Caveolin-1 Alleviates Angiotensin Ⅱ Induced Endothelial Injury and Cerebral Microbleed Via Mitochondrial Protection

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Abstract

Cerebral microbleeds are a manifestation of cerebral small vessel disease (CSVD) and a predictor of cerebral hemorrhage. However, the causes of cerebral microbleeds remain unclear. Research on the mechanism of cerebral microbleeds may provide new ideas for the treatment of CSVD. In a mouse model, we show cerebral microbleeds and cognitive impairment with increasing expression of caveolin-1 (Cav-1). Mechanistically, in an in vitro study, we find that Cav-1 knockdown significantly affects the mitochondrial pathway in the cytoplasm and decreases the endothelial viability. Moreover, Cav-1 knockdown induces mitochondrial membrane potential (MMP) depolarization and mitochondria reactive oxygen species (ROS) production. By contrast, overexpression of Cav-1 alleviates the decrease of cell viability, MMP depolarization and mitochondria ROS production induced by angiotensin Ⅱ (Ang Ⅱ). Furthermore, Cav-1 activation significantly improves cerebral microbleeds and cognitive impairment in CSVD mice. We identify Cav-1 as an endogenous protective molecule via mitochondrial protection, and activation of Cav-1 resists microbleeds and cognitive impairment in an animal model of CSVD. Cav-1 might be a candidate therapeutic target for CSVD.

Introduction

Cerebral small vessel disease (CSVD) is a neglected but very serious disease in modern society that is characterized by cognitive dysfunction, microbleeds, and white matter hyperintensities[1]. CSVD is related to many diseases, such as stroke, Alzheimer's disease (AD), vascular dementia, and depression[2-4]. The incidence of CSVD in people over 60 years old is 6-10 times higher than that of large vessel stroke[4]. Although abundant clinical data on CSVD are available, relatively few basic studies have been conducted. One of the reasons is that no animal model coincides with the pathological manifestations of CSVD. In my previous study, we found that mice infused with Ang Ⅱ for 28 days lead to CSVD phenotype coincided with the early clinical manifestation of the patients[5]. Due to a lack of understanding of its pathogenesis, effective treatment methods have not been developed. Stroke caused by CSVD accounts for 25% of cases, and CSVD increases stroke accidents and worsens the outcome[6,7]. Microbleeds are an independent risk factor for stroke and result from the exudation of red blood cells caused by endothelial cell injury and iron deposition in brain tissue. Therefore, microbleeds are a characteristic of vascular injury. Microbleeds are closely related to cognitive impairment[8], which is a consequence of endothelial dysfunction. The causes of cognitive impairment in individuals with CSVD are mainly vascular factors, such as hypertension, ageing, obesity, and diabetes.

Endothelial cell dysfunction is considered the first stage of CSVD[9]. Damage to endothelial cells leads to blood-brain barrier (BBB) leakage, promotes inflammatory cell infiltration into the brain parenchyma, and triggers neuroinflammatory reactions[10]. Endothelial dysfunction seriously affects the function of vascular nerve coupling and cognitive function[11]. The brain is one of the organs that consumes the largest amount of energy and accounts for 20% of oxygen consumption, especially in the learning and memory period [12]. Based on accumulating evidence, mitochondrial dysfunction plays a crucial role in endothelial cell injury in patients with cardiovascular and cerebrovascular diseases[13,14]. Although

endothelial cells consume less energy than cardiomyocytes and skeletal muscle cells, they also need a large amount of energy to maintain cognitive function. The mitochondrial content of endothelial cells that comprise the BBB is 2-4 times higher than that of endothelial cells in other locations to control the BBB integrity[15]. Furthermore, the production of mitochondrial reactive oxygen species (ROS) induced by mitochondrial dysfunction are an important factor contributing to endothelial dysfunction[16]. Mitochondrial membrane potential (MMP) depolarization results in excess mitochondrial ROS production [17]. As reported in our previous study, the mitochondrial protective drug nicotinamide riboside rescues CSVD, which provides evidence that targeted mitochondrial therapy is a feasible strategy[5]. Therefore, we speculate that mitochondria in endothelial cells play a critical role in the process of CSVD.

Cav-1 is the main protein in caveolae, a membrane structure that invaginates into a flask shape on the cell membrane. Caveolae are rich in sphingomyelin and cholesterol, known as lipid rafts[18,19]. Their size is approximately 50-100 nm, and evidence has shown that vesicle transport is abundant in endothelial cells [20]. Caveolae-mediated vesicle transport is involved in the transport and signal transduction of many macromolecules[21]. Cav-1 not only dominates the function of the BBB but is also critical for maintaining cognitive function [22,20]. The induction of Cav-1 expression effectively improves the cognitive function of individuals with AD, stroke, diabetes, and other diseases [23-25]. Cav-1 is involved in the release of NO from vascular endothelial cells [26] and is related to the synthesis of neurotrophic factors. Both of these processes are associated to the function of the neurovascular unit. The dysfunction of the neurovascular unit leads to the dysfunction of neurovascular coupling and eventually leads to cognitive impairment [27]. Cav-1 is significantly related to mitochondrial function[28]. The absence of Cav-1 leads to mitochondrial dysfunction, MMP depolarization and premature cell ageing[29]. However, the function and role of Cav-1 in the mitochondria of endothelial cells remain unclear, and thus we plan to investigate microbleeds and cognitive function in a model of CSVD by regulating Cav-1. We suspect that Cav-1 is essential for mitochondria to protect endothelial function.

Results

Ang □ induces cerebral microbleeds and cognitive dysfunction in mice.

We tested the cognitive function of mice after modelling by performing the nesting experiment, and the nesting ability of mice in the Ang □ group was significantly decreased compared with the control group (Fig.1 A-B). We used Prussian blue staining to detect cerebral microbleeds in mice after 4 weeks of perfusion of Ang □. The microbleeds in the cortex significantly increased (Fig.1 C-D). Endothelial dysfunction is associated with cognitive function and microbleeds. We examined abnormal endothelial cells around blood vessels using immunofluorescence staining to provide support for this concept. Propidium Iodide (PI) staining showed that endothelial nucleus was wrinkled significantly in Ang □ group (Fig.1 E-F). The sequence of endothelial dysfunction is BBB disruption. The immunofluorescence data showed that the fibrin leaked from blood vessels in Ang □ mice (Fig.1 G-H).

Ang □ increases the expression of Cav-1 in blood vessels.

Cav-1, which is considered an important molecule for neurovascular coupling function, is expressed at high levels in endothelial cells. We observed that Cav-1 was mainly co-labeled with blood vessels by immunofluorescence, and the fluorescence intensity increased in Ang □ group compared with control group (Fig.2 C-D). Western blot results confirmed that the expression of Cav-1 was significantly upregulated (Fig.2 A-B). We speculate that Cav-1 might play an essential role in the process of CSVD.

Cav-1 knockdown affects the mitochondrial function.

We first analyzed the RNA-Seq data from the endothelial cell line bEnd.3 after Cav-1 knockdown in vitro to explore the role of Cav-1 in endothelial cells. In addition to the classical effects on cytoplasm and cell membrane, the GO (gene ontology) term was enriched in mitochondrial function (Fig3. A). KEGG enrichment revealed that Cav-1 knockdown mainly changed the function about cellular community, cell growth and cell death, which provided a view to focus mitochondrial function (Fig3. B). We screened the first 16 differentially expressed genes. The heatmap showed that Ccn1, Nptx1, Cav-1, and Stfa2 were the most significantly altered genes (Fig3. C). Then, we analyzed the genes whose expression was significantly changed at log2>2. The results showed that 25% of the genes were related to mitochondrial function (Fig3. D). Similarly, the genes related to the mitochondrion showed the greatest differences in expression, accounting for 75%.

Cav-1 Knockdown induces mitochondria oxidative injury

We detected mitochondrial function after Cav-1 knockdown by performing live cell staining to obtain more insights into the relationship between mitochondria and Cav-1. Western blot analysis confirmed Cav-1 knockdown was successful (Fig4. A-B). We next detected the cell viability of endothelial cells after Cav-1 knockdown. The results showed that cell viability of endothelial cells in Cav-1 knockdown group decreased significantly compared with control group (Fig4. C). Combining with the results of RNA-Seq data in Fig. 3, We examined the mitochondrial oxidative stress in vitro. The maintenance of mitochondrial function requires normal levels of the MMP and mitochondrial ROS. Indeed, Cav-1 knockdown caused depolarization of the MMP (Fig4. D-E) and increased mitochondrial ROS production (Fig4. F-G) in bEnd.3 cell line, which suggested the mitochondrial dysfunction.

Cav-1 overexpression decreases the Ang □ induced mitochondrial dysfunction in endothelial cells.

We overexpressed Cav-1 (Fig5. A-B) in endothelial cells to examine the protective function of Cav-1 after Ang □ treatment. Cell viability was significantly decreased after Ang □ treatment for 12 or 24 h (Fig5. C). When we overexpression the Cav-1 in endothelial cells, the influence of Ang □ was significantly decreased (Fig5. D). Because of the role of Cav-1 in mitochondrial function, we detected the markers of oxidative stress in mitochondrion. Cav-1 overexpression in endothelial cells decreased the MMP depolarization (Fig5. E-F) and mitochondrial ROS production (Fig5. G-H) induced by Ang □. Thus, these results indicated that Cav-1 overexpression restrains Ang □-induced endothelial dysfunction by protecting mitochondrial function.

Cav-1 activation decreases cognitive dysfunction and cerebral microbleeds induced by Ang □

We injected the Cav-1 activating peptide AP-Cav-1 into mice to further determine the role of Cav-1 in CSVD in vivo. After treatment for 28 days, we performed nesting experiments and found that the nesting function of the mice in the AP-Cav-1 group was significantly improved compared with that of the Ang □ group (Fig6. A-B). Additionally, using Prussian blue staining, we found that the microbleeds of the mice treated with AP-Cav-1 were decreased compared with those of the Ang □ group (Fig6. C-D). Regarding endothelial cells, the immunofluorescence results showed that the number of abnormal endothelial cells and the leakage of fibrin from blood vessels were decreased significantly in AP-Cav-1 group compared with Ang □ group (Fig6. E-H) indicating a significant improvement in BBB function after Cav-1 activation in the animal model of CSVD (Fig6. G-H).

Discussion

With the increasing development of social economics, the incidence of CSVD has hardly increased. In this study, we identified a protective role for Cav-1 in CSVD by maintaining the mitochondrial function of endothelial cells. In an in vitro study, we found that Cav-1 knockdown leads to mitochondrial abnormalities. After overexpression of Cav-1 in endothelial cells, we observed that the mitochondrial dysfunction induced by Ang □ was significantly reversed. Overall, Cav-1 is an endogenous molecule that protects against mitochondrial dysfunction and endothelial dysfunction in CSVD.

Microbleeds and cognitive impairment are considered two important pathological manifestations of CSVD, and microbleeds are the precursor of future bleeding [30]. Vascular dementia accounts for 45% of all types of dementia, and cognitive impairment can cause and aggravate AD [31]. The common cause of these two different pathological processes is endothelial cell dysfunction. We focused on endothelial cells and observed microbleeds, cognitive dysfunction, and endothelial dysfunction in this model. In the present study, the expression of Cav-1, which was originally expressed at relatively high levels in endothelial cells, was upregulated substantially in the CSVD model. First, we postulated that the upregulation of Cav-1 caused endothelial cell damage, but when we knocked down Cav-1, endothelial dysfunction was more serious. We propose that the upregulation of Cav-1 expression exerted an endogenous protective effect.

Clinical data have indicated that microbleeds are robustly relevant to stroke and are a potential risk factor for intracerebral [32]. Both microbleeds and cognitive dysfunction suggest endothelial dysfunction. Although clinical trials have been carried out on some drugs, such as antihypertensive therapy, statin therapy and antiplatelet therapy, treatments for hypertension do not improve cognitive impairment[33]. Furthermore, treatment with statins and antiplatelet drugs has the risk of bleeding transformation [34-37]. Although these treatments are all methods to protect endothelial cells, their ineffectiveness may be due to incorrect treatment targets. A better treatment for CSVD is urgently needed.

Here, we observed significantly increased expression of Cav-1 in endothelial cells. Cav-1 has been reported to be closely related to cognitive function in many diseases; for example, Cav-1 deficiency

causes tentacle reaction abnormalities, which cooperate with eNOS function[22]. Overexpression of Cav-1 in the hippocampus of adult or old rats significantly improves cognitive function. Upon Cav-1 activation, the TrkB receptor is expressed in the hippocampus and relocated to the cell membrane for its activation, which improves cognitive function[38]. On the other hand, depletion of Cav-1 upregulated the expression of the amyloid precursor protein and BACE-1, increasing the β-amyloid Aβ42/40 ratio and hyperphosphorylated tau species, which coincide with the pathological manifestations of AD [23]. In previous studies, researchers found that the function of Cav-1 is associated with mitochondrial function. In fibroblasts, Cav-1 knockdown or knockout decreased mitochondrial respiration, reduced the activity of complex I, inactivated SIRT1, and decreased the NAD+/NADH ratio, which contributed to premature cell senescence[29]. AP-Cav-1, known as the CSD peptide (amino acids 82 to 101 of Cav-1), has been applied to individuals with many diseases and exerts protective effects. In the present study, CSVD mice treated with AP-Cav-1 for 28 days exhibited improved cognitive function in the nesting test and decreased the microbleeds in the brain. Furthermore, after the intervention, the number of PI-labelled endothelial cells were decreased. The CSD peptide treatment might be a new treatment strategy for CSVD, but further clinical study is needed to confirm this hypothesis.

Endothelial dysfunction initiates CSVD [39]. Endothelial dysfunction mainly influences cognitive function and BBB integration. Moreover, Cav-1 is critical for mitochondrial function. Cognitive dysfunction is an energy metabolism disorder. According to a previous study, Cav-1 and eNOS dominate neurovascular coupling, which requires abundant energy. Using two-photon microscopy, Brian W. Chow et al. found that Cav-1 is one of the two proteins that regulates blood flow changes in the tentacle reaction[22]. In the present study, we found that Cav-1 plays a critical role in endothelial mitochondrial function, which might explain why Cav-1 plays a crucial role in cognitive function. On the other hand, BBB maintenance is supported by retaining Cav-1-mediated vesical transport. Here, knockdown with Cav-1 led to an abnormal MMP and increased mitochondrial ROS. A recent study reported that treadmill excise increases the expression of Cav-1 to protect mitochondrial function and improve the outcome of disease models. By analyzing the RNA-Seq data, we found that Cav-1 deficiency mostly influenced the mitochondrial process. Furthermore, 75% of the molecules related to mitochondrial function were significantly altered. In a subsequent study, Cav-1 overexpression reduced Ang -induced MMP depolarization and decreased mitochondrial ROS generation. However, we did not explore the molecular mechanism underlying the effects of Cav-1 on mitochondria. We will explore the molecular mechanism of changes in mitochondria mediated by Cav-1 in future studies.

Conclusions

We found for the first time that the expression of Cav-1, an endogenous protective molecule, was upregulated in a CSVD model. We confirmed that Cav-1 was associated with cognitive function and microbleeds. Mechanistically, Cav-1 was critical for maintaining mitochondrial function. We infer that Cav-1 plays a key role in CSVD. It may be a target for the treatment of CSVD, and further clinical data are needed to prove this hypothesis.

Materials And Methods

Animals

Adult male C57BL/6 mice were used in this study. The mouse model was induced by subcutaneously implanting osmotic minipumps (ALZET® Osmotic Pumps, DURECT Corporation, Cupertino, CA 95041) as previously described[40]. Briefly, the hair of anaesthetized mice was removed from the back, and the skin was disinfected with iodophor and cut with scissors. The osmotic minipumps containing Ang Ⅱ (1000 ng/kg/min, purchased from Absin, Shanghai Hai, China, abs811565) or saline were implanted for 28 days, and the skin was sutured. AP-Cav-1(2mg/kg, synthesized by Sino Biological) was injected intraperitoneally every two days for 28 days. The experimental procedures were approved by the Laboratory Animal Care and Use Committee of the Army Medical University, China. The mice were fed with food and water under a 12 hours light / dark cycle.

Immunohistochemistry

Immunohistochemistry was performed using previously described procedures [41]. Briefly, anaesthetized mice were perfused with saline, and then mice were further perfused with phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA). Brains were postfixed by PBS which contain 4% PFA overnight at 4°C. Then, mice were dehydrated in 30% sucrose at 4°C for 2 days until the brain completely sink to the bottom. For immunofluorescence staining, brains were sectioned coronally at a thickness of 30 μm using a cryostat microtome (CM1860UV, Leica, Wetzlar, Germany). Antibodies against Cav-1 (rabbit, 1:200 Cell Signaling Technology (CST), 3267S) and Alexa Fluor 488- (mouse and rabbit, 1:1000) and Alexa Fluor 555-conjugated secondary antibodies (rabbit, 1:1000) from Invitrogen were used. The nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI; Invitrogen, 62248). The samples were observed with a laser-scanning confocal microscope (Zeiss, LSM780) and analysed using ZEN2012 software.

Immunoblotting

Western blot methods were performed as previously described [5]. Briefly, extracted brain protein was loaded onto an SDS-PAGE gel and then transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% BSA at room temperature for 2 hours. Then, membranes were incubated with primary antibodies overnight at 4°C. After that, the membranes were washed with TBS containing 0.1% Tween-20 for 3 times and then incubated with a horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Immunoreactive bands were detected using a chemiluminescence reagent kit (Thermo Scientific, IL, USA, 34580). The primary antibodies used were as follows: Cav-1 (rabbit, 1:1000 CST, 3267S) and GAPDH (mouse, 1:1000, Proteintech 60004-1-Ig). An HRP-conjugated anti-rabbit secondary antibody (rabbit, 1:5000, Proteintech SA00001-2) was used. Images were photographed and analysed using Image Lab software (Image Lab 3.0; Bio-Rad).

RNA-Seq

The protocol is from LC-Bio. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA, 10296010) according to the manufacturer's procedure. The total RNA quantity and purity were analyzed with a Bioanalyzer 2100 and RNA 6000 Nano Lab Chip Kit (Agilent, CA, USA) with a RIN number >7.0. Approximately 10 μg of total RNA extracted from a specific adipose tissue type was subjected to isolation of poly(A) mRNA with poly-T oligo-conjugated magnetic beads (Invitrogen). Following purification, the mRNA was fragmented into small pieces using divalent cations at an elevated temperature. Then, the cleaved RNA fragments were reverse transcribed to create the final cDNA library in accordance with the protocol for the mRNA Seq sample preparation kit (Illumina, San Diego, USA), and the average insert size of the paired-end libraries was 300 bp (±50 bp). Then, we performed paired-end sequencing on an Illumina sequencing platform.

Cell culture

bEnd.3 cells were purchased from the National Collection of Authenticated Cell Cultures. Cells were maintained at 37°C with 5% CO2 in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, 11965092) supplemented with 10% fetal bovine serum (FBS, Gibco, Australia, 10099141C). For Cav-1 knockdown, the siRNA sequence was provided by GeneChem (GCTTGTTGTCTACGATCTT). For Cav-1 overexpression, pLentai-hEF1a-PuroR-CMV-CAV1-EGFP-3xFlag was transfected into bEnd.3 cells. (Taitool, Shanghai).

Cell Viability assay

Viable cells were measured by Cell Counting Kit-8 (CCK-8, Dojindo, CK04, gift from Si Wang). Protocol are provided by manufacturer. In brief, $1*10^4$cells were seeded onto 96-well plates. Then cells were treated with Ang □ in 1μm for 12 or 24 h. After that, cells were exposed in 10μl CCK-8 for 3 h. The absorbance at 450 nm was measured by microplate.

Mitochondrial Membrane Potential detection

The mitochondrial membrane potential was detected using tetramethylrhodamine (TMRM, Invitrogen, I34361). bEnd.3 cells ($1*10^5$) were plated in a dish for confocal microscopy. After removing the medium, the cells were incubated with TMRM (25 nm) for 30 min at 37°C, and the nuclei were stained with Hoechst (Invitrogen, R37165). The samples were observed with a laser-scanning confocal microscope (Zeiss, LSM780, Germany) and analyzed using ZEN2012 software.

Mitochondrial ROS detection

Mitochondrial ROS were measured using MitoSOX Red (Molecular Probes, USA, M36008), a mitochondrial superoxide indicator. At the end of the experiment, the medium was removed, the cells were washed with PBS and stained with 5 μM MitoSOX Red for 10 min in a humidified atmosphere of 5% CO2 at 37°C, and the nuclei were stained with Hoechst (Invitrogen). After washes with PBS, cell sampling was performed using a confocal microscope (Zeiss LSM 780, Germany).

Behavioral test

Nesting experiment: The nesting experiment was performed as described previously[42]. On the 27th day, a 10 cm × 10 cm square cotton cloth was prepared and placed in the feeding cage (each mouse was placed in a cage separately) at 7 pm. The utilization rate of cotton cloth and nesting situation of mice were observed in the morning of the next day. According to the scoring standard, the scores were 0, 1, 2, 3, 4 and 5.

Statistical Analysis

All results are presented as the means ± standard errors of the means (SEM). Prism 6 software was used for statistical analysis, and one-way ANOVA and Tukey's test were used to analyze data from the three groups. Significant differences are denoted as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; NS, not significant.

Declarations

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Ethics declarations

The experimental procedures were approved by the Laboratory Animal Care and Use Committee of the Army Medical University and performed with the guidelines of Animal Use and Care of the National Institutes of Health.

Informed Consent

Not applicable.

Conflicts of interest

The authors declare no competing interests.

Consent for publication

Not applicable.

Data Availability

Data are available on reasonable request

Competing interests

The authors declare no competing interests.

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Authors' Contributions

H.F., X.W., Y.Y and C.L conceived and designed the study. C.L., X.T., and J.W. performed the animal experiments. C.L., M.X., Y.C., and B.W. conducted the cellular experiments. C.G., C.L., and Y.C. performed the molecular biology experiments. C.L., X.W., and H.D. analyzed the data. C.L. drafted the article. H.F., X.W., and Y.Y. revised and edited the article. All authors have read and approved this article.

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Figures

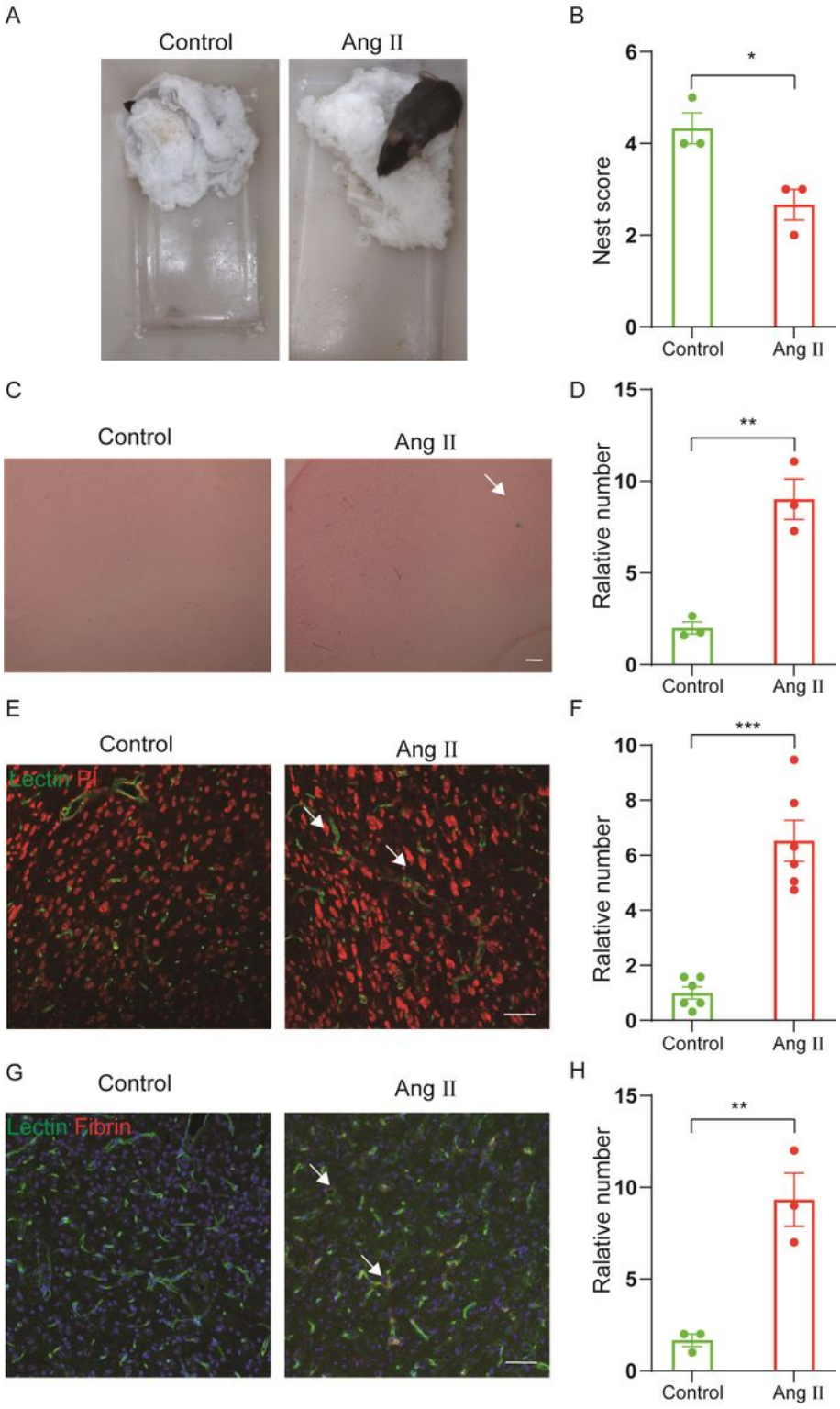

Figure 1

Ang Ⅱ induces cerebral microbleeds and nesting dysfunction in mice. (A-B) Representative images and quantification of the nesting function of mice treated with Ang Ⅱ for 28 days. (C-D) Representative images and quantification of cerebral microbleeds in mice. Arrows indicate microbleeds. (E-F) Representative images and quantification of PI (red, nucleus) and lectin (green, endothelial cells) immunofluorescence staining in brain tissue; arrows indicate endothelial nuclear abnormalities. (G-H) Representative images of

immunofluorescence staining and quantification of fibrin (red) deposition in the extravasculature region; endothelial cells, green; nucleus, blue. Arrows represent fibrin leakage. Student's t-test. Data are mean±sem. Scale bars: E and G = 20 μm, C = 50 μm. *p < 0.05, **p < 0.01 and *** p < 0.001.

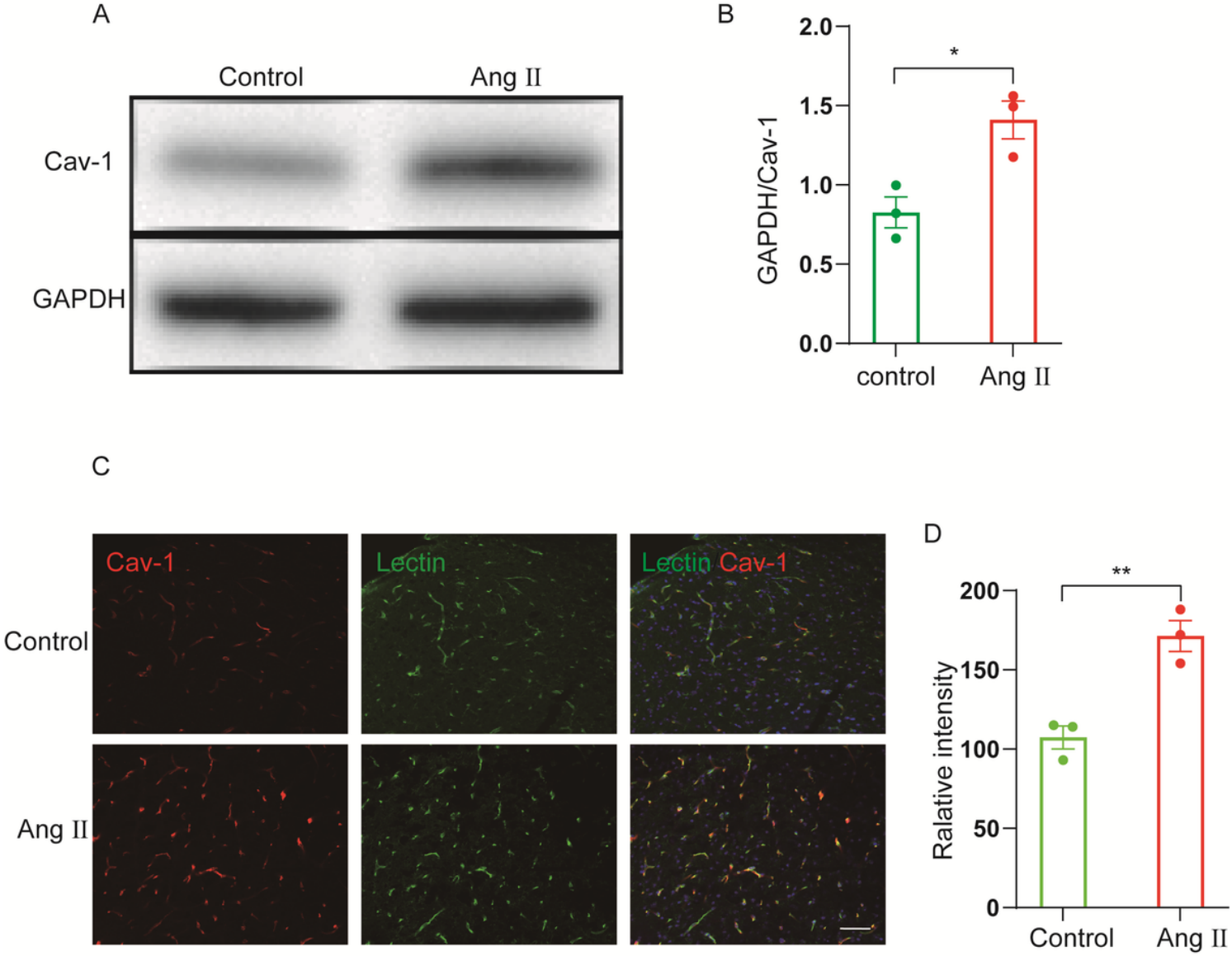

Figure 2

Ang Ⅱ increases the expression of Cav-1 in blood vessels (A-B) Representative images of immunoblots and quantification of Cav-1 levels in brain tissues. (C-D) Representative images of Cav-1 (red) and lectin (green) immunofluorescence staining. Student's t-test. Data are mean±sem. Scale bars: C = 20 μm. *p < 0.05, and **p < 0.01.

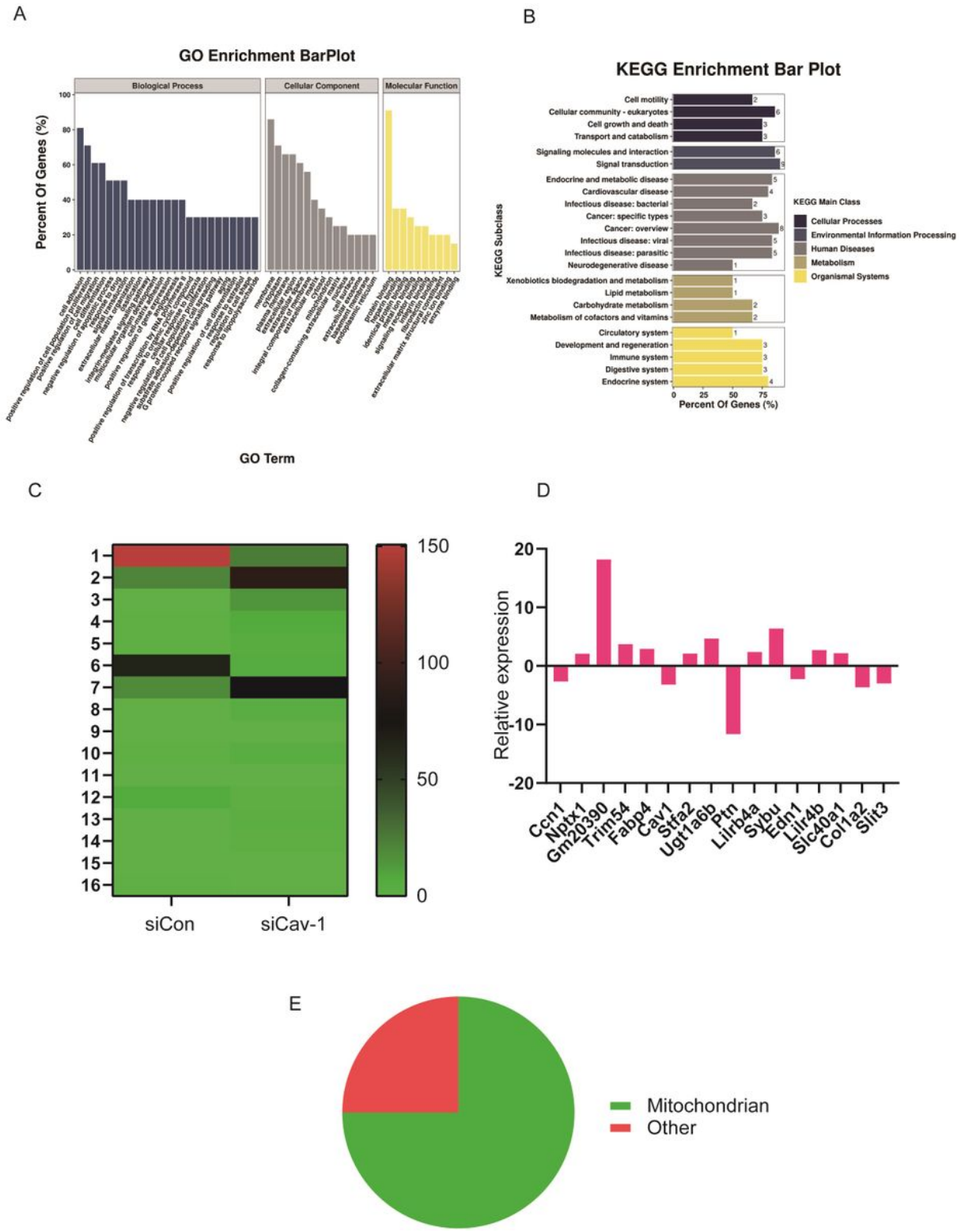

Figure 3

RNA-Seq analysis of the effect of Cav-1 knockdown on endothelial cells (A-B) GO and KEGG enrichment of Cav-1 knockdown and control groups. (C) Heatmap of top 16 significantly differentially expressed genes. (D) The names of the 16 genes. (E) Percentage of mitochondria-related genes of interest.

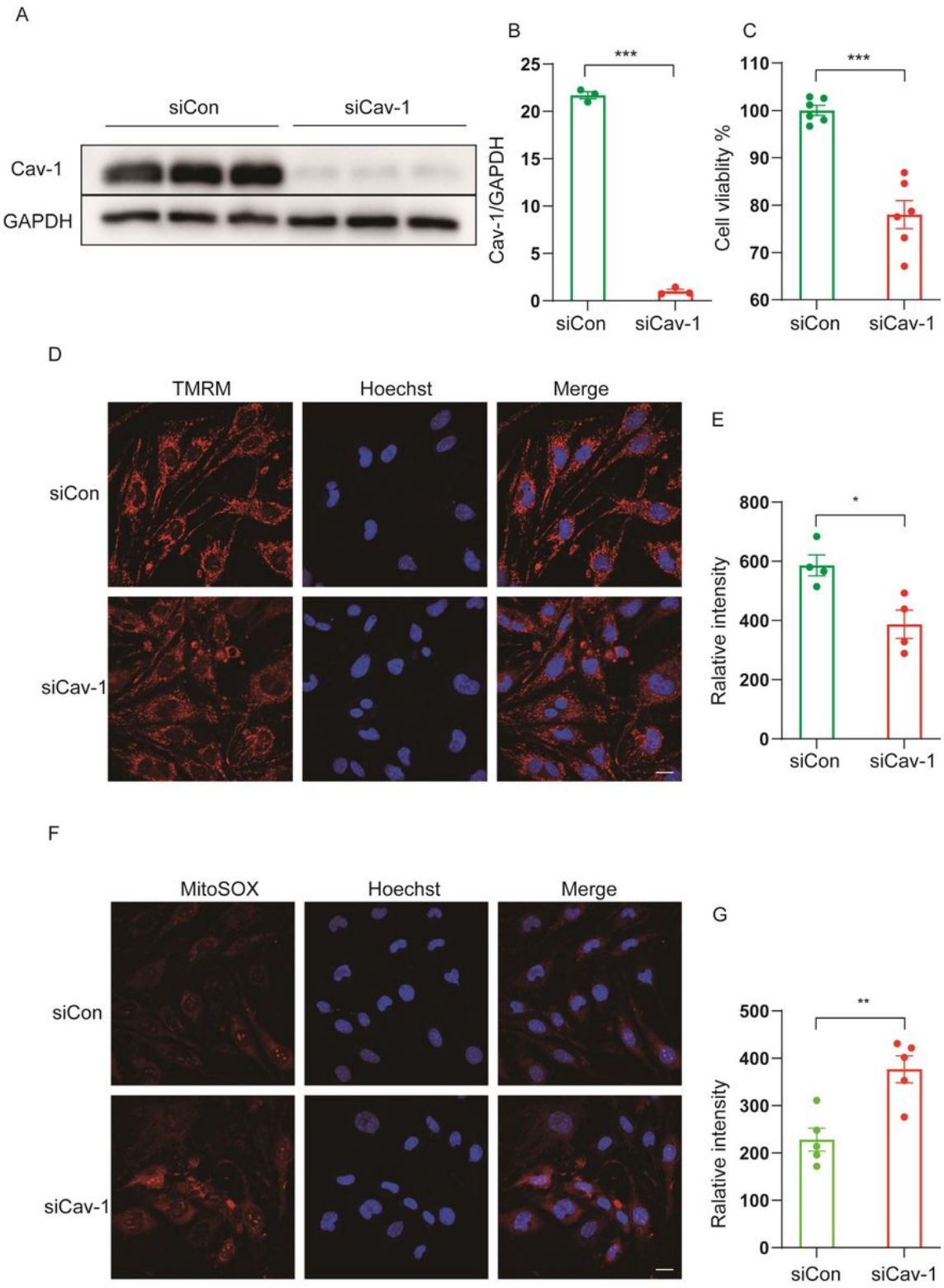

Figure 4

Cav-1 knockdown causes MMP depolarization and increases MitoSOX production (A-B) Representative images of immunoblots and quantification of Cav-1 in bEnd.3 cell line in control and Cav-1 knockdown groups. (C) Cell viability of bEnd.3 cell line in control and Cav-1 knockdown groups. (D-E) Representative images of TMRM (red) in Cav-1 knockdown and control group; nucleus, blue. (F-G) Representative images

of mitoSOX (red) in Cav-1 knockdown and control group; nucleus, blue. Scale bars: D and F = 20 μm. Student's t-test. Data are mean±sem. *p < 0.05, **p < 0.01 and *** p < 0.001.

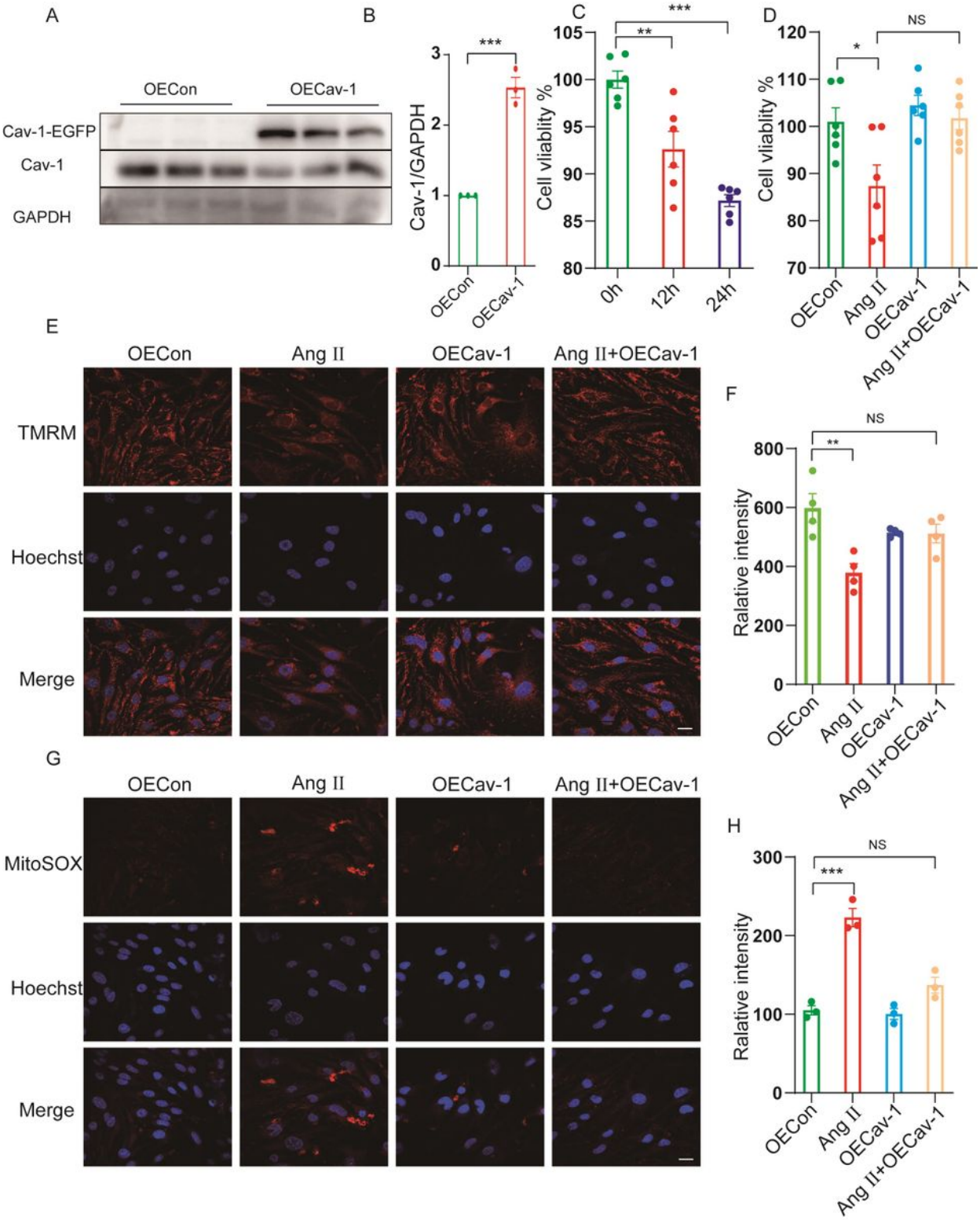

Figure 5

Cav-1 overexpression decreases MMP depolarization and MitoSOX production induced by Ang Ⅱ (A-B) Representative images and quantification of Cav-1 in bEnd.3 cell line in control and Cav-1 overexpression group. (C) Cell viability of bEnd.3 cell line after Ang Ⅱ (1μM) treatment for 0, 12, 24h. (D) Cell viability of

bEnd.3 cell line in control, Ang Ⅱ, Cav-1 overexpression and Cav-1 overexpression plus Ang Ⅱ treatment groups. Ang Ⅱ, 1μM for 24h. (E-F) Representative immunofluorescence images of TMRM (red) in 4 groups; nucleus, blue. (G-H) Representative images of mitoSOX (red) in 4 groups; nucleus, blue. Scale bars: E and G = 20 μm. One-way ANOVA, Tukey's post hoc test. Data are mean±sem. *p < 0.05, **p < 0.01 and *** p < 0.001.

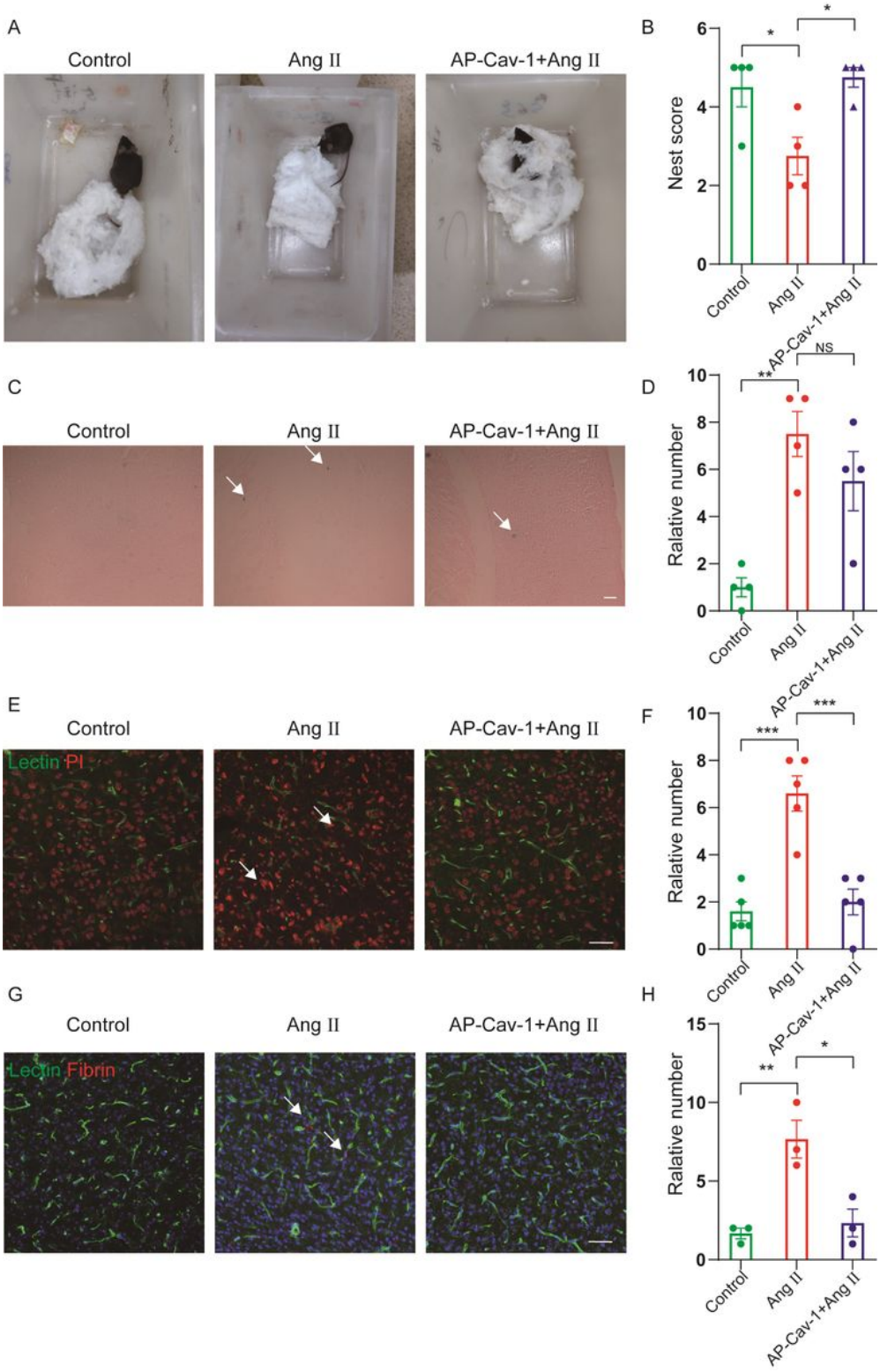

Figure 6

AP-Cav-1 treatment decreases the microbleeds and improves the nesting function (A-B) Representative images and quantification of the nesting function of mice in the control, Ang and AP-Cav-1 groups. (C-D) Representative images and quantification of lectin (green) and PI staining (red) in brain tissues from the three groups. Arrows indicate endothelial nuclear abnormalities. (E-F) Representative images and quantification of Prussian blue (blue) staining. Arrows indicate microbleeds. (G-H) Representative images and quantification of fibrin (red) that was colabelled with lectin (green); nucleus, blue. One-way ANOVA, Tukey's post hoc test. Data are mean±sem. Scale bars: E and G = 20 μm, C =50 μm. *p < 0.05, **p < 0.01, and *** p < 0.001.