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Effect of Danshen on the Zero-Stress State of Rat's Abdominal Aorta

Hui Han[*], David C. C. Lam[†] and Wei Huang[†,‡]

Abstract: The objective of our study was to study the effect of danshen, a Chinese herbal medicine known to prevent hypertension, on the zero-stress state of rat's abdominal aorta. The zero-stress state of a blood vessel represents the release of residual stress on the vessel wall, and is the basic configuration of blood vessel affected solely by intrinsic parameters. At the *in vivo* state, the rat's abdominal aorta was subjected to blood pressure and flow and longitudinal stress. After dissecting from the abdominal aorta, the aortic specimens were cut into small rings at no-load state, in which the internal pressure, external pressure, and longitudinal stress in a short ring-shaped segment were all zero; by cutting radially to release the residual stress in the wall, the vessel ring opened up into a sector quickly, and the sector's configuration would not change at 20 min after cutting and was defined as the zero-stress state of a blood vessel, which was characterized by its residual strain and opening angle. Then aqueous extract of danshen prepared with methanol was added in the Krebs solution, and the changes of the aorta's zero-stress state were monitored by taking photos routinely for analysis to determine the opening angle and residual strain. Additionally, other sets of samples were tested in a Norepinephrine-Krebs solution as positive control or a Krebs solution as negative control, respectively. It was demonstrated that the zero-stress state of rat's abdominal aorta was affected by danshen extract and norepinephrine in two different patterns, while the Krebs solution did not have similar effects. The present work provides a new approach to study the anti-hypertension effect and mechanism of danshen.

Keywords: opening angle, residual strain, residual stress, Chinese herbal medicine.

[*] Dept. of Health Technology and Informatics, Hong Kong Polytechnic University, Kowloon, Hong Kong

[†] Dept. of Mechanical Engineering, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong

[‡] Correspondent author. E-mail: mewhuang@ust.hk

1 Introduction

Danshen, the dried root of *Salvia miltiorrhiza*, is a traditional Chinese herbal medicine and has been widely used for the reduction of hypertension and cardiovascular protection (Bensky, Gamble, and Kaptchuk, 1993; Zhou, Zuo, and Chow, 2005; Yang, Wei, Lee, Chen, and Ueng, 2012).

The anti-hypertensive effects of aqueous extract of danshen include inhibition of angiotensin converting enzyme (Kang, Yun, Ryoo, and Lee, 2002) and generation of endothelial nitric oxide synthase (eNOS) and/or vasodilatation (Kim, Sánchez, Durán, Kanetaka, Durán, 2007). The vasorelaxant actions of danshen and its fractions were found to be related to the inhibition of $Ca_2{}^+$ influx in the vascular smooth muscle cells and a small component was mediated by the opening of K^+ channels (Lam, Yeung, Cheung, and Or, 2006). In addition, the effect of danshen on the reduction of blood pressure was found due to the improvement of erythrocyte rheology (Hou, Tsay, Liang, Lee, Wang, and Liu, 2007). More detailed molecular mechanisms on the effects of danshen are under investigation (Wang, Jiang, Wan, Yang, Zhang, Chen, Wang, Lai Zhao, Jiang, Sun, Zhong Ran, and Lu, 2013). However, how danshen affects the mechanical properties of blood vessel is not clear.

To study the mechanical properties of a blood vessel, one has to know the zero-stress state of the vessel wall, which is very different from the *in vivo* state and the no-load state of the vessel as shown in **Figure 1**. At the *in vivo* state, the blood vessels are subjected to blood pressure and flow and longitudinal stress (**Fig. 1A**). The zero-stress state of a blood vessel is obtained by cutting (Vaishnav and Vossoughi, 1983; Fung, 1984). After dissecting from the body and cutting into small rings, the arterial small rings are at no-load state (**Fig. 1B**), in which the internal pressure, external pressure, and longitudinal stress in a short ring-shaped segment are all zero; however, there are residual stress and strain in the wall of the segment. When such a ring is cut radially to release the residual stress in the wall, it usually opens up into a sector. The sector's configuration would not change after cutting and is defined as the zero-stress state of a blood vessel (**Fig. 1C**). The zero-stress state of a blood vessel is the basic configuration of blood vessel affected solely by intrinsic parameters (Vaishnav and Vossoughi, 1983; Fung, 1984).

The zero-stress state of a blood vessel can be described by its residual strain and opening angle, which is the angle subtended between two radii, with origin located at the midpoint, and tips at the outer edges of the endothelium (**Fig. 1C**). The zero-stress state of blood vessels has been found affected by the tissue remodeling of blood vessels in hypertension (Fung and Liu, 1991; Huang, Sher, Delgado-West, Wu, Peck, and Fung, 2001), diabetes (Liu and Fung, 1992), and cigarette smoking (Liu and Fung, 1993). The residual strain and residual stress in aorta of hyperten-

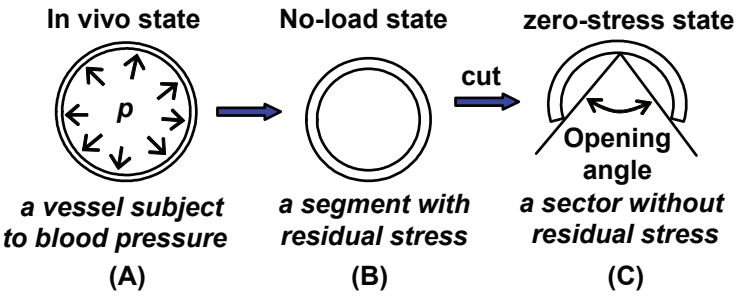

Figure 1: Definitions of *in vivo* state (A), no-load state (B), and zero-stress state (C) and opening angle (C) of a blood vessel

sive rats were changed rapidly and significantly (Liu and Fung, 1989; Matsumoto and Hayashi, 1996). In the present study, we evaluated the effect of aqueous abstract of danshen on the zero-stress state of rat's abdominal aorta.

2 Methodologies

2.1 Extraction of Danshen (Salvia miltiorrhiza)

Dried danshen herb purchased from a local store was used in the present study. To prepare for total extract of danshen, a total of 200 g of the powdered danshen herb was extracted with 250 ml methanol for 30 min by sonication. The supernatant was obtained by suction filtration. The sample was reextracted twice with 250 ml methanol and the supernatants were combined. The residue was then transferred into a 2 L round bottomed flask and refluxed with 1 L of water for 30 min. The suspension was filtered and supernatant was combined with the above methanol solution. The combined solution was dried by a rotary evaporator and 70 g total extract powder was obtained by freeze-drying. For hydrophilic and lipophilic layers, 33.3 g total extract powder was first suspended in 100 ml water and partitioned with 50 ml ethyl actate (EtOAc) 3 times. The two layers were dried by a rotary evaporator respectively. Hydrophilic layer powder of danshen obtained by freeze-drying was prepared for aqueous extract in the present study.

2.2 Tissue Sample Preparation and Experimental Setup

Sprague Dawley rats, male, around 350g, were used. The protocol was approved by the Hong Kong Polytechnic University Committee on Animal Subjects Ethics. Each rat was anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). After a bolus of normal saline with 1000 U/kg body

weight of heparin (1000 U/ml) was injected, the blood in cardiovascular system was washed out by vascular perfusion of 0.1 M physiological saline buffer (PBS) for 5 min under a perfusion pressure of 80 mm Hg in the left ventricle and a draining pressure of 0 mm Hg at the right atrium. Then the abdominal aorta was isolated from surrounding tissues for dissection. After dissecting from the abdominal aorta between the renal artery and the bifurcation of left and right ileal arteries, the aortic specimens were cut into small rings with about 1-mm height; and the rings were placed on the small wells in a sample box filled with Krebs solution bubbled with a gas of 95% O_2-5% CO_2 at 37^{o}C (**Figure 2**). The temperature of Krebs solution in the heating box was kept at 37^{o}C during experiment with a temperature control system consisted of a stirring hot plate (Cole-Parmer, Vernon Hill, Illinois, USA) and an ANC 675 Thermal Controller (Yeou Jenq Electric Co., Taiwan).

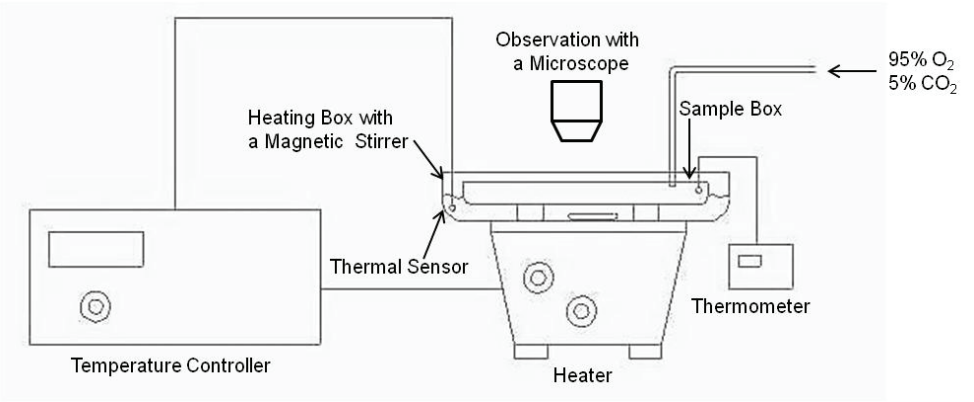

Figure 2: Schematic drawing of experimental setup.

The arterial small rings were at no-load state. By cutting radially to release the residual stress in the wall, the vessel ring opened up into a sector quickly. A series of photos were taken under an Olympus zoom stereo microscope (model: SZH, Olympus Co., Lake Success, New York, U.S.A.) with a Canon digital camera (model: EOS Digital Rebel, Canon Co., Tokyo, Japan). At 20 min after cutting, the sector's configuration would not change and was defined as the zero-stress state of a blood vessel (**Figure 1**).

2.3 Effects of Danshen Aqueous Extract and Norepinephrine

While the vascular sectors were at zero-stress state, the aqueous extract of danshen prepared with methanol was added in the Krebs solution with a final concentration of 1 mg danshen/ml. Additionally, other sets of samples were tested in a solution

of 10^{-5} Mol/L Norepinephrine in Krebs solution as positive control and a Krebs solution as negative control, respectively. After taking photos at time, danshen or norepinephrine was added into Krebs solution. The changes of the aorta's zero-stress state after adding danshen or norepinephrine were monitored by taking photos routinely up to 60 min, respectively, for analysis to determine the opening angle and residual strain of the aorta at the zero-stress state.

2.4 Determination of Residual Strain

For constitutive equations of soft tissue involving finite strain, Green's strain is used (Fung, 1984). In circumferential direction, Green's strain, ε, is related to the circumferential stretch ratio λ by the equation:

$$\varepsilon = \left(\lambda^2 - 1\right)/2. \tag{1}$$

The circumferential stretch ratio for the inner wall, λ_{iw} is

$$\lambda_{iw} = l_{iw}^{no-load}/l_{iw}^{zero-stress}, \tag{2}$$

where $l_{iw}^{no-load}$ is the circumferential length of inner wall at no-load state and $l_{iw}^{zero-stress}$ is the circumferential length of inner wall at zero-stress state. Similarly, the circumferential stretch ratio for the outer wall, λ_{ow} is

$$\lambda_{ow} = l_{ow}^{no-load}/l_{ow}^{zero-stress}, \tag{3}$$

where $l_{ow}^{no-load}$ is the circumferential length of the outer wall at no-load state and $l_{ow}^{zero-stress}$ is circumferential length of the outer wall at zero-stress state. From the difference of the vessel geometry between the zero-stress state and the no-loaded state, we computed the strains in the inner wall, ε_{iw} and outer wall, ε_{ow}, of the blood vessel by

$$\varepsilon_{iw} = \left(\lambda_{iw}^2 - 1\right)/2, \quad \varepsilon_{ow} = \left(\lambda_{ow}^2 - 1\right)/2. \tag{4}$$

$\varepsilon_{\imath\omega}$ and $\varepsilon_{o\omega}$ are the residual strains in the inner wall and outer wall, respectively.

3 Results

The opening angles and the circumferential lengths along the inner and outer wall of the vascular sectors at the zero-stress state of abdominal aorta at time and *0.17, 0.5, 1, 1.5, 5, 10, 20,* and 60 minutes after adding the aqueous extract of danshen (1 mg danshen/ml) in Krebs solution are summarized in **Table 1** and **Table 2**, respectively. The residual strains on the inner wall $\varepsilon_{\imath\omega}$ and outer wall $\varepsilon_{o\omega}$ were computed based on Eqs. (2)-(4), and the results are presented in **Table 3**.

Table 1: Opening angles (degree) at the zero-stress state of abdominal aorta after adding the aqueous extract of danshen (1 mg danshen/ml) after time *0*

Time (minute)	Rat #1 (degree)	Rat #2 (degree)	Rat #3 (degree)	Rat #4 (degree)
0	103	112	169	98
0.17	103	117	172	100
0.5	113	118	188	99
1	132	127	190	135
1.5	132	150	192	144
5	109	133	181	139
10	103	122	176	121
20	103	118	171	112
60	103	116	187	104

Table 2: The circumferential lengths (mm) of inner and outer wall at the zero-stress state of abdominal aorta after adding the aqueous extract of danshen (1 mg danshen/ml) after time *0*

Time	Rat #1		Rat #2		Rat #3		Rat #4	
(min)	inner	outer	inner	Outer	inner	outer	inner	outer
No-load	2.98	3.41	2.98	3.52	2.79	3.36	2.94	3.37
0	3.43	3.49	3.56	3.62	3.10	3.09	3.78	3.82
0.17	3.43	3.49	3.55	3.57	3.11	3.12	3.54	3.64
0.5	3.35	3.41	3.52	3.56	3.07	3.01	3.64	3.73
1	3.34	3.48	3.47	3.51	2.98	2.94	3.57	3.66
1.5	3.42	3.42	3.44	3.48	2.99	2.99	3.40	3.56
5	3.51	3.51	3.46	3.50	2.98	2.98	3.43	3.63
10	3.64	3.65	3.57	3.64	3.13	2.99	3.46	3.79
20	3.64	3.70	3.60	3.66	3.20	3.01	3.50	3.65
60	3.70	3.76	3.57	3.70	3.40	3.34	3.65	3.73

The opening angles at the zero-stress state of abdominal aorta at time *0* and *0.17, 0.5, 1, 1.5, 5, 10, 20,* and 60 minutes after adding norepinephrine (10^{-5} Mol/L) in Krebs solution were summarized in **Table 4** Along with the changes along the time in responding to norepinephrine, the circumferential lengths and the residual strains along the inner and outer wall of the vascular sectors at zero-stress state were changed as well, and their data were presented in **Table 5** and **Table 6**, respectively. The residual strains on the inner wall $\varepsilon_{\iota\omega}$ and outer wall $\varepsilon_{o\omega}$ were computed based

Table 3: The residual strains of inner and outer wall at the zero-stress state of abdominal aorta after adding the aqueous extract of danshen (1 mg danshen/ml) after time *0*

Time	Rat #1		Rat #2		Rat #3		Rat #4	
(min)	inner	outer	inner	Outer	inner	outer	inner	outer
0	0.16	0.02	0.21	0.03	0.12	-0.08	0.33	0.14
0.17	0.16	0.02	0.21	0.01	0.12	-0.07	0.22	0.08
0.5	0.13	0.00	0.20	0.01	0.11	-0.10	0.27	0.11
1	0.13	0.02	0.18	0.00	0.07	-0.12	0.24	0.09
1.5	0.16	0.00	0.17	-0.01	0.07	-0.10	0.17	0.06
5	0.19	0.03	0.17	-0.01	0.07	-0.11	0.18	0.08
10	0.25	0.07	0.22	0.03	0.13	-0.10	0.19	0.13
20	0.25	0.09	0.23	0.04	0.16	-0.10	0.21	0.09
60	0.27	0.11	0.22	0.05	0.24	-0.01	0.27	0.11

Table 4: Opening angles (degree) at the zero-stress state of abdominal aorta after adding norepinephrine (10^{-5} Mol/L) after time *0*

Time (minute)	Rat #1 (degree)	Rat #2 (degree)	Rat #3 (degree)	Rat #4 (degree)
0	60	105	134	116
0.17	62	107	156	146
0.5	70	113	232	161
1	143	207	234	167
1.5	161	262	240	186
5	174	319	248	240
10	172	318	246	236
20	171	320	253	242
60	174	319	252	246

on Eqs. (2)-(4).

The opening angles of the aorta at zero-stress state in Krebs solution were summarized in **Table 7**. As shown in **Figure 3**, the opening angle of the aorta in Krebs solution did not change much by comparing with the ones after adding danshen extract and norepinephrine in Krebs solution, respectively.

The circumferential lengths along the inner and outer wall of the vascular sectors at the zero-stress state of abdominal aorta in Krebs solution are summarized in **Table 8**. The residual strains on the inner wall $\varepsilon_{\iota\omega}$ and outer wall $\varepsilon_{o\omega}$ were computed

Table 5: The circumferential lengths (mm) of inner and outer wall at the zero-stress state of abdominal aorta after adding norepinephrine (10^{-5} Mol/L) after time *0*

Time	Rat #1		Rat #2		Rat #3		Rat #4	
(min)	inner	outer	inner	outer	inner	outer	inner	outer
No-load	3.65	3.87	3.07	3.45	2.85	3.25	3.24	3.85
0	3.81	3.98	3.37	3.58	3.31	3.23	3.59	3.61
0.17	3.78	3.90	3.36	3.56	3.11	2.89	3.36	3.48
0.5	3.73	3.79	3.27	3.54	2.78	2.86	3.14	3.21
1	3.42	3.60	3.16	3.22	2.76	2.84	3.09	3.15
1.5	3.38	3.44	3.07	2.98	2.73	2.68	2.95	2.90
5	3.27	3.41	2.89	2.67	2.71	2.61	2.91	2.89
10	3.14	3.35	2.80	2.67	2.71	2.65	2.95	2.83
20	3.06	3.38	2.88	2.67	2.71	2.63	2.91	2.81
60	3.12	3.38	2.80	2.67	2.70	2.63	2.94	2.88

Table 6: The residual strains of inner and outer wall at the zero-stress state of abdominal aorta after adding norepinephrine (10^{-5} Mol/L) after time *0*

Time	Rat #1		Rat #2		Rat #3		Rat #4	
(min)	inner	outer	inner	outer	inner	outer	inner	outer
0	0.04	0.03	0.10	0.04	0.17	-0.01	0.11	-0.06
0.17	0.04	0.01	0.10	0.03	0.10	-0.10	0.04	-0.09
0.5	0.02	-0.02	0.07	0.03	-0.02	-0.11	-0.03	-0.15
1	-0.06	-0.07	0.03	-0.06	-0.03	-0.12	-0.05	-0.17
1.5	-0.07	-0.10	0.00	-0.13	-0.04	-0.16	-0.09	-0.22
5	-0.10	-0.11	-0.06	-0.20	-0.05	-0.18	-0.10	-0.22
10	-0.13	-0.13	-0.08	-0.20	-0.05	-0.17	-0.09	-0.23
20	-0.15	-0.12	-0.06	-0.20	-0.05	-0.17	-0.10	-0.23
60	-0.13	-0.12	-0.08	-0.20	-0.05	-0.17	-0.09	-0.22

based on Eqs. (2)-(4), and the results are listed in **Table 9**.

As shown in **Figure 3**, after adding the aqueous extract of danshen in Krebs solution, the opening angles of rat's abdominal aorta were increased immediately, but then gradually decreased from its maximum at time 1.5 minutes. The opening angles of the aorta were increased immediately after adding norepinephrine in Krebs solution, and remained constant thereafter for 60 minutes, while the opening angle of the aorta in Krebs solution did not change much. Each point presents with the mean value and its standard deviation of the changes of opening angle (%) at time

Table 7: Opening angles (degree) at the zero-stress state of abdominal aorta in Krebs solution after time *0*

Time (minute)	Rat #1 (degree)	Rat #2 (degree)	Rat #3 (degree)	Rat #4 (degree)
0	39	93	131	90
5	40	94	134	96
10	40	95	130	93
20	38	93	130	86
60	40	95	129	90

Table 8: The circumferential lengths (mm) of inner and outer wall at the zero-stress state of abdominal aorta in Krebs solution

Time	Rat #1		Rat #2		Rat #3		Rat #4	
(min)	inner	outer	inner	outer	inner	outer	inner	outer
No-load	3.17	3.75	2.89	3.28	2.80	3.25	2.83	3.44
0	3.54	3.89	3.62	3.61	2.70	2.84	3.31	3.38
5	3.59	3.87	3.61	3.61	2.70	2.87	3.30	3.36
10	3.56	3.86	3.62	3.61	2.71	2.84	3.30	3.36
20	3.60	3.90	3.62	3.61	2.71	2.86	3.29	3.36
60	3.59	3.98	3.62	3.60	2.71	2.87	3.32	3.37

Table 9: The residual strains of inner and outer wall at the zero-stress state of abdominal aorta in Krebs solution

Time	Rat #1		Rat #2		Rat #3		Rat #4	
(min)	inner	outer	inner	outer	inner	outer	inner	outer
0	0.12	0.04	0.28	0.11	-0.04	-0.12	0.18	-0.02
5	0.14	0.03	0.28	0.11	-0.04	-0.11	0.18	-0.02
10	0.13	0.03	0.28	0.11	-0.03	-0.12	0.18	-0.02
20	0.14	0.04	0.28	0.11	-0.03	-0.11	0.18	-0.02
60	0.14	0.06	0.28	0.10	-0.03	-0.11	0.19	-0.02

t with respect to time 0.

4 Discussion

It was shown in the previous studies that the larger opening angles were associated with larger residual stress in the vessel wall (Vaishnav and Vossoughi, 1983; Fung, 1984; Fung and Liu, 1991; Liu and Fung, 1992; Liu and Fung, 1993). Nore-

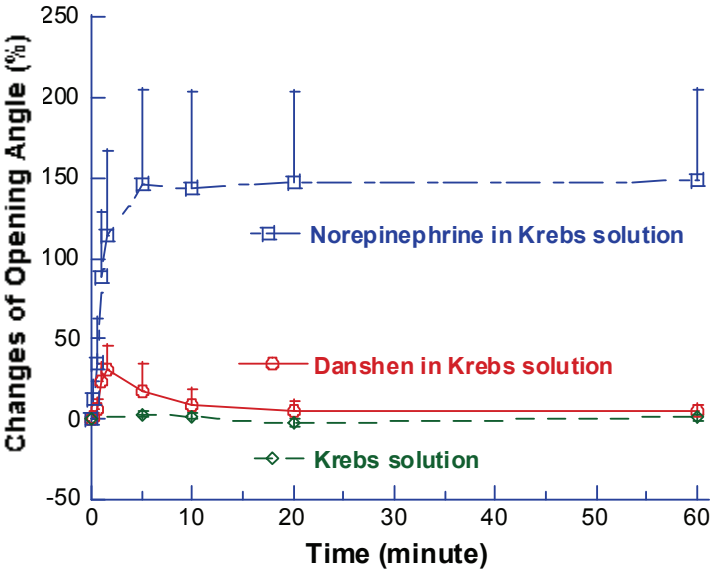

Figure 3: Changes of opening angle (%) at the zero-stress state of rat's abdominal aorta after adding danshen extract or norepinephrine in Krebs solution.

pinephrine binding α_1 receptors cause vasoconstriction, i.e., the contraction of vascular smooth muscle cells decreasing the diameter of the blood vessel (O'Neil, 2006). The aqueous extract of danshen causes vasodilation, i.e., the relaxation of vascular smooth cells increasing the diameter of the blood vessel (Kang, Yun, Ryoo, and Lee, 2002). The mechanisms of vasodilation by danshen include inhibition of angiotensin converting enzyme (Kang, Yun, Ryoo, and Lee, 2002) and generation of endothelial nitric oxide synthase (eNOS) and/or vasodilatation (Kim, Sánchez, Durán, Kanetaka, Durán, 2007), which rely on the existence of endothelium on the vessel wall. Recent studies focused on the components or fractions of danshen (Zhou, Zuo, and Chow, 2005), e.g., Caffeic acid (3,4-dihydroxycinnamic acid) (Jiang, Laua, Hon, Mak, Woo and Fung, 2005), Sodium Tanshinone IIA Sulfonate (Wang, Jiang, Wan, Yang, Zhang, Chen, Wang, Lai, Zhao, Jiang, Sun, Zhong, Ran, and Lu, 2013), etc.

In the present study, the opening angles of rat's abdominal aorta at zero-stress state were increased immediately after adding norepinephrine in Krebs solution and remained constant for 60 minutes (**Figure 3**), which indicates that the increase of opening angle is related to the contraction of vascular smooth muscle cells on the vessel wall. After adding the aqueous extract of danshen in Krebs solution, the opening angle of the aorta at zero-stress state was increase quickly and reached its

maximum around 1.5 minutes, then gradually decreased. The changes in opening angles by the aqueous extract of danshen might cause by the vasoconstriction and vasodilation of danshen. The mechanism to cause transient vasoconstriction effect of danshen is not clear, and it is not clear whether this kind of vascular action relies on endothelium. It is worthy of further studies that may lead to new mechanism discovery.

The present work showed the advantage by monitoring the changes of opening angles and residual strains of blood vessel at zero-stress state to study the effect of danshen in blood vessels, since the changes in zero-stress state reflect the response of vascular smooth muscle cells, and the mechanical properties and tissue remodeling of blood vessel (Fung and Liu, 1991; Liu and Fung, 1992; Liu and Fung, 1993; Huang, Sher, Delgado-West, Wu, Peck, and Fung, 2001). It is not clear how danshen affect the tissue remodeling of blood vessel in hypertension or other cardiovascular diseases. More *in vivo* and *in vitro* studies are needed to help us better understanding the anti-hypertension effect and mechanism of danshen.

5 Conclusion

The approach by monitoring the changes of opening angles and residual strains of blood vessel at zero-stress state was applied to study the effects of aqueous extract of danshen. After adding danshen extract in Krebs solution, the opening angles of rat's abdominal aorta at zero-stress state were increased immediately, but then followed with a pattern differed from the one with norepinephrine. These results may help us better understanding the effects of danshen on the mechanical properties of blood vessels. More *in vivo* and *in vitro* studies are needed to study the anti-hypertension effect and mechanism of danshen on the tissue remodeling and zero-stress state of blood vessels

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