. 11-3).

Tab. 11-3 Effects of LZ on BMNC of normal Mice

LZ (mg/kg)	Number of BMNC (x 10^4/mm^3)		
	d5	d8	d10
Control	1 188 ± 198	987 ± 43	1 044 ± 99
250	1 172 ± 62	1 557 ± 163**	1 566 ± 112*
500	1 343 ± 344	1 377 ± 168**	1 014 ± 106

LZ po. qd. x 6; $\overline{X}$ ± SD; n = 5; *P<0.05; **P<0.01 compared with control

Effect of LZ on Hb in peripheral blood of normal mice

The result was given on Tab. 11-4. The amount of Hb of LZ groups had increasing tendency compared with that of control group on d10, without statistic significance. The data indicated the change of amount of Hb in mice was less distinct than that of the white cells.

Tab. 11-4 Effects of LZ on Hb in peripheral blood of normal Mice

LZ (mg/kg)	Hb (g/100ml)		
	D2	d4	d10
Control	152.8 ± 31.8	151.2 ± 13.4	158.2 ± 12.9
250	140.0 ± 12.1	145.7 ± 17.2	177.9 ± 21.8
500	133.9 ± 11.4	138.6 ± 9.04	170.3 ± 11.1

LZ po. qd. x 6; $\overline{X}$ ± SD; n = 5

Effect of LZ on the number of WBC in peripheral blood of mice treated with CYA

When CYA was injectived the general finding consisted of an initial fall in the number of circulating leukocytes followed by a period of sustained depression and a subsequent return to normal level. When CYA was injectived after CYA was stopped, blood count of LZ groups dirapiy rise on d6 and significantly higher than that of CYA group (Tab. 11-5).

Tab. 11-5 Effects of LZ on the number of WBC in peripheral blood of Mice treated with CYA

LZ (mg/kg)	CYA (mg/kg)	Number of WBC (/mm^3)				
		D0	d2	d4	d6	d0
—	—	10744 ± 1628	11155 ± 2 068	10510 ± 1557	11327 ± 1923	11962 ± 311
—	40	10670 ± 1313	6900 ± 682	3715 ± 992	7562 ± 453	10550 ±1257
250	40	10728 ± 1457	8130 ± 1210	3433 ± 574	11160 ± 1783*	11044 ± 867
500	40	10737 ± 1384	6353 ± 1320	2621 ± 592	10688 ± 3480	9588 ± 1594
1 000	40	10700 ± 1444	8325 ± 1587	2903 ± 567	11271 ± 1827*	11641 ± 2222

LZ po. qd. x 6; CYA ip. d0~d3 and d6. $\overline{X}$ ± SD; n = 5; *P<0.05 compared with CYA group

Effect of LZ on the number of BMNC of mice treated with CYA

The range of number of BMNA was seen in Tab. 11-6. In CYA group from d1 to d8 the BMNC counts dropped rapidly and continued slowly to rise. The number of BMNC in LZ groups was significantly higher than that of CYA group on d10.

Tab. 11-6 Effects of LZ on the number of BMNC of Mice with CYA

LZ (mg.kg)	CYA (mg/kg)	Number of BMNC (x 10^4/mm^3)		
		d5	d8	d10
—	—	1 188 ± 198	987 ± 43	1 044 ± 99
—	40	245 ± 94	668 ± 80	678 ± 199
250	40	245 ± 40	762 ± 161	1 234 ± 155**
500	40	310 ± 77	762 ± 303	1 115 ± 212*
1 000	40	192 ± 20	377 ± 131	1 137 ± 220*

LZ po. qd. x 6; CYA ip. d0~d3 and d6. $\overline{X}$ ± SD; n = 4; , *P<0.05, **P<0.01 compared with CYA group

Effect of LZ on Hb in peripheral blood of mice treated with CYA

On d10, all animals of LZ groups (250, 1 000 mg/kg) showed the amount of Hb similar to normal, but that of CYA group was still in lower degree and significantly lower than that of LZ groups (Tab. 11-7).

Tab. 11-7 Effects of LZ on Hb in peripheral blood of Mice with CYA

LZ (mg.kg)	CYA (mg/kg)	Hb (g/100mml)		
		d2	d4	d10
–	–	152.8 ± 31.8	151.2 ± 13.4	158.2 ± 12.9
–	40	135.1 ± 11.4	143.0 ± 8.0	126.2 ± 13.5
250	40	134.2 ± 19.0	138.7 ± 6.1	151.2 ± 12.0*
500	40	127.8 ± 12.5	131.1 ± 10.6	141.3 ± 7.2
1 000	40	132.0 ± 8.8	131.8 ± 13.9	152.0 ± 19.0*

LZ po. qd. x 6; CYA ip. d0~d3 and d6. $\overline{X}$ ± SD; n = 5; , *P<0.05 compared with CYA group

Discussion

Blood picture depression was a common clinical phenomenon. It was caused by various diseases such as virus infection, malignant tumor or autoimmune diseases. At present, the ordinary methods of killing tumor cells were chemotherapy or radiotherapy. But these methods could cause the decrease of WBC. In our work therapeutic effectiveness of LZ was investigated on the animal model of leukopinia. The data showed that LZ not only enhanced the number of leukocyte and the amount of Hb, but also promoted the proliferation of BMBC. These results were consistent with clinical information.

Our experimental data had also shown, either in normal mice or in immunosuppressed mice by CYA, the therapeutic effectiveness of LZ in low dosage (250 mg/kg) was better than that of higher dosages.

Effects of Ling Zhi on Sex Vitality and Longevity in DROSOPHILA MELANOGASTER

LI Huaiyi* WANG Xinmin* JIANG Minhua** ZHANG Peng**
PENG Hongli** Masao MORI***

Abstract Effects of Ling Zhi (LZ) from Japan on sex vitality and the longevity in Oregon K *Drosophila melanogaster* were studied. The results showed that 0.5% and 1.0% of LZ could significantly increase the number of feeding in female flies and 1.0% of LZ increase the number of mating with dose-effectiveness. In addition, the favorable effects of 0.5% ~ 3.0% of LZ in prolonging the mean life span, the maximum life span and the median life span were observed in tested flies.

Key words Ling Zhi (LZ); *Drosophila melanogaster*; Reproduction; Longevity.

Ling Zhi (LZ) was used in treatment for chronic diseases and improvement of human health. *Drosophila melanogaster* is a model in aging system which has often been used in gerontological studies [1, 2]. Wang et al reported that LZ prolonged the life span in fruit fly [3, 4]. The Vigour Matter 1 and the mixture of rice plumule were tested for their influence on the life span of fruit fly in our laboratory [5, 6]. This study was undertaken to determine the effects of LZ on the reproduction and the longevity in *Drosophila melanogaster.*

Materials and Methods

Animals

Wild type Oregon K flies *Drosophila melanogaster* imported from Holland was used in this study. The methods of stock culture was described as references [5, 6].

Reagents

The extract in hot water of LZ was provided by Wakan Shoyaku Botany Institute, Tokyo, Japan. The drug is the powder in yellow-brown color. In the treated groups, the drug was incorporated into medium and the flies fed on the medium at whole adult stage.

* Dept. of Special Toxicology, Second Military Medical University, Shanghai, China
** School of Pharmacy, Shanghai Medical University, Shanghai, China
*** Wakan Shoyaku Botany Institute, Tokyo, Japan

Tested dosages

LZ proved to be nontoxic as measured by the mortality of adult flies when they were reared on medium containing of 0.5% ~ 5.0% of LZ. Thus, LZ, at doses of 0.5%, 1.0%, 2.0% and 3.0%, were selected for the study.

Survival experiment

The mean life span, maximum life span and median life span were tested in survival experiment. In addition, both tests for the number of feeding and for the number of mating were also conducted to evaluate the effects of LZ on aging process. All tests mentioned above were performed in 4 groups with concentrations of 0.5%, 1.0%, 2.0% and 3.0%, respectively. The flies in control group fed on the basic medium [6]. The each survival experiment at same concentration of LZ was conducted by means of dividing into female flies group and male flies group. The number of feeding in female flies was scored in this study only. Mean body weight range 1.10 mg ~ 1.15 mg in female flies and 0.78 mg ~ 0.82 mg in male ones.

The standard of experiment

Test for the number of feeding: To score the number of feeding at interval of 5 min was performed over a period of 4 h.

Test for sex vitality: To score the number of mating at intervals of 1 min was performed over a period of 11 min.

Survival test: To score the number of natural dead flies at intervals of 6 h was performed over a period of the whole adult stage.

Experimental methods

The experimental methods were done as described in references [5, 6].

Results

Feeding number

LZ significantly increased the number of feeding in female flies compared with that of control group. There was a 5% ~ 24% increase in the number of feeding with dose-effectiveness (Tab. 12-1).

Tab. 12-1 Effect of LZ on the number of feeding in *Drosophila melanogaster*

LZ (%)	Number of feeding
0	343
0.5	463***
1.0	554***
2.0	374*
3.0	350*

χ^2 test; n = 60; ♀ *P >0.05, ***P<0.01 vs control

Sex vitality

LZ significantly increased the number of mating of the flies compared with that of control group. There was a 2% 6 ~ 2% increase in the number of mating with dose-effectiveness (Tab. 12-2).

Tab. 12-2 Effect of LZ on sex vitality in *Drosophila melanogaster*

LZ (%)	Number of mating
0	265
0.5	304*
1.0	329***
2.0	283*
3.0	278*

χ^2 test ♀ : ♂ = 60 : 40; *P >0.05, ***P<0.01 vs control

Life situation

LZ significantly prolonged the mean life span, the maximum life span and the median life span compared with that of the control group with dose-effectiveness. The mean life span was increased 7% ~ 13% in the female flies and 8% ~ 15% in the male flies, the maximum life span increased 3% ~ 23% in the female flies and 13% ~ 21% in the male flies, the median life span increased 3% ~ 10% in the female flies and 6% ~ 13% in the male flies (Tab. 12-3).

Tab. 12-3 Effect of LZ on mean life span, maximum life span and median life span in *Drosophila melanogaster*

LZ %	Mean life span (d) $x \pm s$		Maximum life span (d)		Median life span (d)	
	♀	♂	♀	♂	♀	♂
0	55.12 ± 14.78	50.37 + 14.36	80	72	60	53
0.5	58.82 ± 14.83*	54.33 + 15.16**	90	81	62	56
1.0	62.19 ± 13.20***	55.70 ± 12.54***	98	87	66	57
2.0	59.38 ± 16.43**	57.45 ± 14.42***	82	84	64	59
3.0	61.07 ± 12.86***	57.96 ± 13.69***	83	84	62	60

u test; n = 105; *P >0.05, **P<0.05, ***P<0.01 vs control

As shown in Figure 12-1 and 2, the survival curves of the treated groups were located at the right side of that of the control group. Furthermore, the effects were observed in both female and male flies, and continued for 60 days (i.e. from 20 ~ 30 days to 80 ~ 100 days at adult stage).

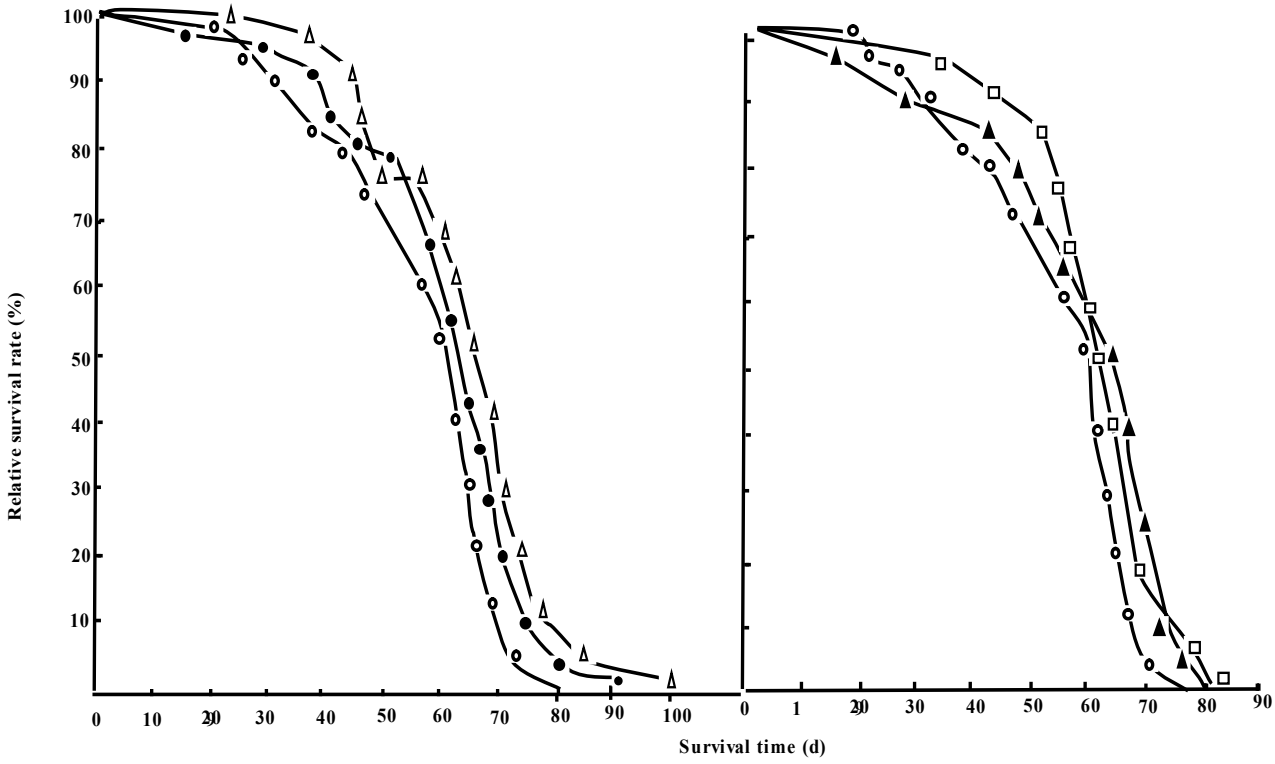

Fig. 12-1 Effect of 0.0% (○), 0.5% (●), 1.0% (△), 2.0% (▲) and 3.0% (□) of LZ on life span of female flies

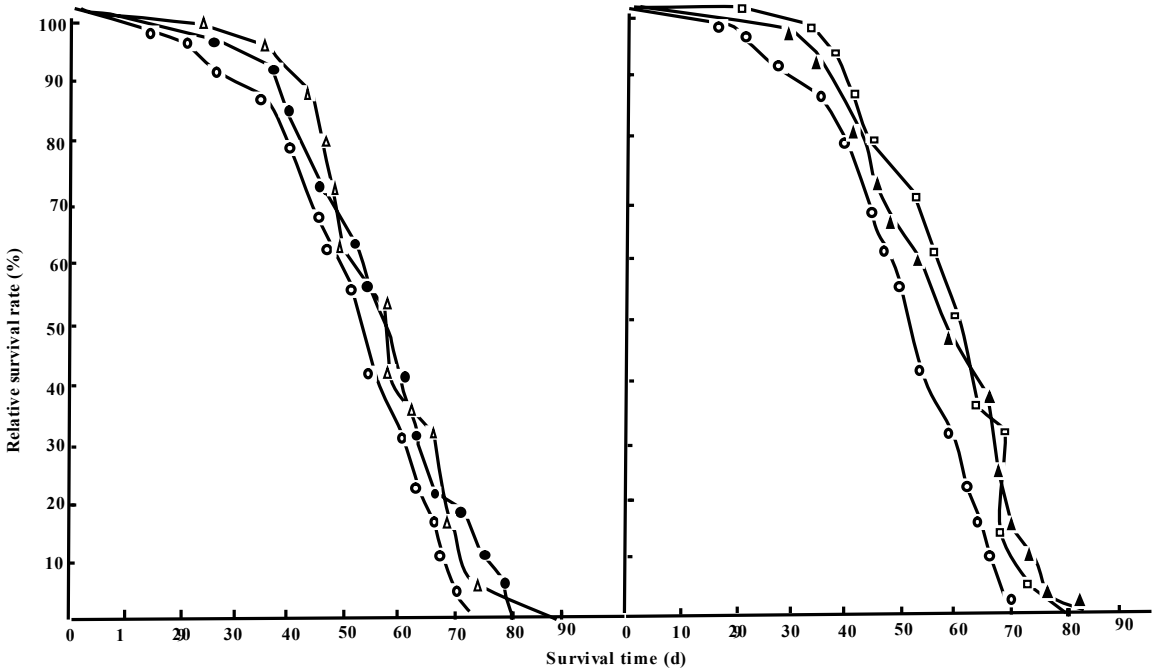

Fig. 12-2 Effect of 0.0% (○), 0.5 (●), 1.0% (△), 2.0% (▲) and 3.0% (□) of LZ on life span of maleflies

Discussion

LZ at concentration less than 5% was proved to be nontoxic and might increased the number of feeding and mating, and might prolong the mean life span, the maximum life span and the median life span in wild type Oregon K *Drosophila melanogaster*. The data analysis showed that LZ had a dose-effectiveness in the antiaging action. Wang et al reported that LZ significantly increased the mean life span but did not significantly prolong the maximum life span in two strains of *Drosophila melanogaster.* (Canton S and American wild type). This data were not consistent with that of Wang's. LZ was not only significantly prolonged the mean life span and the median life span but also significantly prolonged the maximum life span in *Drosophila melanogaster*. The reason for the different results is not clear and it may be: (1) the different strains of *Drosophila melanogaster* respond to LZ differently. (2) LZ used in our study is not the same as that used in their study in efficient ingredient or in preparation techniques. The real cause remains to be determined.

There were the different opinions on the criteria of the antiaging drugs. Walford postulated that the drug so long as prolonged the maximum life span might be considered as the antiaging drug, even it did not prolong the mean life span [(7)]. However, Bindra suggested that the drug which only prolonged the mean life span and did not prolong the

maximum life span might also be considered as the antiaging drug [8]. Overall our results demonstrate that LZ was able to prolong the longevity including the mean life span, maximum life span and median life span in *Drosophila melanogaster*. The authors think that LZ belongs to the antiaging drug according to two criteria mentioned above.

Analgesic, Sedative Effects and Promoting Tolerance Activity of Ling Zhi in Mouse

JIANG Minhua* LIN Chung* FANG Xiang* MIAO Yongsheng*
Masao MORI**

Abstract In this study Ling Zhi (LZ) is efficient to relieve pain sensation induced by chemical stimulus and hot plate stimulus. The ED_{50} of LZ is 4.98 k/kg measured by chemical stimulus method. LZ also cooperates with using Pentobarbitol in sedative dose. The ED_{50} of reducing spontaneous motor activities of LZ is 2.65 g/kg. LZ (3.5 g/kg) is obviously to prolong the swimming time of mice. The ability to stand hypoxia is increased by using 5.0 g/kg and 7.5 g/kg of LZ. These results further confirm that LZ possesses analgesic, sedative effects and promoting tolerance activity.

Key words Ling Zhi (LZ); Analgesia; Sedation; Hypoxia.

LZ has been used in Chinese traditional medicine for centuries. It was reported that LZ had been used clinically to alleviate pain sensation such as headache, backache, neuralgic pain, cancerous pain, and to be a hypnotic as well as a mild energetic remedy. Following the clinical observation, it is suggested that LZ have many pharmacological actions [1, 2].

Recently, we are interested in the evaluation of the analgesic and sedative effects of LZ. The study was designed to investigate the activities of LZ on analgesia and sedation. Meanwhile, the work was carried out the promoting tolerance activity of LZ either.

Materials and Methods

Animals

Kunming (KM) mice, 20 ± 2.0g, supplied by the Animal Center, Shanghai Medical University.

* Dept. of Pharmacology, School of Pharmacy, Shanghai Medical University, Shanghai, China
** Wakan Shoyaku Botany Institute, Tokyo, Japan

Reagents

LZ extracta were provided by Wakan Botany Institute, Tokyo, Japan. It was diluted by 0.5% CMC and prepared in a shaking bath at 80°C, 4 h.

Aminopyrine was purchased from Shanghai Pharmaceutical Company. Acetic acid (analytical grade) was made by Suzhou Jincheng Chemical Factory. Diazepam was the product of Shanghai Haipu Pharmaceutical Factory. Sodium pentobarbitol was obtained from Sigma Chemical Company. Propranolol was the product of Beijing Pharmaceutical Factory.

Writhing inhibition test

Seventy male mice were randomly divided into 6 groups. The mice were pretreatment with LZ 0, 3.4, 4.9, 7, 10 g/kg (0.5% CMC as Vehicle) and aminopyrine 0.3 g/kg po. respectively. 10 min later, the mice were treated with 0.7% acetic acid (prepared with NS) 10 ml/kg ip. to observe the writhing response[3]. The writhing times were counted in 15 min after 5 min injection of acetic acid. The writhing inhibition rate (1%) was calculated as following:

Writhing inhibition rate (%)

$$= \left[1 - \frac{\text{writhing times of treated group}}{\text{writhing times of control group}} \right] \times 100\%$$

Hot plate test

The analgesic activity was accessed by a type Y SD-4 hot plate apparatus with water bath of 55 ± 0.2°C. The first signs of discomfort shown by a mouse on the hot plate are that it stands up on its hind legs and licks or blow its front paw to cool them. In a few seconds the pain is too heavy to be borne by the back paws, and the mouse either kicks its leg and dances around the restraining cylinder, or attempts to jump out of the cylinder. As normal mice with often standing up and grooming their front paws, the licking back paw has been used as the criterion of acute pain (pain threshold)[3]. The standard time of exposure to the pain stimulus has been 30s. It were tested at regular intervals (30 min) after injection, then at intervals of 30 min for at least 1.5 h. The results were expressed as pain threshold increment rate:

Pain threshold increment rate (%)

$$= \left[\frac{\text{pain threshold after treatment}}{\text{pain threshold before treatment}} - 1 \right] \times 100\%$$

For this experiment, forty five female mice were arranged to 3 groups with 0, 5, 7.5 g/kg of LZ po. respectively. The pain threshold were measured before and after treatment of LZ 30, 60, 90 min respectively. Each pain threshold was measured twice, the mean value was taken and the increment rate was calculated.

Photoelectric test

Seventy six mice of either sex were divided into 6 groups and placed in a photoelectric apparatus in order to record their spontaneous motor activities [(3)]. After having drugs 15 min, the spontaneous motor activities in 5 min were measured in groups of mice which have received the following doses of drugs: LZ 0, 2.06, 2.94, 4.2, 6 g/kg and diazepam 10 mg/kg, po. respectively. The ED_{50} of LZ was calculated [(4)].

Righting reflex test

Twenty two mice of either sex were arranged to 2 groups. The mice of the first group were given Pentobarbitol 25 mg/kg ip. The mice of the second group were given LZ 4.2 g/kg po., 20 min later, each mouse was additionally received Pentobarbitol 25 mg/kg ip. Compared the difference of the righting reflex between 2 groups for 20 min after injection with Pentobarbitol [(5)].

Swimming test

Eighty male mice were divided into 6 groups with LZ in the doses of 0, 0.15, 1.5, 3.5, 5, 7.5 g/kg/day x 7 po., respectively. 1 h after the last drug given, the mice with a tail load (10% body weight) were placed in bath (deep 30 cm, 27°C) for swimming until the mice subsided. Recorded the swimming time, i.e., from swimming to subsiding (maintain 10 s) of mice. If the swimming time of mouse was more than 60 min, the test should be stopped [(6)].

Hypoxia test

Forty male mice were randomly divided into 4 groups. The mice were pretreatment with LZ 0, 3.5, 5, 7.5 g/kg, po. bid x 7. 30 min after the last administration of drug, the mice were placed into a tighting cylinder (250 ml) with 10 g of calx natrica. The results were calculated as survival time of mice [(7)].

Results

Analgesic effect of LZ

Tab. 13-1 showed that the writhing response was inhibited by LZ. LZ (10 g/kg) is similar to aminipyrine (0.3 g/kg) in analgesic effect. The ED_{50} of LZ was 4.98 g/kg.

The results by hot plate test was indicated that LZ could significantly increase the pain threshold. It was found that the analgesic effect appeared after LZ given 30 min, and the effect was still maintained until 90 min (Tab. 13-2, Fig. 13-1, 2).

Tab. 13-1 Analgesic effect of LZ on writhing response

	N	Writhing response $\overline{X}$ ± SD	Inhibition (%)
CMC	20	54.5 ± 19.5	
Aminopyrine 0.3 g/kg	10	0.1 ± 0.3	99.8
LZ 10 g/kg	10	4.0 ± 3.6	92.7
LZ 7 g/kg	10	12.3 + 10.9	77.4
LZ 4.9 g/kg	10	30.8 ± 16.7	43.5
LZ 3.4 g/kg	10	41.9 ± 14.4	23.1

Tab. 13-2 Influence of LZ on pain threshold by hot plate test

	n	Pain threshold ($\overline{X}$ ± SD)				Increment of pain threshold (%)		
		0 min	30 min	60 min	90 min	30min	60min	90min
CMC	15	25.9±2.5	26.8±5.4*	26.5±4.6*	28.7±7.3*	3.5	2.3	10.8
LZ 5g/kg	15	23.8±2.7	28.3±7.9**	33.1±8.2***	34.0±10.5***	18.9	39.1	42.9
LZ 7.5g/kg	15	22.5±3.3	36.0±11.9**	38.4±10.5***	40.4±10.3***	60.0	70.7	79.6

0 min: before administration; 30, 60 and 90 min: after administration.
*P > 0.05; ** P > 0.05; *** P > 0.01 compared with control (0 min)

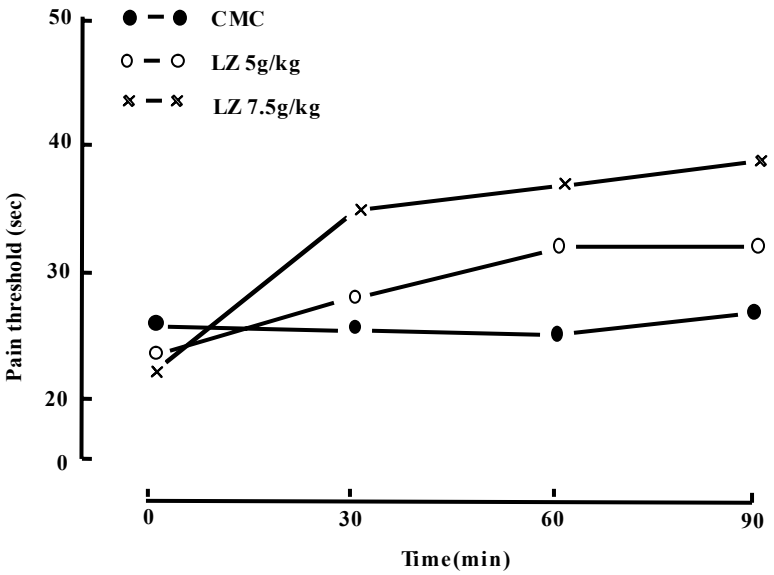

Fig. 13-1 Effect of LZ on pain threshold by hot plate test

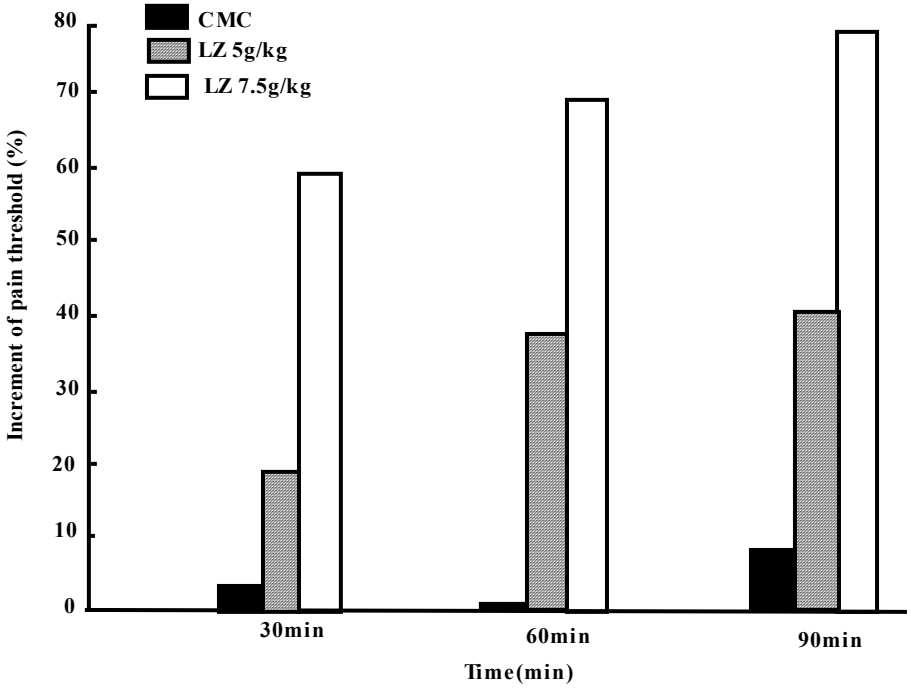

Fig. 13-2 Effect of LZ on increment of pain threshold by hot plate test

Sedative effect of LZ

Tab. 13-3 demonstrated that LZ significantly decreased the spontaneous motor activities in mice. The ED_{50} of LZ was 2.65 g/kg.

It was obvious that LZ could cooperate the effect of pentobarbital. The righting reflex in the group of Pentobarbitol was normal, but it disappeared in the pentobarbitol +LZ group. The disappearing time was 8.5 ± 2.1 min ($\overline{X}$ ± SD).

Tab. 13-3 Effect of LZ on spontaneous motor activities in mice

Group	Animals (n)	Spontaneous motor activities ($\overline{X}$ ± SD)	Inhibition (%)
CMC	10	57.1 ± 16.5	
LZ 2.058 g/kg	14	39.0 ± 22.2	32
LZ 2.94 g/kg	13	20.9 ± 12.8	63
LZ 4.2 g/kg	10	15.2 ± 14.8	74
LZ 6.0 g/kg	17	14.1 ± 10.2	75
Diazepam 10 mg/kg	12	2.2 ± 3.6	95

Promoting tolerance activity of LZ

Tab. 13-4 showed that the swimming time of mice could be prolonged by LZ (3.5 g/kg), but decreased by LZ (5, 7.5 g/kg). It was found that the swimming time of mice on hypoxia test could be prolonged in the group treated with LZ (5, 7.5 g/kg) (Tab. 13-5).

Tab. 13-4 Influence of LZ on swimming time in mice

Group	Animals (n)	Swimming time (min) ($\overline{X}$ ± SD)	P value
CMC	15	26.0 ± 21.7	
LZ 0.15 g/kg	12	12.7 ± 15.0	> 0.05
LZ 1.5 g/kg	12	24.7 ± 26.3	> 0.05
LZ 3.5 g/kg	15	43.1 ± 22.2	< 0.05
LZ 5.0 g/kg	14	5.2 ± 2.1	< 0.01
LZ 7.5 g/kg	12	4.9 ± 1.9	< 0.01

Tab. 13-5 Effect of LZ on hypoxia test

Group	Animals (n)	Survival time (min) ($\bar{X}$ ± SD)	P value
CMC	10	19.65 ± 6.18	> 0.05
LZ 3.5 g/kg	10	22.87 ± 5.50	> 0.05
LZ 5.0 g/kg	14	27.02 ± 4.58	< 0.01
LZ 7.5 g/kg	12	29.47 ± 6.38	< 0.01

Discussion

Most tests for analgesia depend on the application of an external stimulus on an animal and the observation of typical change in behavior by which the animal attempts to escape from the original situation [8]. This study applied the chemical stimulus (acetic acid) and physical stimulus (hot place) to induce pain. It was obvious that LZ could release pain sensation in both tests. The ED_{50} of LZ was 4.98 g/kg (writhing test). However, it is not possible to say that the sensations in the mouse are similar to those experienced by human and the mouse feels pain in exactly the same degree as human does. Results obtained from mice, however, may give some guide to the drug using in human.

The righting reflex in mice can be used to assess whether slept or not. If they are placed on their backs, they normally turn over immediately. If they are given a suitable dose of a drug they can be placed on their backs and the time after the administration of the drug at which they are right themselves is taken as the sleeping time. In this study, it was found that the mice with sedative dose of pentobarbitol (25 mg/kg) could not go to sleep because of their righting reflex was normal. If they additionally received LZ (4.2 g/kg), all of them were asleep with the sleeping time 8.5 ± 2.1 min ($\bar{X}$ ± SD). They were cooperative to combine use. In this way, we may assess the sedative activity of drugs indirectly.

The photoelectric method is widely applied to assess the sedative effect [8]. In this series, the ED_{50} of LZ was 2.65 g/kg.

After administration of LZ, we have observed that the mice can stand hypoxia, and can prolong the swimming time. It is suggested that LZ possesses the promoting tolerance activity. The swimming time was shortened in the larger dose groups of LZ (5, 7.5 g/kg). However, the mechanism of that is unclear. It may be related to the toxicity of LZ.

Effects of Ling Zhi on Stress Ulcer in Mice and Its Antagonism to Acetylcholine

CHENG Zhanghua* WEN Jingyuan* CHEN Binling* Masao MORI**

Abstract The mice were pretreated with Ling Zhi (LZ) 0.4g, 1.0g, 2.0 g/kg, po. 3d respectively. 1 h after last dosage given, mice were kept in restraint plus water immersion stress for 22 h. The results revealed that LZ significantly lowered the mucosal lesion incidence and mucosal hemorrhage induced by stress at doses of 1.0 g/kg and 2.0 g/kg. The contractile responses of acetylcholine on isolated guinea-pig ileum were antagonized by LA. IC_{50} was 8.5×10^{-4} g/ml.

LZ was markedly effective on suppressing the stress ulcer formation. It suggested that LZ might block the peripheral parasympathetic nervous system.

Key words Ling Zhi (LZ, Ganoderma Lucidum, Fr. Karst); Stress ulcer; Anticholinergics.

LZ has depressant effects on the central nervous system. It was reported that LZ reduced spontaneous motor activities and enhanced the effects of Hypnotics in mice [(1)]. It had been used clinically to improve the patients' sleep. In this paper, we observed the effects of LZ on the parasympathetic nervous system.

Materials and Methods

Animals

Kunming (KM) male mice, 22 ± 2g, supplied by the Animal Center, Shanghai Medical University. Guinea-pigs of both sexes, weighing 350 ± 50g, purchased from market.

Reagents

LZ extract was provided from Wakan Shoyaku Botany Institute, Tokyo, Japan. It was diluted by 0.5% CMC and 5% LZ suspension was obtained, oscillated 4 h in 80°C water bath and cooled in 4°C refrigerator for using. Atropine sulphate was the product of Shanghai No. 10 Pharmaceutic Factory. It was diluted by 0.5% CMC and 0.1% atropine solution was prepared and kept at 4°C.

* Dept. of Pharmacology, School of Pharmacy, Shanghai Medical University, Shanghai, China
** Wakan Shoyaku Botany Institute, Tokyo, Japan

Stress ulcer

Fifty male mice were randomly divided into 5 groups, ten mice in each group. The mice were pretreated with LZ 0.4 g, 1.0 g, 2.0 g/kg; atropine sulphate 0.04 g/kg; CMC 0.2 g/kg, po. respectively in the same volume of 0.4 ml / 10 g body weight intragastrically for 3 days. Mice were deprived of food but allowed to take tap water freely for 24 h before stress. 1 h after last dosage given, mice were put into individual close-fitting tubular cages of wire mesh and immersed in a water bath (23°C) to the depth of the xiphoid to be stressed. The mice were killed for autopsy after 22 h stress.

After cardia occluded, the stomach was excised. The gastric contents were squeezed out through pylorus and diluted 10 times with the Hayem's solution for determination of red blood cells. Total number of RBC was counted on the hemocytometer. Then the stomach was inflated with 2 ml of 1% formalin, occluded pylorus and put in 1% formalin for 1 h to fix both inner and outer layers. Stomach was opened along the greater curvature and rinsed with normal saline to remove adherent substance on mucosa. The stomach walls were observed carefully under dissecting microscope and examined for mucosal lesions.

Antagonistic effects on isolated ileum

Guinea-pigs were stunned and the abdomen opened. Isolated ileum 1.5 cm in length was excised, suspended in 20 ml organ bath containing Tyrode's solution bubbled with air at 37°C. The resting tension was 1.0 g. Maximum contractile responses of accetylcholine were recorded by force-displacement transducer. Antagonisms in different dosages of LZ to contraction of acetylcholine were measured.

The data presented were expressed as $\overline{x} \pm$ SD and statistically evaluated by the Student's t test. P value of less than 0.05 was considered significant.

Results

Effects of LZ on stress ulcer

The incidences of ulcer and hemorrhage caused by water-immersing stress were 100%. Mucosal lesions and acute hemorrhagic erosions on the stomach walls were observed. Mucosal lesion with size less than 1 mm in length and hemorrhagic erosion were expressed as the petechial lesions. Mucosal lesion with size more than 1 mm in length was expressed as an ulcer. Three petechial lesions were considered equivalent to an ulcer. Experiments revealed that LZ 1.0 g, 2.0 g/kg, Atropine 0.04 g/kg groups compared with control group markedly decreased ulcer formations and hemorrhage incidences (Tab. 14-1, Tab. 14-2).

Tab. 14-1 Effect of LZ on restraint plus water-immersion stress ulcer

Group	No. of animals	No. of petechial lesions	Decreasing rate (%)	No. of ulcer	Decreasing rate (%)
LZ 0.4g/kg	10	31.4 ± 8.7*	6.5	20.2 ± 5.4*	4.3
LZ 1.0g/kg	10	13.7 ± 7.7***	59.2	8.1 ± 5.8***	61.6
LZ 2.0g/kg	10	13.7 ± 7.1***	59.2	6.8 ± 4.1***	67.8
Atropine 0.4g/kg	10	4.5 ± 3.9***	86.7	1.8 ± 2.0***	91.5
Control CMC 0.2g/kg	10	33.6 ± 13.1	—	21.1 ± 8.8	—

$\overline{X}$ ± SD; *P>0.05, ***P<0.01 and decreasing rate (%), compared with control

Tab. 14-2 Effect of LZ on hemorrhage of stress ulcer

Group	No. of Animals	No. of RBC from mucosal hemorrhage	Decreasing rate (%)
LZ 0.4g/kg	10	357.7 ± 245.8*	20.8
LZ 1.0g/kg	10	66.6 ± 90.1***	85.2
LZ 2.0g/kg	10	32.9 ± 14.5***	92.7
Atropine 0.4g/kg	10	88.1 ± 112.2**	80.5
Control CMC 0.2g/kg	10	451.1 ± 404.9	

$\overline{X}$ ± SD; *P>0.05, **P<0.05, ***P<0.01 and decreasing rate (%), compared with control

Antagonism of LZ to Acetylcholine on isolated guinea-pig ileum preparation

LZ in different dosages could inhibit ileum contractile responses produced by Acetylcholine. Inhibition rate 9%) in each dosage group was the average of five experiments. IC_{50} was the concentration of 50% inhibition in contraction induced by acetylcholine. The results showed that the IC_{50} of LZ was 8.5 x 10^{-4} g/ml. The IC_{50} of Atropine was 4.3 x 10-3 g/ml obtained from the same experimental procedure.

Discussion

The highest incidence of ulcer and lowest mortality of animal were observed when the stress to fix plus water-immersion in mice. Ulcer formation under stress conditions is due to stimulation of the excessive central hypothalamus and parasympathetic nervous system. Especially vagal overactivity plays an important role in stress ulcer formation[2, 3]. The following pathogenetic mechanisms have been reported to account for the stress-induced gastric mucosal lesions: abnormal gastric motility; alteration in gastric secretion; ischaemic changes in the gastric mucosa, muscular contractions and extrinsic compression of intramural vessels, reduction in mucosal resistance to acid-peptic digestion [4]. Atropine is a standard anticholinergic. Itreduced gastric motility and muscular contraction. The increase of acid secretion during water-immersion stress was significantly inhibited by Atropine [5]. In this experiment, Atropine at dose of 0.04 g/kg was markedly effective on suppressing the ulcer formation. LZ at dose of 1.0 g/kg markedly antagonized stress ulcer formation as the same as Atropine. Lin et al reported that LZ has a central depressant effects at dose over 2.0 g/kg [1]. LZ produces the peripheral actions mainly with little effect on the central nervous system at dose of 1.0 g/kg. LZ also showed antagonism to Acetylcholine on isolated ileum preparation. The IC_{50} was 8.5×10^{-4} g/ml.

LZ prevented the stress ulcer formation significantly and antagonized contraction of the smooth muscle induced by Acetylcholine. These results demonstrated that LZ possesses the blocking effects on peripheral parasympathetic nervous system

Effects of Ling Zhi on Isolated Guinea-Pig Trachea

MIAO Yongsheng* ZHANG Luoxiu* JIANG Minghua* Masao MORI**

Abstract The slight relaxation effect of Ling Zhi (LZ, 0.001 ~ 0.1 mg/ml) on isolated guinea-pig trachea was demonstrated in this study. LZ can also antagonize the contraction of the above-mentioned trachea caused by histamine in non-competitive manner. LZ relaxes the contraction of the trachea induced by slow reacting substance anaphylaxis (SRS-A) when the concentration of LZ is higher than 0.001 mg/ml.

Key words Ling Zhi (LZ); Isolated guinea-pig trachea; Relaxation.

It has been well known for more than 2 000 years in clinical medical practice that Ling Zhi (LZ) is effective in the treatment of chronic bronchitis and asthma but its mechanism is not clear so far. This study was designed for clarifying the mechanism, using isolated guinea-pig trachea and taking another Chinese traditional medicine Han Ji Song (Tetrandrini Dimethiodidum) as a positive control.

Materials and Methods

Animals

Guinea-pig, male 250 ~ 300 g, supplied by the Animal Center, Shanghai Medical University.

Reagents

LZ extract was provided by Wakan Shoyaku Botany Institute, Tokyo, Japan. The powder 30 g was treated in 100 ml 80°C distilled water for 2 h in an constant vibrating state. Then it was contrifugalized with 1 600 rpm for 10 min. The upper supernatant was separated and kept at -30°C (Part A). For Part B, after LZ powder was treated in 80°C distilled water for 2 h in constant vibrating state, it was put in a refrigerator at -30°C for 1 h, then thawed at 37°C. This course was conducted for three times. It was centrifugalized with 1 600 rpm for 10 min. The supernatant was taken and kept at -37°C for further use.

SRS-A was retracted form guinea-pig lung[(1)].

* Dept. of Pharmacology, School of Pharmacy, Shanghai Medical University, Shanghai, China
** Wakan Shoyaku Botany Institute, Tokyo, Japan

Han Ji Song (Tetrandrini Dimethiodidum) was provided by Department of Natural Chemistry, School of Pharmacy, Shanghai Medical University.

Isolated trachea prepared

Guinea-pig was sacrificed by head knocked. The trachea was taken and put into Krebs' solution (mmol/L: NaCl 120, KCl 4.5, $MgSO_4$. $7H_2O$ 1.0, KH_2PO_4 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 20, glucose 10.0, pH 7.4 ± 0.5). After the attached connective tissue removed, the trachea was cut into sectors and connected as a chain. The preparation was then put into a bath containing 20 ml Krebs' solution. The solution remained at 37 ± 0.5°C and bubbled with oxygen. One end of the preparation was fixed to a steel needle at the bottom of the bath and the other end was binded to muscle force displacement equipment which connected to recorder. The rest tension was adjusted to 1.5 g and remained unchanging for 2 h. During this period, the Krebs''solution in the bath was replaced every 15 min. After equilibrium, the experiment commenced.

LZ acted on isolated guinea-pig trachea

LZ was added to the bath accumulatively with 5 min interval. The concentration of LZ was from 0.00001 ~ 0.1 mg/ml.

LZ acted on histamine-induced contraction of guinea-pig trachea

Histamine (0.001 ~ 0.1 mmol/ml) was added to the bath accumulatively with 5 min interval. The concentration-response curve of histamine was drawn. The preparation was washed by fresh Krebs' solution for several times and equilibrated for 30 min. LZ in different concentrations (0.001 ~ 0.01, 0.1 mg/ml) or chlorpheniramine (0.00001 mmol/ml) was added to the bath. After the preparation had contacted the drug for 10 min, the concentration-response curves of histamine were made again. The pharmacological parameters pD_2 and pA_2 were calculated with Schild method.

LZ acted on SRS-A-induced contraction of isolated guinea-pig trachea

After equilibrium, the Krebs' solution in bath was replaced and 1 μg/ml Atropine and 0.06 μg/ml Chlorpheniramine were added to the bath to antagonize the possible effect of acetylcholine and histamine. 5 min later, SRS-A 72 unit/ml was added to the bath. When the contraction of the preparation reached to the maximum, LZ (0.001 ~ 0.1 mg/ml) or Han Ji Song (Tetrandrini Dimethiodidum 0.00001 ~ 0.1 mg/ml) were added to bath in an accumulative manner. The SRS-A-induced contraction of the preparation was steady, lasting for 45 min without change.

Results

Effect of LZ on isolated guinea-pig trachea

Both LZ Parts A and B had slight relaxation effects on isolated guinea-pig trachea when their concentrations were higher than 0.0001 mg/ml (Fig. 15-1). The relaxation effect of Part B is stronger than that of Part A.

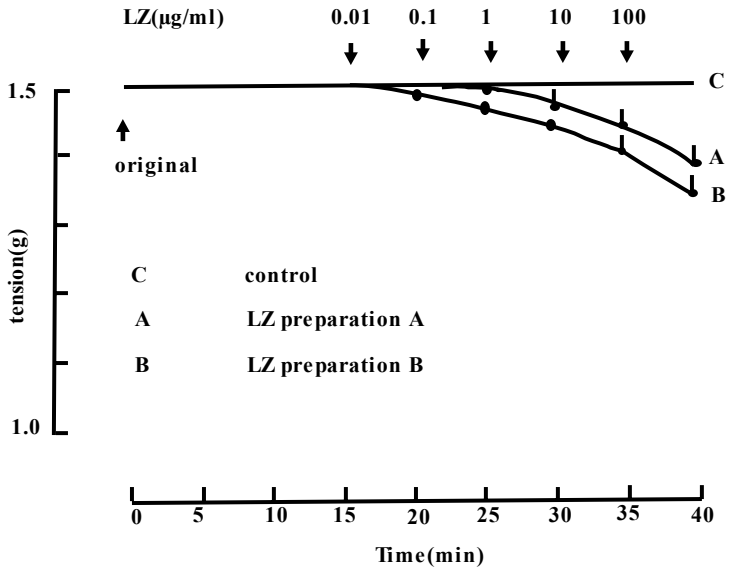

Fig. 15-1 Effect of LZ on isolated guinea-pig trachea (n=4)

Effect of LZ on histamine-induced contraction of guinea-pig trachea

Chlorpheniramine competitively antagonized the histamine-induced contraction of the isolated guinea-pig trachea. The pD_2 value of histamine was 5.9 ± 0.15 and pA_2 value of Chlorpheniramine was 11.21 ± 0.38 closed to the result in references[2]. LZ Part A and B (0.1 mg/ml) both antagonized the contraction of the trachea in a non-competitive manner with the maximum relaxation 37.5% and 29.6% respectively. (Fig. 15-2).

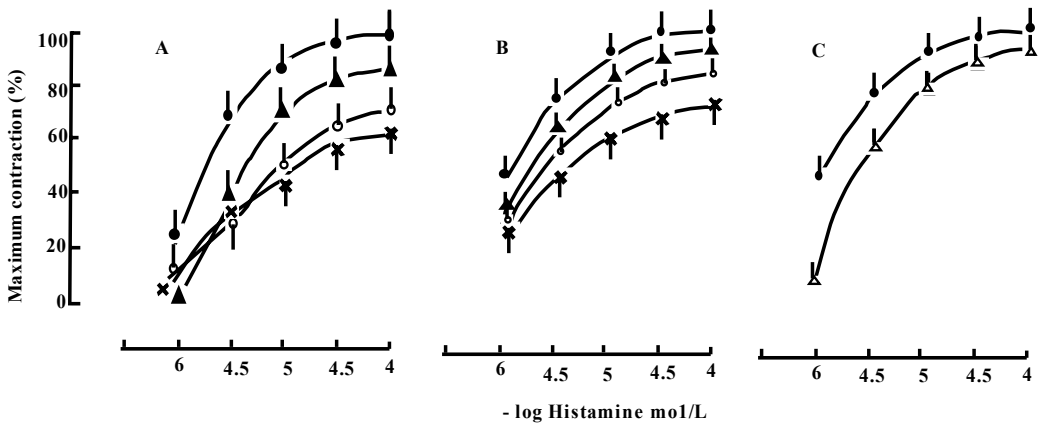

Fig. 15-2 Effect of LZ on the histamine-induced contraction of isolated guinea-pig trachea. The interval between two concentractions is 5 min;
● : Control: ▲: LZ 1 μg/ml; ○ : LZ 10 μg/ml; X: LZ 100 μg/ml; △: Chlorpheniramine 10^{-11}mol/L; A: LZ Part A (n=4); B: LZ Part B (n=6); C: Chlorpheniramine (n=4)

Relaxation effect of LZ on SRS-A-induced contraction of isolated guinea-pig trachea

LZ showed relaxation effect when its concentration was higher than 0.001 mg/ml. LZ Part A (0.1 mg/ml), B(0.1 mg/ml), and Han Ji Song (Tetrandrini Dimethiodidum) (0.1 g/ml) all reduced the maximum contraction of the trachea by 20.4%, 24.2% and 40.5%, respectively (Fig. 15-3).

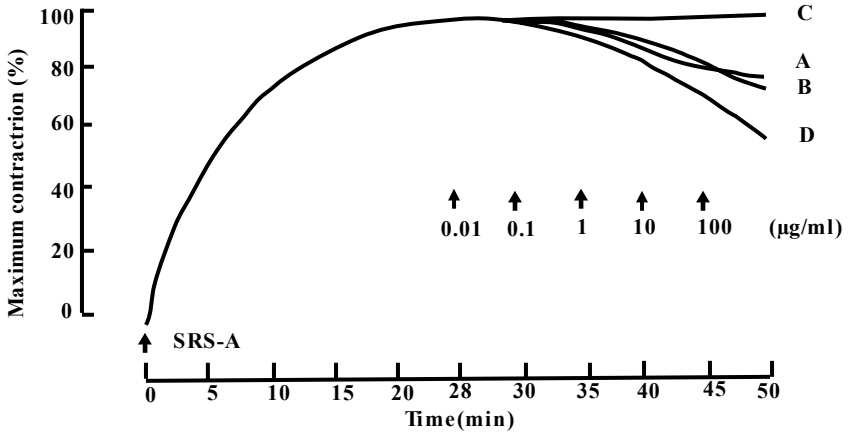

Fig. 15-3 Effect of LZ on SRS-A induced contraction of isolaed guinea-pig trachea (n=4)
A: LZ Part A; B: LZ Part B; C: Control; D: Han Ji Song

Discussion

It is known that bronchial smooth muscle tone is controlled by humoral factors as well as the autonomic nervous system (ANS). The humoral factors, such as histamine, SRS-A, serotonin, prostaglandin and bradykinin may cause bronchoconstriction. It may influence the calibre of the airways in ill situation. In healthy condition the calibre is mainly controlled by the ANS, both parasympathetic and sympathetic. The present study was designed to investigate the model of LZ effect. Even it was carried out on trachea preparation, it is proved to be an easy-doing, valid and reliable experiment used to estimate the effect of a drug on muscle.

This study demonstrated that LZ has relaxation effects on the isolated guinea-pig trachea in normal condition or contacted by histamine or SRS-A. Part A is more effective than Part B in relaxing the trachea contracted by histamine, while preparation B is more effective in relaxing SRS-A induced contraction. The results may be due to the different treatment of LZ, related to the pharmaceutical substances.

LZ shows its antagonism against histamine and SRS-A which are the key factors in asthma. This is likely to be the reason why LZ is effective in the treatment of asthma[3,6]. LZ interfering with other humoral factors or/and ANS remains to be studied.

Effects of Ling Zhi on Superoxide Anion Radicals

JIANG Minghua* MIAO Yongsheng* WANG Zhengyue* HU Tianxi**
CHEN Jiwu** ZHOU Chenming*** GUO Huiju*** Masao MORI****

Abstract The role of Ling Zhi (LZ) in benefit against the damages of the superoxide anion radicals was investigated. It showed that LZ inhibited the xanthine/xanthine oxidase reaction with its dose-dependency in Luminol-dependent chemiluminoscence system (CL system). The LC_{50} of Parts A and B of LZ were 48.5 μg/ml and 30.4 μg/ml, respectively.

Electron spin resonance (ESR) detection was also used to determine the effect of LZ on superoxide anion radicals. It has been indicated that Parts A and B (0.25 g/ml) of LZ can decrease the intensities of the ESR signals about 84% and 86%.

It was suggested that LZ might act as an antioxidant in scavenging the superoxide anion radicals.

Key words Ling Zhi (LZ); Free radicals; Superoxide anion; Luminol-dependent chemiluminoscence; ESR.

It has been reported that LZ were used in the prevention and restoration of senile change, cardiovascular diseases and also possessed anticancer, anti-inflammatory properties[(1)]. In pathological process mentioned above, a common phenomenon occurred owing to free radicals from the metabolism. In this paper, we attempted to elucidate the effects of LZ on superoxide anion radicals (O_2).

ESR detection and CL system were widely used in studying free radicals formed in biological systems. This report describes the application of CL system and ESR detection to investigate the effects of LZ on L_2.

* School of Pharmacy, Shanghai Medical University, Shanghai, China
** East China Normal University, Shanghai, China
*** Shanghai Institute of Organic Chemistry, China Academy of Science, Shanghai, China
**** Wakan Shoyaku Botany Institute, Tokyo, Japan

Materials and Methods

Reagents

Xanthine, luminol were purchased from Sigma Co. Xanthinoxidase was extracted according to the paper[2]. K, O_2 was the product of Alfa Company. Dimethyl sulloxide (DMSO) was purchased from Nan Xin Solvent Factory.

LZ extract was provided by Wakan Shoyaku Botany Institute, Tokyo, Japan. It was diluted by distilled water and prepared 0.25 g/ml in the shaker bath at 80°C for 4 h. The mixture was centrifugalized at 30 000 g for 15 min, the supernatant was taken as Part A. Freezing Part A at -30°C for 1 h, then thawed at 37°C, 3 times, after centrifuge at 30 000 g for 15 min, the supernatant was taken as Part B. Parts A_1 and B_1 was prepared by the Parts A and B with distilled water diluted 20 times.

Equipments

CL measurement were performed in a SHG-1 Luminometer. ESR spectra were recorded at liquid nitrogen 77K on a Varian E 112 X-band spectrometer with a field set a 2 x 1 g, gain of 5.0×10^4, modulation frequency of 100 kHz, and microwave power of 5 mw, time constant of 0.25s.

CL system

The control value was measured with a tube (1.2 x 5 cm) containing 50 μl of xanthinoxidase. The light emission for 10s was record. LC_{50} of LZ was calculated as the paper [3].

ESR detection

For LZ determinations, the following conditions were used: prior to measurement, 400 μl of DMSO were added to a reaction tube containing K, O_2 100 mmol/L, ESR were detected at liquid nitrogen 77K after adding 100 μl of LZ Part A or B. The total volume was 500 μl.

Results

Effect of LZ exhibited on CL system

The result of kinetic light emission has been shown that the max of light emission was occurred at 10s (Fig. 16-1). After adding 5 ~ 100 μg/ml of LZ, the light emission for 10s were in exhibited with dose-dependency (Tab. 16-1, 2). The LC_{50} of Parts A and B were 48.5 μg/ml, respectively.

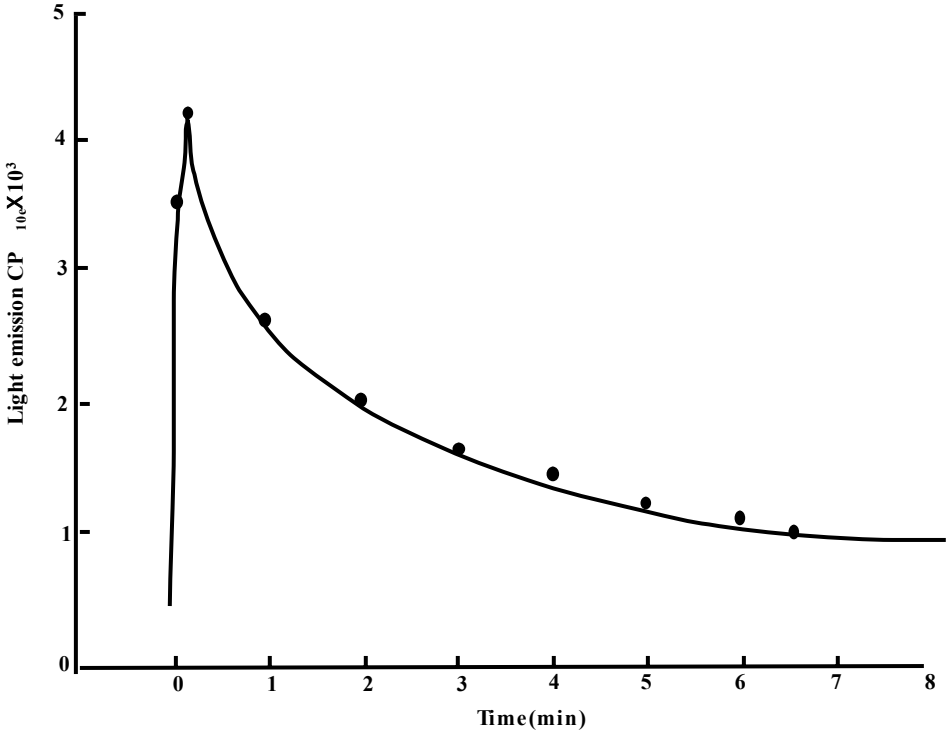

Fig. 16-1 Kinetic curve of light emission

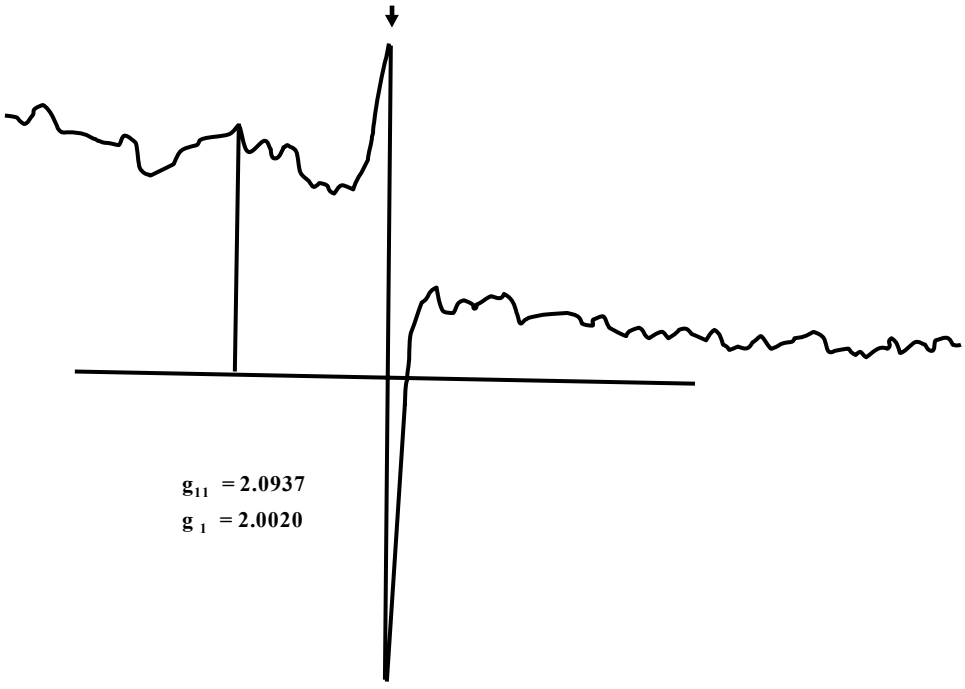

Fig. 16-2 ESR spectra of K,

Tab. 16-1 Dose-depended inhibition of light emission by Preparation A of LZ (n = 3)

LZ concentration (μg/ml)	mean value	Light emission Intensity (%)	Inhibition (%)
0	49 803	100.0	0
100	11 340	22.8	77.2
80	12 067	24.2	75.8
50	24 669	49.5	50.5
40	28 403	57.0	43.0
20	38 528	77.4	22.6

Tab. 16-2 Dose-depended inhibition of light emission by Preparation B of LZ (n = 3)

LZ concentration (μg/ml)	mean value	Light emission Intensity (%)	Inhibition (%)
0	53 114	100.0	0.0
80	9 799	18.4	81.6
40	22 093	41.6	58.4
30	27 230	51.3	48.7
20	29 431	55.4	44.6
10	40 092	75.5	24.5
5	44 078	83.0	17.0

Effect of LZ exhibited on ESR spectra

ESR spectra of O_2 was $g_{11} = 2.0094$, $g_1 = 2.0020$, (Fig, 16-2), and g_1 of Parts A and B were 4 cm and 3.4 cm, g_1 of Parts A_1 and B_1 were 20.5 cm and 11.9 cm, respectively (control = 24.1 cm) (Fig. 16-3 ~ 6).

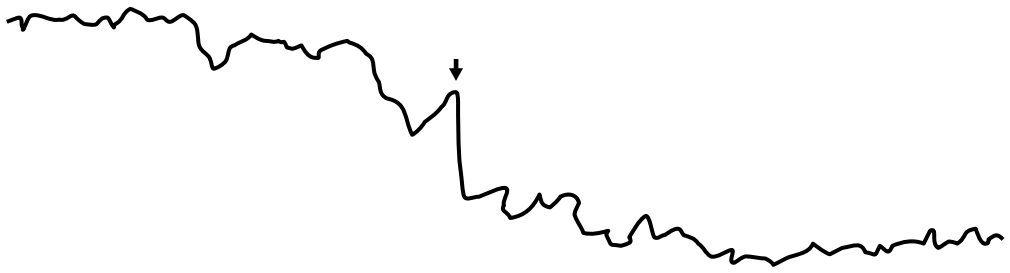

Fig. 16-3 Effect of preparation A of LZ on ESR spectra of K,

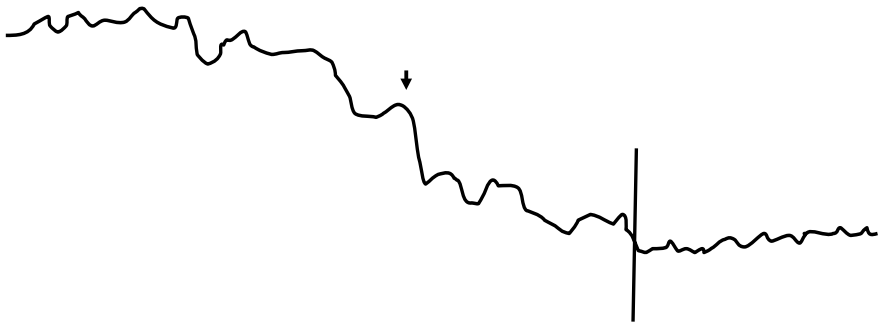

Fig. 16-4 Effect of preparation B of LZ on ESR spectra of K,

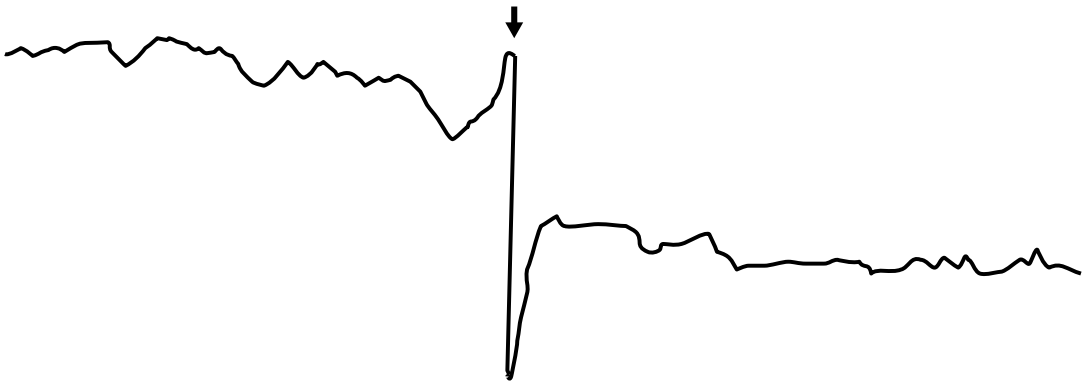

Fig. 16-5 Effect of preparation A_1 of LZ on ESR spectra of K,

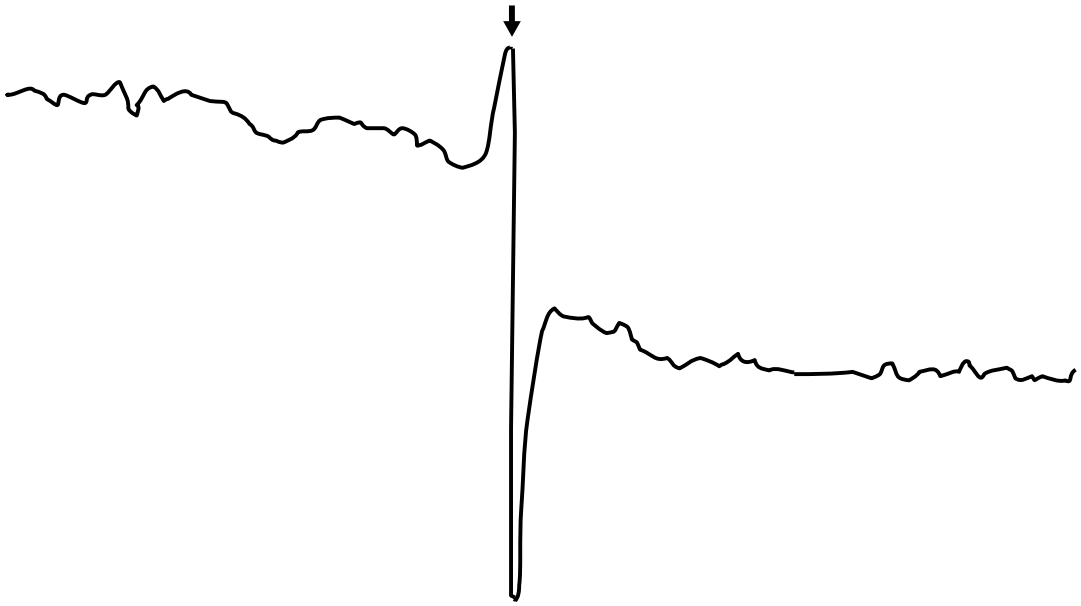

Fig. 16-6 Effect of preparation B_1 of LZ on ESR spectra of K,

It means that the addition of Parts A and B of LZ can decrease the ESR intensities of the O_2 about 84% and 86% respectively.

Discussion

Free radicals are atoms, ions or molecules containing an unpaired electron. Most of oxygen consumed by cells produced water with cytochrome oxidase. However, part of molecular oxygen may change to the superoxide anion radical (O_2) by accepting the single electron in various metabolic pathway[2]:

$$O_2 + e \rightarrow O_2$$

After the formation or O_2, it will undergo secondary reductions to yield hydrogen peroxide (H_2O_2) and the very reactive hydroxyl free radical OH. The oxygen free radicals might damage cellular-constituents, such as to inact enzymes, break DNA, cross-link proteins and peroxidize lipid. Furthermore, it has been postulated that free radicals play a causal role in the aging process [5, 6].

The potentially disastrous consequences of free radical reactions might be expected to protect the cell of plants and animals (i.e. antioxidant defenses). LZ is a well known Chinese traditional medicine herb and available for treatment of some disease related to

free radicals. In order to research the mechanism of LZ, we observe the effect of LZ on superoxide anion radical.

The xanthine/xanthine oxidase (x/xo) reaction, widely used as a superoxide generating system, was employed in the present study. It was demonstrated that LZ could inhibit this reaction in CL system with dose-dependence. The LC_{50} of Preparations A and B were 48.5 μg/ml and 30.4 μg/ml, respectively. However, x/xo reaction may be inhibited by xanthine oxidase inhibitor, it should be carefully considered whether LZ acting as a scavenger of O_2 radicals.

Since ESR technique was employed for direct detection and identification of free radicals, and the sequence of the ESR spectra is essential for successful and correct interpretation of the results. We observed that the effect of LZ exhibited on ESR detection. The results of ESR detection have been indicated that the ESR signal intensities are decreased with LZ. The experiments are related to the various concentrations of LZ shown in Fig. 15-3 ~ 6.

In summary, it suggested that LZ might be used as an antioxidant in possessing the properties for clearance of O_2.