

• BASIC RESEARCH •

# Study on the mechanism of regulation on peritoneal lymphatic stomata with Chinese herbal medicine

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## Abstract

**AIM:** To study the mechanism of Chinese herbal medicine (CHM, the prescription consists of *Radix Salviae Miltiorrhizae*, *Radix Codonopsis*, *Pilosulae*, *Rhizoma Atractylodis Alba* and *Rhizoma Alismatis*, *Leonurus Heterophyllus* Sweet, etc) on the regulation of the peritoneal lymphatic stomata and the ascites drainage.

**METHODS:** The mouse model of live fibrosis was established with the application of intragastric installations of carbon tetrachloride once every three days; scanning electron microscope and computer image processing were used to detect the area and the distributive density of the peritoneal lymphatic stomata; and the concentrations of urinary ion and NO in the serum were analyzed in the experiment.

**RESULTS:** Two different doses of CHM could significantly increase the area of the peritoneal lymphatic stomata, promote its distributive density and enhance the drainage of urinary ion such as sodium, potassium and chlorine. Meanwhile, the NO concentration of two different doses of CHM groups was  $133.52 \pm 23.57 \mu\text{mol/L}$ , and  $137.2 \pm 26.79 \mu\text{mol/L}$  respectively. In comparison with the control group and model groups ( $48.36 \pm 6.83 \mu\text{mol/L}$ , and  $35.22 \pm 8.94 \mu\text{mol/L}$ ,  $P < 0.01$ ), there existed significantly marked difference, this made it clear that Chinese herbal medicine could induce high endogenous NO concentration. The effect of Chinese herbal medicine on the peritoneal lymphatic stomata and the drainage of urinary ion was altered by adding NO donor (sodium nitroprusside, SNP) or NO synthase (NOS) inhibitor (N(G)-monomethyl-L-arginine, L-NMMA) to the peritoneal cavity.

**CONCLUSION:** There existed correlations between high NO concentration and enlargement of the peritoneal lymphatic stomata, which result in enhanced drainage of ascites. These data supported the hypothesis that Chinese herbal medicine could regulate the peritoneal lymphatic stomata by accelerating the synthesis and release of endogenous NO.

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## INTRODUCTION

Numerous investigations have demonstrated that the peritoneal lymphatic stomata are small openings of the subperitoneal lymphatic vessels both in animals and in humans<sup>[1-18]</sup>. It has also been observed that particles, cells and solutions containing vital dyes are absorbed rapidly by the peritoneal lymphatic stomata<sup>[19-22]</sup>. Subsequent researches suggested that the peritoneal cavity is an integral part of the lymphatic system with enormous absorption powers, functioning primarily by means of the subperitoneal lymphatics via the peritoneal lymphatic stomata<sup>[23-29]</sup>. Thus, it has important clinical implications, especially in ascites drainage<sup>[30-32]</sup>. In recent years, therapeutic effect of Chinese herbal medicine (CHM) on the ascites has also drawn world wide attention among the scholars. It is further confirmed that Chinese herbal medicine can regulate the lymphatic stomata and promote the excretion of substance from the peritoneal cavity, which showed a good future in the treatment of the hepatocirrhosis with ascites<sup>[33-44]</sup>. However, it is still unclear how the lymphatic stomata is regulated by the Chinese herbal medicine. This article aimed at the regulation of CHM on the lymphatic stomata in the mouse liver fibrosis model induced by CCl<sub>4</sub>, which provided theoretical evidence on ascites. Furthermore, by the application of NO donor (SNP) and NOS inhibitor (L-NMMA), the effect of NO was studied on the peritoneal lymphatic stomata in order to clarify the mechanism of CHM on the regulation of the peritoneal lymphatic stomata.

## MATERIAL AND METHODS

### Experimental CHM

By examining a computerized media index, the conventional remedies of CHM was selected for the treatment of hepatocirrhosis with ascites. The Chinese composite prescription was supplied by Zhejiang Academy of Traditional Chinese Medicine. The prescription consisted of *Radix Salviae Miltiorrhizae*, *Radix Codonopsis*, *Pilosulae*, *Rhizoma Atractylodis Alba* and *Rhizoma Alismatis*, *Leonurus Heterophyllus* Sweet. The medicament was immersed in the 750ml·L<sup>-1</sup> alcohol for 24 hrs, then purified with rotatory evaporator (ZFQ85A type, produced by Shanghai 11th Factory of Electron Tube). The crude drug content was 15.2 g·ml<sup>-1</sup>.

### Animal and grouping

Ninety healthy mature NIH male mice, weighing 25g-30g, provided by the Experimental Animal Center of Zhejiang Academy of Medical Sciences, were selected and divided at random into six groups (each of 15 mice): the control group (NS group), model group, low dose of CHM group (CHM I), high dose of CHM group (CHMII), the donor group (DR group) and the inhibitor group (IR group).

### Mouse liver fibrosis model

Except the NS group, the other experimental mice were fed freely with 50ml·L<sup>-1</sup> alcohol solution instead of water for 1 week, then the

mice were given 100ml·L<sup>-1</sup> CCl<sub>4</sub> rape-seed oil solution 0.1ml/10g by gastrogavage every 3 days for 6 weeks to induce liver fibrosis. After liver fibrosis was confirmed by pathological examination, low dose of the Chinese herbal medicine (0.1ml/10g/day) was given to CHM I group, high dose of the Chinese herbal medicine (0.2ml/10g/day) was give to CHMII group, normal saline (0.2ml/10g/day) was given to DR group, for 3 weeks respectively. After the 10th week, DR and IR group was additionally injected intraperitoneally with 50μg/10g/day NO donor (SNP) or 80μg/10g/day NOS inhibitor (L-NMMA) for two days respectively. Model group was untreated and NS group was given 0.2ml/10g of normal saline per day.

#### **Preparation of samples for scanning electron microscopic examination and computer image processing**

The diaphragmatic peritoneum on the right side was cut into 5.0×5.0 mm<sup>2</sup> pieces and put into 25ml·L<sup>-1</sup> glutaraldehyde solution for 1 h, then postfixed for 1h in 10ml·L<sup>-1</sup> OsO<sub>4</sub>, dehydrated in a graded series of alcohol, CO<sub>2</sub> critical-point dried, mounted on aluminum tubs and sputter-coated with gold. Specimens were examined with a Stereoscan 260 SEM operated at 25kV. The result was treated with the computer image processing system attached to SEM. The system consists of video, A/D, IBM386. Software was designed for processing and quantitatively analyzing the area and the distributive density of the lymphatic stomata.

#### **Urinary volume and ionic concentration analysis**

Mouse urine was collected in 2 hrs and ionic concentration of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> was measured using auto-biochemical analyzer (Beckman CX ∇7 type).

#### **Measurement of serum NO**

Five hundred microliters of the serum was de-proteinized with 200mL of 75mM zinc sulfate and 250mL of 55mM sodium hydroxid and subsequently were centrifuged at 3000rpm for 10min. One hundred microliters of the deproteinized solution was mixed with 0.3ml ddH<sub>2</sub>O and 0.25g newly-activated Cadmium sufficiently for 1h. The nitrite concentration was determined by mixing 0.1ml of the supernatants from the mixed solution with an equal volume of Griess reagent (1 part of 0.2% N-(1-naphthyl) ethylenediamine dihydrochloride to 1 part of 1% sulfanilamide in 2% phosphoric acid ) for 15min. The absorbance at 545 nm was measured, and the nitrite concentration was determined from a standard curve calibrated with NaNO<sub>2</sub> solution<sup>[45]</sup>.

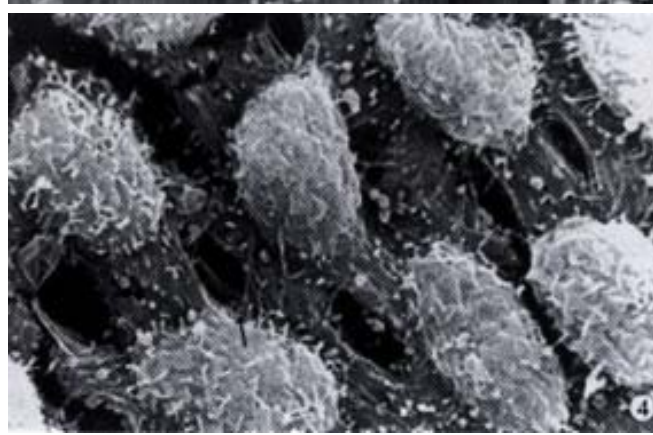
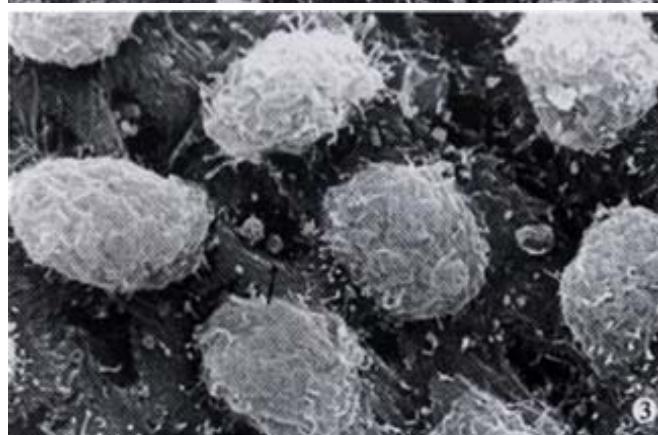
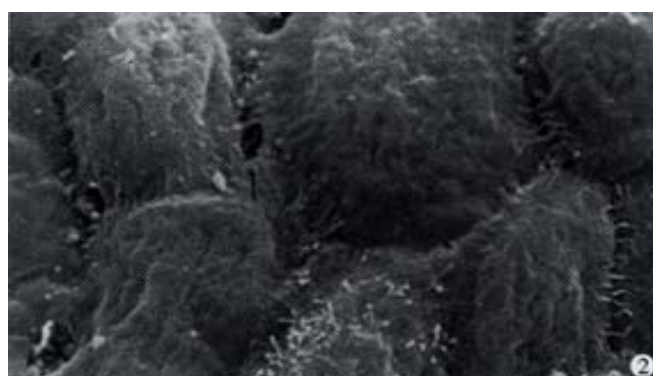
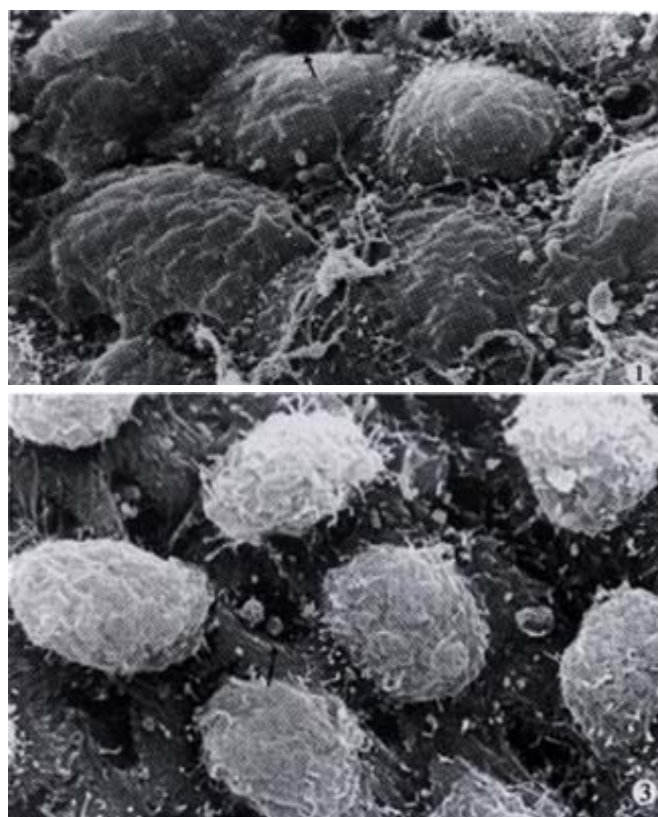
#### **Statistic analysis**

The experimental results were described by  $\bar{x} \pm s$  and the difference among the groups was analyzed by *t* test.

## **RESULTS**

### **Changes of peritoneal lymphatic stomata**

In SEM, there were cuboidal and flattened cells in the mesothelium. The lymphatic stomata which was round or ellipse were located only among the cuboidal cells and most of them were distributed in cluster. In NS group and model groups, there were few and small lymphatic stomata (Figures 1,2). In contrast, there were many and large lymphatic stomata in CHMI and CHMII groups (Figure3, 4). In DR group, there were fewer and larger lymphatic stomata than in the model group (Figure 5), while in the IR group fewer and smaller than that of the CHMII group (Figure 6).

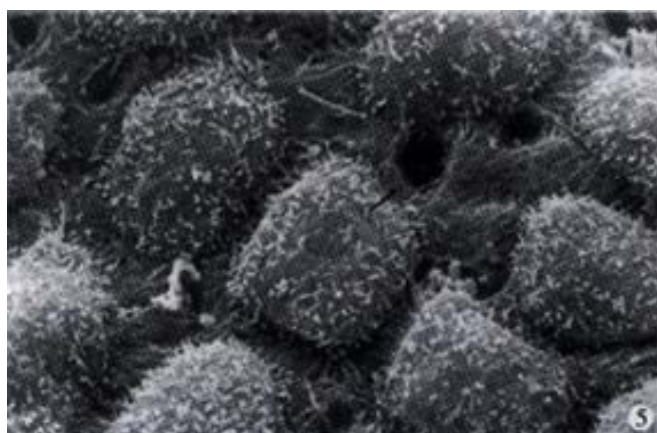


**Figure 1** SEM observation of mouse diaphragmatic peritoneum in the control group. Both the area and distribution density of the peritoneal lymphatic stomata (arrow) are small. ×3500

**Figure 2** SEM observation of mouse diaphragmatic peritoneum in model group showing the peritoneal stomata (arrow).×3500

**Figure 3** SEM observation of mouse diaphragmatic peritoneum in CHMI group. The area and distribution density of the peritoneal lymphatic stomata (arrow) are significantly increased. ×3500

**Figure 4** SEM observation of mouse diaphragmatic peritoneum in CHMII group. The area and distribution density of the peritoneal lymphatic stomata (arrow) are significantly increased. ×3500



**Figure 5** SEM observation of mouse diaphragmatic peritoneum in DR group. Compared with model group, the area and distribution density of the peritoneal lymphatic stomata (arrow) are relatively increased.  $\times 3500$

**Figure 6** SEM observation of mouse diaphragmatic peritoneum in IR group. Compared with CHMII group, the area and distribution density of the peritoneal lymphatic stomata (arrow) are relatively decreased.  $\times 3500$

With image processing, the average area of the stomata was  $3.59 \pm 1.29 \mu\text{m}^2$  in NS group and  $3.02 \pm 1.11 \mu\text{m}^2$  in model group, whereas in CHMI group and CHMII group, the average area of the stomata was  $5.89 \pm 0.33 \mu\text{m}^2$ , and  $5.93 \pm 1.87 \mu\text{m}^2$  respectively. There were significant differences in the enlargement of the stomata between CHMI and CHMII groups, based on the analysis of variance ( $P < 0.01$ ). By comparing the area at the 99% confidence interval of population means, we found that the stomata area of CHMI and CHMII groups were much larger than those of NS and model groups (Table 1).

When the mouse was injected intraperitoneally by SNP, the average area of the stomata was  $4.37 \pm 0.10 \mu\text{m}^2$ , which was larger than that of the corresponding model group. When the mouse was injected intraperitoneally by L-NMMA, the average area of the stomata was  $2.70 \pm 1.30 \mu\text{m}^2$ , which was smaller than that of the corresponding CHMII group. These showed that the area of the stomata could be altered by SNP or L-NMMA significantly ( $P < 0.05$  or  $P < 0.01$ ).

**Table 1** The influence of CHM, NO donor and NOS inhibitor ( $n=15$ ) on the area of the lymphatic stomata

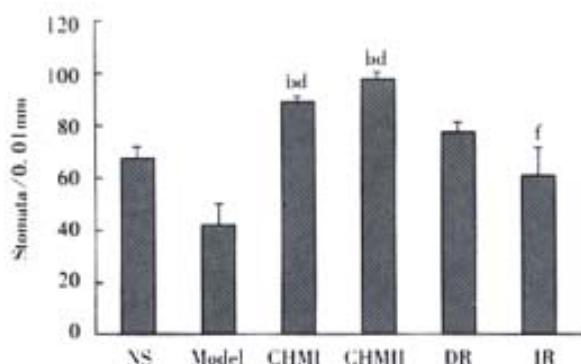
Groups	Mean ( $\mu\text{m}^2$ )	SD	Min. ( $\mu\text{m}^2$ )	Max. ( $\mu\text{m}^2$ )
NS	3.59	1.29	0.91	8.93
model group	3.02	1.11	1.83	7.53
CHMI <sup>bd</sup>	5.89	0.33	2.00	9.82
CHMII <sup>bd</sup>	5.93	1.87	2.08	10.15
DR <sup>c</sup>	4.37	0.10	1.92	8.70
IR <sup>f</sup>	2.70	1.30	1.75	9.19

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs NS group; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , vs model group; <sup>e</sup> $P < 0.05$ , <sup>f</sup> $P < 0.01$ , vs CHMII group

The average distribution density of the stomata of NS group, and model group were  $66.99 \pm 5.43/0.01\text{mm}^2$ ,  $42.80 \pm 13.35/0.01\text{mm}^2$ , whereas those of CHMI and CHMII group were  $92.08 \pm 4.44/0.01\text{mm}^2$ , and  $96.24 \pm 4.62/0.01\text{mm}^2$  respectively. The results showed that CHM can promote the distribution density of the stomata significantly (Figure 7) ( $P < 0.01$ ).

When the mouse was injected intraperitoneally by SNP, the average distribution density of the stomata was  $79.06 \pm 5.37\text{mm}^2/0.01\text{mm}^2$ , which was much higher than that of the corresponding model group. When the mouse was injected intraperitoneally by L-NMMA, the average distribution density of the stomata was  $60.82 \pm 30.79\text{mm}^2/0.01\text{mm}^2$ , which was much lower than that of the corresponding CHM group. The above statistics show that the distribution density of the stomata could be altered by SNP or L-

NMMA significantly ( $P < 0.01$ ).



**Figure 7** The influence of CHM, NO donor and NOS inhibitor on the distribution density of the lymphatic stomata.

### Comparison of urinary ionic concentration

Subsequent experiment showed that the excretion of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  in CHMI and CHMII group was significantly higher than those in NS group and model groups respectively ( $P < 0.01$ ) (Table 2).

When NO donor was injected intraperitoneally, the excretion of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  in DR group was significantly higher than those in the model group ( $P < 0.05$  or  $P < 0.01$ ). When NO inhibitor was injected intraperitoneally, the excretion of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  in IR group decreased significantly in comparison with the corresponding CHMII group ( $P < 0.05$  or  $P < 0.01$ ).

**Table 2** The effect of CHM, NO donor, NOS inhibitor on the urinary ion of the mice (mmol/L,  $n=15$ )

Groups	$\text{Na}^+$	$\text{K}^+$	$\text{Cl}^-$
NS	$91.55 \pm 23.42$	$106.15 \pm 34.16$	$111.18 \pm 30.05$
model group	$97.48 \pm 42.12$	$129.65 \pm 46.91$	$121.90 \pm 41.65$
CHMI	$202.09 \pm 35.30^{\text{bd}}$	$217.30 \pm 57.78^{\text{bd}}$	$176.00 \pm 0.00^{\text{bc}}$
CHMII	$170.78 \pm 17.05^{\text{bd}}$	$210.11 \pm 51.49^{\text{bd}}$	$184.72 \pm 13.81^{\text{bd}}$
DR	$126.74 \pm 51.27^{\text{d}}$	$142.16 \pm 6.33^{\text{c}}$	$134.18 \pm 30.36^{\text{c}}$
IR	$139.28 \pm 26.02^{\text{f}}$	$88.29 \pm 22.59^{\text{f}}$	$154.98 \pm 14.88^{\text{e}}$

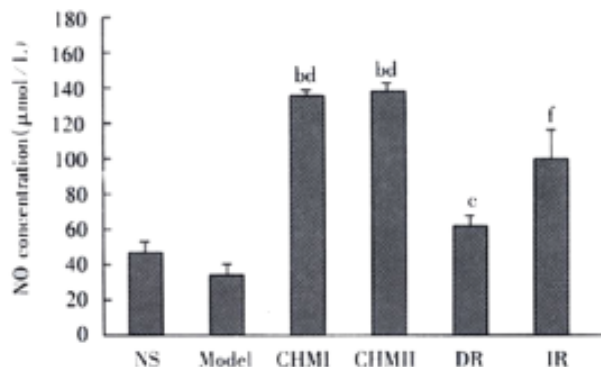
<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs NS group; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , vs model group; <sup>e</sup> $P < 0.05$ , <sup>f</sup> $P < 0.01$ , vs CHMII group

### Comparison of NO concentration

There were significant difference in the concentration of NO between groups. The NO concentration of NS, model groups was  $48.36 \pm$



6.83  $\mu\text{mol/L}$  and  $35.22 \pm 8.94 \mu\text{mol/L}$  respectively, however that of CHMI and CHMII group was  $133.52 \pm 23.57 \mu\text{mol/L}$  and  $137.2 \pm 26.79 \mu\text{mol/L}$ . The results showed that the concentration of NO in CHMI and CHMII groups was higher than that in NS and model groups ( $P < 0.01$ ) (Figure 8). The results indicated that Chinese herbal medicine could induce higher endogenous NO. When NO donor was injected intraperitoneally, NO concentration in DR group was  $62.56 \pm 18.91 \mu\text{mol/L}$ , which was significantly higher than that in the model group ( $P < 0.05$ ). When NO inhibitor was injected intraperitoneally, NO concentration in IR group was  $99.88 \pm 21.03 \mu\text{mol/L}$ , which decreased significantly as compared with that of CHMII group ( $P < 0.01$ ).



**Figure 8** The change of NO concentration by using CHM, NO donor and NOS inhibitor in the mice.

## DISCUSSION

In the mesothelial cells constituting the lymphatic stomata, there exists bundles of actin microfilaments, the contraction and relaxation of the microfilaments could result in the change of the diameter of the lymphatic stomata. Because the lymphatic stomata is the main pathway of the drainage of the material from the peritoneal cavity<sup>[23-29]</sup>, further investigation on regulating mechanism of the lymphatic stomata could promote the treatment of ascites and other associated illness.

With regard to the regulation of patency of the stomata, some authors have proposed that the peritoneal lymphatic stomata open passively when the diaphragm stretches during expiration, and close passively when it contracts during inspiration. Tsilibary and Wissig observed the regulation of the stomata by means of intravenously injecting carbacholine and succinylcholine, which cause the contraction and relaxation of the mouse diaphragm<sup>[3]</sup>. Their experimental results showed that stomata opened and closed with drug-induced relaxation and contraction of the diaphragm. Changes of the intra-abdominal pressure also play an important role on the regulation of the peritoneal lymphatic stomata. When the intra-abdominal pressure is increased experimentally by injecting normal saline intraperitoneally, the amount of the peritoneal lymphatic stomata is much larger than that of the normal group. On the contrary, when the intra-abdominal pressure is decreased experimentally, the patent number of the peritoneal lymphatic stomata is much less than that of the normal group<sup>[3]</sup>.

Li *et al* and Lv *et al* further confirmed the effect of some Chinese herbal medicine such as Radix Salviae Miltiorrhizae, Radix Codonopsis Pilosulae, Rhizoma Atractylodis Alba and Rhizoma Alismatis in the regulation of the peritoneal lymphatic stomata significantly by increasing the average diameter and the average distribution densities respectively, which can enhance fluid drainage into the vascular system<sup>[37-44]</sup>. This is of important clinical significance in treating ascites caused by liver cirrhosis. In the present study, the prescription is made up according to the traditional Chinese medicinal therapeutic principle in treating “hypocondriac pain”, “lump” and “tympanites”. The drug has the effects of activating blood

circulation to remove stasis, strengthening Spleen, supplementing Qi, and smoothening Qi to eliminate fullness. Our study showed that the CHM in the experiment could regulate the peritoneal lymphatic stomata significantly by increasing the average area and the average distribution densities respectively. Meanwhile, the medicines could enhance the drainage of urinary ion such as sodium, potassium and chlorine. The finding has further confirmed Li *et al*'s results<sup>[37-39]</sup> and Lv *et al*'s results<sup>[40-44]</sup>.

It can also be seen that both dosage of CHM could induce higher concentration of NO, that is, the high endogenous NO production is associated with the enlargement of the peritoneal lymphatic stomata and the increase of the drainage of urinary ion. Further results indicated that great changes could occur in the area and the distribution density of the lymphatic stomata when the NO donor or NOS inhibitor was injected intraperitoneally. When NO donor was injected intraperitoneally, i.e., the concentration of the endogenous NO increased, the area and the distribution density of the lymphatic stomata in the NO donor group were much larger than those of the model group ( $P < 0.05$  or  $P < 0.01$ ). Moreover, when NO inhibitor was given, the concentration of the endogenous NO decreased, these indexes of the lymphatic stomata in NO inhibitor group were much less than those of the corresponding large dose of CHM ( $P < 0.01$ ). Thus, it could be seen that the effect of CHM on the peritoneal lymphatic stomata was altered by adding NO donor or NOS inhibitor to the peritoneal cavity.

It has confirmed that the endothelium-derived relaxing factor (EDRF) is nitric oxide<sup>[46-49]</sup>, which has an effect in relaxing the blood vessel. Li *et al* reported that, with the proceeding of the peritoneal dialysis. Clinically, numerous macrophages were found to enter the peritoneal cavity to form milky spots. Damages of mesothelial cells, increased density of their distribution and enlargement of the peritoneal lymphatic stomata were found to be associated with the increase of macrophage NO quantity. Furthermore, increased NO production was related to the enlargement of the peritoneal lymphatic stomata in the long-term peritoneal dialysis. Therefore, Li *et al* proposed that NO could relax the lymphatic stomata which lead to the enhanced lymph absorption or ultrafiltration failure<sup>[50]</sup>. On these grounds, we suggested that the regulation of CHM on the lymphatic stomata may be related to endogenous NO. Chinese herbal medicine may regulate the lymphatic stomata by accelerating the synthesis and release of endogenous NO. Nitric oxide as an endothelium-derived relaxing factor, mediates its biological effects by activating soluble guanylyl cyclase and increasing cyclic GMP synthesis from GTP and decreasing the concentration of  $\text{Ca}^{2+}$ . These reactions result in the strong relaxation of the lymphatic stomata, with the area and the distribution densities of the lymphatic stomata enlarged, which would lead to the drainage of ascites from the peritoneal cavity.

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