



# OPEN Knockdown of LncRNA *MEG3* promotes damage of vascular endothelial cells induced by vibration

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Hand–arm vibration syndrome (HAVS) is caused by long-term exposure to hand-transmitted vibration (HTV), and its pathogenesis has not been elucidated fully. We explored the molecular mechanism of HAVS and provided clues and a theoretical basis for the early prevention and treatment of HAVS. After vibration, samples were collected from the plasma of human workers, plasma of rat tails, and human umbilical vein endothelial cells (HUVECs). ELISAs were used to measure the expression of vasoactive factors. Cell Counting Kit-8 and electron microscopy were used to detect cell damage. Flow cytometry was employed to detect apoptosis. Real-time reverse transcription-polymerase chain reaction was used to measure the expression of long non-coding RNAs (lncRNAs). Western blotting was used to measure the expression of apoptosis-related proteins. Vibration could cause cell damage, apoptosis, and changes in the expression vasoactive factors and lncRNAs. The lncRNA maternally expressed gene 3 (*MEG3*) had a significant regulatory effect on cell damage, apoptotic proteins, and vascular regulatory factors in the HUVEC damage induced by vibration, as shown by the further decrease in viability and aggravation of injury after knockdown of *MEG3* expression in HUVECs treated with vibration. Expression of vasoactive factors and apoptosis-related proteins was changed after interfering with *MEG3* expression. In conclusion, vibration can affect the expression of vasoactive factors and lncRNA, and cause damage to vascular endothelial cells. *MEG3* may be involved in the inflammatory damage to vascular endothelial cells induced by vibration.

About 25% of the 3 billion workers worldwide are exposed to vibration in the workplace<sup>1</sup>. Currently, at least 2-million workers in China are exposed to hand-transmitted vibration (HTV) tools, and the main types of work are polishing, grinding, and sawing<sup>2</sup>. Long-term exposure to HTV can lead to spontaneous hand symptoms and even lesions in the nervous, circulatory, muscular, or skeletal systems. Serious cases can further develop into occupational hand–arm vibration syndrome (HAVS)<sup>3,4</sup>. HAVS can lead to the loss of the ability to work and live, bringing a huge economic burden to employers and the country. Vibration-induced white finger (VWF) is a typical manifestation of HAVS. HAVS development is irreversible. The pathogenesis of HAVS and influence on the vascular function of fingers have not been elucidated fully<sup>5</sup>.

Studies have shown that vibration-induced vascular endothelial dysfunction help to cause HAVS. Destruction of the structure and function of vascular endothelial cells as well as dysregulation of the production and secretion of vasoactive factors may be key factors in vibration-induced vascular injury<sup>6,7</sup>.

Long non-coding RNAs (lncRNAs) are considered to have crucial regulatory roles in the proliferation<sup>8,9</sup>, apoptosis of cells, and invasion by cells<sup>10,11</sup>. Wang et al. showed that a lncRNA, maternally expressed gene 3 (*MEG3*), may have an important role in the progression of endothelial dysfunction induced by hyperglycemia, and that knockout of *MEG3* expression could aggravate inflammatory damage to endothelial cells. Knockout of *MEG3* expression in human umbilical vein endothelial cells (HUVECs) has been shown to regulate the proliferation and apoptosis of endothelial cells by upregulating expression of B-cell lymphoma (Bcl)-2 and downregulating expression of Bax, caspase-3, and P53<sup>12</sup>.

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In the present study, plasma samples from humans and rats and HUVECs were selected to study changes in levels of vasoactive factors induced by vibration. We detected cellular damage in vitro under vibration. We interfered with *MEG3* expression in HUVECs in vitro to further explore the effects of vibration-induced damage and the role of *MEG3* in vibration-induced injury to vascular endothelial cells. Our data could provide: (i) a theoretical basis for revealing the epigenetic mechanism of HAVS; (ii) clues for exploring HAVS pathogenesis and finding early biomarkers. In these ways, early prevention, diagnosis, and treatment of HAVS could be achieved.

## Results

### Vibration alters the viability and subcellular structure to induce HUVEC apoptosis

Compared with the control group, HUVEC viability increased at 1, 1.5, 2, and 4 h, and HUVEC viability in the vibration group at 2.5 h and 3 h decreased (Fig. 1A). These data indicated that this vibration condition elicited the greatest effect upon HUVECs. Exposure to 125-Hz vibration could increase HUVEC viability for 3 h and 4 h. These results suggested that longer exposure could increase HUVEC viability, and could not be considered a beneficial effect of vibration at this frequency. Based on this result, we investigated whether there were other frequencies that reduced HUVEC viability. HUVEC viability decreased at 20 and 200 Hz, and increased at 10, 40, 80, and 130 Hz, compared with the control group, for 2.5 h. After exposure to vibration for 3 h, HUVEC viability decreased at 125 and 130 Hz and increased at 205 Hz (Fig. 1B). Due to the survival status of HUVECs, only a 2-day experiment could be conducted. Compared with the control group, HUVEC viability increased at 10 Hz, and decreased at 20 and 125 Hz for 2.5 h of vibration. HUVEC viability increased at 20 and 125 Hz for 3 h and 4 h of vibration (Fig. 1C). Therefore, we selected a vibration time of 2.5 h and frequencies of 20 and 125 Hz as criteria for subsequent experiments.

Transmission electron microscopy revealed that the control group had normal nuclei, endoplasmic reticulum (ER), and mitochondria, but the structure was faint. The cytoplasm was abundant and dense, and a small amount of vacuolization was visible. In the 20-Hz, 2-day group, pyknosis, chromatin margination, and swollen ER were observed. The cytoplasm was abundant and dense, cell outgrowth was reduced, and some of the cytoplasm was vacuolated. In the 125-Hz, 2-day group, pyknosis, chromatin margination, nuclear-membrane irregularities, and considerable cytoplasm vacuolization were observed. ER were swollen and cell outgrowth was reduced (Fig. 1D).

Compared with the control group, 125-Hz vibration for 2.5 h caused an increase in apoptosis in the 1-day group and 2-day group (Fig. 1E–I). These results suggested that vibration induced cell damage and promoted HUVEC apoptosis.

### Vibration induces changes in the expression of vasoactive factors in the plasma of workers and rats, HUVECs, and HUVEC supernatants

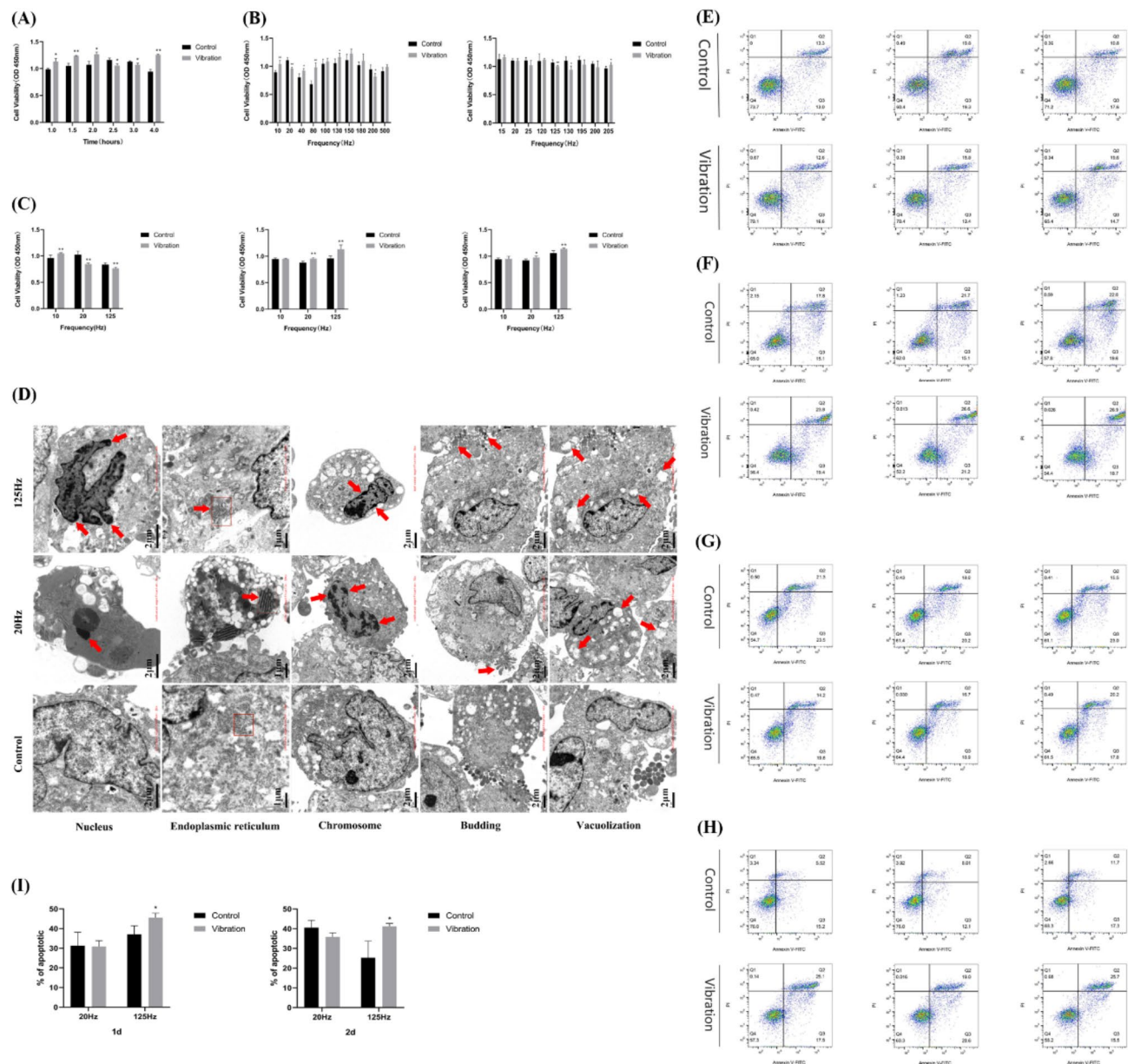
All worker groups comprised 50 males. The mean age of the vibration-white-finger group was  $39.86 \pm 6.70$  years and they had  $11.92 \pm 5.39$  years of service. The mean age of the vibration-non-white-finger group was  $35.92 \pm 6.61$  years, and they had  $6.98 \pm 6.84$  years of service. The mean age of the control group was  $34.50 \pm 5.75$  years and they had  $10.70 \pm 6.21$  years of service. There were significant differences in age, duration of service, and hand symptoms among these three groups (Table 1). Expression of LTB<sub>4</sub>, IL-1 $\beta$ , CGRP, and ET-1 in the vibration-white-finger group was higher than that in the vibration-non-white-finger group and control group. The distribution of MLC2 was reversed in these three groups, and differences in VEGF expression among these three populations was not significant (Fig. 2A).

Changes in expression of vasoactive factors in rat plasma were measured (Fig. 2B). Expression of LTB<sub>4</sub> in 3-, 7-, and 14-day groups was higher than that in the control group and 1-day group. VEGF expression in 7- and 14-day groups was higher than that in control, 1-, and 3-day groups. IL-1 $\beta$  expression in 3-, 7-, and 14-day groups was higher than that in control and 1-day groups. IL-1 $\beta$  expression in the 14-day group was higher than that in 3- and 7-day groups. MLC2 expression in 3-, 7-, and 14-day groups was lower than that in control and 1-day groups. CGRP expression in 7- and 14-day groups was lower than that in control, 1- and 3-day groups. ET-1 expression in 3-, 7-, and 14-day groups was higher than that in control and 1-day groups. ET-1 expression in the 14-day group was higher than that in 3- and 7-day groups.

We also detected the changes in expression of vasoactive factors in HUVECs and HUVEC supernatants (Fig. 2C). Expression of LTB<sub>4</sub>, VEGF, IL-1 $\beta$ , CGRP, and ET-1 was increased in HUVECs and/or HUVEC supernatants in 20- and 125-Hz groups compared with that in the control group. MLC2 expression was reduced in HUVECs and HUVEC supernatants in the 125-Hz, 2-day group compared with that in the control group. LTB<sub>4</sub> expression was decreased in HUVECs and HUVEC supernatants in the 125-Hz group compared with that in the 20-Hz group. Expression of VEGF and ET-1 was increased in HUVECs in the 125-Hz, 1-day group compared with that in the 20-Hz, 1-day group. MLC2 expression was decreased in HUVECs in the 125-Hz, 2-day group compared with that in the 20-Hz, 2-day group. CGRP expression was increased in the HUVEC supernatants of the 125-Hz, 1-day group and HUVECs in the 125-Hz, 2-day group, compared with that in the 20-Hz group with the same number of days of exposure. Thus, vibration could induce changes in the expression and secretion of the vasoactive factors LTB<sub>4</sub>, IL-1 $\beta$ , MLC2, CGRP, and ET-1 in HUVECs and HUVEC supernatants.

### Changes in lncRNA expression induced by vibration in HUVECs

The schematic diagram of the vibration experiment is shown in Fig. 4A. In 1-day groups, expression of the lncRNAs *MEG3*, nuclear-enriched abundant transcript 1 (*NEAT1*), taurine-upregulated gene 1 (*TUG1*), and metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) decreased in the 20-Hz group and 125-Hz group compared with that in the control group. In 2-day groups, expression of *MEG3*, *TUG1*, and *MALAT1* decreased in the 20-Hz group, but *NEAT1* expression increased; *MEG3* had increased expression in the 125-Hz group, but *TUG1* expression decreased (Fig. 3). These data suggested that vibration induced changes in the expression of *MEG3*, *NEAT1*, *TUG1* and *MALAT1* in HUVECs.



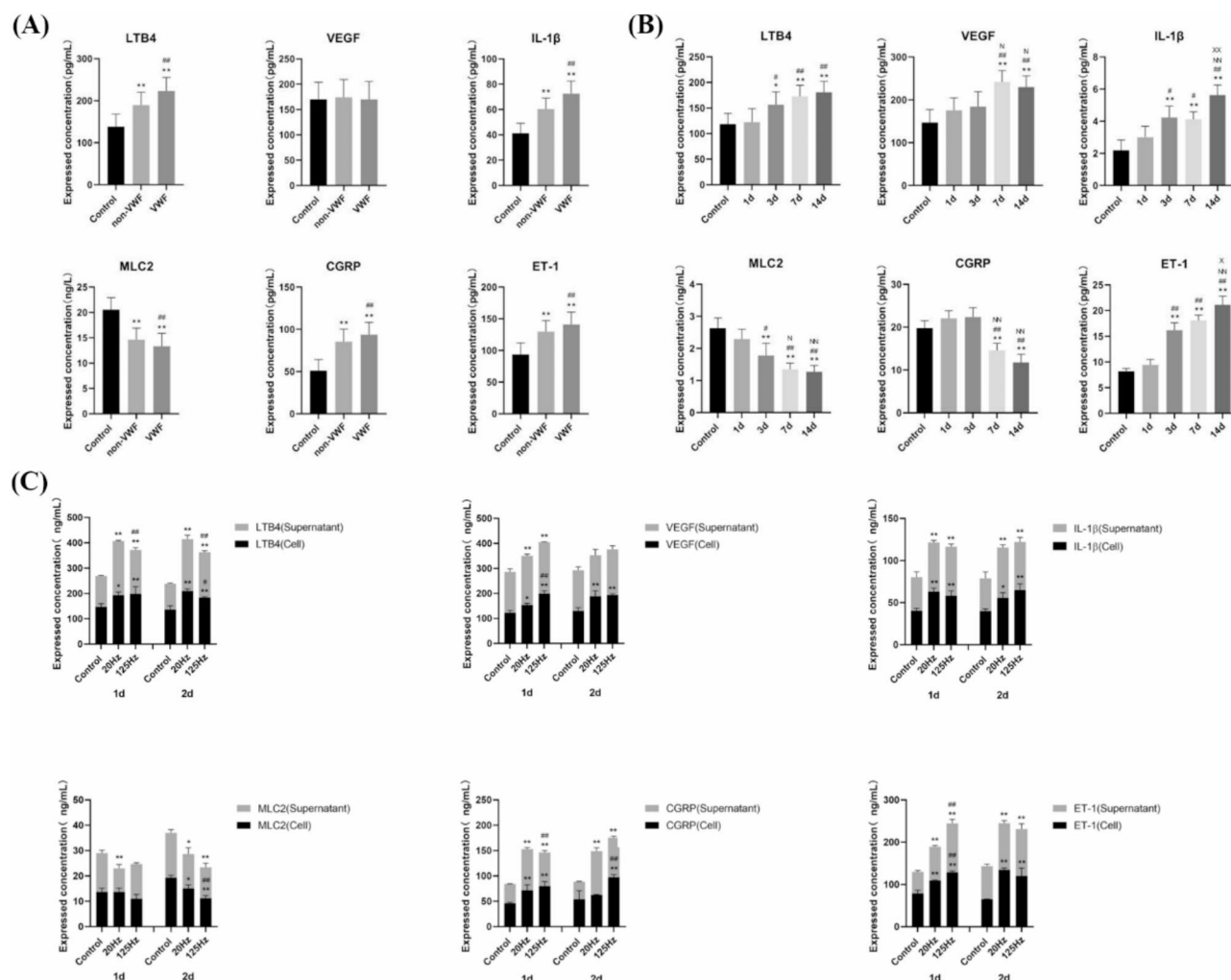
**Fig. 1.** Vibration-induced changes in viability, subcellular structure and apoptosis in HUVECs. **(A)** Effect of vibration at 125 Hz for 1–4 h on HUVEC viability. N = 3 experimental replicates per group. **(B)** Effect of vibration at different frequencies for 2.5 h (left) and 3 h (right) on one-day group HUVEC viability. N = 3 experimental replicates per group. **(C)** Effect of vibration at different frequencies for 2.5 h (left), 3 h (middle) and 4 h (right) on two-day group HUVEC viability. N = 3 experimental replicates per group. **(D)** Intracellular structural changes were observed at different magnifications of 1  $\mu$ m–10 kx, 2  $\mu$ m–5 kx, 2  $\mu$ m–4 kx, and 2  $\mu$ m–5 kx. Nucleus pyknosis, chromatin margination, swollen endoplasmic reticulum and mitochondria, cell budding and vacuolization are indicated with red arrows. **(E–F)** Effect of vibration at 125 Hz **(E)** and 20 Hz **(F)** for 2.5 h on one-day group HUVEC apoptosis. N = 3 experimental replicates per group. **(G–H)** Effect of vibration at 125 Hz **(G)** and 20 Hz **(H)** for 2.5 h on two-day group HUVEC apoptosis. N = 3 experimental replicates per group. **(I)** Flow cytometry was used to measure % of apoptotic cells. N = 3 experimental replicates per group. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the control group.

### Vibration induces changes in *MEG3* expression in HUVECs

In 1-day groups, *MEG3* expression decreased in the 20-Hz group and 125-Hz group compared with that in the control group. In 2-day groups, *MEG3* expression decreased in the 20-Hz group (Fig. 4B). These results indicated that vibration could cause changes in *MEG3* expression. Fluorescence microscopy revealed that intracellular fluorescence could be observed in transfected HUVECs in each group. An appropriate fluorescence effect and transfection efficiency could be observed at the dose of 5  $\mu$ L of liposome (Fig. 4C). Compared with the control group, expression of small interfering (si)RNA *MEG3*-3#5 mRNA was decreased (Fig. 4D–F), indicating that

Variables	Control (n = 50)	non-VWF (n = 50)	VWF (n = 50)	F/ $\chi^2$	P
Age, y	34.50 $\pm$ 5.75	35.92 $\pm$ 6.16	39.86 $\pm$ 6.70	9.979 <sup>a</sup>	<0.001
Length of service, y	10.70 $\pm$ 6.21	6.98 $\pm$ 6.84	11.92 $\pm$ 5.39	16.949 <sup>a</sup>	<0.001
Smoking, n (%)	21(29.6)	24(33.8)	26(36.6)	1.016 <sup>b</sup>	0.048
Drinking, n (%)	32(36.0)	26(29.2)	31(34.8)	1.713 <sup>b</sup>	0.079
Hand symptoms, n (%)	3(4.1)	23(31.1)	48(64.9)	81.348 <sup>b</sup>	<0.001

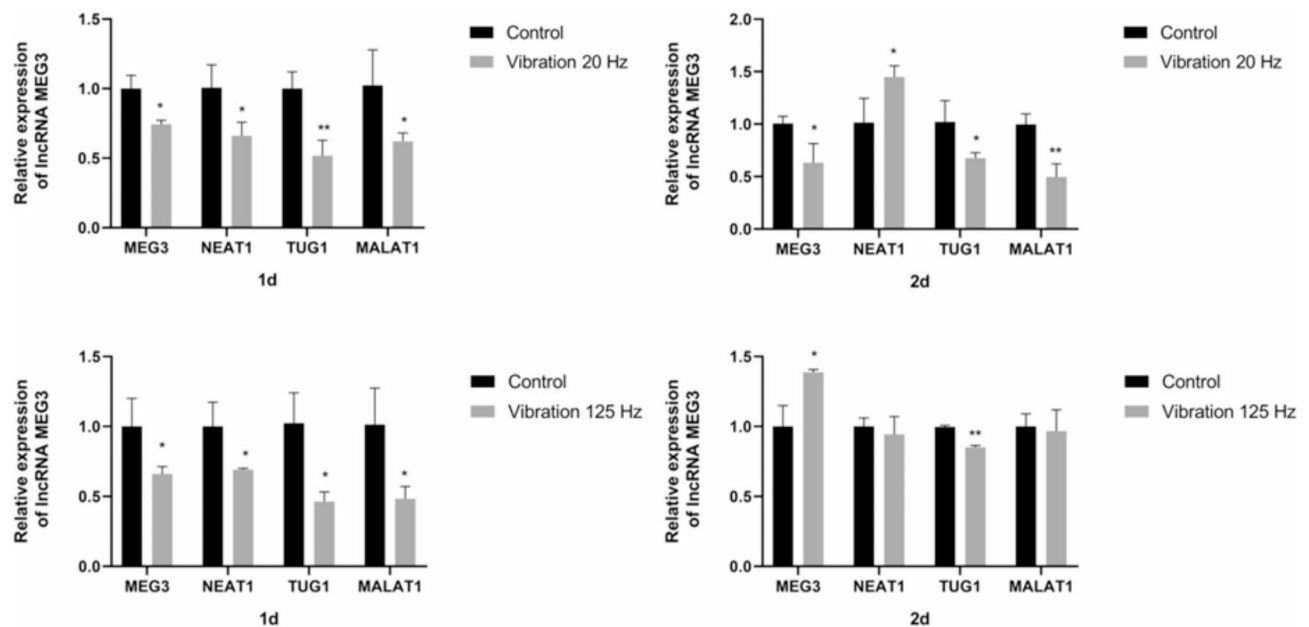
**Table 1.** Comparison of the basic situation of the three groups of workers. [Note]: a is F value; b is  $\chi^2$  value; compared with the control group,  $P < 0.05$ .



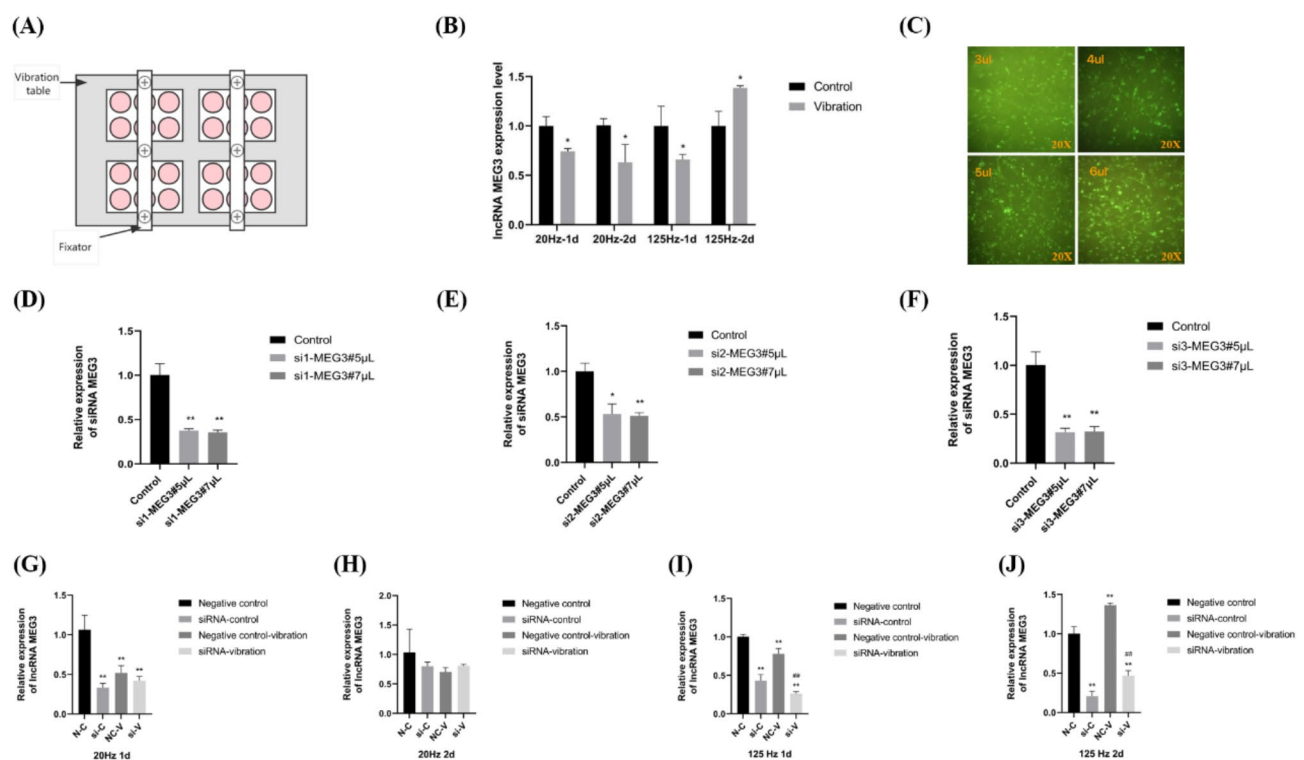
**Fig. 2.** Vibration-induced changes in the expression of vasoactive factors in plasma of workers, plasma of rats, HUVEC and supernatant. (A) Comparison of plasma concentrations of six vasoactive factors among three groups of workers. \*\* $P < 0.01$ , compared with the control group ( $n = 50$ ); # $P < 0.01$ , compared between the vibration white finger (VWF) group ( $n = 50$ ) and the vibration non-white finger (non-VWF) group ( $n = 50$ ). (B) Comparison of plasma concentrations of six vasoactive factors among five groups of rats. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the control group ( $n = 5$ ); # $P < 0.05$ , ## $P < 0.01$ , compared between 1 day ( $n = 5$ ) and 3 days ( $n = 5$ ), 7 days ( $n = 5$ ), 14 days ( $n = 5$ ); X $P < 0.05$ , NN $P < 0.01$ , compared between 3 and 7 days, 14 days; X $P < 0.05$ , XX $P < 0.01$ , compared between 7 and 14 days. (C) Cells and supernatants in one-day group and two-day group were collected after vibration for 2.5 h.  $N = 3$  experimental replicates per group. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the control group; # $P < 0.05$ , ## $P < 0.01$ , compared with the 20 Hz group.

knockdown of *MEG3* expression had been achieved. In the 125-Hz, 1- and 2-day groups, compared with the negative control group, expression in the siRNA-control group and negative control-vibration group decreased; compared with the negative control-vibration group, *MEG3* expression was decreased in the siRNA-vibration group (Fig. 4G–J). In the 20-Hz, 1-day group, expression increased in the negative control-vibration group





**Fig. 3.** Changes in lncRNA expression induced by vibration in HUVEC. Use qPCR to analyse the expression of lncRNA in HUVEC after vibration for 2.5 h. N = 3 experimental replicates per group. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the control group.



**Fig. 4.** Role of knockdown of lncRNA MEG3 in vibration-induced HUVEC injury. (A) Diagram of the vibration instrument used in the experiment. (B) Effect of vibration on the expression of lncRNA MEG3. (C) Expression of FAM-modified siRNA with different doses of Lipofectamine 2000. (D–F) Transfection efficiency of three different doses of siRNA. (G–J) Changes of lncRNA MEG3 expression under different vibration frequencies. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with negative control group (n = 3); # $P < 0.05$ , ## $P < 0.01$ , compared between negative control-vibration group (n = 3) and siRNA-vibration group (n = 3).

compared with that in the negative control group; in the 2-day group, expression in the siRNA-control group decreased, and expression in the negative control-vibration group increased. Compared with the negative control-vibration group, *MEG3* expression in the siRNA-vibration group decreased. These results indicated that vibration could affect *MEG3* expression in HUVECs, and that *MEG3* had an important role in HUVEC damage induced by vibration.

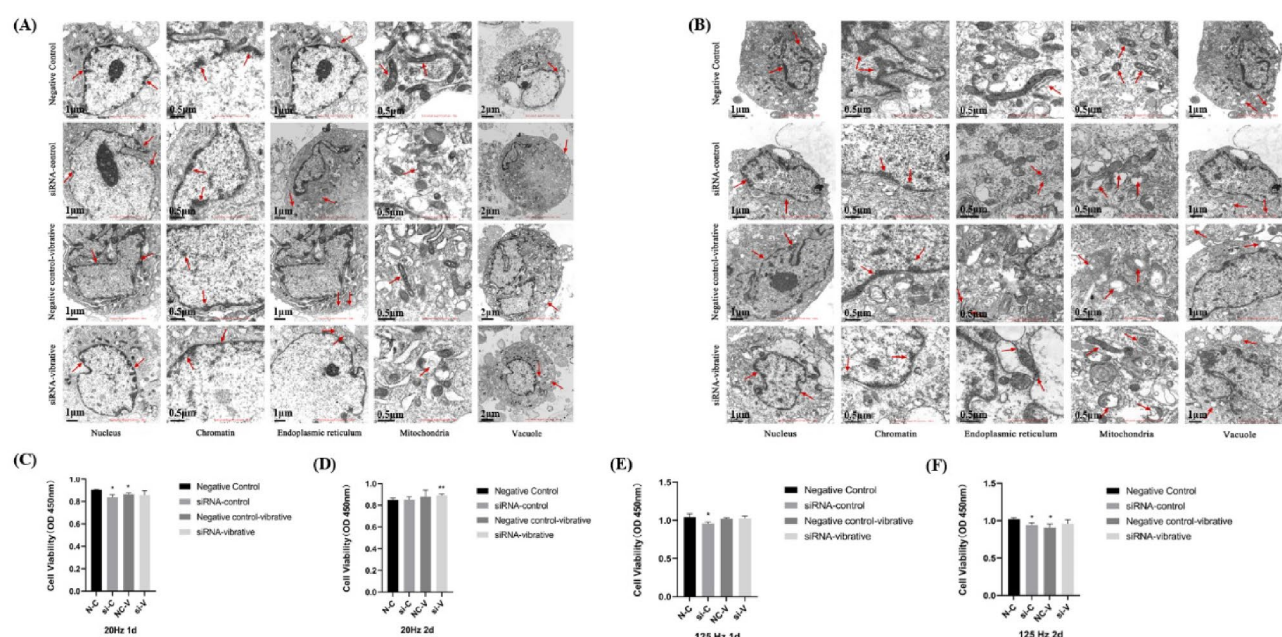
### Effect of *MEG3* on the internal structure in vibration-induced HUVECs injury

Electron microscopy showed that in the 20-Hz, 2-day group, the nucleus, ER, and mitochondria of the negative control group were normal, but the structure was fuzzy. The cytoplasm was abundant and dense, with a small amount of vacuolization. In the siRNA-control group, nuclear pyknosis, malformation, slightly irregular nuclear membrane, inconspicuous chromatin, relatively swollen ER and mitochondria, and some cytoplasmic vacuolization were observed. In the negative control-vibration group, nuclear pyknosis, chromatin margination, no swelling or expansion of ER, no obvious swelling of mitochondria, and some cytoplasmic vacuolization were observed. In the siRNA-vibration group, nuclear pyknosis and malformation, a relatively regular nuclear membrane, and chromatin margination were observed. ER and mitochondria were swollen, and vacuolization was observed in the cytoplasm (Fig. 5A).

In the 125-Hz, 2-day group, the nucleus, ER, and mitochondria were normal in the negative control group, chromatin margination was absent, the cytoplasm was dense, and some vacuolization was observed. In the siRNA-control group, we observed nuclear pyknosis and malformation, slightly irregular nuclear membranes, and inconspicuous chromatin. Rough endoplasmic reticuli (RER) were slightly dilated, ER ribosomes had been shed, mitochondria were swollen and ruptured, and some cytoplasm was vacuolated. In the negative control-vibration group, we observed nuclear pyknosis and chromatin margination. RER were arranged in a lamellar pattern, ribosomes were near ER membranes, mitochondria were swollen and ruptured, and a small part of the cytoplasm was vacuolated. In the siRNA-vibrative group, nuclear pyknosis, malformation, and chromatin margination were observed. Expanded RER were seen in the cytoplasm. Mitochondria were swollen and morphologically abnormal. Extensive vacuolization was observed in the cytoplasm (Fig. 5B).

In the 20- and 125-Hz groups at 1 day, compared with the negative control group, HUVEC viability decreased in the siRNA-control group. In the 125-Hz, 2-day group, compared with the negative control group, HUVEC viability decreased in the siRNA-control group and negative control-vibration group. In the 20-Hz, 2-day group, compared with the negative control group, HUVEC viability increased in the siRNA-vibration group (Fig. 5C–F).

Compared with the negative vibration group, cell damage in the *MEG3* knockdown vibration group was more obvious and severe. These data suggested that *MEG3* may be involved in vibration-induced injury and may inhibit HUVEC damage.

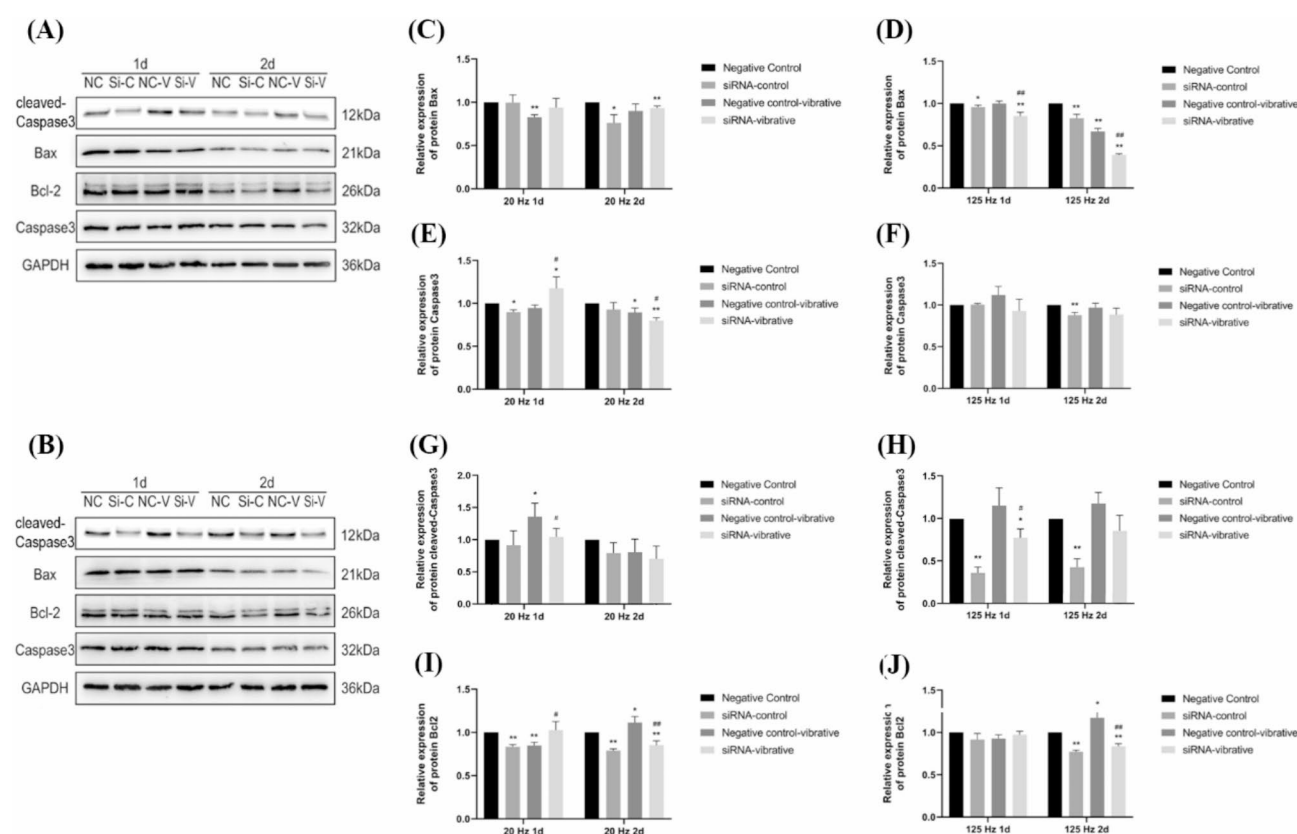


**Fig. 5.** Vibration treatment of *MEG3* knockdown HUVECs induces cells injury. (A and B) Intracellular structural changes of cells in 20 Hz, two-day group (A) and 125 Hz, two-day group (B) were respectively observed at different magnifications of 1 μm-10 kx, 0.5 μm-25 kx, 1 μm-10 kx, 0.5 μm-30 kx, and 2 μm-5 kx. Nucleus pyknosis, abnormal chromatin or chromatin margination, swollen endoplasmic reticulum and mitochondria and vacuolization are indicated with red arrows. (C to F) Effect of vibration on cell viability in each group. N = 5 experimental replicates per group. \*P < 0.05, \*\*P < 0.01, compared with the negative control group.

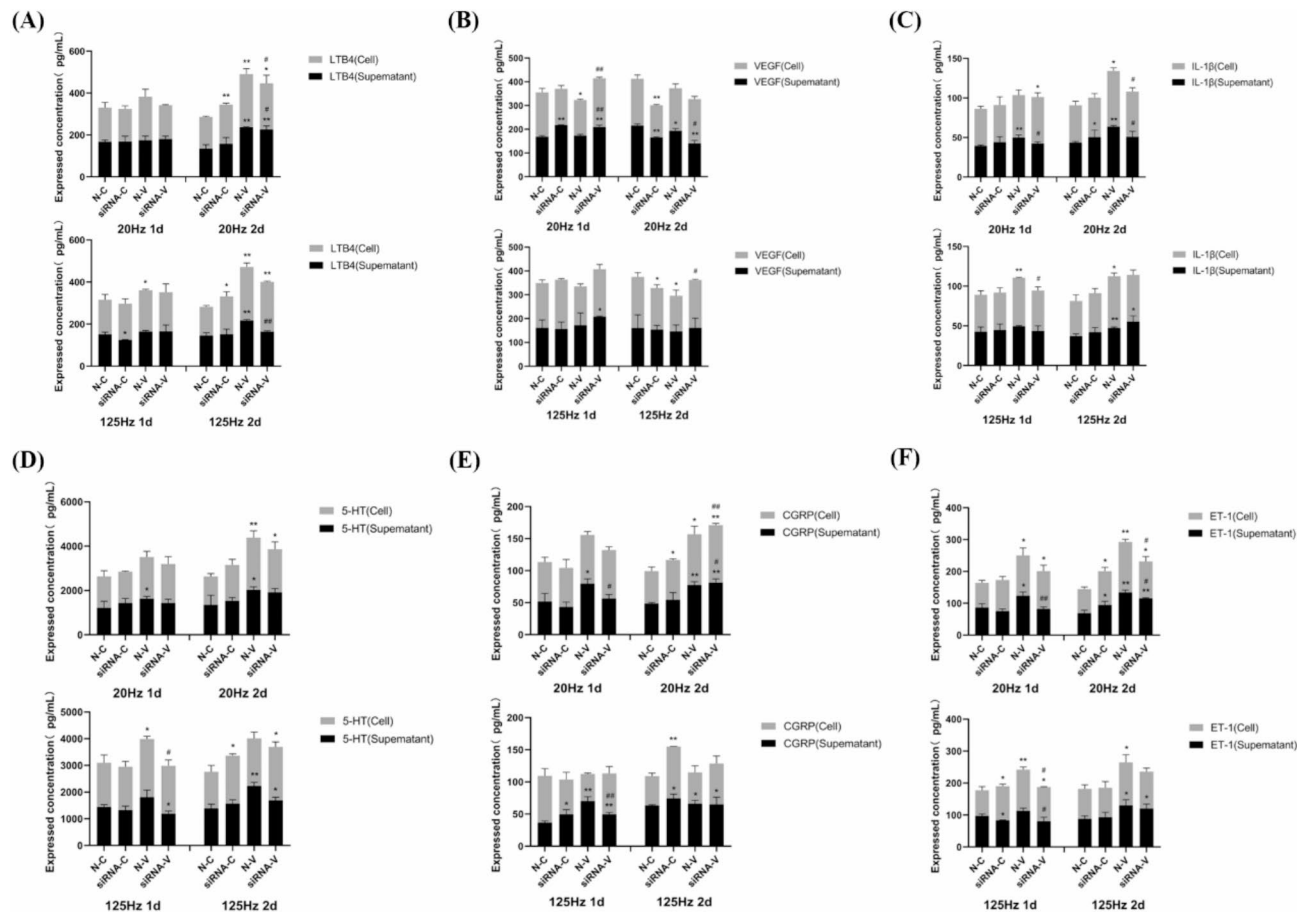
# MEG3 regulates the expression of Bax, Caspase-3, cleaved-Caspase3 and Bcl-2 during vibration-induced damage to HUVECs

In the 20-Hz, 1-day group, compared with the negative control group, Bax expression was decreased in the negative control-vibration group. In the 20-Hz, 2-day group, compared with the negative control group, Bax expression was increased in the siRNA-control group and negative control-vibration group, and Bax expression in the siRNA-vibration group was higher than that in the siRNA-control group. In the 20-Hz, 1-day group, Caspase-3 expression was decreased in the negative siRNA-control group and increased in the siRNA-vibration group compared with that in the negative control group; compared with the negative control-vibration group, Caspase-3 expression was increased in the siRNA-vibration group. In the 20-Hz, 2-day group, compared with the negative control group, Caspase-3 expression was decreased in the negative control-vibration group and siRNA-vibration group; compared with the negative control-vibration group, Caspase-3 expression was decreased in the siRNA-vibration group. In the 20-Hz, 1-day group, compared with the negative control group, cleaved-Caspase-3 expression was increased in the negative control-vibration group; compared with the negative control-vibration group, cleaved-Caspase-3 expression was decreased in the siRNA-vibration group. In the 20-Hz, 1-day group, compared with the negative control group, Bcl-2 expression was decreased in the siRNA-control group and negative control-vibration group; compared with the negative control-vibration group, Bcl-2 expression was increased in the siRNA-vibration group. In the 20-Hz, 2-day group, compared with the negative control group, Bcl-2 expression was reduced in the negative control-vibration group and siRNA-vibration group; compared with the negative control-vibration group, Bcl-2 expression was reduced in the siRNA-vibration group (Fig. 6).

In the 125-Hz, 1-day group, compared with the negative control group, Bax expression was decreased in the siRNA-control group and siRNA-vibration group, and Bax expression in the siRNA-vibration group was lower than that in the siRNA-control group. In the 2-day group, compared with the negative control group, Bax expression was decreased in the negative control-vibration group and siRNA-vibration group. In the 125-Hz, 2-day group, Caspase-3 expression was decreased in the negative siRNA-control group compared with that in the negative control group. In the 125-Hz, 1-day group, compared with the negative control group, cleaved-



**Fig. 6.** *MEG3* regulates the expression of Bax, Caspase-3, cleaved-Caspase3 and Bcl2 in vibration-induced damage in HUVECs. (A and B) The effect of *MEG3* on the expression levels of Bax, Caspase3, cleaved-Caspase3 and Bcl2 in HUVECs injury induced by vibration. (C and D) The effect of *MEG3* on the expression levels of Bax in HUVECs injury induced by vibration. (E and F) The effect of *MEG3* on the expression levels of Caspase3 in HUVECs injury induced by vibration. (G and H) The effect of *MEG3* on the expression levels of cleaved-Caspase3 in HUVECs injury induced by vibration. (I and J) The effect of *MEG3* on the expression levels of Bcl2 in HUVECs injury induced by vibration. N=3 experimental replicates per group. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the negative control group; # $P < 0.05$ , ## $P < 0.01$ , compared between negative control-vibrative group and siRNA-vibrative group.



**Fig. 7.** Knockdown of *MEG3* affects the expression and secretion of vasoactive factors in vibration-induced HUVEC injury. Cells and supernatants were collected after vibration for 2.5 h, and the expression level of vasoactive factors were measured by ELISA. N = 3 experimental replicates per group. N-C: negative control, siRNA-C: siRNA-control, N-V: negative control-vibrative, siRNA-V: siRNA-vibrative. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the negative control group; # $P < 0.05$ , ## $P < 0.01$ , compared between negative control-vibrative group and siRNA-vibrative group.

Caspase-3 expression was decreased in the siRNA-control group and siRNA-vibration group; compared with the negative control-vibration group, cleaved-Caspase-3 expression was decreased in the siRNA-vibration group. In the 20-Hz, 2-day group, compared with the negative control group, cleaved-Caspase-3 expression was decreased in the siRNA-control group. In the 125-Hz, 2-day group, compared with the negative control group, Bcl-2 expression was decreased in the siRNA-control group and siRNA-vibration group, and Bcl-2 expression was increased in the negative control-vibration group; compared with the negative control-vibration group, Bcl-2 expression was decreased in the siRNA-vibration group (Fig. 6).

These results showed that vibration-induced expression of apoptotic proteins (Bax and Caspase-3) decreased, whereas that of an anti-apoptotic protein (Bcl-2) increased. After interfering with *MEG3*, the expression of Bax, Caspase-3, cleaved-Caspase3 and Bcl2 decreased. This finding suggested that *MEG3* may be involved in vibration-induced HUVEC injury by regulating the expression of Bax, Caspase-3, and Bcl-2.

### Effect of *MEG3* on vasoactive factors in vibration-induced damage to HUVECs

In the 20-Hz, 1-day group, compared with the negative control group, ET-1 expression was increased and VEGF expression was decreased in HUVECs; after knockdown of *MEG3* expression, compared with the negative control-vibration group, VEGF expression was increased. Expression of IL-1 $\beta$ , CGRP, and ET-1 was increased in HUVEC supernatants; after knockdown of *MEG3* expression, compared with the negative control-vibration group, VEGF expression was increased but expression of ET-1 and IL-1 $\beta$  decreased (Fig. 7).

In the 20-Hz, 2-day group, compared with the negative control group, expression of IL-1 $\beta$ , 5-HT, CGRP, and ET-1 was increased and VEGF expression was decreased in HUVECs; after knockdown of *MEG3* expression, compared with the negative control-vibrative group, CGRP expression was increased and expression of LTB4, IL-1 $\beta$ , 5-HT, and ET-1 was decreased. Expression of LTB4, IL-1 $\beta$ , 5-HT, CGRP, and ET-1 was increased and VEGF expression was decreased in HUVEC supernatants; after knockdown of *MEG3* expression, compared with the negative control-vibration group, CGRP expression was increased and expression of LTB4, VEGF, IL-1 $\beta$ , 5-HT, and ET-1 was decreased (Fig. 7).



In the 125-Hz, 1-day group, compared with the negative control group, expression of LTB4, IL-1 $\beta$ , 5-HT, and ET-1 in HUVECs was increased; after knockdown of *MEG3* expression, compared with the negative control-vibration group, expression of IL-1 $\beta$ , 5-HT, and ET-1 was decreased. Expression of 5-HT and CGRP was increased in HUVEC supernatants; after knockdown of *MEG3* expression, compared with the negative control-vibration group, VEGF expression was increased and expression of ET-1 and 5-HT was decreased (Fig. 7).

In the 125-Hz, 2-day group, compared with the negative control group, expression of LTB4, IL-1 $\beta$ , and ET-1 was increased and VEGF expression was decreased in HUVECs; after knockdown of *MEG3* expression, compared with the negative control-vibration group, VEGF expression was increased and expression of LTB4 and IL-1 $\beta$  was decreased. Expression of LTB4, IL-1 $\beta$ , 5-HT, and ET-1 was increased in HUVEC supernatants; after knockdown of *MEG3* expression, compared with the negative control-vibration group, expression of LTB4 and 5-HT was decreased (7).

These results indicated that *MEG3* regulated the expression of vasoactive factors in vibration-induced inflammation. Hence, *MEG3* may be involved in vibration-induced damage to HUVECs by regulating the expression of vasoactive factors.

## Discussion

Studies on the pathophysiology of HAVS have focused mainly on its clinical manifestations and diagnosis. Studies on the mechanism of injury to vascular endothelial cells caused by vibration are scarce<sup>13</sup>. Previously, we found that Chinese workers were exposed to high intensity and long duration of vibration without effective protective measures, and that the hazards of vibration were seriously underestimated or ignored<sup>14</sup>. The effect of vibration on peripheral vascular function has been the focus of HAVS research, and vasoactive factors help to regulate vascular function.

We measured expression of the vasoactive factors LTB4, IL-1 $\beta$ , CGRP, MLC2, and ET-1 using in vivo and in vitro models of vibration exposure. This approach provided a theoretical basis for future studies on the pathogenesis of HTV-induced HAVS. In addition, few studies have investigated the effect of vibration on cells in vitro. White et al. proposed that the absolute size of the temporal gradient and the frequency/duration of each pulse cycle had roles in the biomechanical transduction of fluid vibration in endothelial cells<sup>15</sup>. Therefore, we also employed a cellular model to investigate the effects of, and damage of vibration to, cells. By ultrastructural observation we found, for the first time, that vibration could induce changes in the viability of, and damage to, HUVECs.

LTB4 is an important metabolite of arachidonic acid. It is produced by the action of 5-lipoxygenase and other enzymes. It has a powerful activating effect on leukocytes, stimulating their chemotaxis and aggregation, releasing oxygen radicals and lysosomal enzymes, and increasing vascular permeability and vascular-wall constriction<sup>16,17</sup>. Our in vivo experimental results showed that, in workers exposed to HTV, those with a higher plasma level of LTB4 were more likely to suffer from VWF. In rats, the longer the duration of vibration, the higher was the LTB4 expression in plasma, suggesting that there may be a dose–response relationship between LTB4 expression and the duration of vibration exposure. The in vitro HUVEC model revealed LTB4 expression in vibration groups to be higher than that in the control group.

VEGF acts specifically and multilaterally on vascular endothelial cells, causing their proliferation, thereby promoting neovascularization. However, in the present study, VEGF expression was not associated with VWF. This finding may have been due to the small sample size of our study. The human body is a complex system with many influencing factors, and these confounding variables may obscure the changes in VEGF levels caused by vibration. However, the results observed in a single cell model exclude other complex influences. We believe that VEGF plays a significant role in endothelial cells, which is why we continue to monitor this indicator in the animal and cell experiments. The increased VEGF expression in plasma in the 7- and 14-day groups of rats may have been because repair of vascular endothelial cells was at a compensatory stage. The increased VEGF expression in HUVECs in the 2-day vibration group and no change in VEGF expression in HUVEC supernatants may have been due to impaired secretion of VEGF in HUVECs as a result of prolonged vibration.

IL-1 $\beta$  is a key mediator of intercellular communication in the immune system. It is also a key driver of local and systemic immune responses in atherosclerotic cardiovascular disease<sup>18</sup>. Our in vivo results showed that the higher the IL-1 $\beta$  expression in a population exposed to HTV, the greater was the chance of developing occupational HAVS. In rats, the longer the duration of vibration, the higher was the IL-1 $\beta$  expression in plasma, suggesting that there may be a dose–response relationship between IL-1 $\beta$  expression and duration of exposure to vibration.

Myosin consists of one pair of heavy chains and two pairs of light chains. Phosphorylation of myosin light chains is the basis for increasing vascular endothelial permeability<sup>19</sup>. We showed that vibration decreased MLC2 expression in rat plasma and HUVECs in vitro. Also, if the vibration lasted longer, considerable necrosis of HUVEC occurred, and then expression in vibration groups was lower than that in the control group. We speculated that humans have a regulatory function in which short-term vibration increases MLC2 secretion.

CGRP is the main transmitter of sensory nerves. It is distributed widely in central and peripheral tissues, and produces biological effects by interacting with specific receptors. CGRP can elicit vasodilatation, protect vascular endothelial cells, and inhibit the proliferation of smooth muscle cells<sup>20,21</sup>. We observed that vibration decreased CGRP expression rat tails. Vibration increased CGRP expression significantly in human plasma and HUVECs. The different results between humans and rats may have been because stress in humans can be regulated by the immune system, whereas rats exhibit a short-term stress response. The pathology associated with sensory-nerve deficits in patients with HAVS has been described, but the pathogenesis of these deficits has not, which merits requires further investigation.

ET-1 is a potent vasoconstrictor secreted by vascular endothelial cells that mediates vasoconstriction, proinflammatory effects, and mitogenesis. ET-1 overproduction may be important in cardiovascular diseases<sup>22</sup>.

Our *in vivo* and *in vitro* models showed that vibration increased ET-1 expression, which led to HUVEC injury. We elucidated that, under different frequencies and times of damage to HUVECs, changes in the expression of LTb4, VEGF, IL-1 $\beta$ , MLC2, CGRP, and ET-1 in vibration-induced *in vivo* and *in vitro* models may be involved in HAVS pathogenesis. However, quantifying the effects of vibration exposure accurately is challenging, and the number of validated biomarkers of exposure is limited.

The association between lncRNAs and HAVS is not clear. We found that vibration induced altered expression of the lncRNAs *MEG3*, *NEAT1*, *TUG1*, and *MALAT1* in HUVECs. *MEG3* expression has been shown to be significantly higher in the plasma of patients suffering from sepsis compared with that in healthy controls<sup>23</sup>. However, it has also been shown that *MEG3* expression decreases in the retinal epithelial cells of diabetic mice under oxidative stress and high-glucose stimulation *in vitro* and *in vivo*<sup>12</sup>. *MEG3* expression may differ in different conditions or diseases. We suggest that lncRNAs may be affected by the frequency and duration of vibration, which may lead to a different extent of expression. lncRNAs have a very important regulatory role in vascular endothelial inflammation and may be involved in vibration-induced endothelial injury. The specific role of lncRNAs in HAVS progression will be explored further in cellular experiments and animals by our research team, and may reveal HAVS pathogenesis.

We found that *MEG3* was involved in the regulation of damage, apoptotic proteins, and vasoactive factors in HUVECs induced by vibration. Vibration treatment of HUVECs with knockdown of *MEG3* expression caused a further reduction in cell viability and aggravation of injury. On the first day of vibration, the cells were damaged after being exposed to 125 Hz vibration stimulation, resulting in a decrease in *MEG3* expression. After the vibration ceased and the cells were returned to the incubator, they initiated self-regulatory mechanisms to repair the damage, leading to an increase in *MEG3* expression. The damage to the cells may be greater at 20 Hz, and the cell protective response may be weakened. In the future, more different vibration frequencies and times can be grouped to further study the vibration-induced changes in *MEG3* expression levels. Bax expression decreased in the 1- and 2-day groups for 20-Hz vibration. In the 1-day group, caspase-3 expression increased and decreased in the 2-day group, data which align with results from work by Song and colleagues. Vibration induced a decrease in the expression of the apoptotic proteins Bax and caspase-3. After knockdown of *MEG3* expression, the expression of Bax and caspase-3 decreased more significantly, and Bcl-2 expression increased. These findings suggest that *MEG3* may be involved in the vibration-induced injury to vascular endothelial cells by regulating the expression of Bax, caspase-3, and Bcl-2.

Vibration increased the expression of LTb4, 5-HT, IL-1 $\beta$ , CGRP, and ET-1, and decreased the expression of VEGF when *MEG3* was intact. After knockdown of *MEG3* expression, expression of IL-1 $\beta$ , 5-HT, CGRP, and VEGF was increased. Expression of LTb4, IL-1 $\beta$ , ET-1, and CGRP in the siRNA-vibration group was lower than that in the negative control-vibration group. These data suggested that, under the influence of vibration and siRNA, expression of LTb4, IL-1 $\beta$ , ET-1, CGRP, and VEGF was reversed.

After knockdown of *MEG3* expression, VEGF expression was increased in the 1-day group, but decreased in the 2-day group, which may be a way that cells protect themselves from stress. Studies have shown that *MEG3* affects the expression of inflammatory factors. Wang et al. found that knockout of *MEG3* expression increased the expression of VEGF, TNF- $\alpha$ , and IL-6, significantly in HUVECs, suggesting that *MEG3* may inhibit the secretion of inflammatory factors<sup>12</sup>. Conversely, Liu et al. showed that downregulation of *MEG3* expression inhibited the secretion of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in lipopolysaccharide-treated human dental pulp cells<sup>24</sup>. However, our data suggest that downregulation of *MEG3* expression has different effects on the expression of vasoactive factors under different vibration conditions. There were differences between the *in vitro* cell model and actual human exposure to vibration, which merit further study.

Knockdown of *MEG3* expression in HUVECs can regulate the proliferation and apoptosis of endothelial cells by upregulating expression of Bcl-2 and downregulating expression of Bax, Caspase-3, and P53<sup>12</sup>. In contrast, Liu and colleagues found that downregulation of *MEG3* expression in HUVECs induced apoptosis and inhibited cell proliferation<sup>24</sup>. In addition, knockdown of *MEG3* expression reduced neovascularization and VEGF-induced angiogenesis in alkali-burned corneas through stromal cell-derived factor-1/chemokine receptor-4 (SDF-1/CXCR4) and Smad2/3 signaling pathways<sup>25</sup>. *MEG3* may have different roles in the proliferation and apoptosis of endothelial cells and inflammatory injury due to different conditions for cell growth. Therefore, more studies are needed to understand the role and mechanism of action of *MEG3*.

*MEG3* is involved in the physiological and pathologic processes of several vascular diseases. *MEG3* is an effective indicator of changes in vascular structure and function. Increasing numbers of studies have shown that lncRNAs are regulators of protein-coding genes, and that their dysfunction affects the occurrence and development of diseases. We discovered that, according to electron microscopy, downregulation of *MEG3* expression aggravated cell damage. We conclude that *MEG3* has a protective role in the cell damage induced by vibration.

## Methods and materials

### Cell culture and treatments

HUVECs were purchased from Shanghai Cell Bank, Chinese Academy of Sciences. HUVEC culture medium (10% fetal bovine serum, penicillin, 0.25% streptomycin) were purchased from Thermo Fisher (MA, USA) and Bioshark (Beijing, China). Before the start of the experiment, cells were kept in an incubator at 37 °C, 5% CO<sub>2</sub>. After observing cell morphology and growth, aspirate the old culture using a sterile Pasteur pipette, wash the culture flask with PBS. The cells were completely suspended by digestion and washing using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA). Then transfer the cells to a new flask, replenish with the appropriate amount of new medium, and place them in a 37 °C, 5% CO<sub>2</sub> incubator for culture, and the experiment was started when HUVEC were cultured to 70% to 80% abundance. The experimental group was a vibration group with multiple vibrational frequency gradients of 10–100 Hz, 100–200 Hz, and 500 Hz. The vibration frequencies

and accelerations are shown in Table S1, the schematic diagram of the vibration experiment is shown in Figure S1. The acceleration values corresponding to the vibration exposure at each frequency were obtained by instrumental measurements. The control group had the same conditions as the experimental group except that they were not exposed to vibration.

### Cell ultrastructure detection

At the end of the vibration, cells were collected for fixation, embedding, then cutting (ultrathin sections) and staining. Changes in the internal structure of the cells, such as cell shape, mitochondria and endoplasmic reticulum, were observed under an electron microscope (JEM-1400, JEOL Ltd., Tokyo, Japan).

### Cell viability assay

Cell viability was assayed using the Cell Counting Kit CCK-8. At the end of vibration, 10  $\mu$ l CCK-8 reagent (UE Landi, Suzhou, China) was added and incubated for 2.5 h. The absorbance value was measured at 450 nm (Swiss Sunrise Automatic Enzyme Labeler).

### Flow cytometry (FCM) assay

After the vibration treatment, the cells were harvested and stained with FITC-Annexin V and PI according to the FITC-Annexin V apoptosis detection kit (BD Biosciences, San Jose, CA, USA). Flow cytometry (FACScan, BD Biosciences) was performed.

### Molecular epidemiological survey of occupational population

Judgmental sampling was used to divide 150 employees of a factory in Zhongshan City (Guangdong, China) into three groups, of which 50 patients with a chief complaint hospital diagnosis of HAVS were the vibration white finger group, 50 hand-transmitted vibration workers without HAVS in the same period as the vibration non-white finger group, and another 50 non-hand-transmitted vibration workers were selected as the control group. In the early morning, 4 ml/person of median venous blood from the elbow of the research subjects was uniformly collected, placed in an anticoagulation tube (see supplementary materials for population inclusion criteria). All methods were performed in accordance with the relevant guidelines and regulations. The survey was approved by the ethics board of the first affiliated hospital of Guangdong Pharmaceutical University (batch number: (2021) No. [116]).

### Rat tail vibration exposure model

Twenty-five Specific Pathogen Free (SPF) grade male Sprague–Dawley (SD) rats, weighing  $178.3 \pm 6.1$  g and aged 7–8 weeks, were supplied by the Guangdong Medical Experimental Animal Center and randomly divided into five groups: the control group, 1-day vibration exposure group, 3-day vibration exposure group, 7-day vibration group, and 14-day vibration group, with five rats in each group. The vibration exposure conditions were as follows: The tail of each rat was exposed to vibration for 4 h/day at a vibration frequency of 125 Hz and an acceleration of  $6.4 \text{ m/s}^2$ . The exposure conditions were chosen from the information provided by most studies on the frequency-related effects of changes in vibration injury<sup>12</sup>. Rats were anesthetized by intraperitoneal injection of 1 ml 3% pentobarbital sodium per 100 g body weight. After anesthesia, blood was collected from the abdominal aorta of the rats and euthanized by air embolization. All methods were performed in accordance with the relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines for the reporting of animal experiments.

### Detection of vasoactive factors by ELISA

The detection of the vasoactive factors leukotriene B<sub>4</sub> (LTB<sub>4</sub>), 5-hydroxy tryptamine (5-HT), VEGF, interleukin  $\beta$  (IL-1 $\beta$ ), Myosin light chain-2 (MLC2), Calcitonin gene-related peptide (CGRP) and Endothelin-1 (ET-1) in human and rat plasma after thawing at room temperature, in cells, and supernatants was performed according to the ELISA kit procedure produced by MEIMIAN (Jiangsu China). The optical density values of each well were measured at 450 nm (Swiss Sunrise Automatic Enzyme Labeler), the standard curve was plotted, and the concentration levels of the above indicators were calculated.

### Real-time fluorescence quantitative reverse transcription PCR

Total RNAs were extracted using Trizol reagent (Invitrogen), equal amounts of total RNA were reverse transcribed using a cDNA synthesis kit (TaKaRa Bio, Kusatsu, Japan), and real-time fluorescent quantitative PCR (qRT-PCR) was performed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference to detect the relative expression levels. The primers used were as follows supplementary Table S2.

### lncRNA *MEG3* knock-down

Before conducting lncRNA *MEG3* knockdown experiments, the most suitable siRNA (small interfering RNA) transfection dose needs to be determined first. We selected FMA-modified siRNA fragments and used universal liposomes Lipofectamine 2000 (ThermoFisher, Waltham, MA) as transfectants. The fluorescence intensity of each well was observed under a fluorescence microscope, and the liposome concentration with the strongest fluorescence was recorded. *MEG3* siRNA and siRNA were transfected into HUVECs at a concentration of 50 nmol/L using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. *MEG3*-specific siRNA and siRNA were designed and synthesized from GenePharma (Shanghai, China). Cells were harvested after 48 h for qRT-PCR, western blot, cell proliferation and apoptosis analyses.

### Western blot

Cellular protein content in lysates was assessed by the BCA protein assay (Thermo Fisher, Waltham, MA). Cell protein lysates were separated in 10%SDS-PAGE and transferred to a 0.22 µm polyvinylidene difluoride membrane. Membranes were subsequently incubated with specific antibodies (ABCam, Cambridge, UK) overnight at 4 °C. Secondary antibodies were added and incubated for 1 h at 37 °C. The immunoreactive bands were visualized using the ECL kit (Thermo Fisher).

### Statistical analysis

All statistical analyses were performed using the SPSS 21.0. The measured data were tested for normality, and data conforming to a normal distribution were described by ( $\bar{X} \pm S$ ). Median (M) and interquartile spacing (P25 to P75) were used to describe the number of disobedience to normal distribution. One-way ANOVA or two-way ANOVA were used for comparison of means between multiple groups. Before comparing between groups, the sameness test of variance was performed on the data of each index, and the interaction between the treatment factors was analyzed. If the variance was equal, the least significant difference (LSD) rank test was used for pairwise comparison. Otherwise, use Dunnett's T3 for pairwise comparison. Rates of enumeration data were compared using the Pearson  $\chi^2$  test or Fisher's exact test. All tests were two-tailed and  $P < 0.05$  was defined as statistically significant. GraphPad was used for plotting.

### Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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## Author contributions

X.H. contributed to the questionnaire survey, experimental operation and data analysis. F.Z. contributed to the thesis writing and experimental operation. Z.C. contributed to the questionnaire survey and experimental operation. K.H. contributed to the data analysis. Q.C. contributed to the questionnaire design and experimental design. Y.X. contributed to the experimental planning and thesis guidance. H.Y. contributed to the overall planning of the experiment, the technical guidance of the experiment, and the guidance of the writing of the thesis. All authors have read and approved the final manuscript.

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## Declarations

## Competing interests

The authors declare no competing interests.

## Ethical approval and consent to participate

The blood samples of this study population were approved by the Ethics Committee of the First Affiliated Hospital of Guangdong Pharmaceutical University, Guangdong Province, batch number: (2021) No. [116], and the written informed consent of the research subjects was obtained. All methods were performed in accordance with the relevant guidelines and regulations. The animal experiments in this study have been approved by the Laboratory Animal Ethics Committee of Guangdong Pharmaceutical University (acceptance number: gdpulac2021212). All methods were performed in accordance with the relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines for the reporting of animal experiments.

## Consent for publication

Consent obtained from individuals participating in stakeholder interviews.

## Additional information

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