

Holographic focused ultrasound enables multitarget EV and MSC delivery for neurovascular repair after ischemic stroke

Corresponding Author: Professor Bingbing Cheng

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Thank you for the opportunity to review this manuscript. The authors present the work as an extension of their previously published hologram-assisted multi-target BBB opening (HaMBO) platform. However, the current study appears largely to be an application of the existing platform in a stroke model, with limited methodological innovation. This reviewer has the following concerns:

1. A major concern is the treatment schedule, which does not reflect the clinical reality of stroke management. In clinical practice, the therapeutic window is typically within 3–6 hours after stroke onset. In contrast, the authors initiate treatment at 7 days post-stroke, a time point that lacks clinical relevance. By this stage, substantial brain reorganization has already occurred, including polarization and accumulation of neutrophils and M1 macrophages. Additionally, efferocytosis is already underway by day 7.
2. Previous studies have demonstrated transient BBB opening using focused ultrasound (FUS) without microbubbles in stroke models within 24 hours of onset, resulting in enhanced delivery of endothelial progenitor cell–derived extracellular vesicles (EVs). The HaMBO platform would be more impactful if applied at earlier time points (e.g., within 24 hours post-stroke), guided by rapid MRI-based stroke delineation, and used to deliver EVs and cells without microbubbles.
3. The reported accumulation of EVs and MSCs following HaMBO is not compelling. Detection of red blood cells (RBCs) should be validated using Prussian blue staining, with or without DAB enhancement, to strengthen the findings.
4. Figure 4 raises concerns. The microscopic images of EVs in the naïve brain following HaMBO are questionable, particularly given the apparent differences in nuclear size between control and treated groups. This discrepancy requires clarification.
5. Brain atrophy can be more rigorously and clinically assessed using MRI. The authors should consider imaging at later time points (e.g., days 28 and 42) to quantify brain tissue loss and ventricular volume. These measures would provide a more reliable assessment of global brain atrophy and improve the study's translational relevance. The currently presented multi-sectional Nissl staining does not appear consistent across groups.
6. The exclusion of female animals based on estrogen levels is not adequately justified. The study uses adult mice, and based on the reported size, the animals appear to be at least 12 months old. At this age, most females are retired breeders with minimal estrogen influence. The authors should clearly state the age of the animals and include female mice in the study.
7. It is unclear why rat-derived MSCs were used. Mouse MSCs can be readily isolated and expanded. Additionally, the methods do not clearly describe how contaminating macrophages were excluded during early MSC culture, which raises concerns about cell purity.

Reviewer #2

(Remarks to the Author)

This is a highly innovative and strong manuscript. I greatly appreciate the multidisciplinary nature of the work, successfully bridging acoustic holography, targeted delivery, stroke modeling, and functional assessment. Furthermore, the translational value of the study—particularly the human skull-based simulations—is excellent and elevates the impact of the findings. Overall, I support the acceptance of this manuscript after some minor revisions to address a few weak points, clarify data interpretation, and improve the narrative flow.

Specific Comments for Revision:

- The ST analysis is notably underpowered. Figure 6a indicates that the spatial RNA-seq was performed with an $n=1$ per group. Because of this, the downstream analysis is somewhat superficial, and the mechanistic claims are overstated. Additionally, the claim that reduced macrophage infiltration is the driving mechanism behind the therapy is likely overrated; it is more probable that this reduction is a downstream result of overall brain recovery rather than the primary mechanism of action. Considering the strong interdisciplinary scope of the paper, the current depth of this analysis is acceptable, but the authors must clearly state the $n=1$ sample size as a major limitation in the discussion and tone down their mechanistic claims.
- Based on Figure 4, the HaMBO-assisted delivery resulted in relatively small increases in cell accumulation. For example, MSCs only showed a 1.2-fold (naive) and 1.5-fold (MCAO) increase. However, these modest improvements in delivery led to dramatic improvements in the behavioral and histological outcomes in Figure 5. The authors should discuss this discrepancy. Why does a relatively small boost in local delivery translate to such profound biological and functional recovery?
- MSCs are relatively large cells ($\sim 10\ \mu\text{m}$). Forcing cells of this size across the BBB carries a known risk of microvascular embolisms. While the authors used H&E staining to show no erythrocyte leakage at 0.5 MPa using FUS alone, they need to clarify if they assessed microhemorrhages after injecting 1 million MSCs combined with FUS. Furthermore, the confocal images (Figure 4h) show MSCs near blood vessels. Providing high-resolution Z-stack confocal imaging would help to definitively prove that the MSCs crossed the endothelial barrier into the brain tissue, rather than simply getting trapped in the perivascular spaces.
- The experimental timeline shows that the FUS + EVs/MSCs treatment was given at 7 days post-MCAO. It is well known that the BBB is often naturally "leaky" following an ischemic stroke. The authors should briefly discuss the natural dynamics of BBB permeability post-stroke and explain why FUS is still necessary to achieve therapeutic penetration at this specific 7-day mark/or why this timepoint was selected?
- The authors use Nestin/Ki67 co-staining to claim enhanced neurogenesis. Because Nestin/Ki67 only indicates the proliferation of neural stem/progenitor cells, it does not confirm that these cells mature into functional neurons. The authors should adjust their text to state that the treatment enhanced neural progenitor proliferation, or soften their claims about complete neurogenesis.
- The results are presented in a very brief, punchy format. While I appreciate concise writing, the flow of the manuscript would benefit from short introductory and concluding sentences for each results section to better guide the reader through the scientific narrative.
- Undefined Abbreviations: There are several abbreviations introduced without prior explanation that need to be defined at their first use (e.g., DiR-labeled, CFDA SE).

Reviewer #3

(Remarks to the Author)

Dear author,

The manuscript is really interesting and demonstrates an advanced application of acoustic holograms in mice for the multi-focal (more than 2 foci) opening of the blood-brain barrier for drug delivery at the peri-infarct border brain region, and the related positive health outcomes found out for ischemic stroke. I have several minor comments.

Comments:

- Title: please modify it to reflect it is done for mice.
- Line 89: The reference 13 placed here does not reflect limitations for a complex (stroke-related or not) pattern, in fact, shows a bifocal pattern with certain complexity already.
- Line 92: The reference 14 indicated here does not show a research work about these limitations, but the solution to those. It looks that you obtained that information from those papers, therefore, I would suggest to write these lines like: "However, current FUS-BBBO systems face limitations when applied to complex stroke patterns, as reported in Ref. 13." and "...whereas phased-array systems can generate multiple foci simultaneously but remain costly and offer restricted spatial flexibility, as described in Ref. 14."
- Lines 97-99: Instead of iterative IASA, which is time-inefficient for transcranial configurations, did you try performing a time-reversal direct method like in those references (13, 14 and 15), given that the same group showed successful arbitrary-shape patterns using a much larger number of virtual sources to focus the beam along a hippocampus slice and even along a self-bending trajectory?

- Lines 126: regarding the derived hologram thickness, it is not considering the skull aberrations, right? Just in free-field. Please, clarify in the manuscript.
- Line 129: regarding the multifocal FUS fields precisely aligned, there are always skull aberrations if the hologram was designed to focus in free-field and not transcranially. Therefore, these cannot be “precisely aligned” fields. Please, explain in the manuscript.
- Lines 131-132: Are the spatially independent FUS foci produced in free-field or transcranially and by a hologram designed for free-field or transcranial one? Please, clarify in the manuscript.
- Lines 132-133: regarding the hydrophone measurements, you cannot measure with the hydrophone inside a full and closed skull. Please, clarify from the beginning that this paragraph corresponds to free-field case, since it is confusing, because you start mentioning these holograms focus at the pre-infarct zones of mouse brains.
- Line 138: regarding the reported axial focal size of 7.89 mm, please note that when a hologram is designed to compensate for the skull aberrations in a mouse skull model like yours (read the reference 13), the axial size should be very small and similar to the lateral one: the foci become quasi-spherical. What was then the distribution of virtual sources used in your case? Elongated axially for each focus, or a single source point for each focus?
- Line 148: How did you calculate the energy loss through the mouse skull and brain to be compensated in order to ensure those values of PNPs?
- Lines 170-172: Why doesn't the increased EV accumulation have the shape of the four foci and looks more like a single spot in Fig. 4b-c?
- Lines 228-229: regarding the 3D simulations accurately reproducing the intended multifocal ultrasound fields, were these acoustic holograms designed to compensate skull distortion? Otherwise, skull distortion was occurring and you cannot state that the ultrasound fields were accurately reproduced. Please, explain.
- Line 235: In the Discussion section, please, also mention the disadvantages of acoustic holograms, since they are static and no dynamic focusing can be performed once they are manufactured, unlike multi-element devices which have that advantage. In addition, theranostic ultrasound (ThUS) devices provide dynamic focusing (within a fixed plane only) and cavitation imaging in real-time, all in one with the same device (add some reference), unlike the approach of single-element transducer with hologram. You can also mention that for your application, the approach of acoustic holograms and single-element works the best given that no dynamic focusing is required.
- Line 363: Why did you use five points per wavelength? Please add corresponding explanation and reference.
- Line 363: regarding mentioning the FUS working frequency of 1.5 MHz for the first time here, please describe in a dedicated sentence the geometry, holographic focal distance, size and frequency of the flat surface transducer.
- Line 367-368: regarding the Huma-scale simulations, at which frequency? And what was the size of the transducer and holographic focal distance?
- Line 369: regarding the CT images, what is the method used to convert the mouse and human CT maps to sound speed and density maps? Please, add in manuscript.
- Line 370: Therefore, no skull was considered for the measurements? How did you design and validate the transcranial hologram then? This is very confusing from the beginning of the manuscript. Please, clarify and organize better.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Thank you for revising the manuscript to include some important experiments, analysis, and discussion.

Reviewer #2

(Remarks to the Author)

The authors have fully addressed all of my comments. I congratulate them on an excellent study.

Reviewer #3

(Remarks to the Author)

Dear author,

Thank you for the accurate responses and clarifications. I have just a couple details to ask to be incorporated, regarding the higher efficiency of transcranial IASA vs a transcranial direct method on kWave. Please add the incidence angle onto the human skull for your simulated case, simply to let the reader know about this important parameter. And please, add a statement as well noting that IASA highlights for its time-efficiency performing faster for simpler FUS propagation conditions, however k-space provides higher wave propagation accuracy, in particular with higher incidence angles, enclosed skull cavities or when the targets are located closer to the base of the skull where reflections and standing waves generation are more detrimental to the focusing quality.

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Manuscript Number

We sincerely thank the editor and all reviewers for their constructive and insightful comments. We have carefully revised the manuscript accordingly and provided a point-by-point response below. The reviewers' comments are shown in bold, followed by our responses and the corresponding changes to the manuscript, where applicable.

Reviewer #1 (Remarks to the Author):

Thank you for the opportunity to review this manuscript. The authors present the work as an extension of their previously published hologram-assisted multi-target BBB opening (HaMBO) platform. However, the current study appears largely to be an application of the existing platform in a stroke model, with limited methodological innovation.

Response: We greatly appreciate the reviewer's time and efforts in reviewing this manuscript and the constructive comments, which helped us improve the overall quality of this manuscript. We agree that the present work builds on our previously reported AH-SiMBO platform (*Communications Engineering*, 2025, 4(1):99). Compared to that study, the current work includes both methodological and translational advances beyond a simple application of the prior system. Methodologically, we incorporated human skull-inclusive acoustic simulations to evaluate transcranial feasibility, which is essential for the clinical translation. Translationally, we adapted the platform specifically for ischemic stroke by designing customized acoustic patterns that conform to heterogeneous infarct geometries and enable targeted peri-infarct delivery. We further demonstrated the feasibility and therapeutic efficacy of HaMBO-mediated peri-infarct EV/MSC delivery in a mouse stroke model. We have revised the manuscript in the Discussion section to better clarify these disease-specific and translational innovations, and to distinguish the present study from our previous work. For your convenience, the revised text is provided below.

“Compared with our previous work¹⁶, this study incorporates human skull-inclusive simulations to assess transcranial feasibility and tailors the approach for ischemic stroke by designing peri-infarct acoustic fields that conform to diverse infarct geometries. We further demonstrate the feasibility and therapeutic benefits of HaMBO-mediated EV/MSC delivery in a mouse stroke model, supporting the translational potential of this strategy.”

This reviewer has the following concerns:

1. A major concern is the treatment schedule, which does not reflect the clinical reality of stroke management. In clinical practice, the therapeutic window is typically within 3–6 hours after stroke onset. In contrast, the authors initiate treatment at 7 days post-stroke, a time point that lacks clinical relevance. By this stage, substantial brain reorganization has already occurred, including polarization and accumulation of neutrophils and M1 macrophages. Additionally, efferocytosis is already underway by day 7.

Response: We thank the reviewer for this important comment and apologize for not

articulating our objective more clearly in the original manuscript. We agree that thrombolysis and thrombectomy are acute recanalization therapies performed within hours after stroke onset. However, this study was not intended to develop an acute ischemic stroke intervention, but rather to focus on function recovery during the subacute-to-chronic stage. Many stroke patients are ineligible for acute reperfusion or continue to have neurological deficits after successful recanalization, underscoring the need for therapies that promote recovery beyond the acute stage. Although inflammatory remodeling and efferocytosis have already occurred by day 7, the peri-infarct zone remains dynamically evolving and amenable to therapeutic modulation. Therefore, we selected day 7 post-MCAO to target viable but functionally impaired peri-infarct tissue and to promote neurovascular repair. We have revised the manuscript in the Discussion section to clarify our objective and the rationale for selecting day 7 post-MCAO as the treatment timepoint. For your convenience, the revised text is provided below.

“We selected day 7 post-MCAO as the treatment timepoint because the peri-infarct zone remains viable and undergoes dynamic remodeling at this stage, providing a relevant window to promote neurovascular repair and functional recovery in the subacute-to-chronic stage.”

2. Previous studies have demonstrated transient BBB opening using focused ultrasound (FUS) without microbubbles in stroke models within 24 hours of onset, resulting in enhanced delivery of endothelial progenitor cell–derived extracellular vesicles (EVs). The HaMBO platform would be more impactful if applied at earlier time points (e.g., within 24 hours post-stroke), guided by rapid MRI-based stroke delineation, and used to deliver EVs and cells without microbubbles.

Response: We thank the reviewer for this insightful suggestion. We agree that applying HaMBO with EV/cell delivery at earlier time points after stroke, particularly when guided by rapid MRI-based infarct delineation, would be an important and clinically relevant direction. In the present study, however, we focused on the EV/MSC delivery during the subacute recovery phase, when spontaneous BBB permeability has declined and reliable, spatially controlled delivery to the peri-infarct region remains challenging.

The acute phase within 24 h after ischemic stroke differs substantially from the subacute/recovery phase studied here. During the acute stage, BBB permeability, vascular fragility, inflammatory responses, and edema are dynamically evolving, which may substantially influence both delivery efficiency and safety. Therefore, the optimal ultrasound parameters, the need for microbubbles, and the therapeutic window are likely to differ between acute and subacute/chronic stroke.

Although previous studies have shown that FUS without microbubbles can enhance EV delivery within 24 h after stroke, these results may reflect the distinct BBB and vascular conditions present during the acute phase. In contrast, our goal was to establish a geometry-matched and spatially controlled delivery strategy for peri-infarct tissue during the recovery stage, where residual BBB disruption alone was insufficient for robust EV/MSC accumulation.

We have revised the Discussion to acknowledge early MRI-guided and microbubble-free HaMBO/FUS-mediated delivery as an important future direction. For your convenience, the revised text is provided below.

“Future studies should further investigate the application of HaMBO at earlier time points after ischemic stroke, potentially guided by rapid MRI-based infarct delineation. Because BBB permeability, vascular fragility, inflammation, and edema differ markedly between the acute and recovery stages, the optimal ultrasound parameters and the requirement for microbubbles may vary across treatment windows. Although microbubble-free FUS-mediated delivery has been reported within 24 h after stroke¹, systematic evaluation will be needed to determine whether early MRI-guided HaMBO can safely and reproducibly enhance EV or cell delivery in the acute phase.”

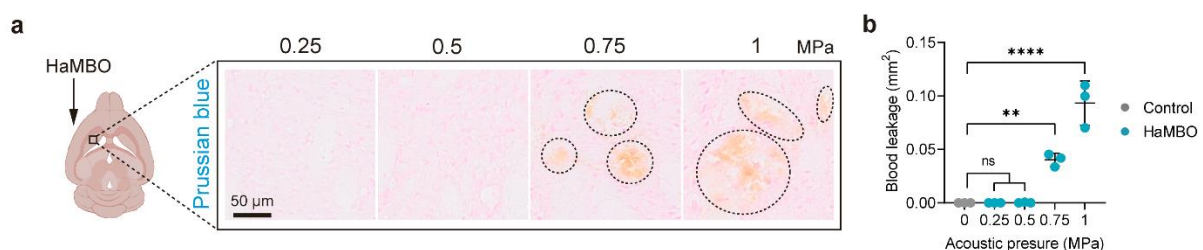
Reference

1. Alptekin A et al. Pulsed Focal Ultrasound as a Non-Invasive Method to Deliver Exosomes in the Brain/Stroke. *J Biomed Nanotechnol* **17**, 1170-1183 (2021).

3. The reported accumulation of EVs and MSCs following HaMBO is not compelling. Detection of red blood cells (RBCs) should be validated using Prussian blue staining, with or without DAB enhancement, to strengthen the findings.

Response: We appreciate the reviewer’s concern. Although EV and MSC delivery across the BBB is intrinsically challenging, our IVIS and fluorescence imaging data demonstrated a statistically significant increase in EV and MSC accumulation in HaMBO-treated groups compared with control groups. We agree that additional histological validation of RBC extravasation would strengthen the safety assessment. As suggested, we performed additional Prussian blue staining after HaMBO to further assess RBC extravasation.

The results showed no Prussian blue-positive deposits at pressures below 0.5 MPa, whereas the 0.75 and 1 MPa groups showed clear evidence of fresh RBC leakage. These additional data support the safety of the selected sonication condition and further validate the pressure-dependent effects observed after HaMBO. We have added Prussian blue staining data to Supplementary Fig. 3 and revised the manuscript accordingly.



Supplementary Fig. 3. Assessment of blood leakage by Prussian blue staining.

a, Prussian blue staining of brain sections at 30 min following HaMBO under 0 (contralateral untreated brain region), 0.25, 0.5, 0.75, or 1 MPa. Dashed circles indicate fresh erythrocyte extravasation. **b**, Quantification of blood leakage. $n = 3$ mice per group. All data are presented as mean $\pm$ SD. Statistical analyses: one-way ANOVA with Tukey’s *post-hoc* test (**b**). $**P < 0.01$; $****P < 0.0001$; ns, not significant.

4. Figure 4 raises concerns. The microscopic images of EVs in the naïve brain following HaMBO are questionable, particularly given the apparent differences in nuclear size between control and treated groups. This discrepancy requires clarification.

Response: We thank the reviewer for pointing out this issue. The apparent difference in nuclear size in the original Fig. 4d was caused by inconsistent display parameters used for the DAPI channel during image processing, rather than a biological or acquisition-related difference. To avoid confusion, we have replaced the representative images with images shown at the same magnification and processed using identical display parameters. Updated images are provided in the revised Fig. 4d. Importantly, the quantitative analysis of the DiR channel was performed using the original image data under consistent analysis criteria and was not affected by the display settings of the representative images. For your convenience, the revised Fig. 4d is provided below.

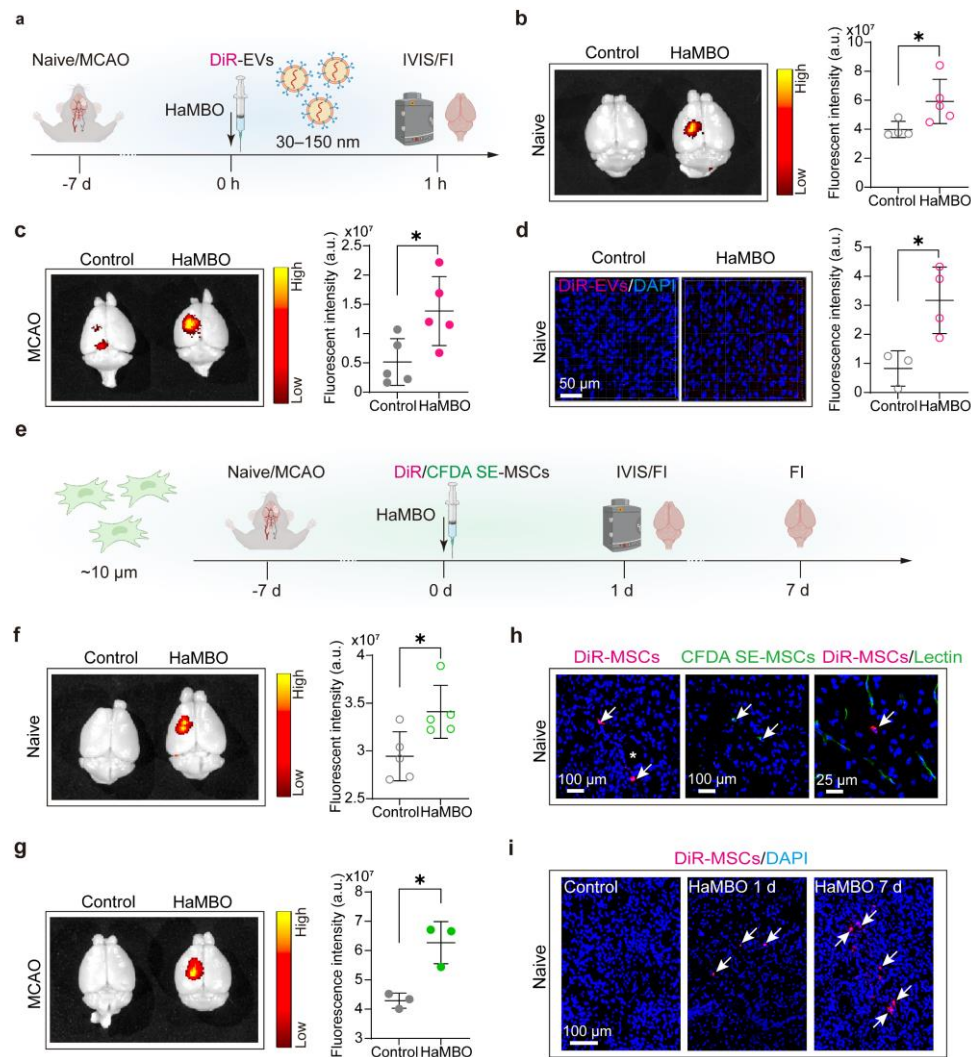


Fig. 4. HaMBO-mediated EV/MSc delivery to peri-infarct zones.

5. Brain atrophy can be more rigorously and clinically assessed using MRI. The authors should consider imaging at later time points (e.g., days 28 and 42) to quantify brain tissue loss and ventricular volume. These measures would provide a more reliable assessment of global brain atrophy and improve the study's translational relevance. The currently presented multi-sectional Nissl staining does not appear consistent across groups.

Response: We fully agree that longitudinal MRI provides a more rigorous and clinically

relevant assessment of brain tissue loss and ventricular enlargement than histological section-based analysis alone. In the initial submission, we quantified brain atrophy using serial Nissl-stained sections at 28 days after MCAO, which is a commonly used endpoint in preclinical stroke studies. However, to address the reviewer's concern and to strengthen the translational relevance of the study, we repeated the experiments and performed T2-weighted MRI at both 28 and 42 days after MCAO.

Using these MRI datasets, we quantified both brain atrophy volume and lateral ventricle volume following HaMBO-assisted EV/MSc therapies. Accordingly, we have replaced the original multi-sectional Nissl-based analysis with the newly acquired T2-weighted MRI images and corresponding quantitative analyses in the revised Fig. 5c, d and Supplementary Fig. 9a, b. We have also revised the Methods section to clarify the procedures used for MRI-based brain atrophy and ventricular volume quantification. For your convenience, the revised Fig. 5 and Supplementary Fig. 9 are provided below.

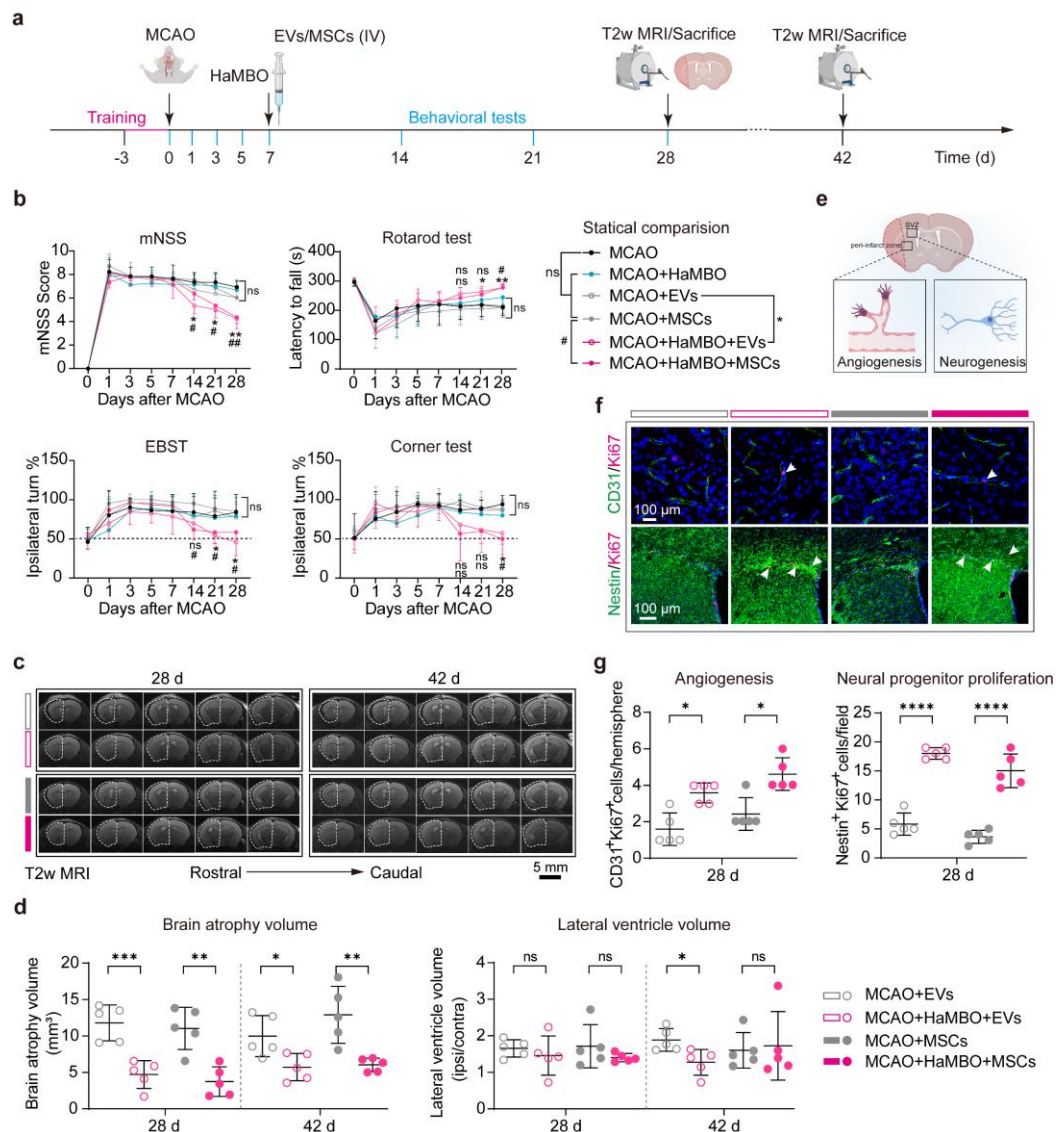
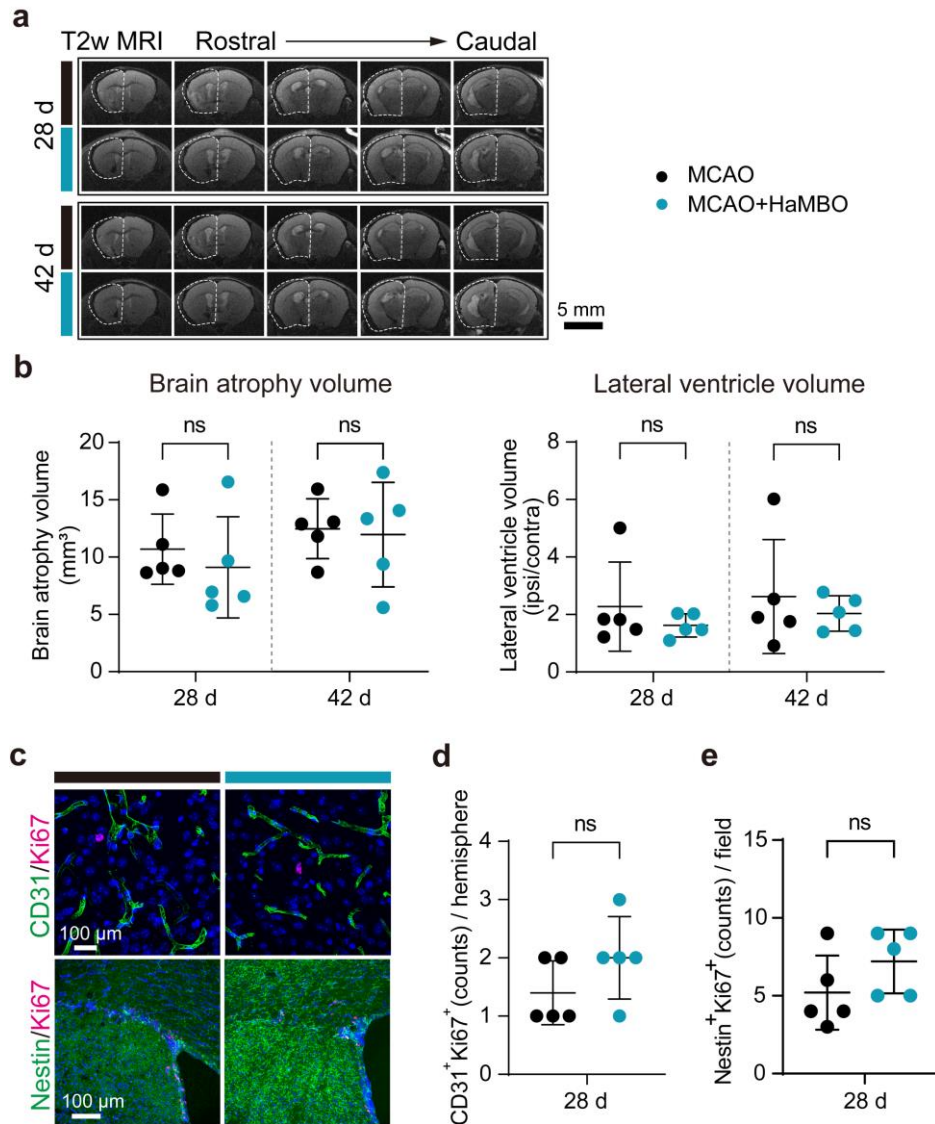


Fig. 5. Therapeutic efficacy of HaMBO-assisted peri-infarct EV/MSc delivery in cerebral ischemia.



Supplementary Fig. 9. HaMBO alone shows neutral efficacy post-MCAO.

6. The exclusion of female animals based on estrogen levels is not adequately justified. The study uses adult mice, and based on the reported size, the animals appear to be at least 12 months old. At this age, most females are retired breeders with minimal estrogen influence. The authors should clearly state the age of the animals and include female mice in the study.

Response: We thank the reviewer for this important comment. We would like to clarify that the animals used in this study were young adult male C57BL/6J mice aged 8–10 weeks and weighing 21–28 g, not 12-month-old animals. Only male mice were included in this study to minimize variability associated with sex-dependent differences in stroke pathophysiology, BBB integrity, and ultrasound-mediated BBB opening. Sex is a key biological variable influencing stroke pathobiology and treatment responses¹, and sex differences have been reported in BBB-related gene expression² and ultrasound-mediated BBB opening³⁻⁴. Because these differences may affect delivery efficiency and therapeutic outcomes, this proof-of-concept study was restricted to male mice to enable a controlled evaluation of HaMBO-assisted

MSC/EV treatment. We have revised the Methods section to clearly state the age, weight, strain, and sex of the animals, and revised the Discussion section to acknowledge the use of only male mice as a limitation. For your convenience, the revised text is provided below.

Revised Methods: “Adult male C57BL/6J mice aged 8–10 weeks and weighing 21–28 g were used in this study. Only male mice were included in this initial proof-of-concept study to minimize variability arising from sex-dependent differences in stroke pathophysiology, BBB integrity, and ultrasound-mediated BBB opening.”

Revised Discussion: “Because this proof-of-concept study was performed in young adult male mice, potential sex-dependent differences in post-stroke responses to HaMBO-assisted EV/MSC therapy remain to be determined in future studies that include both male and female mice.”

References

1. Bushnell, C. D. et al. Sex differences in stroke: Challenges and opportunities. *Journal of Cerebral Blood Flow and Metabolism* **38**, 2179-2191 (2018).
2. Mi, X. et al. Sex- and age- differences in the expression of critical blood-brain barrier regulators: a physiological context. *Biology of Sex Differences* **16** (2025).
3. Angolano, C. et al. Characterization of focused ultrasound blood-brain barrier disruption effect on inflammation as a function of treatment parameters. *Biomedicine & pharmacotherapy* **182**, 117762 (2025).
4. Géraudie, A. et al. Therapeutic effects of blood - brain barrier opening with low - intensity pulsed ultrasounds in tau transgenic P301S mice. *Alzheimer's & Dementia* **19** (2023).

7. It is unclear why rat-derived MSCs were used. Mouse MSCs can be readily isolated and expanded. Additionally, the methods do not clearly describe how contaminating macrophages were excluded during early MSC culture, which raises concerns about cell purity.

Response: We appreciate the reviewer’s comment. We agree that mouse MSCs can also be used. However, rat bone marrow-derived MSCs were selected in this study due to their high cell yield, robust proliferative capability, and stable morphology across passages, as well as their extensive application in preclinical ischemic stroke studies. This rationale has been clarified in the revised manuscript. To address the concern regarding macrophage contamination, we clarified that MSCs were isolated using a modified plastic-adherence method. Non-adherent hematopoietic cells were removed mainly by frequent medium changes. We have revised the Methods section as follows:

“Bone marrow-derived MSCs were isolated from the femurs and tibias of 4–6-week-old Sprague–Dawley rats, which provide a high yield of stromal cells with robust proliferative capability and stable morphology^{1,2}. Rat MSCs were isolated using a modified plastic-adherence method³. Briefly, bone marrow was flushed with low-glucose DMEM (Gibco) supplemented with 10% FBS and antibiotics. Cells were seeded at 5×10^6 cells/cm² and incubated at 37°C in a humidified atmosphere containing 5% CO₂. To reduce hematopoietic cell contamination, the medium was first changed 3 h after seeding, followed by frequent medium changes during the first 72 h.”

References:

1. Tian H et al. Hypoxia-Preconditioned Bone Marrow Mesenchymal Stem Cells Improved Cerebral Collateral Circulation and Stroke Outcome in Mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* **43**, 1281-1294 (2023).
2. Cheng Z et al. Mesenchymal stem cells attenuate blood-brain barrier leakage after cerebral ischemia in mice. *Journal of Neuroinflammation* **15**, 135 (2018).
3. Soleimani, M. et al. A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. *Nature protocols* **4**, 102-106 (2009).

Reviewer #2 (Remarks to the Author):

This is a highly innovative and strong manuscript. I greatly appreciate the multidisciplinary nature of the work, successfully bridging acoustic holography, targeted delivery, stroke modeling, and functional assessment. Furthermore, the translational value of the study—particularly the human skull-based simulations—is excellent and elevates the impact of the findings. Overall, I support the acceptance of this manuscript after some minor revisions to address a few weak points, clarify data interpretation, and improve the narrative flow.

Response: We sincerely thank the reviewer for the positive and encouraging evaluation of our manuscript. We are particularly grateful for the reviewer's time and constructive suggestions, which helped us improve scientific rigor, data interpretation, and narrative flow of the manuscript. In response, we have carefully revised the manuscript to address the concerns raised. We believe these changes have strengthened the rigor, clarity, and impact of the study. Thank you again for your thoughtful assessment and supportive recommendation.

Specific Comments for Revision:

1. The ST analysis is notably underpowered. Figure 6a indicates that the spatial RNA-seq was performed with an $n=1$ per group. Because of this, the downstream analysis is somewhat superficial, and the mechanistic claims are overstated. Additionally, the claim that reduced macrophage infiltration is the driving mechanism behind the therapy is likely overrated; it is more probable that this reduction is a downstream result of overall brain recovery rather than the primary mechanism of action. Considering the strong interdisciplinary scope of the paper, the current depth of this analysis is acceptable, but the authors must clearly state the $n=1$ sample size as a major limitation in the discussion and tone down their mechanistic claims.

Response: We thank the reviewer for this thoughtful and constructive comment. We fully agree that the spatial transcriptomic analysis, performed on one representative brain section per group, is underpowered and should be interpreted as exploratory and hypothesis-generating rather than as definitive mechanistic evidence. Accordingly, we have revised the Introduction, Results, and Discussion sections to tone down the mechanistic claims. Specifically, we no longer state that “Reduced macrophage infiltration is a key mechanism” or that it “underlies the therapeutic efficacy”. Instead, we use more cautious language, such as “may contribute to the therapeutic benefit” and “is associated with improved recovery.” We also acknowledge that the observed reduction in macrophage-related signatures may reflect a downstream consequence of improved tissue recovery rather than a primary mechanism of action. In addition, we have explicitly stated the sample size of $n=1$ brain section per group in the Fig. 6 legend and Methods section, and added this as a major limitation in the Discussion section, as follows:

“There are several limitations in this study. First, spatial transcriptomics was performed on only one representative brain section per group. Therefore, the observed macrophage reduction should be interpreted as exploratory and associative rather than as definitive mechanistic

evidence. Causal validation will require larger sample sizes and functional studies.”

2. Based on Figure 4, the HaMBO-assisted delivery resulted in relatively small increases in cell accumulation. For example, MSCs only showed a 1.2-fold (naive) and 1.5-fold (MCAO) increase. However, these modest improvements in delivery led to dramatic improvements in the behavioral and histological outcomes in Figure 5. The authors should discuss this discrepancy. Why does a relatively small boost in local delivery translate to such profound biological and functional recovery?

Response: We agree that the measured increase in MSC accumulation appears modest relative to the therapeutic effects. This may be explained by two factors. First, in vivo imaging system (IVIS)-based quantification may underestimate local MSC delivery due to tissue scattering, depth-dependent signal attenuation, and background fluorescence. Second, MSCs act mainly through paracrine mechanisms and possess self-renewal and proliferative capabilities, so even a modest increase in local accumulation may amplify reparative responses. We have added the following text to the Discussion section:

“Although HaMBO induced only a modest increase in total MSC accumulation, it produced significant therapeutic benefits. This discrepancy may partly reflect limitations of IVIS-based quantification, which can underestimate local delivery because of tissue scattering, depth-dependent signal attenuation, and background fluorescence. Moreover, MSCs act mainly through paracrine signaling and possess self-renewal and proliferative capabilities¹. Therefore, even a modest increase in local delivery may amplify downstream reparative responses.”

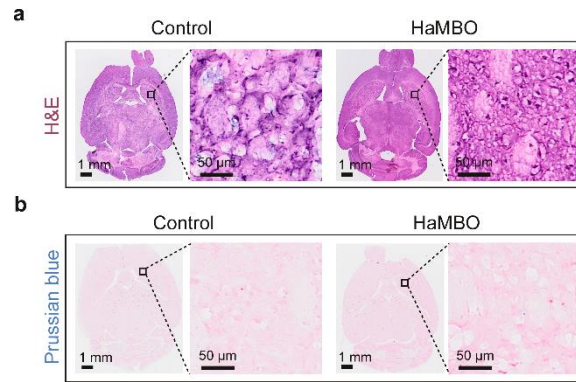
References:

1. Trabulo A et al. Mesenchymal Stem Cell-Based Therapies Applied in Neurological Diseases: A Systematic Review. *Biomedicines* **14**, 475 (2026).

3. MSCs are relatively large cells (~10 µm). Forcing cells of this size across the BBB carries a known risk of microvascular embolisms. While the authors used H&E staining to show no erythrocyte leakage at 0.5 MPa using FUS alone, they need to clarify if they assessed microhemorrhages after injecting 1 million MSCs combined with FUS. Furthermore, the confocal images (Figure 4h) show MSCs near blood vessels. Providing high-resolution Z-stack confocal imaging would help to definitively prove that the MSCs crossed the endothelial barrier into the brain tissue, rather than simply getting trapped in the perivascular spaces.

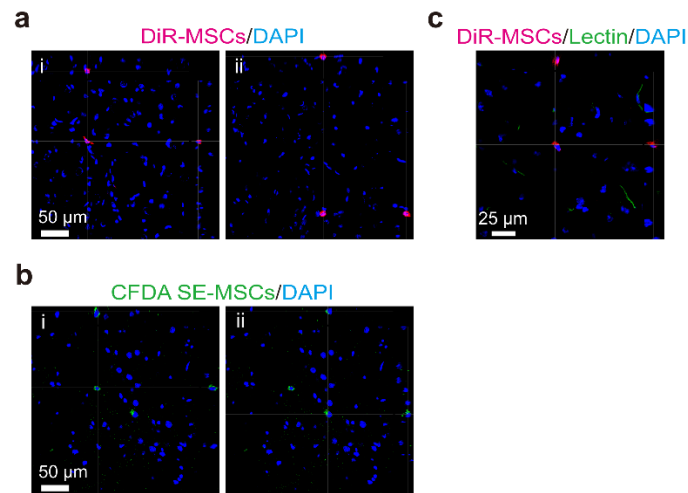
Response: We thank the reviewer for raising this important safety concern. We agree that MSC delivery requires more careful evaluation given the relatively large cell size and the potential risks of microvascular obstruction or hemorrhage. To address this concern, we performed additional experiments. In the revised manuscript, we added a safety assessment following combined HaMBO treatment and intravenous administration of 1 million MSCs. H&E and Prussian blue staining showed no detectable microhemorrhage or obvious tissue damage at 0.5 MPa on day 1 after treatment (Supplementary Fig. 7). Regarding MSC localization, we agree that the original 2D images cannot definitively demonstrate MSC

delivery into the brain parenchyma. We therefore added high-resolution Z-stack confocal images (Supplementary Fig. 6) and a 3D reconstruction movie (Supplementary Movie 1) to confirm MSC localization. These data show DiR-labeled MSCs located adjacent to blood vessels, with fluorescence signals surrounding the nuclei. In addition, we performed an additional DiR-MSC delivery experiment with lectin-labeled vessels, which confirmed successful delivery of DiR-MSCs into the brain parenchyma (Fig. 4h).



Supplementary Fig. 7. Histological analysis following combined HaMBO treatment and MSC delivery.

a, Representative H&E images from the control and HaMBO groups. $n = 3$ mice per group. **b**, Representative Prussian blue images from the control and HaMBO groups. $n = 3$ mice per group.



Supplementary Fig. 6. Z-stack confocal images of HaMBO-assisted MSC delivery.

a, Z-stack confocal images of DiR-MSCs from Supplementary Movie 1. **b**, Z-stack confocal images of CFDA SE-MSCs shown in Fig. 4h. **c**, Z-stack confocal images of DiR-MSCs with lectin-labeled blood vessels from Fig. 4h. i–ii indicate delivered MSCs with DiR or CFDA SE signals surrounding the nuclei.

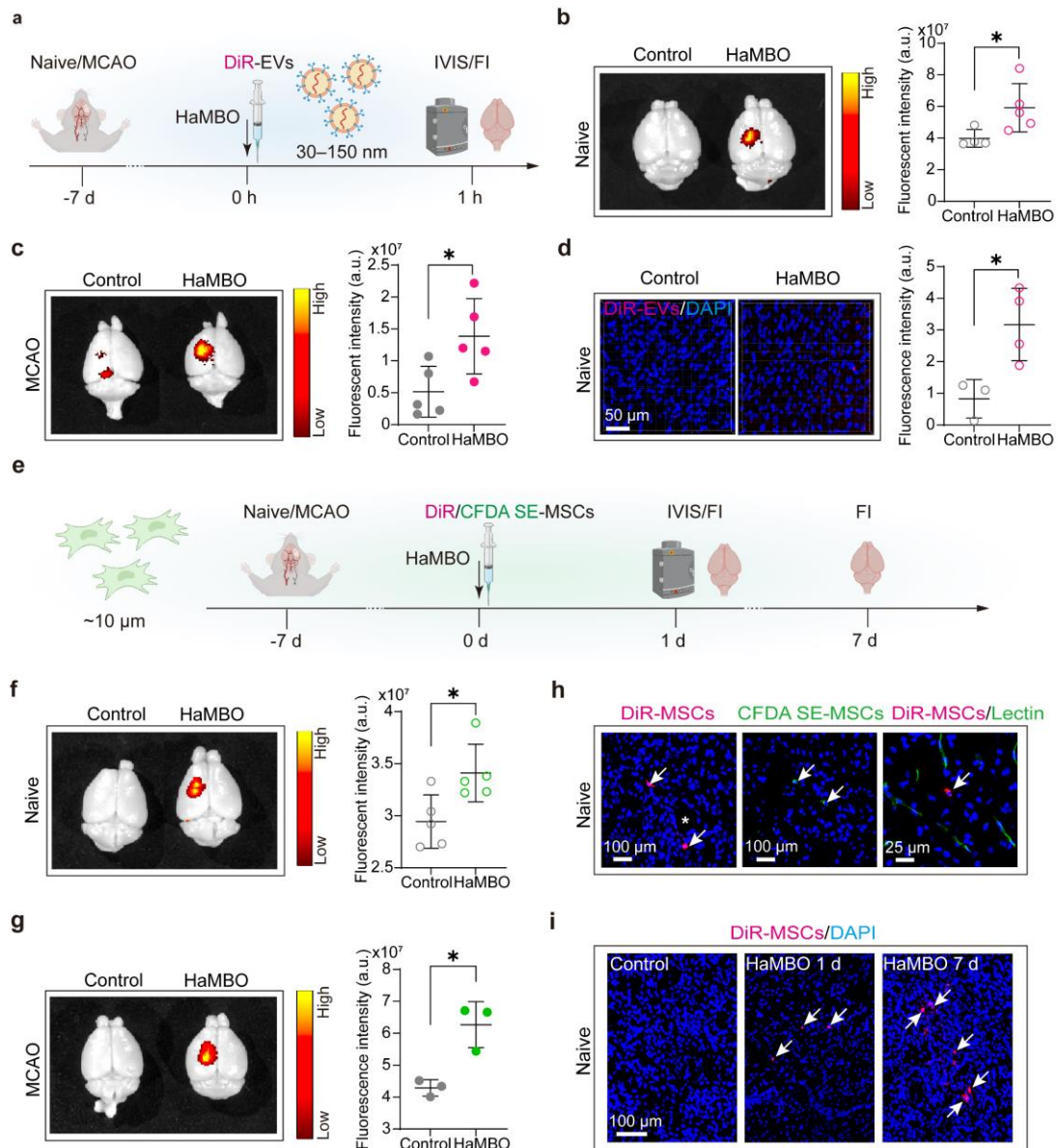


Fig. 4. HaMBO-mediated EV/MSc delivery to peri-infarct zones.

4. The experimental timeline shows that the FUS + EVs/MSCs treatment was given at 7 days post-MCAO. It is well known that the BBB is often naturally "leaky" following an ischemic stroke. The authors should briefly discuss the natural dynamics of BBB permeability post-stroke and explain why FUS is still necessary to achieve therapeutic penetration at this specific 7-day mark/or why this timepoint was selected?

Response: As suggested, we have added a brief discussion of post-stroke BBB dynamics and clarified the rationale for performing HaMBO-mediated delivery on day 7 post-MCAO, as follows:

"Although BBB leakage occurs early after ischemic stroke and may persist for weeks, this spontaneous permeability is highly variable and declines over time¹⁻². Even when pathologically disrupted, the BBB remains much less permeable than peripheral vessels, thereby limiting brain-targeted drug delivery¹. Our IVIS and fluorescence data showed that

residual BBB disruption on day 7 post-MCAO was insufficient for effective EV/MSC accumulation in the peri-infarct zone, whereas HaMBO significantly enhanced targeted delivery. Thus, despite the presence of residual post-stroke BBB disruption, HaMBO remains necessary to achieve sufficient, spatially controlled, and reproducible therapeutic delivery to the peri-infarct region.”

References:

1. Castillo-González J et al. Beyond wrecking a wall: revisiting the concept of blood-brain barrier breakdown in ischemic stroke. *Neural Regeneration Research* **20**, 1944-1956 (2025).
2. Bernardo-Castro S et al. Pathophysiology of Blood-Brain Barrier Permeability Throughout the Different Stages of Ischemic Stroke and Its Implication on Hemorrhagic Transformation and Recovery. *Frontiers in Neurology* **9**, 594672 (2020).

5. The authors use Nestin/Ki67 co-staining to claim enhanced neurogenesis. Because Nestin/Ki67 only indicates the proliferation of neural stem/progenitor cells, it does not confirm that these cells mature into functional neurons. The authors should adjust their text to state that the treatment enhanced neural progenitor proliferation, or soften their claims about complete neurogenesis.

Response: We appreciate the reviewer’s constructive comment. As suggested, we have revised all relevant sections of the manuscript to replace the term “neurogenesis” with “neural progenitor proliferation”, as our experimental results specifically demonstrate enhanced proliferation of neural progenitors rather than complete neurogenesis.

6. The results are presented in a very brief, punchy format. While I appreciate concise writing, the flow of the manuscript would benefit from short introductory and concluding sentences for each results section to better guide the reader through the scientific narrative.

Response: We thank the reviewer for this constructive suggestion. In the revised manuscript, we have added brief introductory and concluding sentences to each Results section to improve the narrative flow and better guide the reader through the study. We believe these revisions help connect the sequential components of the work, including acoustic validation, safety assessment, targeted delivery, therapeutic efficacy, exploratory spatial transcriptomics, and human skull-based translational simulations. Detailed changes are summarized in the table below.

Results section	Introductory sentences	Concluding sentences
1	We first developed acoustic holograms under free-field conditions to generate multiple FUS foci spatially matched to the peri-infarct zones of ischemic mouse brains.	These results collectively identified the four-focus hologram as the optimal design for peri-infarct targeting in subsequent BBBO experiments (Fig. 1e-f).
2	We next optimized the acoustic	Taken together, these findings

	parameters required for spatially confined and safe BBB opening.	demonstrate that 0.5 MPa achieves spatially accurate tracer delivery without inducing detectable behavioral or histological abnormalities.
3	The delivery feasibility of M2 microglial EVs and rat bone marrow-derived MSCs with HaMBO were validated.	Thus, HaMBO safely enhances EV and MSC accumulation in peri-infarct zones, with MSC retention detectable for at least 7 days.
4	Therapeutic efficacy of HaMBO-assisted EV/MSC delivery was evaluated in a transient focal ischemia mouse model (Fig. 5a).	These results demonstrate that HaMBO-assisted EV/MSC delivery markedly improves neurovascular repair and functional recovery after stroke.
5	To characterize the spatial transcriptomic changes associated with the therapeutic benefit of HaMBO-assisted EV therapy, we performed a comparative analysis of high-quality spatial transcriptomics on mouse brains collected 28 d post-MCAO (Fig. 6a, Supplementary Fig. 10a–b).	Together, these findings suggest that reduced macrophage infiltration may contribute to the therapeutic benefit of HaMBO-assisted EV therapy after stroke.
6	Translating HaMBO to the clinic requires generating customized transcranial acoustic fields that conform to patient-specific stroke lesions.	Collectively, human skull-based simulations suggest the potential feasibility of customizing HaMBO acoustic fields for diverse stroke patient-specific infarct patterns.

7. Undefined Abbreviations: There are several abbreviations introduced without prior explanation that need to be defined at their first use (e.g., DiR-labeled, CFDA SE).

Response: We thank the reviewer for pointing this out. We have systematically checked the entire manuscript and added the full definitions for DiR-labeled, CFDA SE, and all other previously undefined abbreviations at their first appearance in the revised manuscript.

Reviewer #3 (Remarks to the Author):

Dear author,

The manuscript is really interesting and demonstrates an advanced application of acoustic holograms in mice for the multi-focal (more than 2 foci) opening of the blood-brain barrier for drug delivery at the peri-infarct border brain region, and the related positive health outcomes found out for ischemic stroke. I have several minor comments.

Response: We sincerely thank the reviewer for their time, efforts, and constructive comments. The reviewer's expertise in acoustic holography and transcranial ultrasound helped us improve the technical accuracy of the manuscript. Following these suggestions, we have clarified the distinction between free-field mouse simulations and human simulations with a skull model, revised several statements to avoid overinterpretation, and added missing methodological details related to acoustic field design, pressure calibration, sampling resolution, and CT-based acoustic modeling. We also discussed the advantages and limitations of the hologram-based approach more carefully. We believe these revisions have improved the clarity and rigor of the manuscript, and we are grateful for the reviewer's valuable guidance.

Comments:

1. Title: please modify it to reflect it is done for mice.

Response: We have revised the title as suggested:

“Holographic focused ultrasound enables multi-target EV/MSC delivery for neurovascular repair after ischemic stroke in mice”

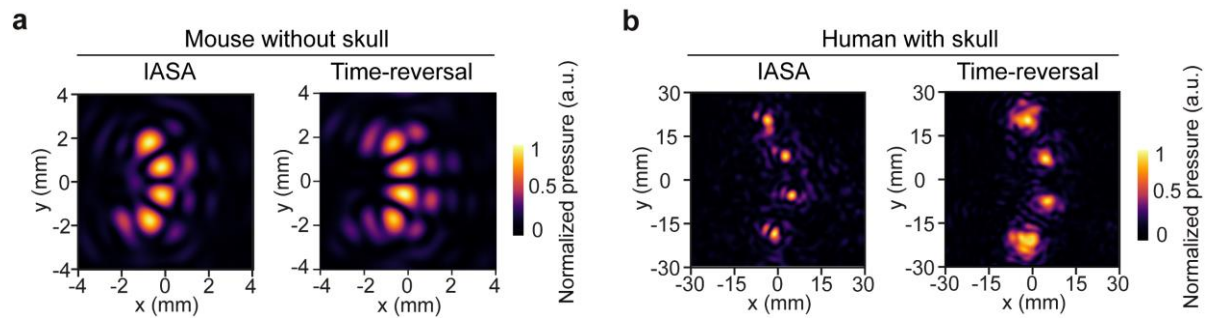
2. Line 89: The reference 13 placed here does not reflect limitations for a complex (stroke-related or not) pattern, in fact, shows a bifocal pattern with certain complexity already. Line 92: The reference 14 indicated here does not show a research work about these limitations, but the solution to those. It looks that you obtained that information from those papers, therefore, I would suggest to write these lines like: "However, current FUS-BBBO systems face limitations when applied to complex stroke patterns, as reported in Ref. 13." and "...whereas phased-array systems can generate multiple foci simultaneously but remain costly and offer restricted spatial flexibility, as described in Ref. 14.".

Response: The reviewer's suggestions are greatly appreciated. We agree that the previous wording did not accurately reflect the specific contributions of Refs. 13 and 14. We have revised the relevant sentences as suggested:

“However, current FUS-BBBO systems face limitations when applied to complex stroke patterns, as reported in a previous study¹³” and “...whereas phased-array systems can generate multiple foci simultaneously but remain costly and offer restricted spatial flexibility, as described in a previous report¹⁴”.

3. Lines 97-99: Instead of iterative IASA, which is time-inefficient for transcranial configurations, did you try performing a time-reversal direct method like in those references (13, 14 and 15), given that the same group showed successful arbitrary-shape patterns using a much larger number of virtual sources to focus the beam along a hippocampus slice and even along a self-bending trajectory?

Response: We thank the reviewer for this important technical comment. We acknowledge that time-reversal-based methods are powerful for designing acoustic holograms that generate arbitrary transcranial acoustic fields and have been successfully demonstrated in prior acoustic holography studies. In the present work, we used our modified IASA framework because of its substantially higher computational efficiency in acoustic hologram design. To address the reviewer's suggestion, we conducted a comparison between the time-reversal method and IASA in both mouse and human, as shown in the figure below. These results show that the acoustic holograms designed using either the time-reversal method or IASA generated comparable acoustic field patterns, whereas the IASA-based method substantially reduced the hologram design time (IASA: 30 s and 15 min for mouse and human, respectively; time-reversal method: 2 h and 1.5 h for mouse and human, respectively).



Comparison of simulated acoustic fields using IASA and time-reversal methods.

a, Simulated acoustic fields in mouse without skull. **b,** Simulated acoustic fields in human with skull.

4. Lines 126: regarding the derived hologram thickness, it is not considering the skull aberrations, right? Just in free-field. Please, clarify in the manuscript.

Response: The reviewer is correct. The hologram thickness used in the mouse experiments was calculated under free-field conditions and did not compensate for skull-induced aberrations. We did not incorporate mouse skull aberration correction because the mouse skull is very thin and has been demonstrated to introduce only minimal distortion during BBB opening in mice (*Communications Engineering*, 2025, 4(1):99). We have revised the Results section accordingly to clarify this condition. The revised text is as follows:

“Using a modified IASA method, we derived the hologram thickness profiles under free-field conditions and then fabricated the hologram using a high-performance 3D printer (**Fig. 1a**).”

5. Line 129: regarding the multifocal FUS fields precisely aligned, there are always skull aberrations if the hologram was designed to focus in free-field and not transcranially. Therefore, these cannot be “precisely aligned” fields. Please, explain in the manuscript.

Response: We agree with the reviewer. The original phrase “precisely aligned” was too strong

because the murine holograms were designed under free-field conditions and without compensation for skull-induced aberrations. Although aberrations induced by the thin mouse skull are expected to be relatively small, they are still present. We therefore replaced “precisely aligned” with more cautious wording “spatially matched to”. The revised text is as follows:

“When coupled to a single-element planar transducer, these customized holograms are supposed to create multifocal FUS fields spatially matched to the peri-infarct border (**Fig. 1b**).”

6. Lines 131-132: Are the spatially independent FUS foci produced in free-field or transcranially and by a hologram designed for free-field or transcranial one? Please, clarify in the manuscript.

Response: We thank the reviewer for this comment. We would like to clarify that the murine hologram thickness profiles were designed under free-field conditions and without compensation for skull-induced aberrations. Mouse simulations were performed without including the skull. We have revised the Results section accordingly. The revised text is as follows:

“We designed holograms capable of generating three, four, or five foci under free-field conditions, with 3D simulations confirming that acoustic holography can produce spatially independent FUS foci (**Fig. 1c, Supplementary Fig. 1a**).”

7. Lines 132-133: regarding the hydrophone measurements, you cannot measure with the hydrophone inside a full and closed skull. Please, clarify from the beginning that this paragraph corresponds to free-field case, since it is confusing, because you start mentioning these holograms focus at the pre-infarct zones of mouse brains.

Response: We thank the reviewer for pointing out this ambiguity. We clarify that the hydrophone measurements were performed under free-field conditions in degassed water. We have explicitly stated this at the beginning of the Results section. The revised text is as follows:

“We first developed acoustic holograms under free-field conditions to generate multiple FUS foci spatially matched to the peri-infarct zones of ischemic mouse brains.”

8. Line 138: regarding the reported axial focal size of 7.89 mm, please note that when a hologram is designed to compensate for the skull aberrations in a mouse skull model like yours (read the reference 13), the axial size should be very small and similar to the lateral one: the foci become quasi-spherical. What was then the distribution of virtual sources used in your case? Elongated axially for each focus, or a single source point for each focus?

Response: We have carefully examined the reference 13. As the reviewer correctly pointed out, in Section “IV. Discussion” of that paper, the authors explicitly state that when the hologram is designed to compensate for skull aberrations in mice, the axial depth of field is significantly reduced, resulting in a “quasi-spherical” focal spot. Its axial dimension (≈ 0.5 mm) is comparable to the lateral one (≈ 0.6 mm).

In our work, a single virtual point source was used for each target focus, as in Reference 13. Therefore, the source type is not the factor causing the discrepancy. The actual difference is

that our holographic metasurface was designed in water (without skull compensation), and we did not incorporate compensation for the acoustic aberrations (phase distortion, attenuation, etc.) induced by the mouse skull in our algorithm. The rationale for this simplification is that, for the mouse model, the skull is relatively thin, and the resulting aberrations and attenuation are much smaller than those in primates or humans (*Ultrasound in Medicine and Biology*, 2011, 37 (11): 1930–1937). Hence, neglecting skull compensation has a negligible impact on the accuracy of our experimental results, while substantially reducing design complexity and cost.

In contrast, the design in the reference 13 not only records the back-propagated wavefront from the target points but also encodes the multiple reflections and reverberations generated by the transducer aperture, the central hole, and the “leaky cavity” formed by the skull. This approach effectively uses the full acoustic information within the cranial cavity for time-reversal, allowing the focal spot to be “compressed” to a quasi-spherical shape approaching the diffraction limit.

Consequently, the axially elongated ellipsoidal focus we observed (axial size ≈ 7.89 mm), as opposed to the quasi-spherical focus in the reference 13 (axial size ≈ 0.5 mm), is an inevitable outcome of our different design goals and strategies. Our work primarily aims to achieve uniform and controllable acoustic field distribution across multiple targets to cover the peri-infarct region, while the reference 13 focuses on exploiting the cavity effect to maximise spatial resolution.

9. Line 148: How did you calculate the energy loss through the mouse skull and brain to be compensated in order to ensure those values of PNPs?

Response: We apologize for not making this clear in the original manuscript. In the murine *in vivo* experiments, the reported PNP values refer to free-field peak negative pressures measured at the target plane after the hologram in degassed water. We did not calculate or compensate for acoustic attenuation through the mouse skull and brain, and therefore these values should not be interpreted as estimated in situ intracranial pressures. We have clarified this in the Methods section as follows:

“PNP values refer to free-field pressures measured at the target plane downstream of the hologram in degassed water.”

10. Lines 170-172: Why doesn't the increased EV accumulation have the shape of the four foci and looks more like a single spot in Fig. 4b-c?

Response: We thank the reviewer for this insightful comment. The increased EV accumulation in Fig. 4b-c appears as a single enhanced region rather than as four discrete foci primarily because IVIS provides a macroscopic whole-brain fluorescence readout with limited spatial resolution. Given the close spacing of the four acoustic foci in the mouse brain, IVIS imaging is not expected to reliably distinguish the individual targets. In addition, tissue light scattering and absorption further broaden the detected fluorescence signal and blur its spatial distribution. Therefore, IVIS is suitable for assessing overall EV accumulation in the targeted brain region, but it is not intended to resolve the fine spatial pattern of individual acoustic foci.

11. Lines 228-229: regarding the 3D simulations accurately reproducing the intended multifocal ultrasound fields, were these acoustic holograms designed to compensate skull

distortion? Otherwise, skull distortion was occurring and you cannot state that the ultrasound fields were accurately reproduced. Please, explain.

Response: We thank the reviewer for raising this point. In the human feasibility simulations, the skull was included in the numerical propagation model. The hologram phase profiles were optimized to reproduce the desired target patterns after transcranial propagation through the skull model. We have clarified the skull-compensated simulations in the Results section as follows:

“Using human skull models, our 3D simulations accurately reproduced the intended multifocal ultrasound fields while accounting for skull-induced distortions (Fig. 7c).”

12. Line 235: In the Discussion section, please, also mention the disadvantages of acoustic holograms, since they are static and no dynamic focusing can be performed once they are manufactured, unlike multi-element devices which have that advantage. In addition, theranostic ultrasound (ThUS) devices provide dynamic focusing (within a fixed plane only) and cavitation imaging in real-time, all in one with the same device (add some reference), unlike the approach of single-element transducer with hologram. You can also mention that for your application, the approach of acoustic holograms and single-element works the best given that no dynamic focusing is required.

Response: We sincerely thank the reviewer for this insightful comment. We have added the following discussion and inserted relevant references in the revised manuscript:

“Unlike multi-element phased arrays capable of electronic focusing and beam steering, most acoustic holograms remain static once fabricated, making it difficult to dynamically steer or refocus the acoustic field. Emerging theranostic ultrasound (ThUS) systems integrate therapy with real-time cavitation imaging in a single device¹, whereas the single-element transducer-hologram approach used in this study lacks real-time cavitation monitoring and closed-loop feedback. Nevertheless, for predefined lesion-conforming exposures requiring no dynamic focusing, this low-cost hologram-based approach provides a simple and scalable solution.”

References:

1. Batts AJ et al. Using a novel rapid alternating steering angles pulse sequence to evaluate the impact of theranostic ultrasound-mediated ultra-short pulse length on blood-brain barrier opening volume and closure, cavitation mapping, drug delivery feasibility, and safety. *Theranostics* **13**, 1180-1197 (2023).

13. Line 363: Why did you use five points per wavelength? Please add corresponding explanation and reference.

Response: Thank you for pointing this out. We apologize for the mistake in the original manuscript. The simulations were performed using 100 μm (ten points per wavelength) to adequately resolve the spatial variations of the acoustic pressure field and ensure numerical accuracy. A similar spatial discretization of one-tenth of the wavelength has been adopted in previous numerical studies of transcranial focused ultrasound propagation¹. The corresponding description in the Methods section has been corrected, and the relevant reference has been

added.

References:

1. Alec, H et al. Design of patient-specific focused ultrasound arrays for non-invasive brain therapy with increased trans-skull transmission and steering range. *Physics in Medicine and Biology* **62**, L9-L19 (2017).

14. Line 363: regarding mentioning the FUS working frequency of 1.5 MHz for the first time here, please describe in a dedicated sentence the geometry, holographic focal distance, size and frequency of the flat surface transducer.

Response: As suggested, we have added a dedicated sentence describing the geometry, focal distance, aperture size, and operating frequency of the flat plane-wave transducer in the Methods section as follows:

“The flat plane-wave transducer had a 30-mm circular aperture and a 1.5-MHz center frequency, and the acoustic holograms focused the acoustic field onto a target plane 20 mm from the transducer surface.”

15. Line 367-368: regarding the Human-scale simulations, at which frequency? And what was the size of the transducer and holographic focal distance?

Response: We apologize for not providing this information clearly in the original manuscript. We have clarified the simulation parameters for the human-scale simulations in the revised manuscript as follows:

“The human-scale simulation was performed at 500 kHz using a planar transducer with a 200-mm aperture, and the holographic focal distance was set to 80 mm from the transducer surface.”

16. Line 369: regarding the CT images, what is the method used to convert the mouse and human CT maps to sound speed and density maps? Please, add in manuscript.

Response: We have added the CT-to-acoustic property conversion method to the revised manuscript as follows:

“Human CT images were converted into acoustic property maps using a CT-density-based method. The skull density map was obtained by linearly scaling the skull CT values between the Hounsfield unit (HU) values of air and water:

$$\rho = \frac{\rho_w}{H_{water} - H_{air}} H - \frac{\rho_w H_{air}}{H_{water} - H_{air}}$$

where H denotes the CT value within the skull mask, H_{water} and H_{air} denote the HU values of water and air (approximated as 0 and -1000 HU, respectively), and ρ_w denotes the density of water¹. The sound speed map was then calculated from the density map using the empirical relationship $c = 2.06\rho - 1540$ ².”

References:

1. Connor CW et al. A unified model for the speed of sound in cranial bone based on genetic

- algorithm optimization. *Physics in Medicine and Biology* **21**, 3925-44 (2022).
2. Clement GT et al. Correlation of ultrasound phase with physical skull properties. *Ultrasound in Medicine & Biology* **28**, 617-24 (2002).

17. Line 370: Therefore, no skull was considered for the measurements? How did you design and validate the transcranial hologram then? This is very confusing from the beginning of the manuscript. Please, clarify and organize better.

Response: We sincerely thank the reviewer for pointing out this ambiguity. We have clarified and reorganized the relevant description in the revised manuscript. The hydrophone measurements were free-field validations of the fabricated holograms and were conducted without a skull. The mouse simulations were performed without the skull. The human simulations included the skull in the numerical propagation model. We did not experimentally validate the transcranial acoustic field using an *ex vivo* skull in the current study.

Manuscript No

We greatly appreciate the constructive comments and positive feedback from the reviewers and editor. We have carefully revised the manuscript accordingly and provided a point-by-point response below. The reviewers' comments are shown in bold, followed by our responses and the corresponding revisions to the manuscript, where applicable.

Reviewer #1 (Remarks to the Author):

Thank you for revising the manuscript to include some important experiments, analysis, and discussion.

Response: We thank the reviewer for recognizing our revisions. We believe that these revisions have substantially improved the overall quality and completeness of the manuscript.

Reviewer #2 (Remarks to the Author):

The authors have fully addressed all of my comments. I congratulate them on an excellent study.

Response: We sincerely appreciate the reviewer's positive assessment and encouragement. We are pleased that our revisions have fully addressed all previous concerns.

Reviewer #3 (Remarks to the Author):

Dear author, Thank you for the accurate responses and clarifications. I have just a couple details to ask to be incorporated, regarding the higher efficiency of transcranial IASA vs a transcranial direct method on k Wave. Please add the incidence angle onto the human skull for your simulated case, simply to let the reader know about this important parameter. And please, add a statement as well noting that IASA highlights for its time-efficiency performing faster for simpler FUS propagation conditions, however k-space provides higher wave propagation accuracy, in particular with higher incidence angles, enclosed skull cavities or when the targets are located closer to the base of the skull where reflections and standing waves generation are more detrimental to the focusing quality.

Response: Thank you for this helpful suggestion. We have incorporated both points into the revised Methods section. First, the central acoustic axis was oriented at a 45° lateral angle toward the right side of the head relative to the superior – inferior axis. Second, we have added the statement clarifying the respective strengths of the IASA and k -space methods, particularly in terms of computational efficiency and wave-propagation accuracy. For your convenience, we copied the revised text below.

“The central acoustic axis was oriented at a 45° lateral angle toward the right side of the head relative to the superior – inferior axis.”

“IASA provides high computational efficiency under relatively simple FUS propagation conditions, whereas k -space methods may offer higher wave-propagation accuracy in more complex scenarios, such as large incidence angles, enclosed skull cavities, or targets located near the skull base.”