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OESOPHAGOGASTROINTESTINAL DISEASES

A sclerosant with astringent properties developed in China for oesophageal varices: Comparison with ethanolamine oleate and polidocanol

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Abstract

Background: Xiao zhi ling (XZL), which consists of Chinese nutgalls and aluminium potassium sulphate, is used as a local injection for the treatment of internal haemorrhoids in China. It is also used for endoscopic sclerotherapy of oesophageal varices. To date, however, it has not been compared with other sclerosants with regard to its safety and efficacy.

Methods: In the present study, the effect on the endothelium and the haemolytic and thrombosing effects of XZL were compared with those of 5% ethanolamine oleate and 1% polidocanol, using an endothelial cell line and red blood cells taken from rats and the dorsal marginal ear vein of rabbits. In addition, XZL was injected into the area surrounding varices in rats and its efficacy was studied endoscopically and histologically.

Results: Xiao zhi ling reduced the size of varices in rats after causing severe damage in the injected region. Compared with the other two sclerosants, however, XZL had little effect on the endothelium and was the least haemolytic compound. Furthermore, XZL did not cause thrombosis in the injected vein of a rabbit.

Conclusions: These results suggest that XZL is another type of sclerosant with astringent rather than detergent properties. This compound should be used to treat oesophageal varices by paravariceal injection in smaller doses than 5% ethanolamine oleate and 1% polidocanol.

Key words: oesophageal varices, sclerosing solutions, xiao zhi ling.

INTRODUCTION

Endoscopic sclerotherapy is currently accepted as one approach to the treatment of oesophageal varices. The sclerosants used, however, vary from one country to another. In Europe and Japan, 5% ethanolamine oleate and 1% polidocanol are commonly used as sclerosants, while sodium morrhuate and sodium tetradecyl sulphate are generally employed in the USA. Xiao zhi ling (XZL) was developed in China where it is used as a local injection for the treatment of internal haemorrhoids. Its active constituents are Chinese nutgall and aluminium potassium sulphate. This compound is injected into the submucosal layer around internal haemorrhoids, causing them to disappear. It is also used for endoscopic sclerotherapy of oesophageal varices in China.

Characterization of sclerosants is important to establish their proper use and so that effective, safe treatments can be developed. Ethanolamine oleate and polidocanol have characteristics similar to those of detergents and induce intravariceal thrombus by damaging the endothelium. In a previous study, we developed an oesophageal endoscopy system for performing sclerotherapy in rats[1] and compared the efficacy of 5% ethanolamine oleate and 1% polidocanol in this system.[2] Endoscopy is the most suitable method for diagnosis of oesophageal varices, because the oesophagus can be observed directly and repeatedly in living animals. In the present study, the effect of three scle-

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rosants (XZL, 5% ethanolamine oleate and 1% polidocanol) on the endothelium and their haemolytic and thrombosing potential were compared using an endothelial cell line and red blood cells taken from rats and the dorsal marginal ear vein of rabbits. In addition, XZL was injected into the area around varices in rats and its efficacy was studied both endoscopically and histologically and compared with that of 5% ethanolamine oleate and 1% polidocanol in the previous study.[2]

METHODS

Chemical analysis of xiao zhi ling

Xiao zhi ling was found to contain tannic acid, according to *The Pharmacopoeia of Japan* issued by the Japanese government in 1986. In brief, ferric chloride solution was added to XZL to confirm the production of a blue-black precipitate. Albumin solution was also added to XZL to precipitate albumin tannate.

Aluminium and potassium were quantified by atomic absorption spectrophotometry. Sulphate was precipitated with a 12% barium chloride solution as barium sulphate after decomposition of the organic matter in XZL with nitric acid and perchloric acid. The precipitate was then weighed.

Glycerin was identified according to the procedures for identifying iodine glycerin as listed in *The Pharmacopoeia of Japan*. Xiao zhi ling was dried at 105°C for 2 h after evaporation in a water-bath. The residual substance was weighed. The residue was dissolved in ethanol and the insoluble aluminium potassium sulphate was removed by filtration. Tannic acid was precipitated with an albumin solution as albumin tannate and was also removed by filtration. The filtrate was dried under reduced pressure and then weighed. Sodium hydroxide solution and cupric chloride in ethanol were added to the dried viscous filtrate to confirm the presence of glycerin by a colour change to blue.

Cytotoxicity assay

An adherent endothelial cell line (CPAE, American Type Culture Collection CCL 209), derived from the main-stem pulmonary artery of a young female cow, was used to provide the target cells for the cytotoxicity assay. The cell line had been passaged less than 30 times before use in this assay. The living cells were quantified using the tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide).[3]

The CPAE cells were first grown to confluency in 24-well culture trays. The cells were then incubated for 1 min at room temperature with the test solutions (0.4 mL/well), which were prepared by diluting the sclerosants with either saline or serum from rabbits. Two wells were treated with a test solution. After treatment, the CPAE cells were washed gently three times in Eagle's minimum essential medium (MEM) containing 1% foetal calf serum (FCS). Medium containing 10% FCS (0.4 mL/well) and 5 mg/mL MTT dye solution (40 µL/well; Sigma Chemicals, St Louis, MO, USA) was then added to the wells and the CPAE cells were incubated for 2 h at 37°C in an atmosphere of air with 5% CO_2. After incubation, the medium and dye were removed and dimethyl sulphoxide (2 mL/well) was added to the wells to solubilize the reduced MTT-formazan produced by the living cells. The absorbance of each well was measured at 550 nm and cytotoxicity was calculated using the following formula:

$$\% \text{ cytotoxicity} = (1 - (\text{the absorbance of wells treated with test solution/the absorbance of wells treated with serum or saline})) \times 100$$

Haemolysis assay

Red blood cells from the venous blood of rats which had been treated with citrate were suspended in saline (1:10, vol/vol). Various concentrations of the sclerosants, diluted in saline, were then added to equal volumes of the red blood cell suspension and the mixture incubated at 37°C for 30 min. Intact red blood cells were removed by centrifugation and the absorbance of the supernatant was measured at 540 nm. Haemolysis was expressed as a percentage of that caused by distilled water alone.[4]

Thrombosis assay

The three sclerosants were injected at a dose of 0.1 mL/kg bodyweight, into the dorsal marginal ear vein of three Japanese white rabbits (Kiwa Experimental Animal Lab. Co. Ltd, Wakayama, Japan) weighing between 3.1 and 3.5 kg. The injected veins were observed macroscopically after 3, 6 and 24 h.

Endoscopic sclerotherapy in rats

Partial ligation of the portal vein (PVL) was performed to induce oesophageal varices in 9-week-old male Wistar/ST rats (Japan SLC Inc., Shizuoka, Japan) weighing between 280 and 338 g. At 2 weeks after PVL, oesophageal varices protruding into the lumen were induced in 10 of 20 rats. One rat had moderately protruding, rosary-like varices and nine had slightly protruding, linear or tortuous varices. Another 10 rats had venous dilatation. Two weeks after PVL, 50 µL XZL was injected paravariceally into the oesophageal wall at one site, using the oesophageal endoscopic system for endoscopic sclerotherapy of oesophageal varices in rats.[1] The device for the endoscopic sclerotherapy of oesophageal varices in rats consists of a microsyringe with a 14 cm long 30-gauge needle, a thin, rigid endoscope with a diameter of 1.7 mm (Selfoscope SES-1717D; Olympus Optical Co., Ltd, Tokyo, Japan) and a cannula with a guide tube for the needle. Oesophagoscopy was carried out on days 1 and 3 and 1, 2, 3 and 4 weeks after sclerotherapy.

Two to five rats were killed with a bolus of potassium chloride at 3 days and 1, 2, 3 and 4 weeks after sclerotherapy. The inferior vena cava was occluded and the superior vena cava was cut off immediately. The oesophageal veins were perfused via the distal superior mesenteric vein with a warmed mixture of gelatin and barium sulphate. After being cooled with ice water, the stomach and oesophagus were removed in one piece and the paraoesophageal vessels were exfoliated. The stomach and oesophagus were opened along the greater curvature and fixed in 10% buffered formalin. The oesophagus was then embedded in paraffin and stained with haematoxylin and eosin.

RESULTS

Analysis of xiao zhi ling

Xiao zhi ling had an odour very similar to that of *d*-camphor and was paler in colour than a 1% solution of tannic acid. The presence of tannic acid, aluminium potassium sulphate and glycerin in XZL were confirmed. The residual substance left after drying was 15–16% of XZL. Aluminium potassium sulphate and the viscous substance considered to be glycerin made up 4.0–4.5% and 9–10% of XZL, respectively.

Cytotoxicity

When 5% ethanolamine oleate was diluted 32-fold in saline it was found to have a cytotoxicity of 89%. When diluted 100-fold, its cytotoxicity was approximately 50% (Fig. 1). When ethanolamine oleate was diluted with serum, the cytotoxicity weakened when compared with that diluted in saline. A 16-fold dilution of 5% ethanolamine oleate in serum had a cytotoxicity of only approximately 30% (Fig. 2).

Polidocanol (1%) was found to be less cytotoxic than 5% ethanolamine oleate. A 32-fold dilution of 1% polidocanol in saline had a cytotoxicity of only 27% (Fig. 1). When polidocanol was diluted in serum, a reduction in the cytotoxicity was observed; no cytotoxicity was observed when 1% polidocanol was diluted eight-fold in serum (Fig. 2).

Undiluted XZL had a cytotoxicity of more than 90%, whereas a four-fold dilution of XZL in saline or serum had no cytotoxicity (Figs 1,2). It was the least cytotoxic of the three sclerosants.

Haemolysis

When 5% ethanolamine oleate was diluted 500-fold, it caused approximately 50% haemolysis, whereas the same amount of haemolysis was obtained with 1% polidocanol diluted 80–100-fold. Hence, the haemolytic effect of 1% polidocanol was also weaker than that of 5% ethanolamine oleate (Fig. 3).

When XZL was diluted 10-fold, 25% haemolysis was observed. At higher concentrations of XZL, however, the amount of haemolysis decreased (Fig. 3) and precipitation was observed after incubation. Aggregated red blood cells were seen on microscopic examination of the precipitate after incubation with XZL.

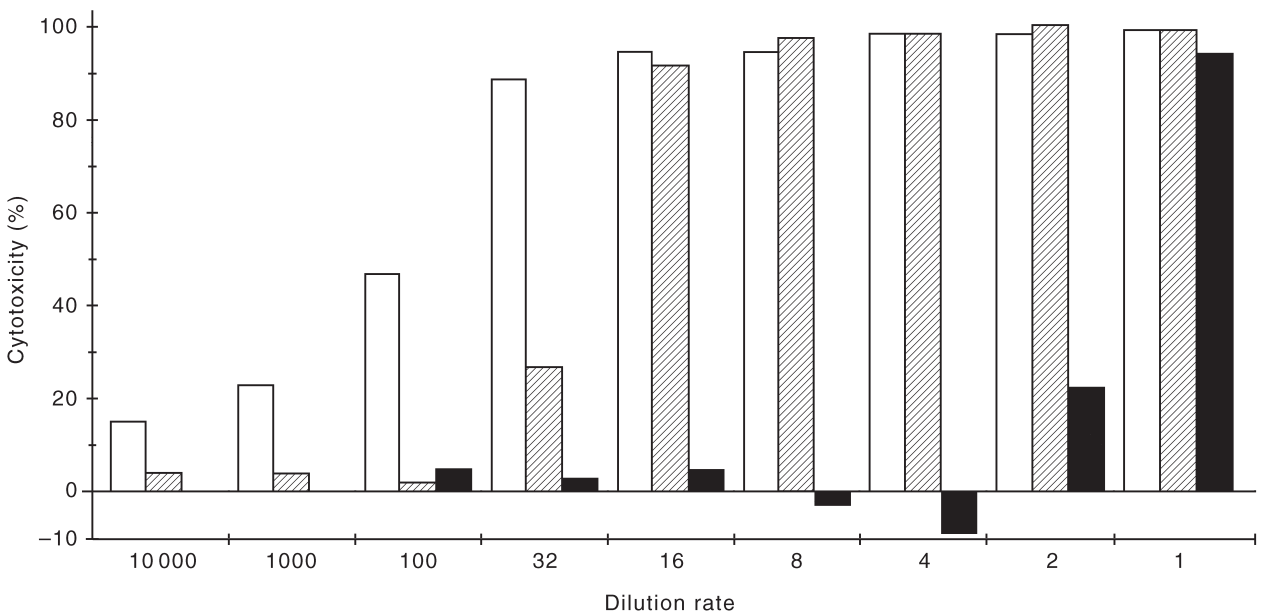

Figure 1 Effect on the endothelium of (□) 5% ethanolamine oleate, (▨) 1% polidocanol and (■) xiao zhi ling diluted in saline. Adherent endothelial cell line (CPAE) cells were incubated for 1 min at room temperature with sclerosants diluted in saline. The percentage cytotoxicity was calculated by determining the number of living cells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye. Each column indicates the average of two tests.

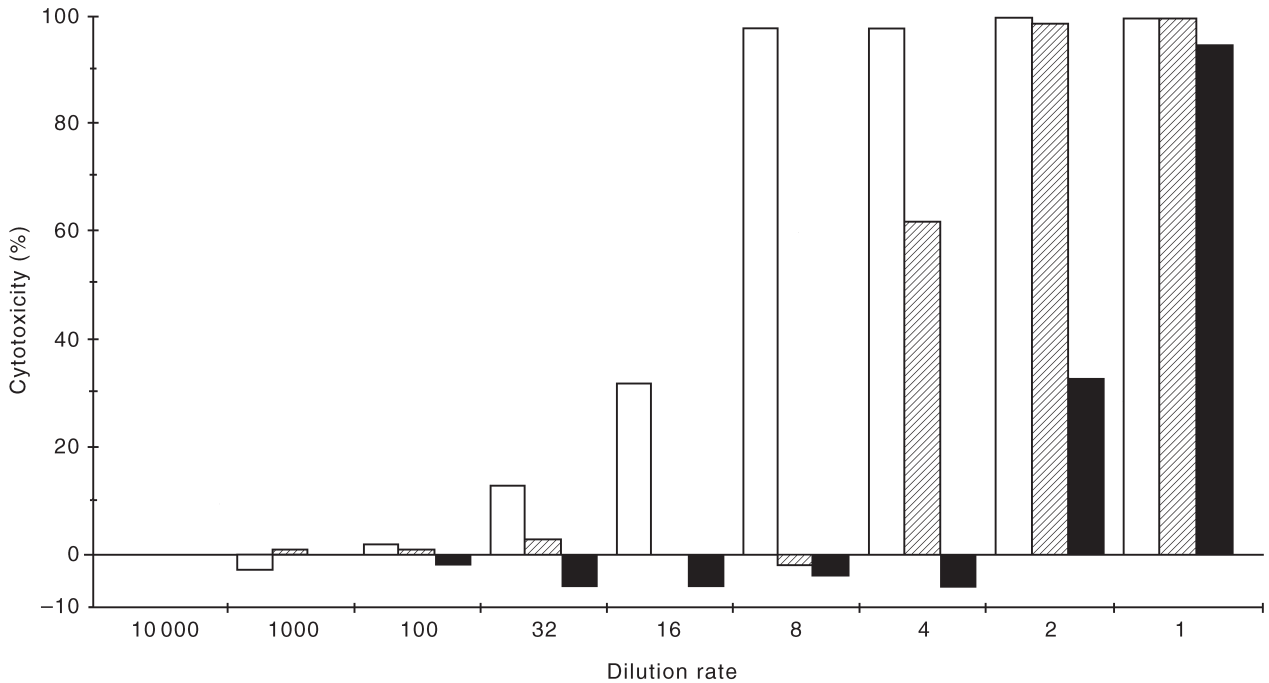

Figure 2 Effect on the endothelium of (□) 5% ethanolamine oleate, (▨) 1% polidocanol and (■) xiao zhi ling diluted in serum. Adherent endothelial cell line (CPAE) cells were incubated for 1 min at room temperature with sclerosants diluted in serum. The percentage cytotoxicity was calculated by determining the number of living cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye. Each column indicates the average of two tests.

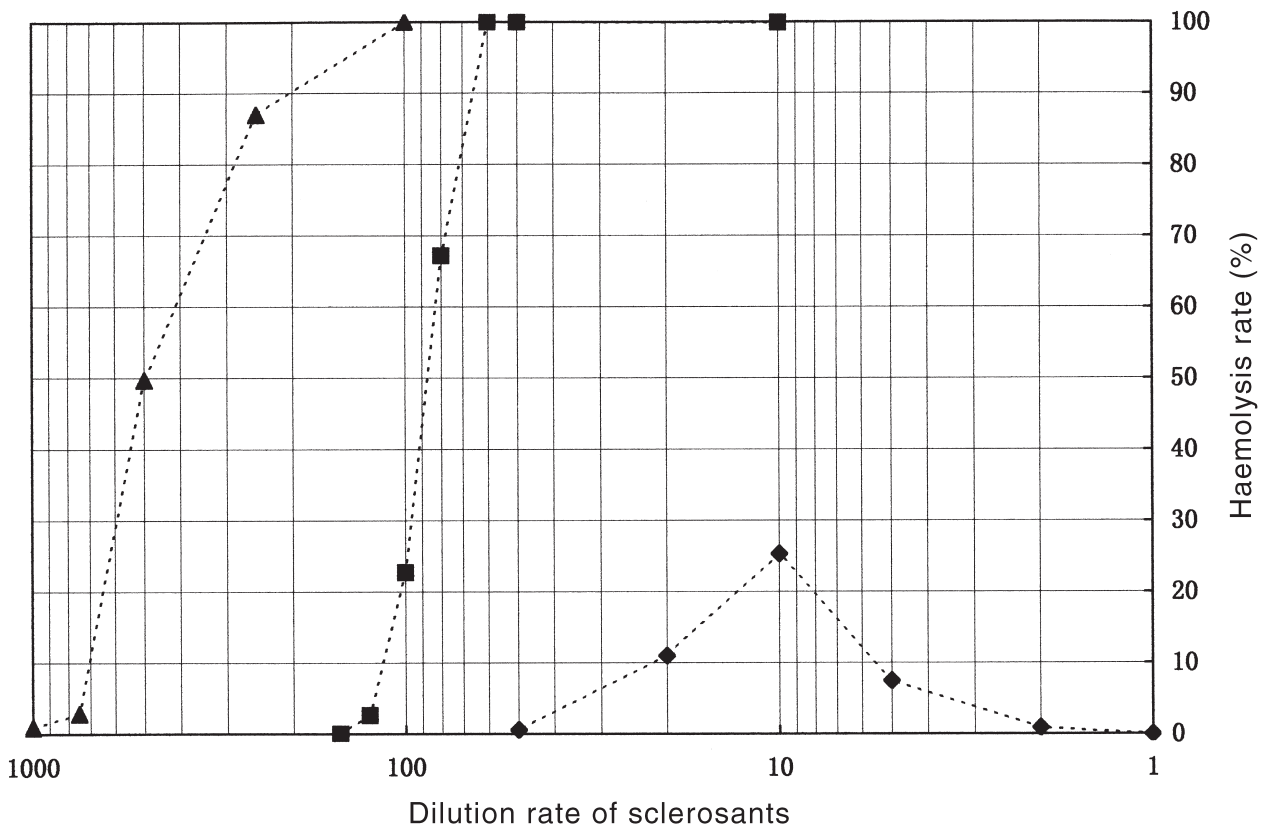

Figure 3 Haemolytic effect of (▲) 5% ethanolamine oleate, (■) 1% polidocanol and (◆) xiao zhi ling. Sclerosants were diluted in saline and an equal volume added to suspensions of red blood cells. The absorbance of the supernatant was measured at 540 nm. Haemolysis was expressed as a percentage of that caused by distilled water.

Thrombosing effect

Ethanolamine oleate (5%) caused thrombus formation in the injected vein and oedema around it 3 h after injection. After 24 h, thrombus and local oedema with slight redness were observed.

Polidocanol (1%) induced only a slight oedema 3 h after injection. After 6 h, the oedema caused by 1% polidocanol was more severe than that induced by 5% ethanolamine oleate and was accompanied by the production of heat. After 24 h, oedema with redness was observed, but a thrombus did not appear to be present.

With XZL, neither thrombus nor oedema were observed except for very mild oedema 6 h after the injection.

Sclerotherapy with xiao zhi ling for oesophageal varices in rats

Twenty rats with PVL were treated by sclerotherapy with XZL. Three rats died during oesophagoscopy 1 day and 1 and 4 weeks after sclerotherapy. Table 1 summarizes the endoscopic findings in the oesophagus after sclerotherapy. Ulceration at the site of injection of XZL was observed after 3 days in all of the 19 rats examined. After 3–4 weeks, the ulcer had healed and the varices in the injected region were reduced in size (Fig. 4).

Figure 5 shows the typical histological appearance of the oesophagus. Loss of epithelium and cellular infiltration were observed 3 days after sclerotherapy with XZL. After 1 week, hypertrophy of the mucosa around the ulcer was observed, with some cellular infiltration. After 3 weeks, extensive fibrosis of the submucosa was seen and this was also apparent 4 weeks after sclerotherapy. Mediastinal adhesion was observed macroscopically in 11 of 16 rats dissected between 1 and 4 weeks after sclerotherapy.

DISCUSSION

Endoscopic variceal ligation (EVL) was developed by Stiegmann *et al*. in 1986 for the endoscopic treatment of oesophageal varices[5] and has become a widely accepted approach because it is easier to perform and is safer than sclerotherapy. In Japan, however, a multicentre questionnaire conducted by Hagiwara *et al*. in 1994 showed that more than 90% of institutions performing EVL for oesophageal varices chose combined therapy with sclerotherapy, because satisfactory eradication could not be achieved with EVL alone.[6] Sclerotherapy, therefore, plays an important part in the endoscopic treatment of oesophageal varices. We set out to look for a safer and more effective sclerosant for endoscopic sclerotherapy than those currently in use.

The two sclerosants used most commonly in Japan are 5% ethanolamine oleate and 1% polidocanol. Ethanolamine oleate is an anionic detergent and is used mainly for intravariceal injection. When it is injected intravariceally, ethanolamine oleate damages the endothelium by its detergent action and induces the formation of intravariceal thrombi. Haemolysis also occurs and this may induce acute renal failure.[7] When 5% ethanolamine oleate is injected paravariceally, severe tissue damage occurs.

Polidocanol is a non-ionic detergent and 1% polidocanol has less effect on the endothelium than 5% ethanolamine oleate. When it is injected paravariceally, polidocanol causes inflammation and fibrosis of the tissue surrounding the varices, resulting in their eradication.

Xiao zhi ling is a sclerosant used for the treatment of oesophageal varices and haemangioma, as well as internal haemorrhoids, in China.[8] The active principles of XZL are Chinese nutgall and aluminium potassium sulphate. Chinese nutgall contains 65–75% tannic acid. The presence of tannic acid, aluminium, potassium and sulphate in XZL was confirmed in this study. Tannic acid and aluminium potassium sulphate have astringent properties which result in precipitation of protein and contraction of tissue. To our knowledge, XZL has not been compared with other sclerosants with regard to its safety and efficacy. In the present study, the effects of XZL, 5% ethanolamine oleate and 1% polidocanol on the endothelium and their haemolytic and thrombosing effects were compared.

Ethanolamine oleate (5%) had a greater effect on the endothelium than 1% polidocanol as shown in the cyto-

Table 1 Endoscopic findings after sclerotherapy of oesophageal varices with xiao zhi ling

	Time after sclerotherapy				
	1 day	3 days	1 week	2 weeks	3–4 weeks
n	20	19	16	10	6
Ulcer/erosion (%)	80	100	81	20	0
Bronzed varices* (%)	60	63	25	20	0
Scar contracture (%)	0	0	6	0	17
Stricture(%)	0	0	0	0	0
Varices					
In the injected region	–		<		<
On the opposite side	<		=		=

–, No; <, less; =, the same varices than before sclerotherapy. *n*, Number of animals studied endoscopically. *Bronzed varices are blackish varices suggesting intravariceal thrombus formation.

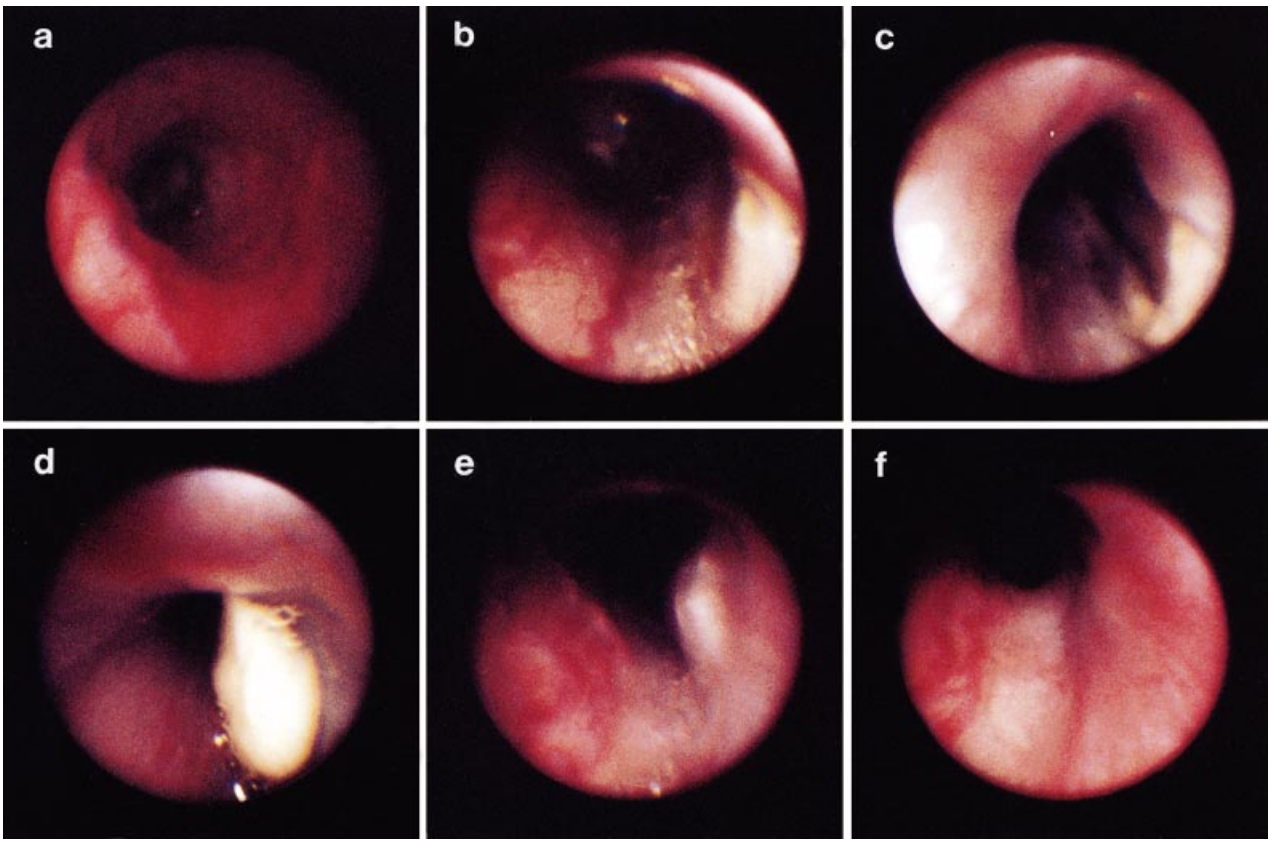

Figure 4 Endoscopic appearance of the oesophagus after sclerotherapy with xiao zhi ling (XZL). (a) Before sclerotherapy (pre), some trunks of slightly protruding, linear or tortuous varices were observed at 3–5 and 6–9 o'clock. (b) One day, (c) 3 days, (d) 1 week and (e) 2 weeks after sclerotherapy. (f) Three weeks after sclerotherapy with XZL, the ulcers had healed and the varices in the injected region had reduced in size.

Table 2 Endoscopic findings after sclerotherapy of oesophageal varices with 5% ethanolamine oleate and 1% polidocanol

	Time after sclerotherapy									
	Ethanolamine oleate					Polidocanol				
	1 day	3 days	1 week	2 weeks	3–4 weeks	1 day	3 days	1 week	2 weeks	3–4 weeks
n	12	10	8	4	4	12	6	8	4	4
Ulcer/erosion (%)	83	40	25	0	0	67	17	0	0	0
Bronzed varices* (%)	17	20	0	0	0	33	33	50	0	0
Scar contracture (%)	0	0	6	25	50	0	0	13	0	0
Stricture (%)	0	0	0	0	0	0	0	0	0	0
Varices										
In the injected region	–		<		<	–		<		=
On the opposite side	<		=		>	=		=		=

–, No; <, less; =, the same; >, greater varices than before sclerotherapy. *n*, Number of animals studied endoscopically. *Bronzed varices are blackish varices suggesting intravariceal thrombus formation. Source: Nishida *et al*.[2]

toxicity assay, and caused thrombosis in the dorsal marginal ear vein of a rabbit. The cytotoxicity of both ethanolamine oleate and polidocanol was weaker when these two compounds were diluted in serum than when they were diluted in saline. The results from the cytotoxicity assay are consistent with the results from the [^{51}Cr]-releasing assay used by Orikasa.[9] Serum proteins contribute to the reduction in the cytotoxicity of ethanolamine oleate and polidocanol.[10] Xiao zhi ling had the least effect on the endothelium and did not cause thrombosis in the dorsal marginal ear vein of a rabbit. A reduction in the cytotoxicity of XZL with

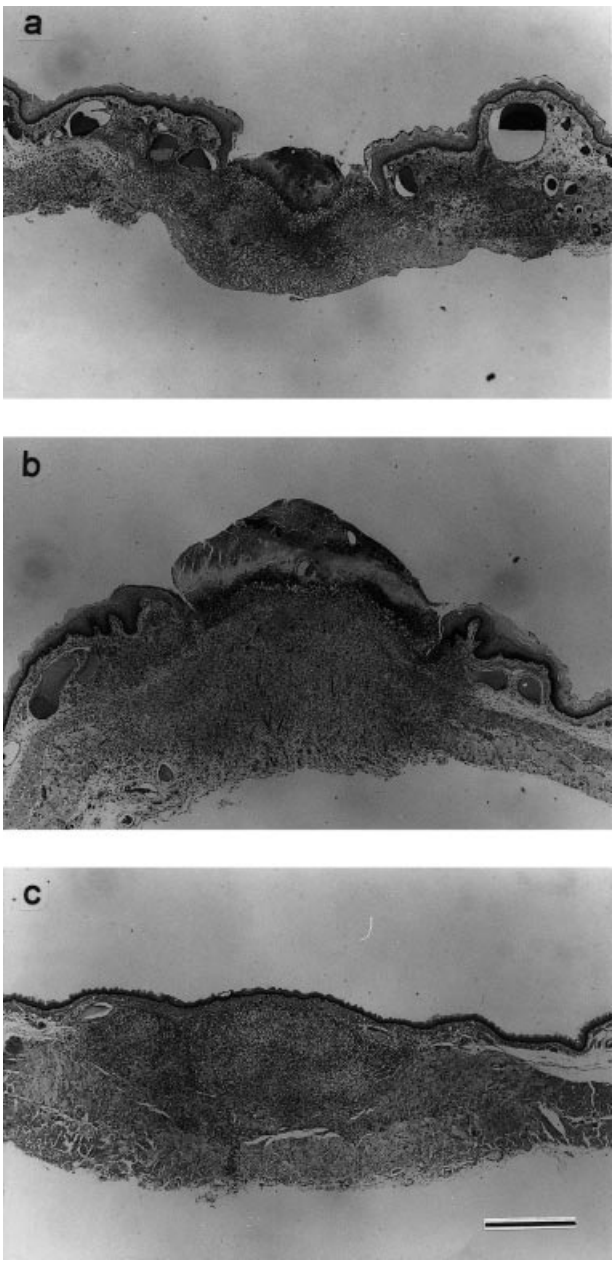

Figure 5 Histological appearance of the oesophageal wall after sclerotherapy with xiao zhi ling (XZL). The oesophageal wall was examined histologically (a) 3 days, (b) 1 week and (c) 3 weeks after sclerotherapy with XZL. Ulceration with cellular infiltration was seen after 3 days and 1 week and extensive fibrosis of the submucosa was observed after 3 weeks. Bar 0.5 mm, HE.

serum was not observed. The results from the thrombosis assay with XZL suggest that this compound is not suitable for intravariceal injection.

Five per cent ethanolamine oleate was a more potent haemolytic agent than 1% polidocanol, whereas XZL had the weakest activity in this assay. After incubation with high concentrations of XZL, the amount of haemolysis was reduced and aggregated red blood cells were observed. Aggregation of the red blood cells may be due to the astringent action of XZL. The absence of haemolysis with XZL is an advantage as acute renal failure caused by haemolysis can be avoided.

In a previous study,[2] we developed and used an oesophageal endoscopic system for sclerotherapy in rats to compare the efficacy of 5% ethanolamine oleate and 1% polidocanol. Ulcers were observed in 10 of 12 rats (83%) 1 day after paravariceal injection of 5% ethanolamine oleate and remained in two of eight rats (25%) 1 week after injection. The ulcers caused by ethanolamine oleate became scarred after 2 weeks. Polidocanol (1%) induced ulcers in eight of 12 rats (67%) after 1 day and scarring was seen after 1 week. Eradication of varices was achieved in the scarred regions in both 5% ethanolamine oleate- and 1% polidocanol-treated animals (Table 2). The results in the previous study[2] were consistent with the known properties of these agents.

In the present study, the same method was used to evaluate the efficacy of XZL. Ulceration was observed 3 days after injection of XZL in all 19 rats (100%) examined and the ulcers remained in two of 10 rats (20%) 2 weeks after injection. The healing of the ulcers by XZL appeared to occur later when compared with 5% ethanolamine oleate and 1% polidocanol, although extensive fibrosis of the submucosa was induced and the varices reduced in the injected region. Stricture did not occur, but mediastinal adhesion was found at high frequency. These results suggest that XZL causes more severe damage at the injected site than 5% ethanolamine oleate and 1% polidocanol and that smaller amounts of XZL should be injected paravariceally at each site.

Bronzed varices, suggesting thrombus formation, were observed frequently in rats treated with XZL, although XZL did not induce thrombosis in the injected vein in a rabbit. These results suggest that XZL has no direct action to coagulate, although it can precipitate, blood proteins and that XZL induces thrombosis in varices following severe tissue damage surrounding the varices by paravariceal injection.

In conclusion, XZL is another type of sclerosant that has astringent rather than detergent properties. It is suggested that this compound is suitable for paravariceal injection, although it does not induce haemolysis. Xiao zhi ling should be injected paravariceally in smaller doses than 1% polidocanol and 5% ethanolamine oleate, because it is likely to cause more severe damage at the injection site.

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