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Original Article

Hemodynamic study about laminar shear stress extenuate cerebral ischaemia-reperfusion injury

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Abstract: Objective: In this study, we explored whether laminar shear stress (LS) had an effect on the apoptosis of rat brain microvascular endothelial cells (rBMECs) during the course of cerebral ischaemia-reperfusion injury (CIRI). Methods: First, blood flow changes in CIRI were studied by Laser Doppler flowmetry *in vivo*. Then, the oxygen/glucose deprivation (OGD) model was adopted to cause ischaemia in rBMECs for 6 hours *in vitro*. Flow intervention without nutrition and an LS force magnitude of 0.8±0.05 dynes/cm^2 were delivered for the last 2 hours to simulate the single factor effect of LS on reperfusion. Results: We found that cell apoptosis was significantly relieved under haemodynamic intervention with Annexin V-FITC/PI and Hoechst 33258 analysis. In terms of molecular mechanisms, it is possible that LS could activate the cytomembrane surface receptor Tie-2 and cause downstream inhibition of the caspase-3 apoptosis pathway. Conclusion: LS should be a protective factor for CIRI.

Keywords: Cerebral ischaemia-reperfusion injury, laminar shear stress, rat brain microvascular endothelial cells, apoptosis

Introduction

Ischaemic stroke is a common disease characterized by a series of pathological changes in brain tissue and neurological disorders. The main cause of ischaemic stroke is thrombus formation in the cerebrovasculature, which blocks the blood supply to the brain [1]. Although recovering or increasing the blood supply to the ischaemic region is one of the essential treatments for the disease [2], immediate reperfusion may lead to cerebral ischaemia-reperfusion injury (CIRI) [3]. CIRI is a syndrome in which brain function deteriorates further as the blood supply recovers after ischaemia. Previous studies indicating that multiple mechanisms, including excessive formation of free radicals, excitotoxicity, intracellular calcium overload, and immune vascular inflammatory reactions, play key roles in CIRI [4]. However, there are few reports on the effects of laminar shear stress (LS) on CIRI.

As the major component of blood vessel endothelium, brain microvascular endothelial cells (BMECs) make direct contact with blood flow. Beyond supplying nutrients, blood flow has unavoidable mechanical effects on BMECs [5]. Among the various types of mechanical force produced by haemodynamics, the function of LS is crucial in the study of mechanics [6]. LS induced by blood flow is a tangential component of force parallel with the vessel long axis and flow direction [7]. The function of LS is constant while the blood is flowing and is in proportion to flow velocity when other conditions are constant [8]. There have been extensive studies about the power transmission mechanism of LS. These studies indicated that certain receptors on the cell membrane could directly sense changes in LS and then transform the mechanical signal into a biological signal to affect endothelial structure, function and survival [9-12]. Interestingly, previous studies showed that increasing LS could extend

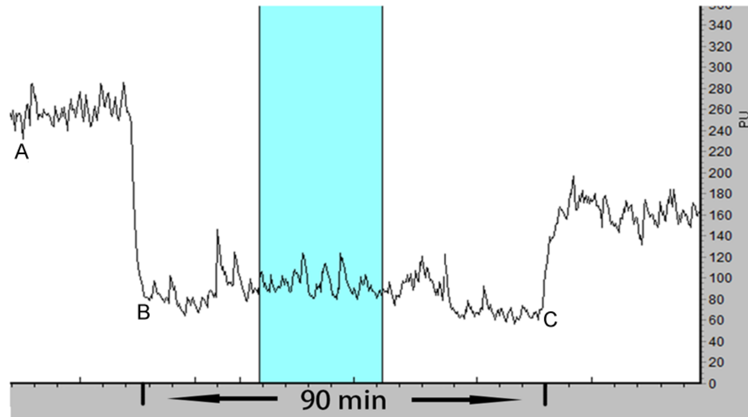


Figure 1. Cerebral blood flow tested with Laser Doppler flowmetry. A: Detected normal blood flow before MCAO in rats. B: Blood flow after MCAO. C: Blood flow after reperfusion. B, C: Ischaemia lasted for 90 minutes.

the normal life cycle of cardiovascular endothelial cells, reduce the speed of metabolism, decrease intracellular activity and slow the response to outside stimulation. When cells were in ischaemic status, such as heart failure or myocardial infarction, the speed and degree of apoptosis were also reduced, and LS had a protective effect [8, 13]. On the basis of these findings, we investigated the function and mechanism of LS in CIRI.

Apoptosis is a dynamic process and can be regulated by a series of genes and proteins [14, 15]. In ischaemic stroke studies, apoptosis usually occurred in the penumbra region. Previous research indicated that at certain times after ischaemia, brain tissue in the ischaemic core region would become necrotic, which occurred rapidly and was not reversible. Around the core region was the penumbra region, and cells in this portion would become apoptotic, which could be blocked by emergency measures [11, 16, 17]. Cell apoptosis is the most important injury type in CIRI [18], and BMECs are the most common cells involved. Tie-2 is a tyrosine kinase receptor expressed especially on the membrane of endothelial cells [19, 20]. Studies have proved that Tie-2 participated in regulation of apoptosis in endothelial cells through inhibition of nucleus lysing by the caspase family [21, 22]. Another open question is whether LS could affect apoptosis of BMECs in CIRI.

To identify the effect and mechanism of LS on BMECs when CIRI occurs, blood flow changes in CIRI were studied with Laser Doppler flowmetry *in vivo*. Then, the oxygen/glucose deprivation (OGD) model [23] was adopted to induce apoptosis of rat BMECs (rBMECs) for 6 hours *in vitro*. The OGD method involves simulating ischaemia by removing oxygen, glucose, and sodium pyruvate, which are necessary for aerobic metabolism and respiration. A parallel-plate flow chamber [24] was used to deliver appropriate LS on rBMECs during the last 2 hours of the 6 hour OGD intervention. This technique has been extensively applied to investigate the function of LS [25, 26]. Cell apoptosis was observed with Annexin V-FITC/PI [27] and Hoechst 33258 [28], and the protein expression of Tie-2 and Caspase-3 were detected with a western blot.

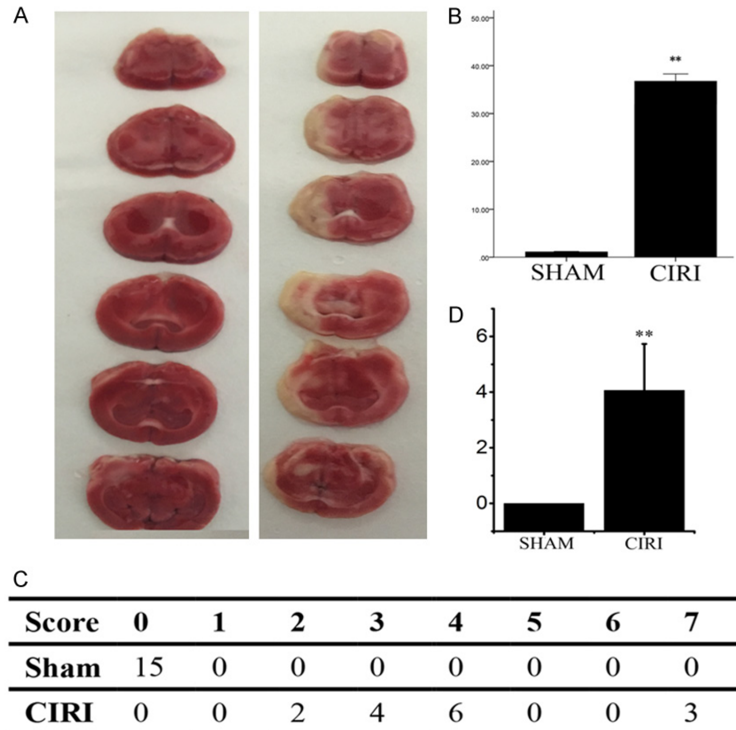
Materials and methods

Middle cerebral artery occlusion model built in rats [29]

Adult male Sprague-Dawley rats (250-270 g) were used and randomly grouped into the sham group and CIRI group (n=15 rats in each group). Right middle cerebral artery occlusion (MCAO) was used to simulate CIRI in rats. The method was established by Longa et al. [29]: when the rats were anaesthetized, an intraluminal nylon monofilament entered the middle cerebral artery following the external carotid artery and internal carotid artery. At this time, due to the middle cerebral artery occlusion, cerebral infarction was induced. The blood flow was recovered for reperfusion with the removal of the nylon monofilament after 90 minutes. The sham group underwent an identical process without arterial occlusion.

Cerebral blood flow was detected in the process of CIRI [30]

The probe of the Laser Doppler flowmetry (PriFlux System 5000, PerimedAB company) was fixed on the surface of the rat brain motor cortex after anaesthesia. The brain blood flow of the rats was observed by the device to record the normal blood flow levels. Then, MCAO was



Score	0	1	2	3	4	5	6	7
Sham	15	0	0	0	0	0	0	0
CIRI	0	0	2	4	6	0	0	3

Figure 2. The brain injury of CIRI. A, B: Infarct volume between the sham group and the CIRI group at the 7th day and the statistics (**$p<0.01$). C, D: Neurobehavioral scores described. The score of each rat in the sham group and CIRI group before MCAO was 0. At the 7th day, the score of each rat in the sham group was also 0. However, the scores of the rat in the CIRI group were 2 points for 2 rats, 3 points for 4 rats, 4 points for 6 rats and 7 points for 3 rats. Statistical analysis of scores between two groups at the 7th day (**$p<0.01$).

given to the rats, when the nylon monofilament was inserted into the middle cerebral artery, and the blood flow value was recorded again. After 90 minutes, the nylon monofilament was removed, and the blood flow after reperfusion was also recorded.

The determination of hemiplegia in rats [31]

Severity of hemiplegia caused by the right MCAO can be evaluated based on the Rogers method [31]. The score was divided into 0-7 points. A 0 point score indicated no hemiplegia. The higher the score, the more serious the hemiplegia. Most rats scored 3-4 points at 7 days after MCAO injury.

2,3,5-triphenyltetrazolium chloride (TTC) was used to observe the infarction volume [32]

At 7 days after MCAO, rat brain tissues were obtained and then stained with 2% TTC perfusion after the hemiplegia assessment. Six coronal slices of 2 mm thickness were cut and captured. Under normal circumstances, normal brain tissue is bright red, while the infarct tissue is white.

Culture of rBMECs

rBMECs were cultured according to the relevant literature [33, 34]. The brain tissues of Sprague-Dawley (SD) rats (50-60 g) were cut, homogenized and extracted over an 80 mesh screen. Cells were isolated and purified with collagenase digestion. At last, cells were cultured in high glucose DMEM medium of 20% FBS.

Identification of rBMECs angiogenesis with tube-form test [35]

The culture plate was pre-coated with Matrigel (BD Biosciences) at 37°C for 30 min. Cells were suspended in high glucose DMEM medium and incubated in the culture plate and underwent tube formation after 18 h.

OGD and LS intervention

The OGD model was adopted for cell ischaemia intervention [23]. The cells were cultured in the parallel-plate flow chamber [24]. Sugar-free culture DMEM medium liquid with 5% CO_2 and 95% N_2 gas was used to provide shear stress during the OGD period of 4-6 hours and the LS force was 0.8 + 0.05 dynes/cm^2.

Annexin V-FITC/PI apoptosis analysis [27]

Cultured cells were dyed with Annexin V-FITC and PI at room temperature for 15 min, and flow cytometry was used to detect the staining.

Hoechst 33258 apoptosis analysis [28]

Cultured cells were dyed with Hoechst 33258 at 4°C for 30 min, and a fluorescence microscope was used to detect the staining.

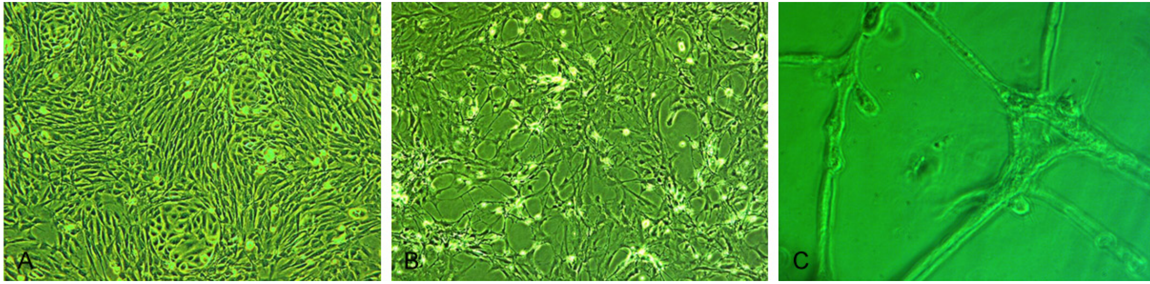


Figure 3. Characterization of rBMECs. A: The primary cells formed a monolayer at the 14th day (100×). B: Apoptotic cells shrinkage (100×). C: The cells cultured on Matrigel formed a tube shape (400×).

Western blot

After extraction of total protein, the protein was incubated with first and two resistances successively after electrophoresis and transfer membrane. The proteins of Tie-2 and caspases-3 were detected on the SDS-PAGE gel with the bioautography technique. The reference protein was β-actin.

Statistical analysis

Data were evaluated with a SPSS 20.0 statistical package. ANOVA was used to determine differences among the groups. $P<0.05$ was considered statistically significant.

Results

Cerebral blood flow changes during CIRI in rats

A model of CIRI was induced by middle cerebral artery occlusion (MCAO) [29] with an intraluminal nylon monofilament to block the middle cerebral blood vessel and reperfusion by removing the monofilament in rats. The probe of the Laser Doppler flowmetry system [30] (PriFlux System 5000, PerimedAB company) was fixed on the surface of the rat brain motor cortex to record the blood flow. As shown in **Figure 1**, when the monofilament was inserted into the middle cerebral artery (**Figure 1B**), blood flow fell sharply and stabilized at approximately 10%-20% of the original volume. After 90 minutes, the monofilament was removed, and blood flow increased rapidly (**Figure 1C**). Blood flow during the stable period did not return to the original levels and only reached approximately 70%-90% of the original levels. Our test confirmed that the reperfusion flow was lower than the normal level before MCAO.

Pathological and behavioural effects of CIRI in rats

The brain injury was stable for 7 days after MCAO. The Rogers method (0-7 scores) was used to assess neural function. The results showed that 15 rats had no action defects and hemiplegia in the CIRI group, and the score of each mouse was 0 before MCAO. However, 7 days after CIRI, there was significant hemiplegia, with a statistically significant difference ($P<0.01$) between the CIRI and sham groups (**Figure 2C**, **2D**). We also detected cerebral infarction volume with TTC staining. We saw that the cerebral infarction was not coloured after CIRI. Compared with the normal rat brain, the volume of cerebral infarction was obvious ($p<0.01$) (**Figure 2A**, **2B**). The severity of the brain injury was evident from the behavioural and anatomical assessments. Thus, With the occurrence of CIRI, the decrease of blood flow would lead to pathological and behavioral damages in rats.

Characterization of rBMECs and the ischaemic response

The morphological features of rBMECs under the microscope were polygonal or fusiform with an irregular arrangement and had a typical paving stone structure when integrated (**Figure 3A**). These characteristics are consistent with previous reports [12, 34, 35]. Cells shrink during apoptosis (**Figure 3B**). The components of Matrigel were close to the substrate of the growth environment of endothelial cells, and the cells seeded on it became tubular [35]. As shown in **Figure 3C**, we could see that the endothelial cells cultured on Matrigel had the characteristics of tube formation. Then we had an anoxic intervention on these active cells. we

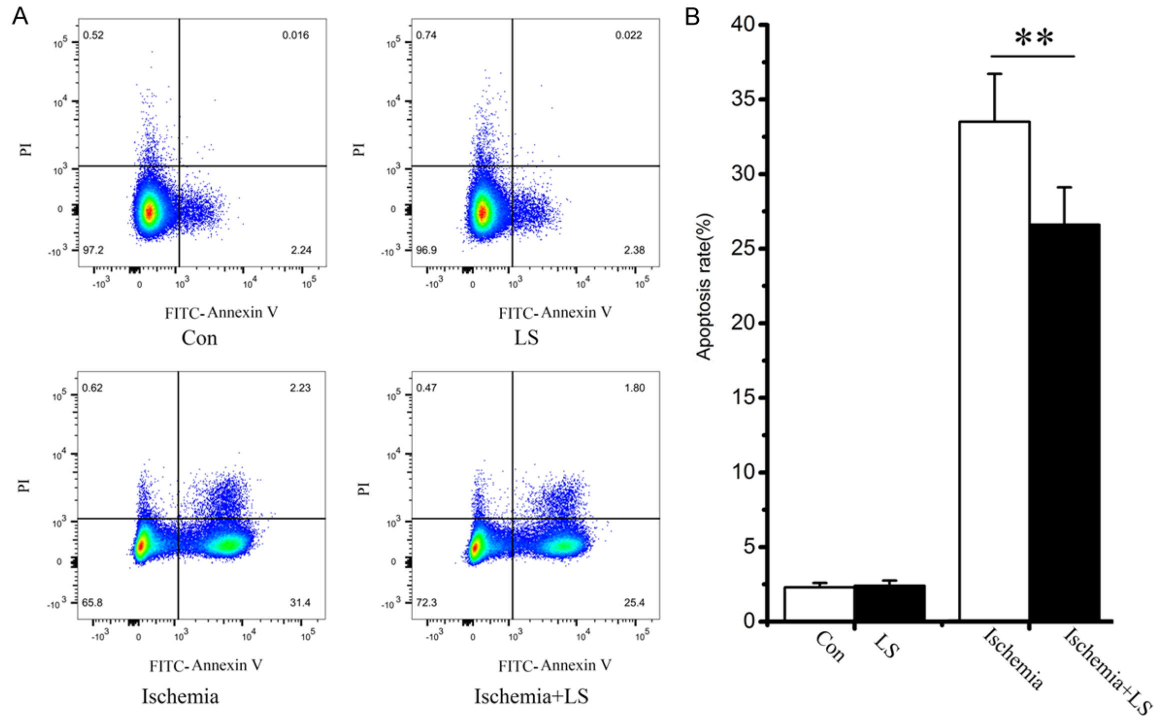


Figure 4. Effect of haemodynamics with Annexin V-FITC/PI staining. A: Cells were incubated with Annexin V-FITC/PI and analysed by flow cytometry; B: The apoptotic trend between groups (**p<0.01).

used OGD to induce cell apoptosis at different times. Apoptosis after 2 h of OGD was not obvious, but it increased significantly at 4-6 h. After 8 h, the cells were almost completely dead and few were affixed to the culture plate. The most appropriate LS intervention time was between 4 and 6 hours for following experiment.

Effects of haemodynamics on normal cells and CIRI cells

We found the recovery level of brain blood flow in rats was below 90% of the normal level when reperfusion after CIRI happened (**Figure 1**). LS in the microvasculature must be smaller than 1 dynes/cm^2 [8]. Therefore, in this *in vitro* experiment, a flow shear stress at the level of 0.8±0.05 dynes/cm^2 was chosen.

Normal cultured cells (control group/Con group) were given haemodynamic intervention for 2 hours (LS group), and the shear stress was 0.8 + 0.05 dynes/cm^2. Apoptosis was detected by Annexin V-FITC/PI flow cytometry and Hoechst 33258 staining. As shown in **Figures 4** and **5**, there was no statistically significant difference in apoptosis under the action of flow force. OGD was used to simulate ischaemia for a total of 6 hours (Ischaemia group). At the same time, the cells were given haemodynamic intervention for 2 hours after 4-6 hours of OGD (Ischaemia + LS group/CIRI group), and the force was 0.8 + 0.05 dynes/cm^2. The intervention liquid had no oxygen, glucose or other nutrients to ensure that the haemodynamics was the only intervening factor during CIRI. Apoptosis as detected by Annexin V-FITC/PI and Hoechst 33258 is shown in **Figures 4** and **5**. The apoptosis rate in the Ischaemia + LS group was decreased ($P<0.05$) compared with the Ischaemia group. These data suggest that haemodynamics might play a protective role in CIRI.

Effects of haemodynamics on protein expression

The cell's intervention is consistent with previous. We detected protein expression during apoptosis. As shown in **Figure 6**, compared with the Ischaemia group, the expression of Tie-2 increased, and the expression of Caspase-3 decreased in the Ischaemia + LS group ($P<0.05$). Caspase-3 is an important factor in the apoptotic pathway, and its downregulation

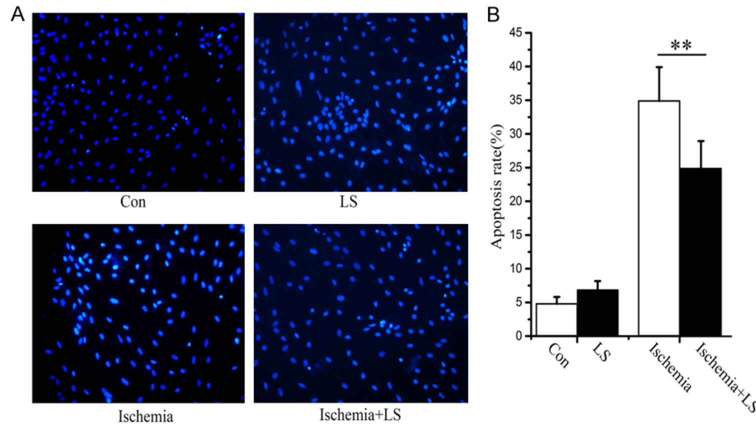


Figure 5. Effect of haemodynamics with Hoechst 33258 staining. A: Cells were incubated with Hoechst 33258 staining and examined with fluorescence microscopy; B: The apoptotic trend between groups (**p<0.01).

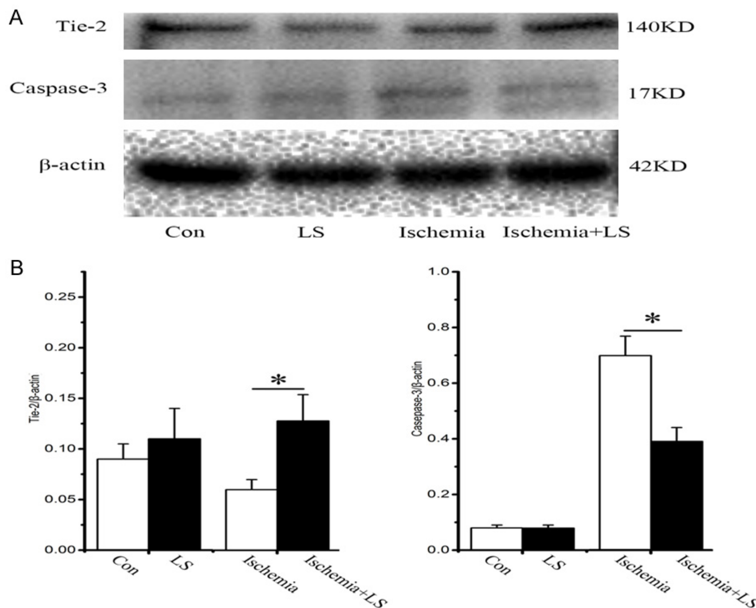


Figure 6. The statistics between groups about Tie-2 and Caspase-3 protein expressions by western blot (*p<0.05).

is an important marker of anti-apoptosis. Tie-2 in the cell membrane of endothelial cells is greatly influenced by haemodynamics. The upregulation of Tie-2 induced some mechanisms and ultimately affect cell apoptosis.

Discussion

In this study, we found that reperfusion of cerebral blood flow could only reach approximately 70%-90% of the normal levels. LS could reduce the apoptosis of rBMECs resulting from CIRI. This effect was associated with activation of receptor Tie-2 on the cell membrane by LS and downstream activation of the anti-caspase-3 apoptosis pathway. Haemodynamics is a protective external signal in CIRI.

Haemodynamics plays an essential role in the development of CIRI. Until now, there has been no research indicating the velocity of flow and the force of LS in cerebral microvasculature. However, previous research showed that the ranges of LS are 1-6 dynes/cm^2 in the vein system and 10-70 dynes/cm^2 in the artery system [8]. Therefore, LS in microvasculature must be smaller than 1 dynes/cm^2 in the vein system. In this work, we found that the recovery level of blood flow in rat brain was below 90% when reperfusion occurred in CIRI. This might be related to the self-regulatory function of blood vessels. When CIRI occurred, even though the blocked thrombus was effectively cleared at an early stage, the regulatory function of the impaired vessels could not recover instantaneously. The normal blood supply was also not restored for a long time, and even the blood flow volume decreased progressively [6, 36].

The transformation of mechanical force into chemical signals is the only mechanism of haemodynamic transmission [8, 37]. The function of LS also follows this principle. Studies indicated that LS activated the sensitive factors on the cells membrane at first and then activated intracellular genes and proteins through signal transduction chains, and altered the structure and function of the nucleus, mitochondria, endoplasmic reticulum and even the whole cell [9, 11, 38]. If the signal transduction pathways were blocked, mechanical signals would never be translated [39]. Previous studies also indicated that mechanical signal transduction pos-

sesses tissue and organ specificity, with cells in different tissues having different responses to LS [12]. Meanwhile, it has been revealed that Tie-2 is closely associated with the function and structure of the cell membrane. Tie-2 is a tyrosine kinase receptor that is mainly expressed on the vascular endothelial cells. Its major function is promoting communication between vascular smooth muscle cells, perithelial cells and endothelial cells to form the integral vascular wall to facilitate angiogenesis and maintain the stabilization of vascular structure [21, 40]. Studies in recent years found that Tie-2 could activate various protective factors to play an anti-apoptotic role when endothelial cells were impaired. For instance, Yin [41] found that Tie-2 could act on the PI3K/Akt pathway and Harfouche et al. [42] found that it influenced the MAPKs pathway. These pathways exerted an anti-apoptotic effect by inhibiting caspase lysing of the nucleus. Caspases were discovered to be a group of structurally relevant cysteine proteases in the cytosol. They participated in apoptosis through cascading activation of different subtypes [43]. For example, caspases 2, 8, 9 and 10 take part in the initiation of apoptosis, and then caspases 3, 6 and 7 execute the final steps of apoptosis. Among them, caspase 3 is the key factor that could inhibit DNA repair and initiate DNA degradation [22].

Until now, studies on LS and blood flow have mainly been conducted *in vitro*. This is because there is no suitable way to detect blood flow deep inside of the brain. Furthermore, in the blood circulation of the whole body, material substances, inflammatory factors and so on disturb the haemodynamic results [4]. Therefore, LS research is unrealistic *in vivo*. The parallel-plate flow chamber is a traditional research tool for studying LS *in vitro* [24-26]. In this study, it was found that 0.8±0.05 dynes/cm^2 LS did not lead to apoptosis of normal cultured endothelial cells. When LS was applied to the cells for 2 hours after 4-6 hours of ischaemia, the degree of apoptosis was less than that of cells subjected to ischaemia for 6 hours. Although LS could not completely reverse apoptosis in endothelial cells after CIRI, it shows delayed action to a certain extent. In this experiment, the expression of Tie-2 was in inverse proportion to the degree of apoptosis, while caspase-3 expression was in direct proportion to apoptosis. Tie-2 is a special receptor on the endothelium membrane and may be activated by haemodynamic forces, which then activates a series of anti-apoptosis pathways. Caspase-3 would be suppressed as well. Lysing of the nucleus and cell apoptosis would also be reduced. At later stages of the experiment, we also tried to add a Tie-2 inhibitor to study the relationship between Tie-2 and Caspase-3. However, due to the large amount of liquid that LS required, it was difficult to achieve.

In this paper, the role of haemodynamics in CIRI was studied. Studies *in vitro* have excluded the inevitable interference factors in animal studies.

Acknowledgements

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Disclosure of conflict of interest

None.

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