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Original Article

Synergistic effect of lidocaine with pingyangmycin for treatment of venous malformation using a mouse spleen model

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Abstract: Aims: To explore whether lidocaine has the synergistic effect with pingyangmycin (PYM) in the venous malformations (VMs) treatment. Methods: The mouse spleen was chosen as a VM model and injected with different concentration of lidocaine or PYM or jointly treated with lidocaine and PYM. After 2, 5, 8 or 14 days, the mouse spleen tissues were acquired for hematoxylin-eosin (HE) staining, transmission electron microscopy (TEM) analysis, TUNEL assay and quantitative RT-PCR analysis to examine the toxicological effects of lidocaine and PYM on splenic vascular endothelial cells. Results: 0.4% of lidocaine mildly promoted the apoptosis of endothelial cells, while 2 mg/ml PYM significantly elevated the apoptotic ratios. However, the combination of 0.2% lidocaine and 0.5 mg/ml PYM notably elevated the apoptotic ratios of splenic cells and severely destroyed the configuration of spleen, compared to those of treatment with 0.5 mg/ml PYM alone. Conclusion: Lidocaine exerts synergistic effects with PYM in promoting the apoptosis of mouse splenic endothelial cells, indicating that lidocaine possibly promotes the therapeutic effects of PYM in VMs treatment via synergistically enhancing the apoptosis of endothelial cells of malformed venous lesions.

Keywords: Venous malformation, lidocaine, pingyangmycin, splenic endothelial cells, apoptosis

Introduction

Venous malformations (VMs) are the most frequent slow-flow vascular malformations and account for approximate two-thirds of vascular malformations. VMs are light-to-dark-blue lesions that can be emptied by compression and present at birth and grow proportionally with the patient [1-3]. VMs can occur at any tissue and organ without sex preponderance. Because of the bleeding, expansion or obstruction of vital structures, VMs can also threaten life.

The treatment of VMs depends on their site and size, and the traditional options were surgical resection, sclerotherapy and laser [2, 4, 5]. Due to the complexity of anatomical structure of some organs such as oral and maxillofacial regions, it is difficult to perform surgical resection and the VMs are impossibly eradicated by surgical excision. Meanwhile, because surgical resection often leads to massive bleeding and scar, it is always unacceptable for patients. Percutaneous sclerotherapy is the first line and gold standard treatment to diminish the volume of VMs [6]. For sclerotherapy of VMs, absolute ethanol is clinically identified as the most effective sclerosing agent until now, however, it also always results in serious local and systemic side effects including tissue necrosis and peripheral nerve injury [7, 8].

In China, pingyangmycin (PYM) sclerotherapy is applied as a routine treatment for VMs and has notably curative effects [9, 10]. PYM, also known as bleomycin A5, was screened out and developed from many components of bleomycin produced by *Streptomycespingyangensisn.* The chemical structure of PYM is similar to bleomycin and has been adopted for chemotherapy

of sqamous cell carcinoma, malignant lymphoma, Hodgkin's disease and lymphamgioma [11]. In addition, mounting evidence demonstrate that PYM is an effective sclerosing agent for sclerotherapy of VMs with little local reactions and few complications [12]. Lidocaine is an amide of local anaesthetics with strong penetrability and diffusivity that can block the sensory nerve, leading to anesthesia [13]. Lidocaine is often used in a combination with PYM for VMs treatment to alleviate the reflective vasospasm caused by the pain due to embolisation with PYM and promote the filling of PYM at the lesions more completely [14]. However, it remains elusive whether lidocaine has synergetic effects with PYM during the processes of VMs treatment.

So far, there are no ideal animal models for VMs studies. The organs and tissues with rich and circuitous veins such as comb, rat saphenous vein and animal spleen were adopted for animal VM models at home and aboard [15-17]. After considering the advantage and disadvantage of each model, the mouse spleen was chosen as the cavernous VM model for studying the PYM treatment in this study. Compared to other models, the mouse spleen possesses the following advantages in this study: the spleen has rich splenic venous sinusoid with long rod-shaped endothelial cells arranged along the longitudinal axis of sinusoids, which are similar to the structures of cavernous VM in particular [18]; the sample is easy to acquired; surgical operation is feasible and the technical requirement level is not high; the bleeding after surgery can be controlled well.

In this study, the mouse spleen was adopted as the cavernous VM model and injected with different concentration of lidocaine or/and PYM, to assess the effects of lidocaine treatment on the vascular endothelial cells. This purpose of this study was to explore whether lidocaine has synergetic effects with PYM during the processes of VMs treatment, trying to provide the theoretical basis for the clinical drug preparation during the therapy of VMs.

Materials and methods

Experimental animals

The mice were obtained from the Experimental Animal Center of Southern Medical University (Guangzhou City, Guangdong Province, China), and all mice were female, 8~9 weeks and 19~21 g. Mice were anesthetized by intraperitoneal injection with 10% chloral hydrate (3 ml/kg) and fixed on the operation platform. The left upper abdomen of mice was disinfected with iodine complex for three times and then cut open with an incision of 1 cm long. Lift up the stomach to expose the spleen. The spleen was fixed gently and 0.3 ml of saline or different concentrations of lidocaine (Shanghai Xudong Haipu Pharmaceutical Co., Ltd., Shanghai, China) or PYM (Tianjin Taihe Pharmaceutical Co. Ltd., Tianjin, China) or the mixture of lidocaine and PYM was injected along the long axis direction of spleen. Then the surgical wound was stitched up and the mice were fed continuously. The day of injection treatment was considered as 0 day. All mice had free access to water and standard rodent chow and were exposed to 14 h light: 10 h darkness photoperiods.

Tissue samples preparation

Different periods (2, 5, 8 or 14 days) after the surgery, mice were anesthetized by intraperitoneal injection and the spleens were separated. For real-time PCR analysis, a part of spleen tissues were frozen immediately in liquid nitrogen and stored at -80°C, and the other parts of spleen tissues were fixed with 10% neutral formalin or 2.5% glutaraldehyde solution. After the fixation with 10% neutral formalin for 24 h, the spleen tissue samples underwent the dehydration with gradient ethanol, the transparency with dimethylbenzene and the paraffin-embedding with automatic tissue-embedding machine (ZMN-7803, Changzhou Huali Electronics Co. Ltd., Jiangsu, China). Then paraffin-embedded samples were sliced into 2 μm of sections for hematoxylin-eosin (HE) staining or 4 μm of sections for TUNEL staining. For transmission electron microscope (TEM) analysis, the spleen tissue samples underwent the fixation with 2.5% glutaraldehyde solution for 2 h and then with osmic acid, the dehydration with ethanol and acetone, and the paraffin-embedding with epoxy resin (EPON-812, Microscopy Sciences, Inc.). Thereafter, the paraffin-embedded samples were sliced into ultrathin sections and then subjected to uranium-lead staining before observation.

HE staining

For HE staining, sections were deparaffinized and rehydrated, and then stained with hema-

toxylin for 3 min. Thereafter, rinse the samples in tape water and differentiate the sections with 1% HCl in 70% alcohol for 10 s. Wash the slides in running tape water for 15 min. Subsequently, samples were stained with eosin for 1 min before dehydration with gradient ethanol and differentiation with xylene twice. Then mount the slides with resinene before microscopic examination.

TUNEL assay

To analyze the cell apoptosis, TUNEL assay was performed using the In Situ Cell Death Detection Kit, POD (Roche, Switzerland) according to the manufacturer's instructions. Briefly, sections were deparaffinized and rehydrated, and then incubated with 100 μl of protease K at room temperature for 10~30 min. Wash slides 3 × 5 min with phosphate buffered saline (PBS) and then cover sections with TUNEL reaction mixture (50 μl TdT + 450 μl fluorescein-dUTP) for 1 h at 37°C. The negative control sections were incubated with 50 μl fluorescein-dUTP while the positive control sections were incubated with 100 μl DNase 1 for 10 min at 15~25°C first and then incubated with TUNEL reaction mixture (50 μl TdT + 450 μl fluorescein-dUTP) for 1 h at 37°C. Rinse the slides with PBS for three times and then cover sections with 0.3% H_2O_2 for 3~5 min. After 3 × 5 min of rinse with PBS, sections were covered by 50 μl converter-POD for 30 min at 37°C away from light. Rinse the slides with PBS for three times, followed by incubation with 50~100 μl DAB for 10 min at 15~25°C. Thereafter, the sections were lightly counter-stained with hematoxylin and then dehydrated and mounted with resinene before microscopic examination.

RNA extraction and quantitative real-time PCR analysis

Total RNA was isolated from the frozen spleen tissues using TRIzol (Invitrogen) according to the manufacturer's instructions, and then quantified by the absorbance ratio at A260/280. Thereafter, 2 μl of total RNA was reversely transcribed into cDNA using PrimeScriptTM RT reagent Kit (TaKaRa, DRR037A). Then 1 μl cDNA was acquired for real-time PCR amplification with SYBR Premix ExTaqTM Kit (TaKaRa, DRR081A) in a volume of 20 μl mixture containing 10 μl of 2 × SYBR Premix ExTaq™, 0.8 μM of each primer and 1.0 μl of cDNA template. The primers for mouse caspase-3 were forward: 5'-TCATGCACATCCTCACTCGT-3' and reverse: 5'-CGGGATCTGTTTCTTTGCAT-3'. The real-time PCR amplification was performed on the ABI 7500 (ABI, USA) referred to the following procedures: Step 1: 95°C for 5 min; Step 2: 94°C for 30 s; Step 3: 60°C for 30 s; Step 4: 72°C for 90 s, run 30 cycles, and then 72°C for 7 min. The specificity of PCR amplification was confirmed by melt-cure analysis, agarose gel electrophoresis and sequencing of PCR products. The quantification of the mRNA expression level was determined using a standard curve established by a tenfold serial of dilution of plasmid containing the corresponding DNA fragment from 10^2 to 10^6 copies/μl. The mRNA expression level of mouse caspase-3 was presented as the copy number.

Statistical analysis

Data were analyzed by One-Way Analysis of Variance (ANOVA) or Kruskal-Wallis H Rank-Sum test followed by the S-N-K multiple-comparison test using the SPSS16.0 software (SPSS, Inc., Chicago, IL, USA). Data were presented as mean ± standard deviation ($\bar{x} \pm S$). Statistical significance was set at $P < 0.05$.

Ethics statement

We confirmed that all the operations using the experimental animals were performed in accordance with the the authors' institutional ethics committee approval.

Results

High concentration of lidocaine induced apoptosis of mouse splenic vascular endothelial cells

Total 88 mice were randomly divided into 4 groups, 16 mice for saline while 24 mice for 0.1%, 0.2% and 0.4% lidocaine respectively. The mice in each group were randomly divided into 4 subgroups that mice were treated with saline or lidocaine for 2, 5, 8 and 14 days.

After the treatment of saline for 2, 5, 8 and 14 days, the appearances of spleens had no significant changes, manifesting that the capsule was smooth and complete, the edge was tidy without swelling. There was no obvious difference between control group and every experimental group that treated with different concentrations of lidocaine for different periods.

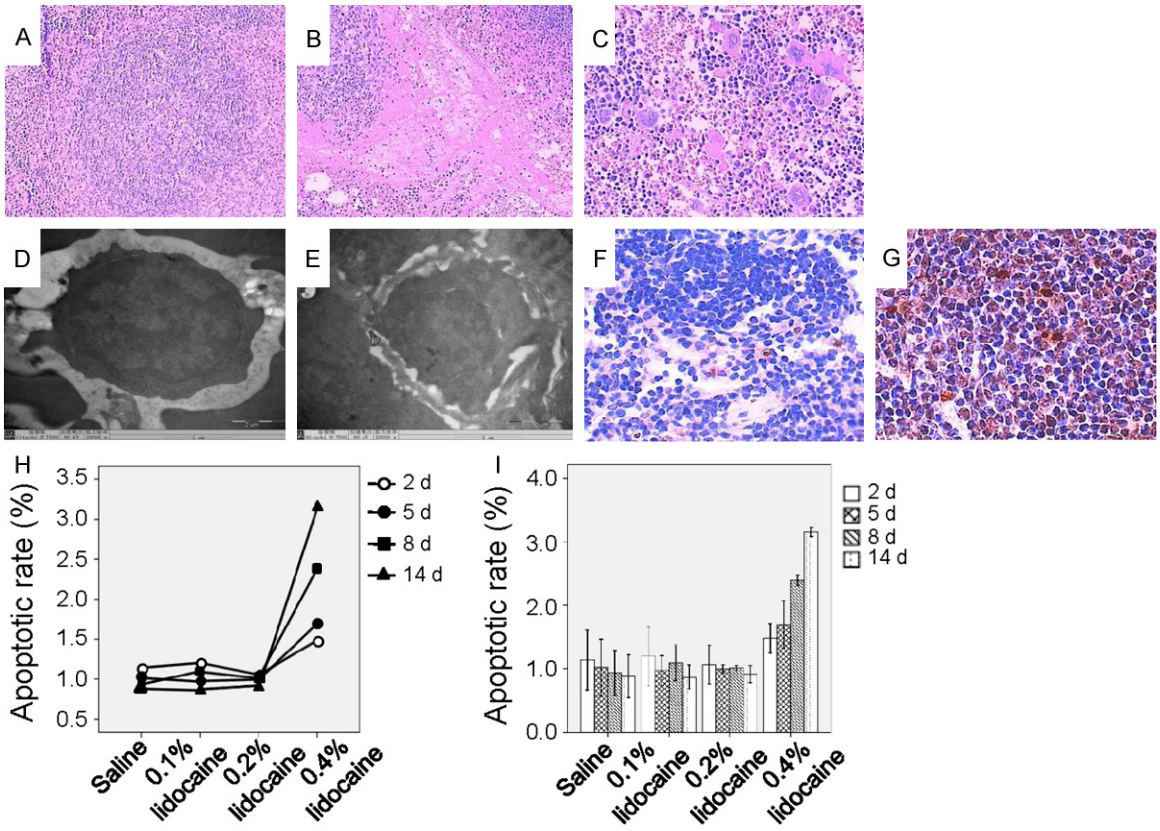

Figure 1. High concentration of lidocaine induced apoptosis of mouse splenic vascular endothelial cells. HE staining of the mice spleen tissues treated with lidocaine (× 100). A. In the saline treatment group, the spleen tissue exhibited normal; B. In the 0.2% lidocaine treatment group, minor hemorrhage of splenic sinus and interstitial fibrin deposition were observed; C. In the 0.4% lidocaine treatment group, minor hemorrhage of splenic sinus and macrophages aggregation were observed. Transmission electron microscope analysis of the mice spleen tissues treated with lidocaine (EM × 20000). D. In the saline treatment group, normal and integrate configuration of splenic sinus was presented; E. In the 0.4% lidocaine treatment group, destroyed blood sinus fascia and apoptotic bodies were observed. TUNEL analysis of the mice spleen tissues treated with lidocaine (× 400). F. In the saline treatment group, the apoptotic splenic vascular endothelial cells were rarely observed; G. In the 0.4% lidocaine treatment group, the apoptotic ratio of splenic vascular endothelial cells increased notably. Statistical analysis of the apoptotic ratio in each group treated with lidocaine. H. The outline of the interactive effects; I. Comparison of the apoptotic ratio in each group presented in histogram.

Under the microscope, the spleens treated with saline for different periods were all normal: the spleen surface was coated with a thin layer of fibrous tissue and the outer layer was a monolayer of mesothelial cell; under the surface, the red and white pulps were clear, and the blood sinus was not dilated while splenic sinus was lined with the integral monolayer of endothelial cells; the blood sinus was filled with appropriate amount of erythrocyte, and there were no inflammatory cells infiltrated in the mesenchyme; the histiocyte had no hyperplasia, and the structure of splenic corpuscle was clear and the germinal center was not dilated (**Figure 1A**).

From the observation of HE staining, 0.1% lidocaine group: no notable difference was observed compared with control group at each time point. 0.2% lidocaine group: in the 2nd day group, the spleen appeared normal; in the 5th day group, the splenic sinus bled occasionally; in the 8th day group, few inflammatory cells such as mononuclear or polynuclear macrophage, occasionally infiltrated into the mesenchyme; in the 14th day group, the infiltrated inflammatory cells were more than those in the 8th day group, and exudation of fibrous protein and polynuclear macrophages that had swallowed cell debris were observed occasionally (**Figure 1B**). 0.4% lidocaine group: in the 2nd day group, the splenic sinus was occasionally dilated and hyperemic, and few inflammatory cells occasionally infiltrated into mesenchyme; in the 5th day group, splenic sinus was dilated and obviously hyperemic while the number of inflam-

Table 1. Comparison of the apoptotic ratios of splenic vascular endothelial cells treated with lidocaine (%, $\bar{x} \pm S$)

Group	N	2 d	5 d	8 d	14 d	Total	F/x^2	P
saline group	16	1.14 ± 0.48	1.02 ± 0.45	0.93 ± 0.35	0.89 ± 0.34	0.99 ± 0.38	0.296*	0.828
0.1% lidocaine	24	1.20 ± 0.46	0.97 ± 0.24	1.10 ± 0.28	0.87 ± 0.19	1.04 ± 0.32	3.687#	0.297
0.2% lidocaine	24	1.06 ± 0.30	1.00 ± 0.07	1.01 ± 0.04	0.92 ± 0.14	1.00 ± 0.17	4.167#	0.244
0.4% lidocaine	24	1.48 ± 0.23	1.69 ± 0.37^{A,B,C}	2.39 ± 0.08^{a,b,A,B,C}	3.15 ± 0.07^{a,b,c,A,B,C}	2.18 ± 0.70	19.747#	0.000
Total		1.23 ± 0.38	1.18 ± 0.42	1.40 ± 0.66	1.51 ± 1.05	1.33 ± 0.68	4.725	0.005
F/x^2		1.386*	11.186#	13.717#	12.753#	103.676	13.682$^{\Delta}$	
P		0.279	0.011	0.003	0.005	0.000		0.000$^{\Delta}$

$^{\Delta}$: interaction *F-value* and *P-value*; *: analysis of variance; #: rank-sum test; ^{a}: vs 2 d, $P < 0.05$; ^{b}: vs 5 d, $P < 0.05$; ^{c}: vs 8 d, $P < 0.05$; ^{A}: vs saline group, $P < 0.05$; ^{B}: vs 0.1% lidocaine, $P < 0.05$; ^{C}: vs 0.2% lidocaine, $P < 0.05$.

matory cells infiltrated into mesenchyme increased; in the 8th day group, interstitial cell infiltration, exudation of fibrous protein and polynuclear macrophages that had swallowed cell debris were observed, and the partial spleen trabecular structure was slightly obscure; in the 14th day group, the interstitial cell infiltration and fibrous protein exudation and the number of polynuclear macrophages increased, and the structures of partial red and white pulps were obscure (**Figure 1C**).

From the transmission electron microscope (TEM) observation, the configurations of spleens were similar in those groups that treated with saline, 0.1% or 0.2% lidocaine for different periods: massive red pulps existed with clear blood sinus and integrated cell junction; a large number of erythrocytes aggregated in the splenic sinus, and lymphocytes distributed uniformly with integrate organelles; no apoptotic bodies were observed (**Figure 1D**). 0.4% lidocaine group: 2 or 5 days after treatment, configurations of spleens did not change significantly; however, the fascia tissues of splenic sinus were damaged and apoptotic bodies were observed at the 8th and 14th day (**Figure 1E**).

The results of TUNEL assay showed that apoptotic cells were rarely visible in the splenic tissues treated with saline, 0.1% or 0.2% lidocaine for different periods (**Figure 1F**). However, apoptosis markedly increased in splenic tissues treated with 0.4% lidocaine (**Figure 1G**).

The data of apoptotic ratios in each group were collected from the results of TUNEL assay and presented in **Table 1**. Factor analysis was performed to examine the apoptotic effects of lidocaine on endothelial cells. The differences of apoptotic ratios were statistically significant compared among different groups (F_{group} = 107.676, P_{group} = 0.000) and different time points (F_{time} = 4.725, P_{time} = 0.005), meanwhile, the interaction between group and time was also significant ($F_{group\times time}$ = 13.682, $P_{group\times time}$ = 0.000) (**Figure 1H**).

From the data analysis, the difference of apoptotic ratio had no statistical significance when spleens were treated with saline for different periods (F = 0.296, P = 0.828). From the Kruskal-Wallis H analysis, the difference of apoptotic ratio had no statistical significance when spleens were treated with 0.1% (x^2 = 3.687, P = 0.297) and 0.2% lidocaine (x^2 = 4.167, P = 0.244) for different periods. However, in the group that spleens were treated with 0.4% lidocaine for different periods, the difference of apoptotic ratios were statistically significant (x^2 = 19.747, P = 0.000), and the apoptotic ratio increased along with the longer periods of treatment (**Figure 1I**; **Table 1**). The difference of apoptotic ratios had no statistical significance when spleens were treated with saline or different concentrations of lidocaine for 2 days (F = 1.386, P = 0.279). However, the apoptotic ratios in 0.4% lidocaine group were notably higher than those in saline, 0.1% or 0.2% lidicaines when spleens were treated for 5, 8 and 14 days (P < 0.05) (**Figure 1I**; **Table 1**).

High concentration of PYM induced apoptosis of mouse splenic vascular endothelial cells

Total 88 mice were randomly divided into 4 groups, 16 mice for saline while 24 mice for 0.5, 1 and 2 mg/ml PYM respectively. The mice in each group were randomly divided into 4 subgroups that mice were treated with saline or PYM for 2, 5, 8 and 14 days.

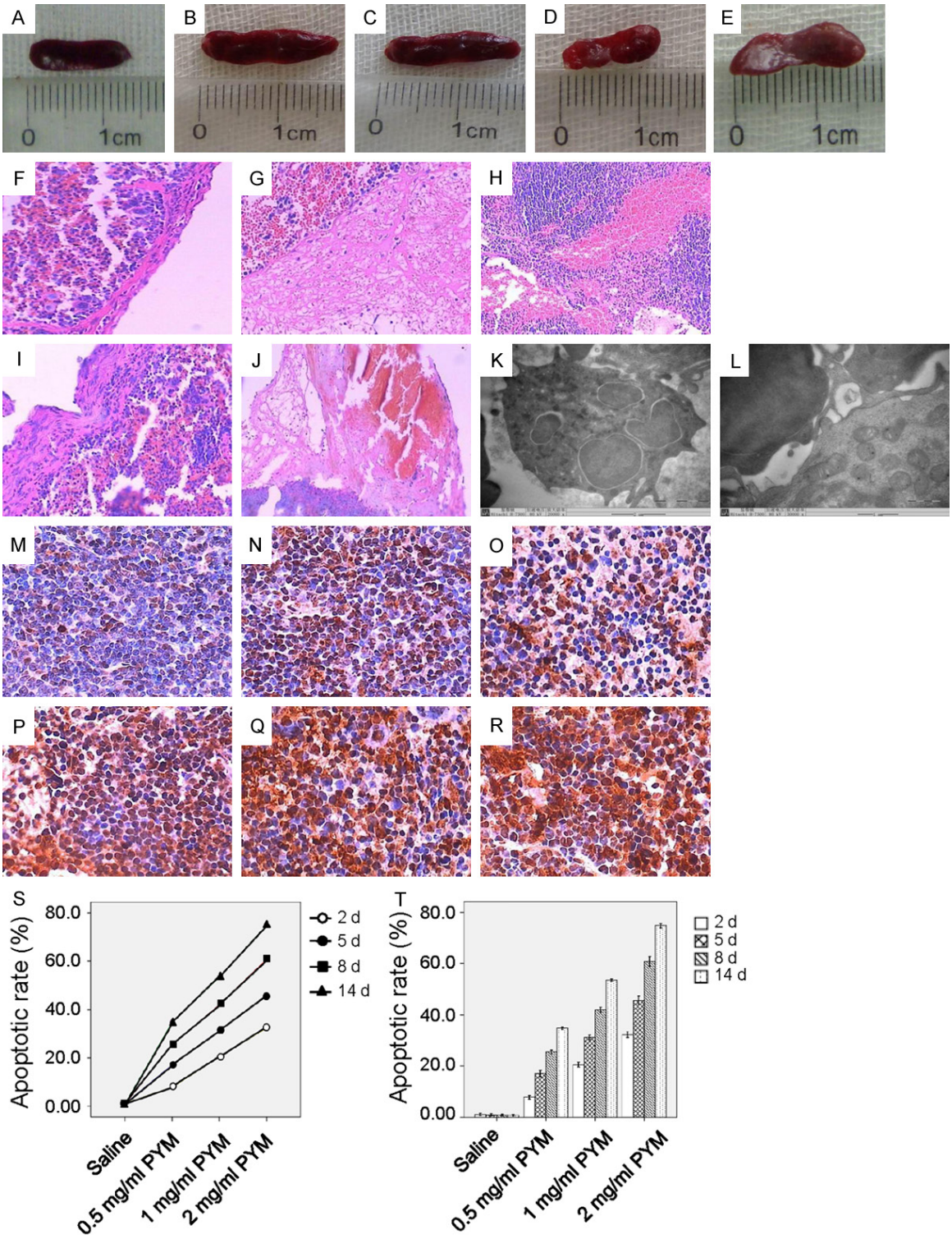

Figure 2. High concentration of PYM induced apoptosis of mouse splenic vascular endothelial cells. The appearance of spleens treated with PYM. (A) The normal spleen treated with saline; (B) The spleen treated with 2 mg/ml PYM for 2 days, the edges were thickening and hardening; (C) The spleen treated with 2 mg/ml PYM for 5 days, the surface was uneven and atrophic; (D) The spleen treated with 2 mg/ml PYM for 8 days, white striped cicatrices were observed on the surface; (E) The spleen treated with 2 mg/ml PYM for 14 days, a large area of cicatrices was observed. HE staining of the mice spleen tissues treated with PYM. (F) The normal capsule of spleen treated with saline (× 200); (G) In the 0.5 mg/ml PYM treatment group, minor hemorrhage of splenic sinus and fibrin exudation were observed (× 100); (H) In the 1 mg/ml PYM treatment group, fresh bleeding of splenic sinus, fibrin deposition

and histiocytosis were observed (× 100); (I) In the group of 2 mg/ml PYM treatment for 8 days, thicken splenic capsule and histiocytosis were observed (× 200); (J) In the group of 2 mg/ml PYM treatment for 14 days, hemorrhage and fibrin exudation were observed (× 100). Transmission electron microscope analysis of the mice spleen tissues treated with 2 mg/ml PYM (EM × 20000). (K) The blood sinus wall was destroyed and apoptotic bodies were obviously observed; (L) Vacuoles changing of mitochondrion and expanded endoplasmic reticulum were also observed. TUNEL analysis of the mice spleen tissues treated with PYM (× 400). (M) In the saline treatment group, the apoptotic splenic vascular endothelial cells were rarely observed; (N) In the group of 1 mg/ml PYM treatment for 5 days, the apoptotic ratio of splenic vascular endothelial cells increased notably; in the group of 2 mg/ml PYM treatment for 2 days (O), 5 days (P), 8days (Q) and 14 days (R), the apoptotic ratio increased significantly along with the rising treatment periods. Statistical analysis of the apoptotic ratio in each group treated with PYM. (S) The outline of the interactive effects; (T) Comparison of the apoptotic ratio in each group presented in histogram.

After the treatment of saline for 2, 5, 8 and 14 days, the appearances of spleens had no significant changes (**Figure 2A**). When spleens were treated with 0.5 mg/ml PYM: 2 days after, the splenic tissue was dull-red and bright without obvious changes; 5 days after, few specimens exhibited slightly dark color and the capsule was a little strained; 8 days after, the margin of few specimens was slightly blunt with mild swelling; 14 days after, most specimens became dark, slightly sunken, and the tesion of splenic capsule disappeared. When spleens were treated with 1 mg/ml PYM: 2 days after, the capsule of some spleens was a little strained with mild swelling; 5 days after, the spleens became dark and the margin was slightly blunt with mild swelling; 8 days after, the tesion of splenic capsule disappeared and partial areas of spleen were sunken; 14 days after, a partial of spleen tissues atrophied and the margin was uneven with incisures, and white striped cicatrices were observed on the surface, adhered to the surrounding tissues. When spleens were treated with 2 mg/ml PYM: 2 days after, the capsule of some spleens was a little strained with different degrees of swelling, and the margin was slightly blunt (**Figure 2B**); 5 days after, the spleens became dark and the tesion of splenic capsule disappeared while partial areas of spleen were sunken (**Figure 2C**); 8 days after, a spleen tissues atrophied to some extent and incisures were observed on the margin, and the partial areas of spleen were sunken with white striped cicatrices on the surface, adhered to the surrounding tissues (**Figure 2D**); 14 days after, most specimens were hard and dull-red, the capsule was concave-convex and atrophic with white striped cicatrices on the surface, severely adhered to the surrounding tissues, and the spleen margins were sharp with irregular incisures (**Figure 2E**).

From the observation of HE staining, there were no significant changes when spleens were treated with saline for different periods (**Figure 2F**). When spleens were treated with 0.5 mg/ml PYM: 2 days after, the splenic sinus was occasionally dilated and hyperemic, and few inflammatory cells occasionally infiltrated into mesenchyme; 5 days after, interstitial inflammatory cell infiltration and exudation of fibrous protein were observed, and few sinusoidal endothelial cells were swelled and transformed; 8 days after, the splenic sinus was significantly dilated and hyperemic, while many inflammatory cell infiltration, exudation of fibrous protein and histiocytosis were observed, and the partial splenic cord structure was obscure; 14 days after, fibrous protein exudation, degeneration, fibrous tissue proliferation and erythrocytes diffusion were observed, and a part of splenic sinuses were sunk and destroyed while splenic corpuscle shrunk with uneven morphology (**Figure 2G**). When spleens were treated with 1 mg/ml PYM: 2 days after, the splenic sinus was dilated and hyperemic, while few inflammatory cells infiltrated and polynuclear macrophages were observed occasionally; 5 days after, the splenic sinus was significantly hyperemic, interstitial inflammatory cell infiltration and exudation of fibrous protein were observed, and few sinusoidal endothelial cells were swelled and transformed, in addition, the number of polynuclear macrophages increased; 8 days after, splenic cord structure was obscure with fibrous protein exudation and histiocytosis, associated with fresh hemorrhage (**Figure 2H**); 14 days after, fibrous protein exudation, degeneration, fibrous tissue proliferation and histiocytosis were observed, and splenic sinuses were sunk and destroyed while splenic corpuscle shrunk with uneven morphology, in addition, many monocytes or macrophages aggregated. When spleens were treated with 1 mg/ml PYM: 2 days after, the splenic sinus was dilated and hyperemic, and few sinusoidal endothelial cells were swelled and transformed, the inflammatory cell infiltration and histiocytosis were

Table 2. Comparison of the apoptotic ratios of splenic vascular endothelial cells treated with PYM (%, $\bar{x} \pm S$)

Group	N	2 d	5 d	8 d	14 d	Total	*F*	*P*
saline group	16	1.14 ± 0.48	1.02 ± 0.45	0.93 ± 0.35	0.88 ± 0.34	0.99 ± 0.38	0.296	0.828
0.5 mg/ml PYM	24	7.86 ± 0.72^{A}	17.13 ± 1.20^{a,A}	25.54 ± 0.79^{a,b,A}	34.82 ± 0.46^{a,b,c,A}	21.34 ± 10.23	1144.825	0.000
1 mg/ml PYM	24	20.57 ± 0.97^{A,B}	31.27 ± 1.02^{a,A,B}	41.92 ± 1.03^{a,b,A,B}	53.52 ± 0.41^{a,b,c,A,B}	36.82 ± 12.53	1494.281	0.000
2 mg/ml PYM	24	32.37 ± 1.15^{A,B,C}	45.69 ± 1.69^{a,A,B,C}	60.84 ± 1.89^{a,b,A,B,C}	74.83 ± 0.84^{a,b,c,A,B,C}	53.43 ± 16.34	963.028	0.000
Total		16.79 ± 11.98	25.84 ± 16.16	35.16 ± 21.27	44.66 ± 25.99	30.61 ± 21.89	2499.782	0.000
F		1232.831	1201.614	2134.600	14886.604	9549.392	279.087$^{\Delta}$	
P		0.000	0.000	0.000	0.000	0.000		0.000$^{\Delta}$

$^{\Delta}$: interaction *F-value* and *P-value*; ^{a}: vs 2 d, $P < 0.05$; ^{b}: vs 5 d, $P < 0.05$; ^{c}: vs 8 d, $P < 0.05$; ^{A}: vs saline group, $P < 0.05$; ^{B}: vs 0.5 mg/ml PYM, $P < 0.05$; ^{C}: vs 1 mg/ml PYM, $P < 0.05$.

Table 3. Comparison of the apoptotic ratios of splenic vascular endothelial cells treated with a combination of PYM and lidocaine (%, $\bar{x} \pm S$)

Group	N	2 d	5 d	8 d	14 d	Total	F/x^2	*P*
Saline group	16	1.14 ± 0.48	1.02 ± 0.45	0.93 ± 0.35	0.88 ± 0.34	0.99 ± 0.38	0.296*	0.828
0.5 mg/ml PYM	24	7.77 ± 0.88^{A}	18.37 ± 3.67^{a,A}	25.54 ± 0.79^{a,b,A}	35.12 ± 0.46^{a,b,c,A}	21.70 ± 10.37	21.600$^{\#}$	0.000
0.5 mg/ml PYM + 0.1% lidocaine	24	8.15 ± 0.57^{A}	17.30 ± 1.16^{a,A}	26.14 ± 1.32^{a,b,A}	35.31 ± 0.55^{a,b,c,A}	21.73 ± 10.35	876.169*	0.000
0.5 mg/ml PYM + 0.2% lidocaine	24	13.96 ± 0.18^{A,B,C}	27.44 ± 1.79^{a,A,B,C}	36.46 ± 1.96^{a,b,A,B,C}	44.53 ± 1.05^{a,b,c,A,B,C}	30.60 ± 11.67	21.600*	0.000
Total		8.36 ± 4.39	17.40 ± 9.19	24.21 ± 12.21	31.51 ± 15.34	20.37 ± 13.85	917.257	0.000
x^2		17.360	17.202	17.715	17.146	1510.583	88.540$^{\Delta}$	
P		0.001	0.001	0.001	0.001	0.000		0.000$^{\Delta}$

$^{\Delta}$: interaction *F-value* and *P-value*; *: analysis of variance; $^{\#}$: rank-sum test; ^{a}: vs 2 d, $P < 0.05$; ^{b}: vs 5 d, $P < 0.05$; ^{c}: vs 8 d, $P < 0.05$; ^{A}: vs saline group, $P < 0.05$; ^{B}: vs 0.5 mg/ml PYM, $P < 0.05$; ^{C}: vs 0.1% lidocaine + 0.5 mg/ml PYM, $P < 0.05$.

Table 4. Comparison of the copy numbers of caspase-3 in spleen tissues treated with a combination of PYM and lidocaine ($\bar{x} \pm S$)

Group	N	2 d	5 d	8 d	14 d	Total	x^2	*P*
0.5 mg/ml PYM	24	4911.28 ± 590.30	11304.42 ± 2821.66^{a}	23396.20 ± 7155.49^{a,b}	45188.16 ± 3167.89^{a,b,c}	21200.01 ± 16163.21	21.600	0.000
0.5 mg/ml PYM + 0.1% lidocaine	24	6874.47 ± 842.61^{A}	12539.72 ± 921.33^{a}	25625.60 ± 4000.36^{a,b}	46147.63 ± 2863.12^{a,b,c}	22796.85 ± 15604.83	21.600	0.000
0.5 mg/ml PYM + 0.2% lidocaine	24	18158.41 ± 8013.87^{A,B}	34515.95 ± 3557.15^{a,A,B}	46947.88 ± 921.92^{a,b,A,B}	94380.37 ± 7544.24^{a,b,c,A,B}	48500.65 ± 29499.23	21.600	0.000
Total		9981.39 ± 7434.97	19453.36 ± 11255.93	31989.89 ± 11804.42	61905.38 ± 24095.55	30832.51 ± 24578.89	479.417	0.000
F/x^2		15.158$^{\#}$	11.415$^{\#}$	11.474$^{\#}$	127.582*	294.028	25.348$^{\Delta}$	
P		0.001	0.003	0.003	0.000	0.000		0.000$^{\Delta}$

$^{\Delta}$: interaction *F-value* and *P-value*; *: analysis of variance; $^{\#}$: rank-sum test; ^{a}: vs 2 d, $P < 0.05$; ^{b}: vs 5 d, $P < 0.05$; ^{c}: vs 8 d, $P < 0.05$; ^{A}: vs saline group, $P < 0.05$; ^{B}: vs 0.5 mg/ml PYM, $P < 0.05$; ^{C}: vs 0.1% lidocaine + 0.5 mg/ml PYM, $P < 0.05$.

observed, splenic cord structure was obscure, the splenic cord fibrocytes were occasionally transformed or the cell nucleus concentrated and crushed; 5 days after, the splenic sinus was significantly hyperemic, many sinusoidal endothelial cells and splenic cord fibrocytes were transformed with concentrated and crushed nucleus, splenic cord structure was obscure, the aggregation of monocytes or macrophages, inflammatory cell infiltration and histiocytosis were frequently observed; 8 days after, splenic sinuses were sunk and destroyed, the karyolysis and crush of endothelial cell nucleus, fibrous protein exudation, degeneration, fibrous tissue proliferation and erythrocytes diffusion were observed, a large number of monocytes or macrophages aggregated, splenic corpuscle shrunk with uneven morphology, the margin of spleens was hyperemic and bleeding (**Figure 2I**); 14 days after, large areas of splenic sinus endothelial cell apoptosis, collapsed and shrunken splenic corpuscle, fibrous tissue hyperplasia and thicken capsule were observed (**Figure 2J**).

From the TEM observation, the configurations of spleens treated with saline were as the same as described in (**Figure 1D**). However, in the groups that were treated with PYM, the blood sinus wall was destroyed and apoptotic bodies were obviously observed (**Figure 2K**); vacuoles changing of mitochondrion and expanded endoplasmic reticulum were also observed (**Figure 2L**).

TUNEL assay was performed to examine the apoptotic effects of PYM on splenic endothelial cells. The results showed that apoptotic cells were rarely visible in the saline treatment group; however, the number of apoptotic cells significantly increased along with the rising dose or period of the treatment (**Figure 2M-R**).

The data of apoptotic ratios in each group were collected from the results of TUNEL assay and presented in **Table 2**. Factor analysis was performed to examine the apoptotic effects of PYM on endothelial cells. The differences of apoptotic ratios were statistically significant compared among different groups (F_{group} = 9549.392, P_{group} = 0.000) and different time points (F_{time} = 2499.782, P_{time} = 0.000), meanwhile, the interaction between group and time was also significant ($F_{group\times time}$ = 279.087, $P_{group\times time}$ = 0.000) (**Figure 2S**).

From the data analysis, the difference of apoptotic ratio had no statistical significance when spleens were treated with saline for different periods (F = 0.296, P = 0.828). However, the difference of apoptotic ratios were statistically significant when spleens were treated with 0.5 mg/ml (F = 1144.825, P = 0.000), 1 mg/ml (F = 1494.281, P = 0.000) and 2 mg/ml PYM (F = 963.028, P = 0.000) for different periods, and the apoptotic ratios increased along with the longer periods of relative treatment (**Figure 2T**; **Table 2**). The difference of apoptotic ratios were statistically significant when spleens were treated with different concentrations of PYM for 2 days (F = 1232.831, P = 0.000), 5 day (F = 1201.614, P = 0.000), 8 days (F = 2134.600, P = 0.000) and 14 days (F = 14886.604, P = 0.000). In addition, the apoptotic ratios in 2 mg/ml PYM treatment group were notably higher than those in other groups when spleens were treated with saline, 0.5 mg/ml or 1 mg/ml PYM for different periods (**Figure 2T**; **Table 2**).

Lidocaine had synergistic effects on apoptosis of splenic vascular endothelial cells induced by PYM

Total 88 mice were randomly divided into 4 groups, 16 mice for saline while 24 mice for 0.5 mg/ml PYM, 0.5 mg/ml PYM + 0.1% lidocaine and 0.5 mg/ml PYM + lidocaine respectively. The mice in each group were randomly divided into 4 subgroups that mice were treated with saline or drugs for 2, 5, 8 and 14 days.

The injection of saline did not obviously affected the morphology and configuration of spleens, but the injection of 0.5 mg/ml PYM significantly caused apoptosis of splenic vascular endothelial cells and destroyed the configuration of spleens as described above. Then we examine the effects of a combination of lidocaine and PYM on spleens via HE staining and TUNEL assay as well as TEM observation. The results showed that compared with the treatment of 0.5 mg/ml PYM alone, the combination of lidocaine and PYM further promoted the apoptosis of splenic vascular endothelial cells and severely destroyed the configuration of spleens. In addition, the combination of 0.2% lidocaine and PYM exerted much more intense apoptotic effects than 0.1% lidocaine + PYM (**Table 3**).

The data of apoptotic ratios in each group were collected from the results of TUNEL assay and

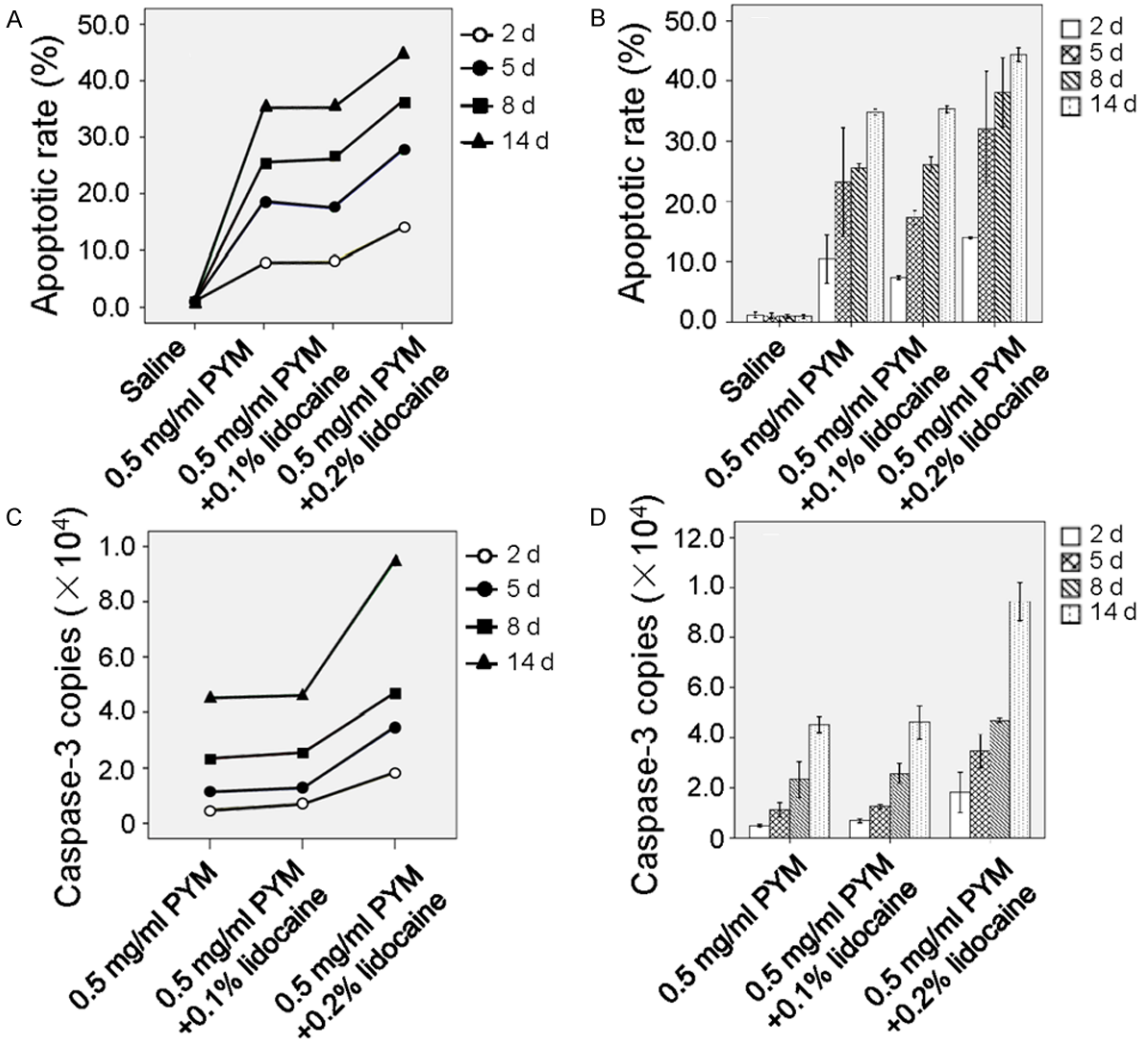

Figure 3. Lidocaine had synergistic effects on apoptosis of splenic vascular endothelial cells induced by PYM. Statistical analysis of the apoptotic ratio in each group treated with PYM or a combination of PYM and lidocaine. A. The outline of the interactive effects; B. Comparison of the apoptotic ratio in each group presented in histogram. Statistical analysis of the copy number of caspase-3 in each group treated with PYM or a combination of PYM and lidocaine. C. The outline of the interactive effects; D. Comparison of the copy number of caspase-3 in each group presented in histogram.

presented in **Table 3**. Factor analysis was performed to examine the apoptotic effects of lidocaine + PYM on endothelial cells. The differences of apoptotic ratios were statistically significant compared among different groups (F_{group} = 1510.583, P_{group} = 0.000) and different time points (F_{time} = 917.257, P_{time} = 0.005), meanwhile, the interaction between group and time was also significant ($F_{group\times time}$ = 88.540, $P_{group\times time}$ = 0.000) (**Figure 3A**).

From the data analysis, the difference of apoptotic ratio had no statistical significance when spleens were treated with saline for different periods (F = 0.296, P = 0.828). However, the difference of apoptotic ratios were statistically significant when spleens were treated with 0.5 mg/ml PYM (x^2 = 21.600, P = 0.000), 0.5 mg/ml PYM + 0.1% lidocaine (F = 876.169, P = 0.000) and 0.5 mg/ml PYM + 0.2% lidocaine (F = 21.600, P = 0.000) for different periods, and the apoptotic ratios increased along with the longer periods of relative treatment (**Figure 3B**; **Table 3**). The difference of apoptotic ratios were statistically significant when spleens were treated with PYM or a combination of lidocaine and PYM for 2 days (x^2 = 17.360, P = 0.001), 5 day (x^2 = 17.202, P = 0.001), 8 days (x^2 =

17.715, $P = 0.001$) and 14 days ($x^2 = 17.146$, $P = 0.001$). In addition, the apoptotic ratios in 0.5 mg/ml PYM + 0.2% lidocaine treatment group were notably higher than those in other groups when spleens were treated with saline, 0.5 mg/ml PYM or 0.5 mg/ml PYM + 0.1% lidocaine for different periods (**Figure 3B**; **Table 3**).

To further examine the apoptosis of splenic vascular endothelial cells in each group, the mRNA expression levels of caspase-3 were determined by real time RT-PCR and the data were presented in **Table 4**. The results of factor analysis showed that the differences of copy numbers of caspase-3 mRNA were statistically significant compared among different groups ($F_{group} = 9549.392$, $P_{group} = 0.000$) and different time points ($F_{time} = 2499.782$, $P_{time} = 0.000$), meanwhile, the interaction between group and time was also significant ($F_{group\times time} = 279.087$, $P_{group\times time} = 0.000$) (**Figure 3C**).

Via the Kruskal-Wallis H analysis, the differences of copy numbers of caspase-3 mRNA were statistically significant when spleens were treated with 0.5 mg/ml PYM ($x^2 = 21.600$, $P = 0.000$), 0.5 mg/ml PYM + 0.1% lidocaine ($x^2 = 21.600$, $P = 0.000$) and 0.5 mg/ml PYM + 0.2% lidocaine ($x^2 = 21.600$, $P = 0.000$) for different periods, and the copy numbers of caspase-3 increased along with the longer periods of relative treatment (**Figure 3D**; **Table 4**). The difference of copy numbers of caspase-3 were statistically significant when spleens were treated with PYM or a combination of lidocaine and PYM for 2 days ($x^2 = 15.158$, $P = 0.001$), 5 day ($x^2 = 11.415$, $P = 0.003$), 8 days ($x^2 = 11.474$, $P = 0.003$) and 14 days ($x^2 = 127.582$, $P = 0.000$). In addition, the copy numbers of caspase-3 in 0.5 mg/ml PYM + 0.2% lidocaine treatment group were notably higher than those in other groups when spleens were treated with saline, 0.5 mg/ml PYM or 0.5 mg/ml PYM + 0.1% lidocaine for different periods (**Figure 3D**; **Table 4**).

Discussion

Lidocaine is an amide of local anaesthetics firstly synthesized in 1943, and widely used in clinical treatment because of its fast-acting function in relieving the pain [13]. However, people gradually discovered the side effects of lidocaine, such as cytotoxicity, delaying wound recovery, leading to thrombosis and inflammation. Lidocaine induced cytotoxicity has attracted extensive attention and many animal and *in vitro* experiments has been conducted, but the mechanisms remain elusive [19]. Some studies unraveled that lidocaine may influence the function of the mitochondria, resulting in damage to the mitochondria, and thus induce apoptosis. Lidocaine stimulated the imbalance of mitochondrial electron conduction and aggregation of endogenous mitochondrial free radicals, leading to mitochondrial DNA damage and even mutations, ultimately resulting in cell dysfunction and cell death [20, 21]. In this study, we found that lidocaine exerted toxicity on splenic vascular endothelial cells, and promoted cell apoptosis in a dose- and time-dependent manner. However, the mechanisms of lidocaine-induced apoptosis of splenic vascular endothelial cells need further studies to figure out.

PYM is a broad-spectrum anti-tumor antibiotic produced by Pingyang Streptomyces and belongs to bleomycin, without immunosuppressive effects and suppressing bone marrow function. In the body, PYM can promote the oxidation of Fe^{2+} to Fe^{3+}, and produce OH^-, which is the most active and powerful oxygen free radicals that can combine and uncoil the DNA [22]. In addition, PYM also induced cell apoptosis. Therefore, local application of PYM may cause vascular endothelial cell injury, cell hydropic, degeneration and hyperplasia, the occurrence of inflammation, local tissue degeneration and necrosis, micro-thrombosis, leading to wall thickening and luminal occlusion, ultimately resulting in fibrosis to promote the regression of lesions. In this study, we found that 0.5 mg/ml of PYM induced apoptosis of mice splenic vascular endothelial cells, and along with the rising concentrations of PYM and treatment periods, the ratios of PYM-induced apoptosis significantly increased. After the treatment with 2 mg/ml of PYM for 14 days, a large area of apoptotic cells in spleen were observed and the configuration of splenic tissue was severely destroyed.

PYM sclerotherapy is applied as a routine treatment for VMs in China and has a good therapeutic effect. However, high concentrations of PYM treatment often causes the patient's pain during the treatment of VMs, while lidocaine is often used as an auxiliary anesthetic to relieve the pain caused by PYM. Besides the anesthetic effect, whether lidocaine has the synergistic

effect with PYM during the treatment of VMs? We chose the mouse spleen as the VM model, a combination of lidocaine and PYM were applied in treatment. The results showed that the apoptotic ratios in the joint use of lidocaine and PYM were notably higher compared to those in the treatment with PYM alone, indicating that lidocaine exerted synergistic effect on apoptosis of endothelial cells induced by PYM. However, the mechanisms remain unclear. Two reasons may account for that, first, lidocaine itself has cytotoxicity; second, lidocaine promoted the penetration and absorption of PYM, thus exhibiting stronger pro-apoptotic ability.

Of course, it requires further study to figure out whether lidocaine has a synergistic effect with PYM in the clinical treatment of VMs. Moreover, we have to look for better animal models for VMs study. Overall, this study investigated the synergistic effects of lidocaine and PYM on inducing apoptosis of endothelial cells in the VM mouse model, trying to provide experimental and theoretical basis for clinical drug preparation of PYM and lidocaine during the therapy of VMs.

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Disclosure of conflict of interest

None.

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References

[1] Boon LM, Mulliken JB, Enjolras O, Vikkula M. Glomuvenous malformation (glomangioma) and venous malformation: Distinct clinicopathologic and genetic entities. Arch Dermatol 2004; 140: 971-976.

[2] Dompmartin A, Vikkula M, Boon LM. Venous malformation: Update on aetiopathogenesis, diagnosis and management. Phlebology 2010; 25: 224-235.

[3] Richter GT, Friedman AB. Hemangiomas and vascular malformations: Current theory and management. Int J Pediatr 2012; 2012: 645678.

[4] Boon LM, Vanwijck R. Medical and surgical treatment of venous malformations. Ann Chir Plast Esthet 2006; 51: 403-411.

[5] Zhi K, Wen Y, Li L, Ren W. The role of intralesional pingyangmycin in the treatment of venous malformation of facial and maxillary region. Int J Pediatr Otorhinolaryngol 2008; 72: 593-597.

[6] Rosenblatt M. Endovascular management of venous malformations. Phlebology 2007; 22: 264-275.

[7] Berenguer B, Burrows PE, Zurakowski D, Mulliken JB. Sclerotherapy of craniofacial venous malformations: Complications and results. Plast Reconstr Surg 1999; 104: 1-11; discussion 12-15.

[8] Revencu N, Boon LM, Mulliken JB, Enjolras O, Cordisco MR, Burrows PE, Clapuyt P, Hammer F, Dubois J, Baselga E, Brancati F, Carder R, Quintal JM, Dallapiccola B, Fischer G, Frieden IJ, Garzon M, Harper J, Johnson-Patel J, Labreze C, Martorell L, Paltiel HJ, Pohl A, Prendiville J, Quere I, Siegel DH, Valente EM, Van Hagen A, Van Hest L, Vaux KK, Vicente A, Weibel L, Chitayat D, Vikkula M. Parkes weber syndrome, vein of galen aneurysmal malformation, and other fast-flow vascular anomalies are caused by rasa1 mutations. Hum Mutat 2008; 29: 959-965.

[9] Liu Y, Liu D, Wang Y, Zhang W, Zhao F. Clinical study of sclerotherapy of maxillofacial venous malformation using absolute ethanol and pingyangmycin. J Oral Maxillofac Surg 2009; 67: 98-104.

[10] Zheng JW, Yang XJ, Wang YA, He Y, Ye WM, Zhang ZY. Intralesional injection of pingyangmycin for vascular malformations in oral and maxillofacial regions: An evaluation of 297 consecutive patients. Oral Oncol 2009; 45: 872-876.

[11] Ho YC, Tai KW, Chang YC. Synergistic effects of verapamil on pingyangmycin-induced cytotoxicity and apoptosis in kb cells. Oral Dis 2007; 13: 40-44.

[12] Spence J, Krings T, TerBrugge KG, Agid R. Percutaneous treatment of facial venous malformations: A matched comparison of alcohol and bleomycin sclerotherapy. Head Neck 2011; 33: 125-130.

[13] Khaliq W, Alam S, Puri N. Topical lidocaine for the treatment of postherpetic neuralgia. Cochrane Database Syst Rev 2007; CD004846.

[14] Lee SH, Hahn ST, Park SH. Intraarterial lidocaine administration for relief of pain resulting from transarterial chemoembolization of hepatocellular carcinoma: Its effectiveness and optimal timing of administration. Cardiovasc Intervent Radiol 2001; 24: 368-371.

[15] Anvari B, Tanenbaum BS, Hoffman W, Said S, Milner TE, Liaw LH, Nelson JS. Nd: Yag laser irradiation in conjunction with cryogen spray cooling induces deep and spatially selective photocoagulation in animal models. Phys Med Biol 1997; 42: 265-282.
[16] Blenkinsopp WK. Choice of sclerosant: An experimental study. Angiologica 1970; 7: 182-186.
[17] Jin XY, Zhao L, She XL, Jing J. Experimental study on borrelidin-mediated apoptosis of endothelial cells of mice spleen sinus as venous malformation mode. J Modern Stomatol 2011; 25: 45-49.
[18] Mulliken JB, Glowacki J. Hemangiomas and vascular malformations in infants and children: A classification based on endothelial characteristics. Plast Reconstr Surg 1982; 69: 412-422.
[19] Johnson ME. Potential neurotoxicity of spinal anesthesia with lidocaine. Mayo Clin Proc 2000; 75: 921-932.
[20] Dabadie P, Bendriss P, Erny P, Mazat JP. Uncoupling effects of local anesthetics on rat liver mitochondria. FEBS Lett 1987; 226: 77-82.
[21] Kamiya Y, Ohta K, Kaneko Y. Lidocaine-induced apoptosis and necrosis in u937 cells depending on its dosage. Biomed Res 2005; 26: 231-239.
[22] Moseley PL. Augmentation of bleomycin-induced DNA damage in intact cells. Am J Physiol 1989; 257: C882-887.