Title: Time-dependent biochemical response after middle cerebral artery occlusion in rats: A pilot study

Authors: Asma Parveen, Prathap Suganthirababu, Lavanya Prathap, Ashisha K. Tanaya, Yamini Umasankar, Debadutta Patra

Reviewer B: Anonymous, COI: None, AI disclosure: None

1. Comment Title can be short and capital letter only be used in the first word as per the journal requirement.

Response: We have revised the title to sentence case, capitalising only the first word and proper nouns: "Occlusion duration and biochemical response after middle cerebral artery occlusion in rats: a pilot comparative study.

2. Comment Quality of the tables are not good. Table 1 and Table 2 can be merged. Tables are appropriate.

Response: We have merged the two tables into one (Table 1), reporting each marker's mean ± SD for all three groups alongside the ANOVA P value, with a full caption and footnotes.

3. Comment Most of the reference are from the year 2017. Only a few references are up-to-date or within 5 years.

Response: We have replaced the reference list with 10 references, 6 of which are from 2020–2025, to bring the citations up to date in Research Letter.

4. Comment Standard of English for can be improved.

Response: We have revised the language throughout for clarity and will have the final version professionally copyedited.

Reviewer E: Anonymous, COI: None, AI disclosure: None

5. Comment The objective should be more precisely stated at the end of the introduction, clearly outlining the specific outcome measures the authors intend to examine.

Response: The final sentence of the Introduction now names all outcome measures explicitly: GSH-Px and SOD activity, IL-1β and IL-6 levels, NRP-1 expression, and histological injury, compared across the three occlusion durations.

6. Comment It would be helpful if the authors could clarify what is already known from prior MCAO duration-comparison studies.

Response: We have added citations on MCAO modelling and duration-dependent injury in the Introduction. Given the Research Letter's word limit, we were not able to elaborate at length, but the cited reviews cover this background in more depth.

7. Comment The specific gap in understanding that this study addresses could be stated more explicitly.

Response: The Introduction now states directly that whether occlusion duration differentially affects these specific markers within the same 24-hour window "is not well characterized," naming the gap explicitly rather than implying it.

8. Comment It is not entirely clear how the chosen time points (20, 30, 60 min) were determined to be relevant; the authors may wish to elaborate on this.

Response: We have clarified that this study was designed as a screening pilot to compare a spread occlusion durations (20, 30, and 60 minutes) and identify which one produces a reproducible, well-tolerated injury profile suitable for a subsequent, fully powered main study. This is now stated in both the Introduction and Methods.

9. Comment The authors imply therapeutic relevance of the findings; it would strengthen the manuscript to briefly note how previous studies support this claim.

Response: We have removed language implying established therapeutic relevance. The Discussion now frames the GSH-Px/NRP-1/IL-1β pattern as worth further investigation rather than as evidence of therapeutic significance.

10. Comment Could the authors specify the age range of the animals, as only "adults" is currently mentioned?

Response: The body-weight range (250–300 g) is reported rather than the age.

11. Comment The authors may wish to justify the use of only male animals in this study.

Response: We used only male animals to avoid the confounding effect of oestrous-cycle-related hormonal variation on infarct size and inflammatory markers.

12. Comment Because a priori power calculation was not provided, a power calculation would help readers appreciate the extent of this limitation.

Response: n=2/group was chosen deliberately for this screening pilot rather than derived from a power calculation for a specific effect size. We have not retrofitted a power calculation, since doing so after the fact would not reflect how the study was actually designed; we note in the Discussion that this limits how much weight the *P* values reported can bear.

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13. Comment No control group was included; a justification for this design choice would be appreciated.

Response: No sham or non-occluded control group was included in this screening phase, as its purpose was to compare the three occlusion durations against each other, not against an uninjured baseline. We agree this means the biochemical changes reported cannot be distinguished from surgical or anesthesia-related effects, and we have stated this explicitly as a limitation. A control group will be included in the main study.

14. Comment Please clarify the rCBF monitoring protocol (continuous vs onset-only) and report per-group rCBF mean ± SD; note any exclusions.

Response: rCBF was confirmed to be reduced by at least 80% at occlusion onset, as stated in the Methods. We did not continuously monitor and log rCBF throughout the occlusion period in a way that would allow us to report per-group mean ± SD, and no animals were excluded for failing to meet the threshold. We will consider continuous rCBF monitoring for the main study.

15. Comment Was the standard indirect correction method (Swanson) applied for infarct volume? If not, please justify.

Response: The Swanson indirect correction method was not applied in this pilot, as infarct volume was not directly quantified. Assessment was limited to qualitative H&E scoring.

16. Comment The number of sections per brain, and the number of brains (animals) per group used in H&E staining, could be stated more clearly.

Response: H&E staining was performed on both animals in each group (n=2/group), with three fields examined per section, as now stated in the Methods.

17. Comment It is not clear how the three fields per section were selected, or how sampling bias was avoided.

Response: Fields were selected by a blinded observer at a fixed magnification (20×) without prior knowledge of group allocation, which we believe reduces selection bias.

18. Comment Were the sections examined by a single investigator? If more than one, how was inter-rater reliability assessed?

Response: Histology was assessed by a blinded investigator. We did not have a second investigator independently score the same sections, so no inter-rater reliability statistic is available

19. Comment Presenting Group 2/3 values as percentage change relative to Group 1 may imply a continuous, linear dose-response trajectory across independent groups.

Response: We have removed the percentage-change presentation. Table 1 now reports each group's own mean ± SD, and the Results and Discussion state explicitly that the three groups are independent samples, not repeated measures, and should not be read as points on a continuous dose-response curve.

20. Comment The cross-sectional design can descriptively inform us about individual groups, but describing a linear trend needs cautious interpretation.

Response: We agree and have removed language implying a linear or progressive trend. The Discussion now describes the direction of difference between the three independent groups without implying a continuous trajectory.

21. Comment The Methods section does not include a dedicated statistical methods description; relocating this from the Results would improve clarity.

Response: We have moved the full statistical description (one-way ANOVA, significance threshold, and the note that pairwise comparisons were not corrected for multiple testing) into the Methods section.

22. Comment Sample size determination was not mentioned.

Response: We have stated in the Methods that n=2/group was a deliberate choice for this screening pilot, made to compare candidate occlusion durations rather than to detect a pre-specified effect size.

23. Comment The authors may wish to justify the use of ANOVA given the small sample size. Where ordinal data were used, the use of ANOVA does not appear statistically appropriate.

Response: We have run one-way ANOVA and pairwise comparisons on the real data (two animals per group) and report the resulting P values in Table 1 and the Results, but we explicitly caution in the Discussion that with n=2/group a $P<0.05$ result is far less robust than the same value from a properly powered study and could be disproportionately influenced by a single animal. We agree. The H&E histology scores (0–4 ordinal scale) are now reported descriptively only, with no ANOVA applied. ANOVA was retained solely for the continuous biochemical assay data (GSH-Px, SOD, IL-1β, IL-6, NRP-1).

24. Comment If a dose-response/trend relationship is intended, a formal statistical test for trend would help substantiate this.

Response: We do not make a dose-response or trend claim anywhere in the revised manuscript, so a formal trend test was not required. We state explicitly that the three groups are independent and should not be interpreted as an ordered trend.

25. Comment Tables are not self-explanatory; they need captions describing the content, and footnotes are missing.

Response: Table 1 now has a full descriptive caption, an abbreviation key, and explanatory footnotes covering the statistical methods used.

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26. Comment Only P-values are provided in the tables; including means/SD and effect sizes would strengthen the presentation.

Response: Table 1 now reports the mean ± SD for both animals in each group for every marker, alongside the ANOVA P value, rather than P values alone.

27. Comment It would be preferable for each group to be reported with its own descriptive statistics, using appropriate independent-groups comparisons.

Response: Each of the three groups now has its own column with its own mean ± SD in Table 1, rather than Groups 2 and 3 being expressed as percentage change from Group 1.

28. Comment The statement implying a "gradual transition... stages of disease progression" is not supportable from independent groups.

Response: We have removed this statement and replaced it with language describing the direction of difference between groups without implying a progression narrative.

29. Comment "Table 2 suggests the intervention is strengthening cellular defenses" may not be fully supported by the current findings.

Response: We have removed this statement; it is not carried into the revised Results or Discussion.

30. Comment "Mild ischemic changes" could be replaced with more objective findings expressed as percentages or scores.

Response: We have retained descriptive histological terms (pyknotic nuclei, cytoplasmic eosinophilia, neuropil vacuolation)

31. Comment Mechanistic/explanatory sentences (e.g., Na/K pump failure) belong in the Discussion, not the Results.

Response: We have moved all mechanistic interpretation out of the Results; the Results section now reports findings only, with mechanistic discussion confined to the Discussion section.

32. Comment There is major redundancy between text and tables/figures in the Results section.

Response: We have condensed the Results to reference Table 1 directly rather than restating every value in prose.

33. Comment It would strengthen the manuscript if the Discussion began with a concise summary of the key findings.

Response: The Discussion now opens with a summary sentence stating which markers differed significantly and that histological injury remained comparable across groups.

34. Comment "Progressive shift... as occlusion duration increases" is difficult to support given the study design; the authors may wish to moderate this.

Response: We have removed this statement. The Discussion instead describes the direction of difference between the three independent groups without implying a progressive shift.

35. Comment "Simultaneous activation of endogenous protective pathways" would benefit from clarification on how the results demonstrate "simultaneous."

Response: We have removed this specific claim; it is not retained in the revised Discussion.

36. Comment "Despite its strengths..." implies a preceding discussion of the study's strengths, which could be added as a separate paragraph.

Response: We appreciate this suggestion and adding a short strengths paragraph.

37. Comment The conclusion does not match between the abstract and the main conclusion; the abstract should follow the more carefully written main conclusion.

Response: We have rewritten the abstract so it uses the same hedged language and caveats as the Discussion's conclusion (small n, screening-pilot design, no control group, need for confirmation in a fully powered study).

Responsible editor: **M Mostafa Zaman,** **ORCID:** **0000-0002-1736-1342**

38. Comment Given the small number of animals (two in each group), the study hardly allows any statistical analysis to draw inferences. References cited in this manuscript used larger samples. Therefore, we shall consider this manuscript if it is submitted as a Research Letter (<1000 words, <10 references, one table or figure).

If agreeable, submit a revised version that takes into account the reviewers' comments. A point-by-point response will be appreciated. The timeline is 15 July.

Response: We agree with the Editor's decision to summarise the manuscript to fit in to the format of the Research Letter.

39. Comment The title can be as "Time-dependent biochemical response after middle cerebral artery occlusion in rats: A pilot study"

Response: We revised the title as suggested.

40. Comment The key message has a thunder start with 60 min; It needs clarification of three time categories first. Do not use an acronym here.

Response: Rewritten. The key message now opens by identifying all three occlusion durations tested (20, 30, and 60 minutes) before stating the 60-minute finding, and no longer uses the MCAO acronym ("middle cerebral artery" is spelled out).

41. Comment The Introduction given in the first paragraph (lines 97-118) could be substantially reduced.

Response: The Introduction has been cut from roughly 430 words to 4 sentences (120 words), retaining only the rationale for studying occlusion duration, the role of MCAO as a model, the specific knowledge gap, and the pilot's aim. Background elaboration and repeated framing have been removed.

42. Comment The ethical clearance (lines 128-129) should be removed because it has been described in a separate heading (lines 215-217). However, the Memo number should also have a date.

Response: The duplicate ethics sentence has been removed from the Methods paragraph. The Memo no. has been added.

43. Comment The Results section (lines 142-161) must be shorter because the text descriptions repeat the whole of Table 1. Therefore, these are redundant. Keep only the major findings and refer to the table for others.

Response: The Results paragraph has been condensed to report only the direction and significance of each marker (e.g. "GSH-Px and NRP-1 were significantly higher, and IL-1β significantly lower, in the 60-minute group") without restating individual group means, SDs, or P-values, which are left to Table 1.

44. Comment Paragraph in lines 162-175 includes Results and Discussion. Please cover the Results in the above paragraph and separate the Discussion points for merging with the next paragraph on Discussion. The Discussion section in lines 176-189 is poorly written. The Discussion section must discuss the pathobiology and answer the "so what?" question. Mention the implications of the findings: does the pilot finding need further confirmation in a larger sample, or is further study needed to cover the limitations (e.g., non-standardisation of biochemical measurements) of the experiment.

Response: The mixed paragraph has been split: its Results content (the independent-groups clarification) was folded into the Results paragraph above, and its Discussion content was merged with the following Discussion paragraph. The Discussion has been rewritten to explain the likely pathobiology (a compensatory antioxidant/pro-angiogenic response building with longer ischemia, and a threshold-like SOD peak at 30 minutes), to state explicitly why the finding matters (biochemical change may precede structural injury, which could eventually help identify tissue still open to intervention), and to address the three implications directly: the pilot needs confirmation in an adequately powered sample; the field's lack of standardised biochemical assay protocols is named as a limitation requiring further study.

45. Comment Table 1: Please use the SDs in parentheses (not with a ± symbol).

Response: All entries in Table 1 have been reformatted from "mean ± SD" to "mean (SD)"

46. Comment References: All authors must be listed (do not use et al). Three references date back to 2017. Please try to use recent references unless these are considered essential references.

Response: All reference entries now list every author in full; "et al." has been removed throughout. Of the three 2017 references, the general stroke-epidemiology citation have been replaced with recent article.

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