Post-ischemic intra-arterial infusion of liposome-encapsulated hemoglobin can reduce ischemia reperfusion injury

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ABSTRACT

Despite successful revascularization, reperfusion after prolonged ischemia causes ischemia reperfusion (I/R) injury. Recruitment and activation of neutrophils is thought to be a key event causing I/R injury. We examined whether post-ischemic intra-arterial infusion of liposome-encapsulated hemoglobin (LEH), an artificial oxygen carrier without neutrophils, could reduce I/R injury in a rat transient middle cerebral artery occlusion (MCAO) model. Male Sprague-Dawley rats were subjected to 2-hour MCAO and then were divided into three groups: 1) LEH group (n=7) infused with LEH (Hb concentration of 6g/dl, 10ml/kg/h) through the recanalized internal carotid artery for 2 hours, 2) vehicle group (n=8) infused with saline (10ml/kg/h) in the same manner as the LEH group, and 3) control group (n=9) subjected to recanalization only. After 24-hour reperfusion, all rats were tested for neurological score and then sacrificed to examine infarct and edema volumes, myeloperoxidase (MPO) expression, matrix metalloproteinase-9 (MMP-9) expression and activity, and reactive oxygen species (ROS) production. Compared with the control group and the vehicle group, the LEH group showed a significantly better neurological score and significantly smaller infarct and edema volumes. MPO expression, MMP-9 expression and activity, and ROS production in the LEH group were also significantly lower than those in the control and vehicle groups. The results in the present study suggest that post-ischemic intra-arterial infusion of LEH can reduce I/R injury through reducing the effect of MMP-9, most likely produced by

neutrophils. This therapeutic strategy may be a promising candidate to prevent I/R injury after thrombolysis and/or thromboectomy. (246 words)

1. Introduction

Intravenous thrombolysis with tissue plasminogen activator (tPA) and mechanical thromboectomy are effective treatments to achieve recanalization in ischemic stroke. Recanalization is the most important action to obtain a favorable outcome in ischemic stroke patients. However, recently, it has been demonstrated that a couple of clinical trials of ischemic stroke treated with mechanical thromboectomy with or without tPA therapy failed to show a favorable outcome (Broderick et al. 2013; Ciccone et al., 2013; Kidwell et al., 2013). Despite successful revascularization after treatment, reperfusion after prolonged ischemia causes infarct enlargement with severe brain edema and hemorrhagic transformation rather than tissue recovery due to so-called “ischemia reperfusion (I/R) injury” (Molina et al., 2009). It is necessary to develop novel treatments for I/R injury to prevent brain edema and hemorrhagic transformation.

The mechanism of I/R injury has been extensively studied using animal transient focal ischemic models (Wong et al., 2008; Pan et al., 2007). Experimentally, focal I/R induces recruitment and activation of inflammatory cells, which are thought to be key events causing I/R injury, represented by blood brain barrier (BBB) breakdown brain edema, neural death, and hemorrhagic transformation (Amantea et al., 2009; Jin et al., 2010). Many studies have shown that systemic suppression of neutrophils attenuated I/R injury in a transient middle cerebral artery occlusion (MCAO) model (Matsuo et al., 1994; Mori et al., 1992; Zhang RL et al., 1994; Connolly et al., 1996).

Although suppression of neutrophils would be an attractive therapeutic strategy for I/R injury, systemic suppression may induce adverse effects on the defense mechanism against infection. We therefore developed a new therapeutic approach by reducing neutrophil influx into the I/R region. For this purpose, we used liposome-encapsulated hemoglobin (LEH), which was developed as an artificial oxygen carrier. LEH is composed of liposome capsules containing purified human hemoglobin without any blood cells, including neutrophils. We examined whether post-ischemic selective intra-arterial infusion of LEH to reduce localized inflow of neutrophils can ameliorate I/R injury in a rat transient MCAO model.

2. Results

2.1. *Physiological parameters*

Physiological parameters were monitored twice at the beginning of MCAO and the end of reperfusion. There was no statistically significant difference in the physiological parameters among the three groups, as shown in the table.

2.2. Neurological score

The rats in the three groups were re-evaluated using their Bederson score after 24-hour reperfusion. While rats in the control and vehicle groups showed no improvements and retained a 3-point Bederson score, some rats in the LEH group showed score improvements. The scores were

significantly better in the LEH group (2.51 ± 0.49) than in the control group (3.0 ± 0) and in the vehicle group (3.0 ± 0) (Figure 1).

2.3. *Infarct and edema volumes*

Brain infarct and edema volumes after 24-hour reperfusion were measured using 2,3,5-triphenyltetrazolium chloride (TTC) staining. Infarct volume of the LEH (34.3 ± 7.9%) group was significantly smaller than that of the control (51.4 ± 5.2%) and vehicle (49.6 ± 6.4%) groups. Edema volume of the LEH group (114.6 ± 4.8%) was significantly smaller than that of the control (121.8 ± 5.8%) and vehicle (121.3 ± 9.4%) groups (Figure 2).

2.4. *Myeloperoxidase (MPO) Western blotting*

MPO Western blotting was performed to assess neutrophil infiltration into the I/R region. MPO expression in the LEH group was significantly lower than that in the control and vehicle groups (Figure 3). MPO expression levels in the LEH group were less than 20% of those in the control group. MPO expression in the vehicle group was somewhat lower than that in the control group, but the difference was not statistically significant.

2.5. *Matrix metalloproteinase-9 (MMP-9) Western blotting and zymography*

The result of MMP-9 Western blotting showed a similar pattern to that of MPO Western blotting. MMP-9 expression in the LEH group was significantly lower than that in the control and vehicle groups (Figure 4a). The result of gelatine zymography for MMP-9 activity showed the same pattern

of results as MMP-9 Western blotting. MMP-9 activity in the LEH group was also significantly lower than that of the control and vehicle groups (Figure 4b).

2.6. *4-hydroxy-2-nonenal (4-HNE) Western blotting*

Western blotting of 4-HNE, which is a marker of lipid peroxidation, was performed to assess ROS production. 4-HNE expression in the LEH group was significantly lower than that in the control and vehicle groups (Figure 5).

3. Discussion

The results in the present study demonstrated that post-ischemic selective intra-arterial infusion of LEH contributed to neurological improvement and reduction of infarct volume and brain edema. This LEH treatment also suppressed neutrophil infiltration, represented by MPO expression, MMP-9 expression and activity, and ROS production in the I/R region. Meanwhile, intra-arterial infusion of saline did not have the same effect. This means that no hemodilution effect was related to the reduction of I/R injury by LEH treatment.

The development and progression of I/R injury is thought to depend on a series of reactions mediated by neutrophils. Histological and enzymatic MPO techniques revealed that marked neutrophil infiltration occurred throughout the infracted cortex after transient MCAO compared with permanent MCAO. (Barone et al., 1992) Depletion of neutrophils by administration of an anti-neutrophil antibody completely inhibited the increase of MPO activity and attenuated infarction and edema size

in a rat transient MCAO model (Matsuo et al., 1994). Inhibition of neutrophil adherence to endothelial cells also attenuated I/R injury; that is, administration of anti-CD18 antibody improved the microvascular no-reflow phenomenon in a baboon transient MCAO model (Mori et al., 1992), administration of anti-ICAM-1 antibody reduced neutrophil infiltration and infarct volume in a rat transient MCAO model (Zhang et al., 1994), and knockout of the ICAM-1 gene reduced infarct volume and increased the survival rate in a mouse transient MCAO model (Connolly et al., 1996). Infiltrated neutrophils in the I/R region are thought to contribute to inflammatory reactions, such as ROS production, release of cytokines and chemokines, release of elastase and matrix metalloproteinases (MMPs), and enhanced expression of integrin and adhesion molecules (Jin et al., 2010). In these sequential reactions, ROS is thought to affect the signaling pathways that induce the expression and activation of MMPs directly and/or indirectly (Jian Liu et al., 2005). Among MMPs, MMP-9 is relavant to BBB disruption, leading to increased vascular permeability and extravasation of plasma and blood cells, and finally resulting in severe brain edema and hemorrhagic transformation (Lakhan et al., 2013). A well-designed study with MMP-9-/- mice and chimeric mice with either MMP-9-deficient or MMP-9-containing leukocytes clearly demonstrated that MMP-9 released from neutrophils promoted further recruitment of neutrophils and caused BBB disruption secondary to collagen IV degradation (Gidday et al., 2005). Instead of neutrophil suppression, MMP-9 suppression by antibody, inhibitor, or gene knockout also reduced infarction size in a transient MCAO model (Romanic et al., 1998; Asahi et al., 2000). In addition to animal experiments,

a human pathological study demonstrated that MMP-9-positve neutrophil infiltration was associated with BBB breakdown and collagen IV degradation during hemorrhagic transformation (Rosell et al, 2008).

Although therapeutic approaches with systemic suppression of neutrophils and MMP-9 are effective in animal experiments, there remains concern about systemic immune insufficiency and other adverse effects in their clinical use. A clinical trial of anti-ICAM-1 therapy indeed demonstrated that intravenous administration of a murine ICAM-1 antibody was not an effective treatment for ischemic stroke and may significantly worsen stroke outcome (Enlimomab Acute Stroke Trial Investigators 2001). To eliminate the problems due to systemic suppression, we attempted to reduce localized neutrophil influx by selective intra-arterial infusion of LEH. In practice, we infused LEH from a tube wedged into the ipsilateral internal carotid artery for 2 hours. Although longer LEH infusion ensured more thorough reduction of neutrophil inflow, we chose 2 hours for LEH infusion in this study because of the difficulty of maintaining a steady cardiorespiratory state in prolonged anesthesia. This LEH treatment reduced MPO expression, with neutrophil infiltration at 20% of the control. It also reduced MMP-9 expression and activity and ROS production, and moreover reduced infarct volume and brain edema. Meanwhile, vehicle treatment, that is, selective intra-arterial infusion of saline, also somewhat reduced MPO expression (not statistically significantly), which was not accompanied by a reduction of MMP-9 expression, ROS production, and infarction size. This implies that not only

reduction of neutrophil influx but also oxygen supply by LEH was important for preventing I/R injury to the brain.

LEH was originally developed as a blood substitute without consideration of blood type in order to decrease transfusion risks and enable longer storage than the usual donated blood (Kaneda et al., 2009). Since LEH can deliver oxygen more efficiently to peripheral tissues because of its smaller diameter (200–250 nm; 1/40th the size of RBC), several studies have attempted to assess the therapeutic effects of LEH on brain ischemia. Intravenous administration of LEH reduced the extent of infarction in the cortex of a rat thrombotic MCAO model (Kawaguchi et al., 2007; Fukumoto et al., 2009) and in a nonhuman primate transient MCAO model (Kawaguchi et al., 2010). Another therapeutic effect of LEH on cognitive impairment was revealed in the analysis of hippocampal dysfunction after transient two-vessel or four-vessel occlusion (Kakehata et al., 2007; Hamadate et al., 2009). These therapeutic effects were explained by efficient oxygen supply via collateral circulation of nanometer-sized LEH and/or the ROS scavenging capacity of LEH in previous papers.

In the present study, intra-arterial LEH infusion was maintained for the first 2 hours but not for the whole reperfusion period. Although neutrophils could flow into the I/R region during the residual reperfusion period, the final neutrophil infiltration was suppressed at 20% of the control. So far, we are not sure whether the reduction of infarction size demonstrated in this study solely depends on the suppression of neutrophil infiltration due to the reduction of localized neutrophil influx for 2 hours. From the results of previous studies on LEH, we may have to consider efficient oxygen supply by

LEH as another reason, such as prevention of endothelial dysfunction, for the reduction of infarction size. This issue needs to be further addressed in future studies. Another issue to address is the durability of the protective effect of intra-arterial LEH infusion. If the cellular inflammation continues after 24 hours, the outcome in the chronic phase may differ from the outcome at 24 hours in the present study.

In conclusion, we have demonstrated that post-ischemic intra-arterial infusion of LEH can reduce ischemic reperfusion injury through inhibition of neutrophil infiltration, MMP-9 expression and production of ROS. LEH may be a promising candidate to prevent infarct enlargement with severe brain edema and hemorrhagic transformation after tPA thrombolysis and/or mechanical thrombectomy.

4, Experimental procedures

4.1. Animals

Male Sprague-Dawley rats (270-320 g) were purchased from CLEA Japan, Inc. (Tokyo, Japan). The protocol of this study was approved by the Animal Studies Ethics Committee at Hokkaido University Graduate School of Medicine. All procedures used in this study were performed in accordance with the institutional guidelines for animal experiments.

4.2. LEH

LEH was developed, manufactured and provided by Terumo Co. Ltd (Tokyo, Japan). LEH contains inositol hexaphosphate used as an allosteric effector to increase the oxygen affinity of Hb compared to usual RBC. The average size of liposomes ranged from 200 to 250 nm, which is 1/40th the size of RBC. The liposomal surface was modified polyethylene glycol to increase LEH stability during storage and use in blood by preventing aggregation. LEH was suspended in saline at Hb concentration of 6g/dl. LEH was oxygenated by mixing with room air before use, so that mean PaO_2 of LEH was increased to 110 ± 10mmHg.

4.3. MCAO model

Focal cerebral ischemia was induced by right MCAO by a modified method of intraluminal suture occlusion (Longa et al., 1989). Briefly, anesthesia was induced with 4.0% isoflurane in N_2O:O_2 (70:30) and maintained with 2.0% isoflurane in N_2O:O_2 (70:30) through a facial mask. Blood pCO_2, pO_2, pH, and Ht were monitored twice at the beginning of MCAO and the end of reperfusion. Rectal temperature was monitored and maintained at 37.0 ± 0.5°C using a heating pad during the MCAO procedure. The right common carotid artery was surgically exposed, the external carotid artery was ligated distally from the common carotid artery, and a silicone rubber-coated nylon monofilament with a tip diameter of 0.37 or 0.39 ± 0.02mm (Doccol Corp., Redlands, CA, USA) was inserted into the external carotid artery and advanced into the internal carotid artery to block the origin of the MCA. Rats recovered from anesthesia and only rats that showed a Bederson score of 3 points were

used for further experiments. The success rate in producing MCAO with Bederson score of 3 points was 50% (27/54).

4.4. Neurological score

Neurological evaluation was performed under awake conditions after the MCAO operation and 24-hour reperfusion using the Bederson score (Bederson et al., 1986). Scores were graded on a 3-point scale: 0, no observable deficit; 1, forelimb flexion; 2, decreased resistance to lateral push without circling; and 3, same behavior as 2 with circling.

4.5. LEH infusion

The rats subjected to 2-hour MCAO were divided into 3 groups: 1) LEH group (n=7) infused with LEH (10ml/kg/hr) through the catheter for 2 hours, 2) vehicle group (n=8) infused with saline in the same manner as the LEH group, and 3) control group (n=9) subjected to recanalization only without infusion. In the LEH and vehicle groups, a PE-10 catheter was inserted into the right external carotid artery immediately after withdrawing the suture and advanced into the internal carotid artery to over the pterygopalatine artery, and then intra–arterial infusion was performed. LEH was oxygenated by mixing with room air just before use, so that mean PaO2 of LEH was increased to 100 ± 10mmHg. The infused LEH and saline were warmed to 37 °C before infusion to avoid hypothermic neuroprotective effects due to low-temperature fluid infusion.

We determined the duration, speed, and total volume of the LEH infusion not to debilitate the I/R-treated rats due to long-time anesthesia and over-hydration. Two hour of anesthesia for

I/R-treated rats was the appropriate time limit not to cause respiratory trouble due to long-time anesthesia. Ten ml/kg/hr infusion for 2 hours, approximately 6 ml infusion per a rat, was the appropriate volume limit not to cause physiological harmful effects accompanied with lung and liver congestion. Therefore we chose 10ml/kg/hr infusion for 2 hours.

In order to speculate whether the 10ml/kg/hr LEH infusion for 2 hours can really reduce localized inflow of neutrophils, it is important to assess the flow relationship between the inter-arterial infusion and the collateral flow in brain parenchyma during the LEH infusion. For this purpose, we have done immunofluorescent analysis on distribution of LEHs labeled with anti-human hemoglobin antibody and rat erythrocytes labeled with anti-rat hemoglobin antibody in the cortical parenchymal microvessels of the MCA territory in the ipsilateral hemisphere during the LEH infusion (Figure 6). Parenchymal vessels in the ipsilateral hemisphere were more filled with LEH than rat erythrocytes. Quantitative analysis showed a significant reduction in the ratio of rat hemoglobin positive microvessels to human hemoglobin positive microvessels suggesting that the collateral flow was suppressed by the flow of inter-arterial infusion of LEH in the ipsilateral hemisphere (Figure 6). From this result, we speculate that the 10 ml/kg/hr infusion for 2 hours could reduce neutroprhil influx into the I/R region to some extent, even it could not completely prevent the collateral flow.

After intra-arterial infusion, the catheter was withdrawn, and normal reperfusion was established in the LEH group and vehicle groups. All rats were sacrificed 24 hours after reperfusion and the brains were collected to examine brain pathological changes.

Dead animals before the analysis set in the acute phase in each group were 0 in the control group, 2 (1; cause unknown, 1; subarachnoid hemorrhage) in the vehicle group, and 1 (cause unknown) in the LEH group, respectively. There was no significant difference among the three groups.

4.6. Immunofluorescent analysis

Frozen sections of the brain harvested during the inter-arterial LEH infusion were used for double immunofluorescent staining. The sections were labeled with primary antibodies (anti-human hemoglobin monoclonal antibody; 1:200, [Institute of Immunology, Tokyo, Japan] and anti-rat hemoglobin polyclonal antibody; 1:100, [Mitsubishi Chemical Medicine, Tokyo, Japan]) for 1 hour. Thereafter, the sections were treated with secondary antibodies (Alexa Fluor 488-conjugated goat anti-mouse IgG, 1:200, [Life Technologies, Carlsbad, CA, USA] and Alexa Fluor 594-conjugated goat anti-rabbit IgG, 1:200, [Life Technologies, Carlsbad, CA, USA]) for 1 hour. Finally, the sections were embedded in SlowFade Gold Antifade Reagent with DAPI (Life Technologies, Carlsbad, CA, USA). The fluorescent signals were observed by a fluorescent microscope (BX51; Olympus, Tokyo, Japan). Cortical parenchymal microvessels labeled with anti-human hemoglobin monoclonal antibody or with anti-rat hemoglobin polyclonal antibody were counted in the same random fields at x100 magnification. The ratio of rat hemoglobin positive microvessels to human hemoglobin positive microvessels were calculated in the bilateral hemispheres (n=3) (Figure 6).

4.7. Evaluation of infarction and edema volume

Infarct volumes were measured using 2,3,5-triphenyltetrazolium chloride (TTC) staining. The collected brains were sectioned coronally into 2mm-thick sections and were then immersed in 2% TTC (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for 20 minutes at 37°C. Infarct volumes were quantified with a standard computer-assisted image analysis system (Image J 1.37v; NIH, Bethesda, MD, USA). Infarct volume was calculated as a percent volume of the normal left hemisphere according to the following formula: (left hemisphere volume − right non-infarct volume)/left hemisphere volume × 100%. Brain swelling was calculated as a percent volume of the normal left hemisphere according to the following formula: (right hemisphere volume − left hemisphere volume)/left hemisphere volume × 100%.

4.8. Western blotting

Western blotting was performed using anti-myeloperoxidase (MPO) antibody (Cell Signaling Technology, Danvers, MA, USA), anti-MMP-9 antibody (Cell Signaling Technology, Danvers, MA, USA) and anti-4-hydroxy-2-nonenal (4-HNE) antibody (Abcam, Cambridge, MA, USA) to evaluate neutrophil infiltration into the brain, and the expressions of MMP-9 and ROS, respectively. Proteins were extracted from brain tissue of the ipsilateral hemisphere by homogenizing in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). An equal amount total protein (10ug) was electrophoresed in a NuPage 4-12% Bis-Tris Gel (Life Technologies, Carlsbad, CA, USA) and then transferred to nitrocellulose membranes using iBlot gel transfer device (Life Technologies, Carlsbad, CA, USA). The membrane was blocked with 10% nonfat dry milk in phosphate-buffered saline

(PBS) containing 0.05% Tween-20 for 1 hour at room temperature, followed by incubating with the primary antibody overnight at 4°C (anti-MMP-9 rabbit polyclonal antibody; 1:2000, anti-MPO mouse monoclonal antibody; 1:2000, anti-4-HNE rabbit polyclonal antibody; 1:2000). After washing with PBS containing 0.05% Tween-20, the membrane was incubated with peroxidase-conjugated secondary antibody at room temperature for 1 hour. Labeled proteins were detected using chemical luminescence (ECL Advanced Western Blotting Detection Kit; GE Healthcare Life Sciences).

4.9. Gelatin zymography

The activity of MMP-9 was measured by gelatin zymography. MMP-9-positive controls and 20μg protein samples extracted in the same manner as for Western blotting were loaded onto Novex zymogram gels (Life Technologies, Carlsbad, CA, USA). After separation by electrophoresis, the gels were renatured and incubated with development buffer at 37°C overnight. The gels were then stained using Coomassie blue dye for 1 hour and destained in washing solution (30% methanol, 10% acetic acid). Areas of MMP-9 activity appeared as clear bands against a dark background, and the gels were scanned and quantified by densitometry using an image analysis system (Image J 1.37v; NIH, Bethesda, MD, USA).

4.10. Statistical analyses

All quantitative measurements were made by blinded investigators. Data were expressed at the mean ± SD. Statistical differences among groups were analyzed using 1-way ANOVA followed by the Tukey-Kramer test. Differences at $p < 0.05$ were considered significant.

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Legends

Figure 1

Effect of intra-arterial infusion of LEH on neurological function.

Neurological function was evaluated by the Bederson score after 24-hour reperfusion. The bar graph shows the Bederson score of each group. *p <0.05.

Figure 2

Effect of intra-arterial infusion of LEH on infarction and edema volume.

Infarction and edema volume was evaluated by TTC staining after 24-hour reperfusion. Bar graph shows the infarction and edema volume of each group. *p <0.05 and **p <0.01.

Figure 3

Effect of intra-arterial infusion of LEH on MPO expression.

MPO expression representing neutrophil infiltration was evaluated by Western blotting after 24-hour reperfusion. Bar graph shows MPO expression of each group. *p <0.05 and **p <0.01.

Figure 4

Effect of intra-arterial infusion of LEH on MMP-9 expression and activity.

MMP-9 expression (A) and activity (B) was evaluated by Western blotting and gelatin zymography respectively after 24-hour reperfusion. Bar graphs show MMP-9 expression and activity of each group. *p <0.05 and **p <0.01.

Figure 5

Effect of intra-arterial infusion of LEH on 4-HNE expression

4-HNE expression representing ROS production was evaluated by Western blotting after 24-hour reperfusion. Bar graph shows 4-HNE expression of each group. **p <0.01.

Figure 6

Immunofluorescent analysis on distribution of LEHs and rat erythrocytes in cerebral parenchymal microvessels.

Double immunofluorescent staining for human hemoglobin contained in LEHs and rat hemoglobin contained in rat erythrocytes was performed with frozen sections of the brain harvested during the intra-arterial LEH infusion. A, Many cortical parenchymal microvessels were filled with LEH (green) in the ipsilateral brain hemisphere of the intra-arterial LEH infusion. B, A few cortical parenchymal microvessels were filled with rat erythrocytes (red) in the same field of panel A. C, A moderate number of cortical parenchymal vessels were filled with LEH (green) in the contralateral brain hemisphere of the intra-arterial LEH infusion. D, A moderate number of cortical parenchymal

vessels were filled with rat erythrocytes (red) in the same field of panel C. Bar graph shows Ratio of rat hemoglobin positive vessels to human hemoglobin positive vessels. **p <0.01.

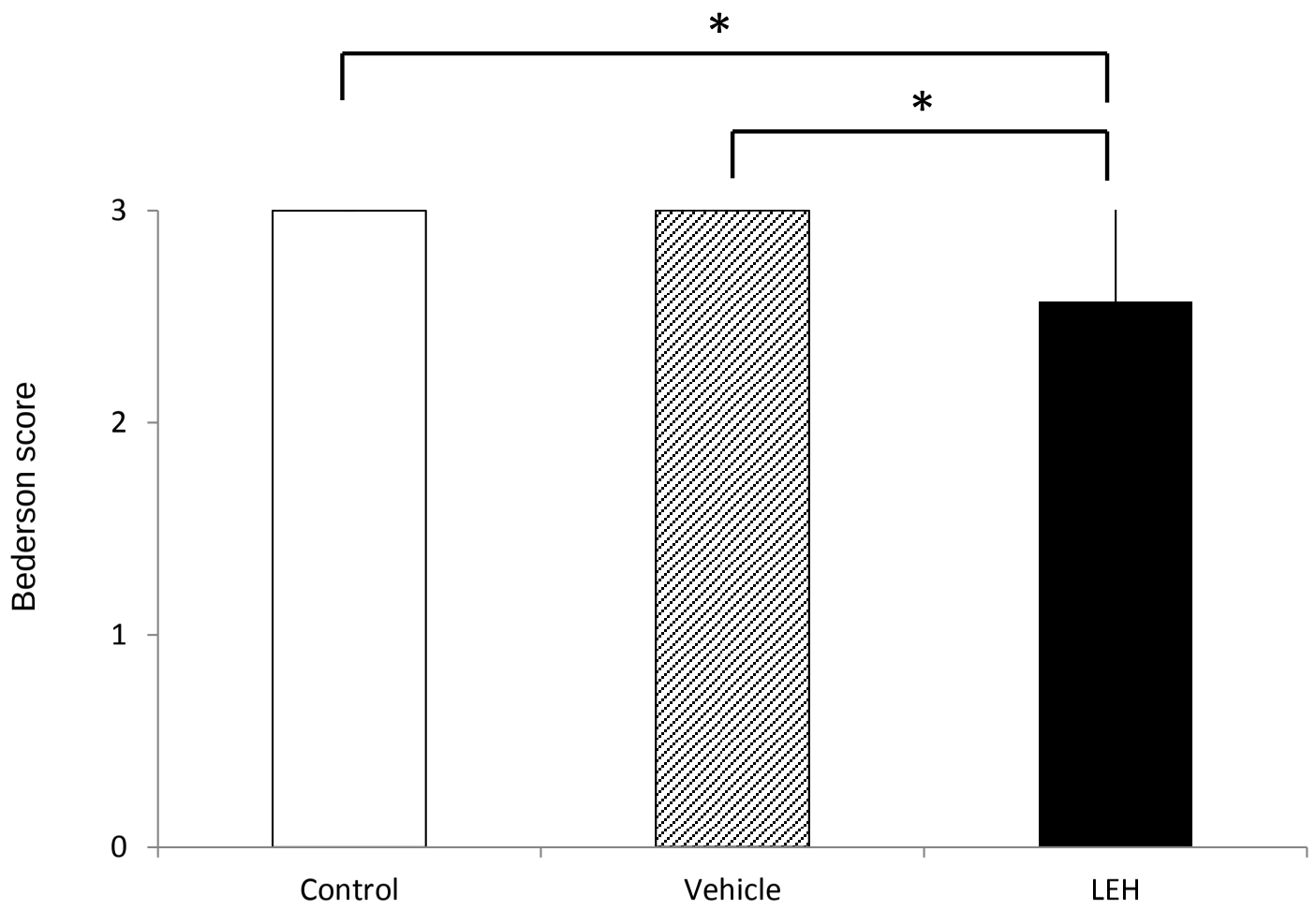

Figure 1

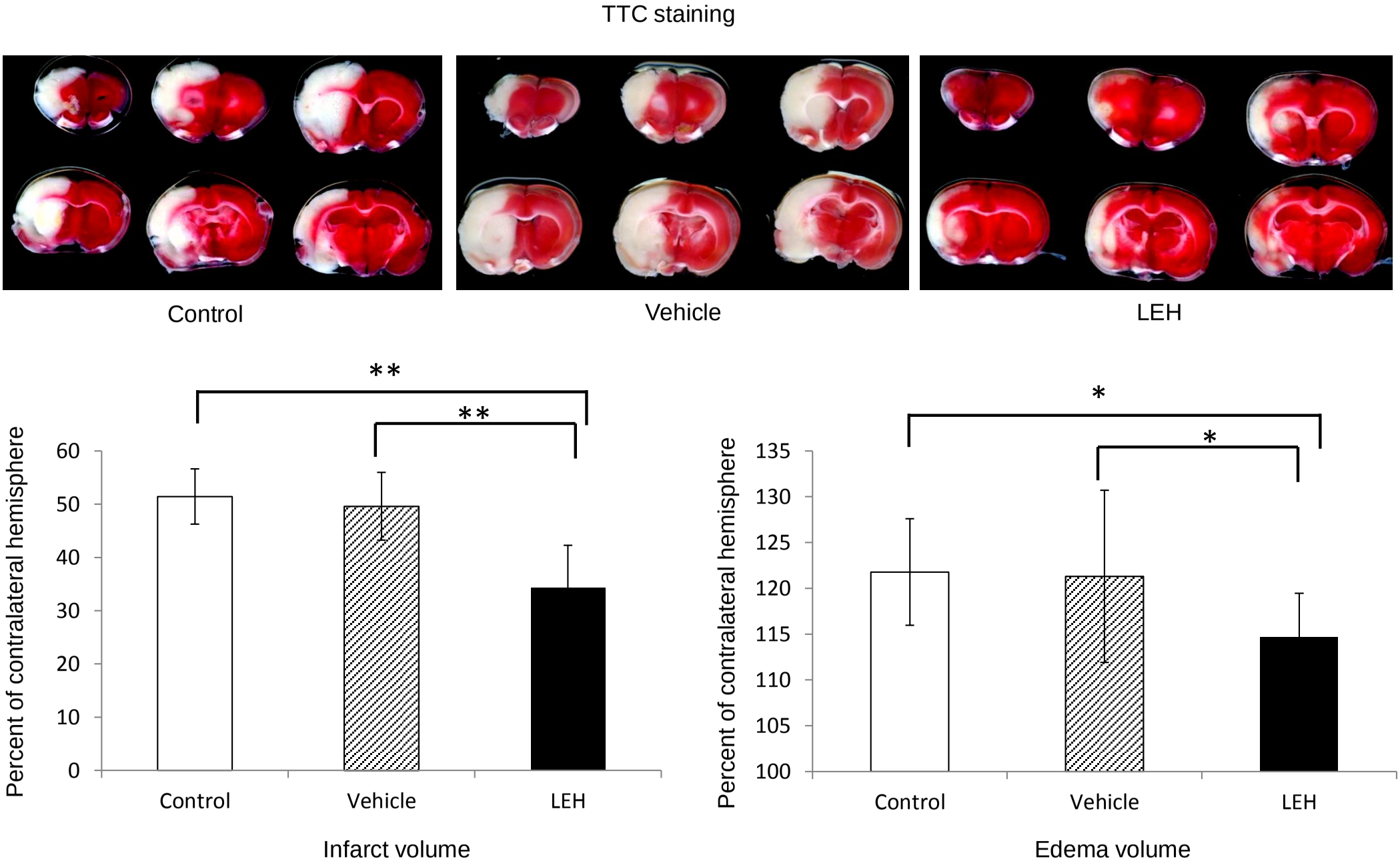
TTC staining
Control
Vehicle
LEH
**
**
Percent of contralateral hemisphere
60
50
40
30
20
10
0
Control
Vehicle
LEH
Infarct volume
*
*
Percent of contralateral hemisphere
135
130
125
120
115
110
105
100
Control
Vehicle
LEH
Edema volume

Figure 2

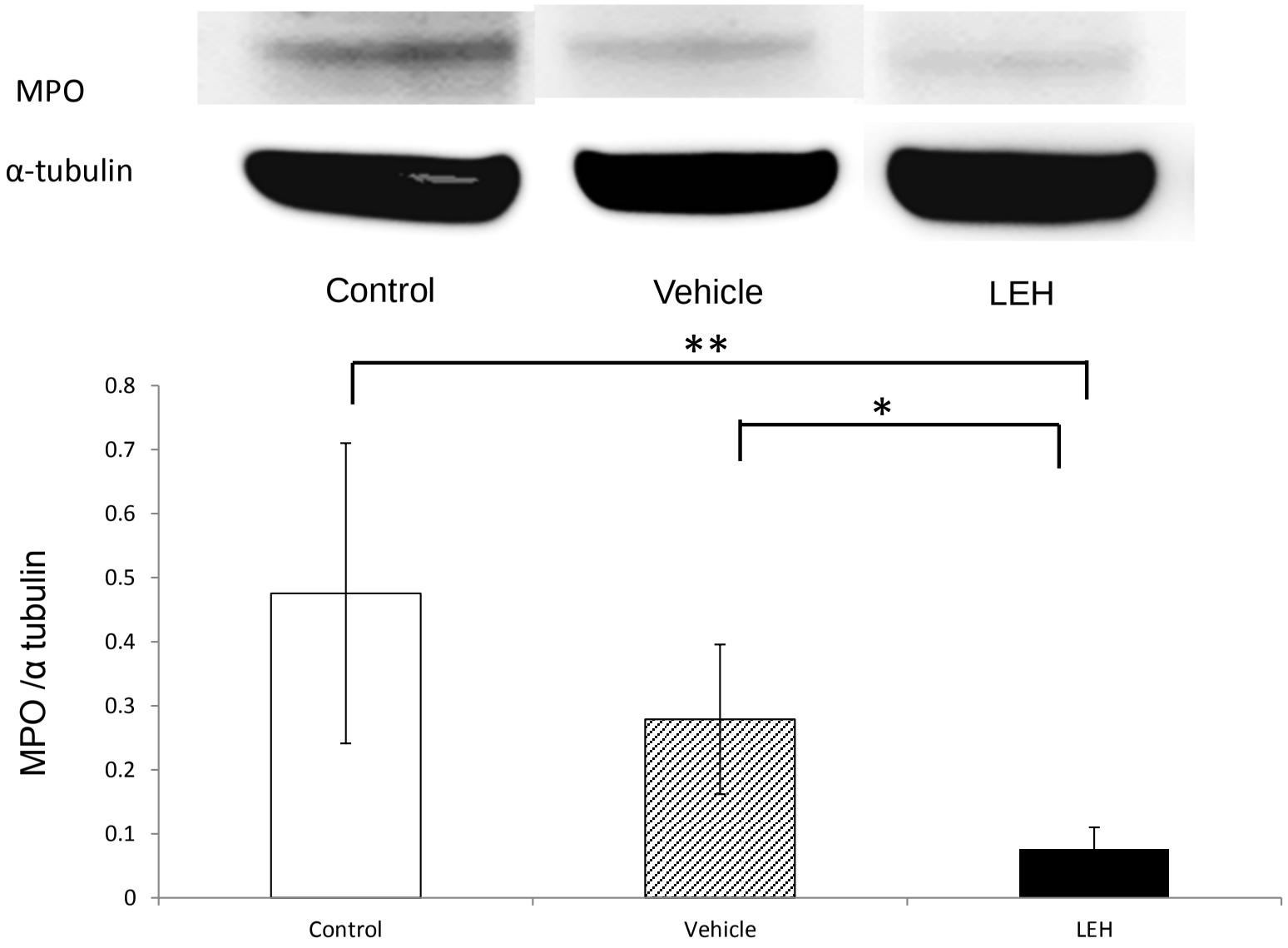
MPO
α-tubulin
Control
Vehicle
LEH
**
*
MPO /α tubulin
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Vehicle
LEH

Figure 3

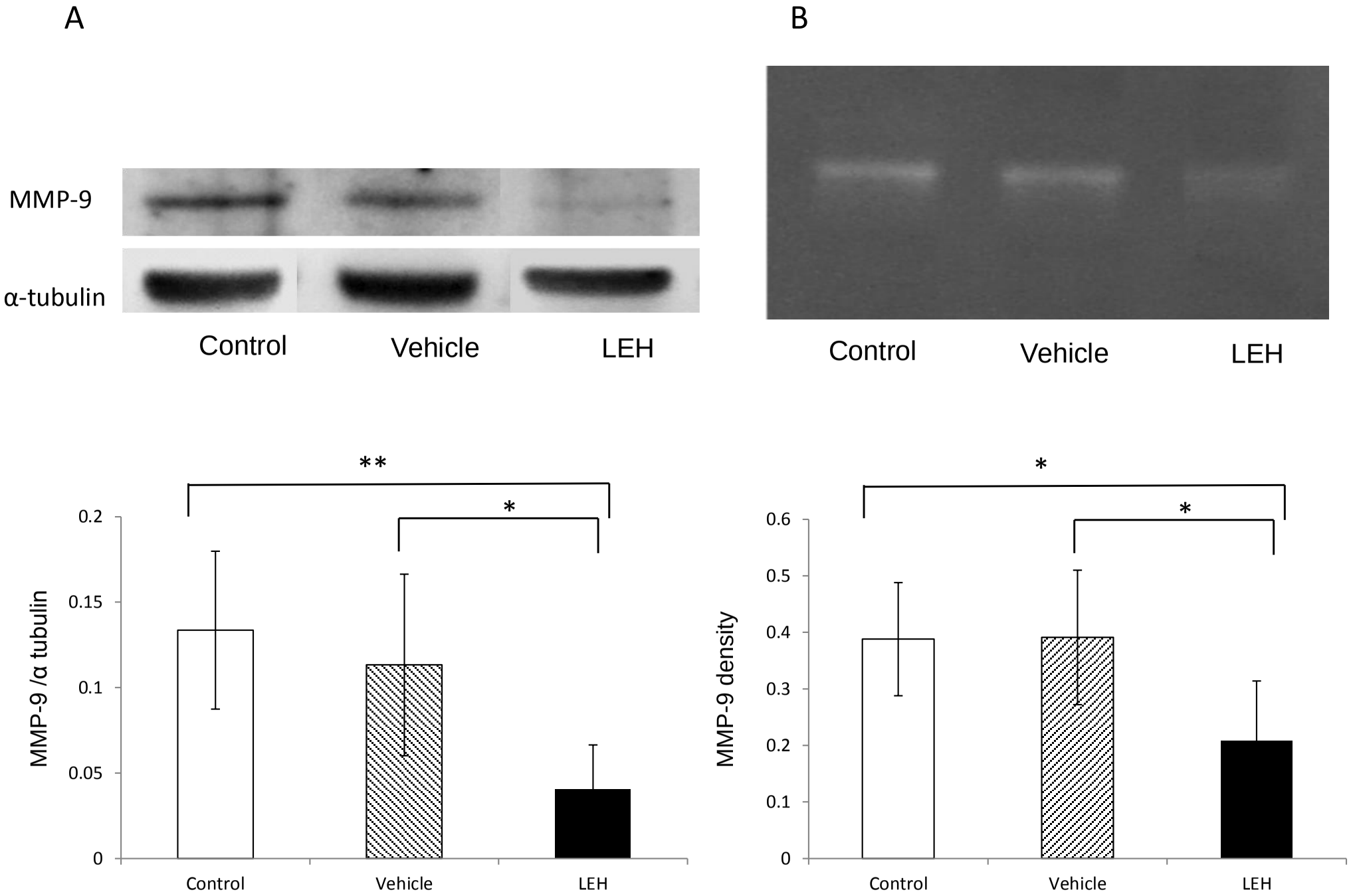
A
B
MMP-9
α-tubulin
Control
Vehicle
LEH
Control
Vehicle
LEH
**
*
0.2
0.15
0.1
0.05
0
MMP-9 /α tubulin
Control
Vehicle
LEH
*
*
0.6
0.5
0.4
0.3
0.2
0.1
0
MMP-9 density
Control
Vehicle
LEH

Figure 4

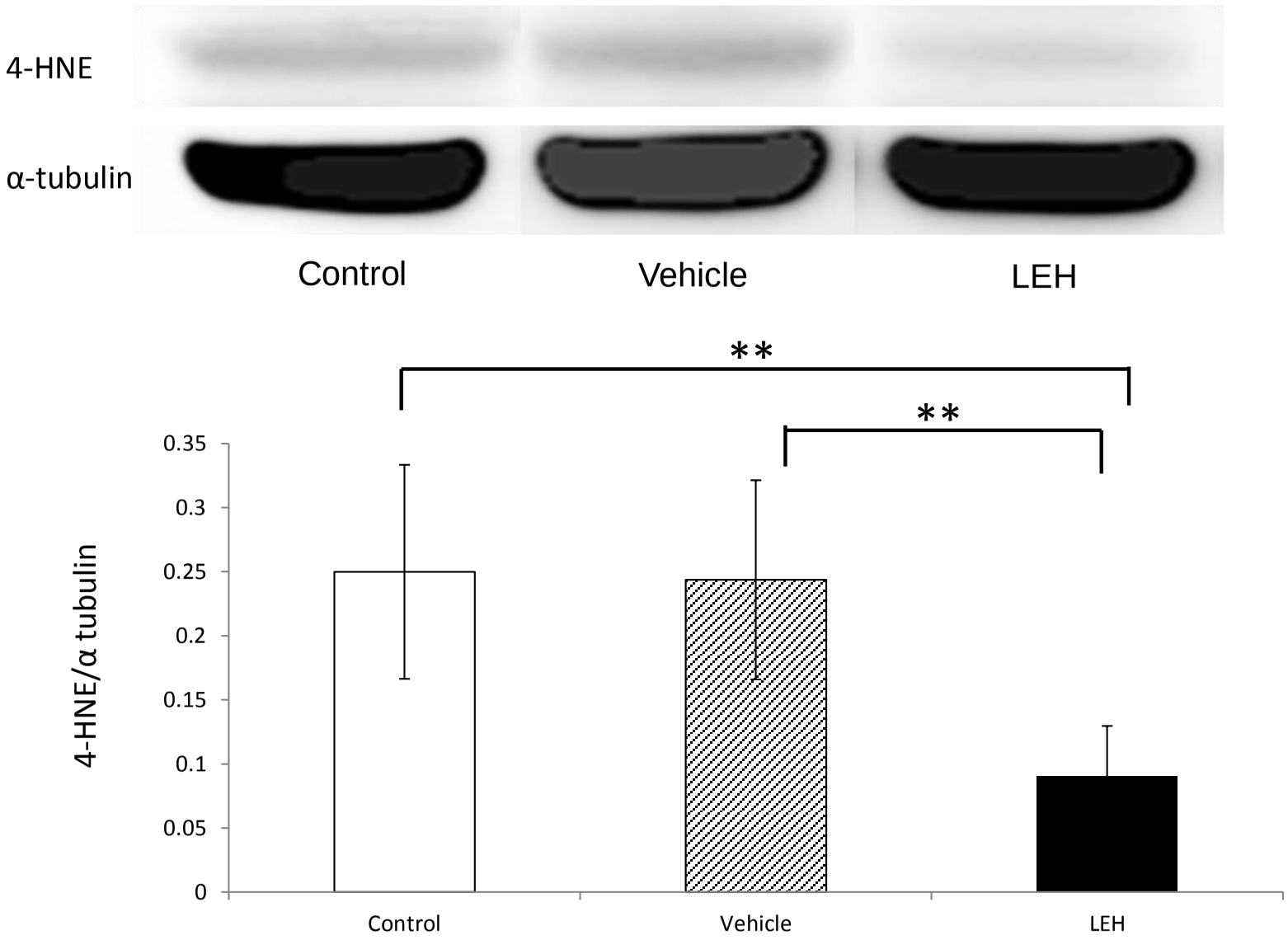
4-HNE
α-tubulin
Control
Vehicle
LEH
**
**
4-HNE/α tubulin
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
Vehicle
LEH

Figure 5

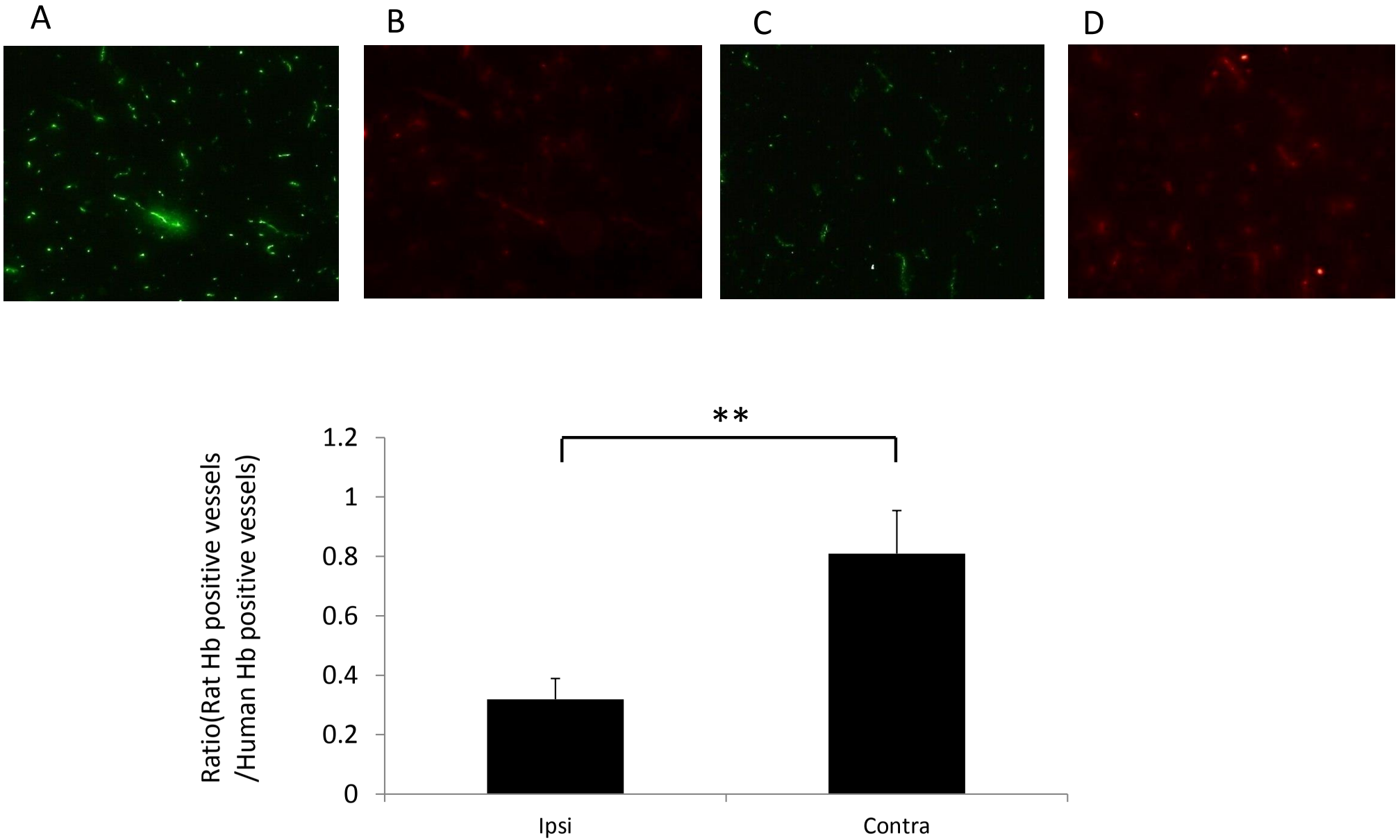
A
B
C
D
**
1.2
1
0.8
0.6
0.4
0.2
0
Ratio(Rat Hb positive vessels /Human Hb positive vessels)
Ipsi
Contra

Figure 6