

Organic-inorganic covalent selenium reversing ischemic reperfusion injury

Corresponding Author: Professor Tianfeng Chen

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)

This manuscript reported a unique Se atom reprogramming strategy to treat ischemic stroke. The authors elucidated in detail the chemical structure and ROS-scavenging activity of Or/In-Se NPs. This design is novel and the physicochemical characterization and cellular level experiments are detailed. However, the mechanisms of Or/In-Se NPs are unattractive and data at the rodent experimental level are insufficient. There are some important issues that need to be addressed as follows.

1. The action mechanisms and therapeutic efficacy of Or/In-Se NPs are unattractive. The evidence for in vivo therapeutic efficacy is very weak and data presentation is not standardized.

1) The data of Figure 5 indicated there were not statistically different.

2) The authors should provide NMR results on progression of cerebral infarction.

3) The authors must provide the quantitative results of TTC (Figure 5).

4) The results of the MRI and the TTC were not well match.

5) The In-Se NP treatment should be as control group in vivo and in vitro experiments.

2. The MCAO mouse model damage is insufficient because time to block the middle cerebral artery is too short.

3. The data about the process of blood circulation, the distribution of the tissues and potential toxicity of Or/In-Se NP in vivo should be provided.

Reviewer #2

(Remarks to the Author)

Reviewer's comments:

This study presents a new selenium-based compound that combines inorganic and organic selenium for a more efficient delivery and use by the cells. They tested the effects of the compound in vitro and in vivo in a ischemic stroke model. This work is of great significance to the field, as it opens avenues for a selenium-based therapy for cases of extreme oxidative stress production. However, the authors must be clearer when presenting their results, providing P-values and writing the results according to the statistical findings. Further, I suggest exploring two other mechanisms that can be involved in the benefits of the compound, and highlight the importance of discussing the clinical relevance and limitations of the findings.

Overall comments

Although the role of selenoproteins in the redox balance, research has pointed out the effects of selenoproteins in neurogenesis (PMID: 35120590), which would also be relevant for stroke. The authors could explore this effect in the animal model. Further, I'd also suggest exploring the effects of the treatment on ferroptosis – this is of particular importance given that the authors reported upregulation of Gpx4 after the treatment.

Introduction

Overall, I recommend the introduction to mention the narrow safe therapeutic window of selenium, with both too little and too much exposure having detrimental health effects. Some papers to consider: PMID: 31388899, PMID: 34262926

L28-30: Could the authors be more specific regarding the confusion around the clinical use of selenium?

L44-49: Even though the explanation given for how organic x inorganic selenium would be driven to different roles is clear, it sounds to me that it lacks rationale. Could the authors provide background information to justify the notion that organic selenium would scavenge free radicals while inorganic selenium would be used for selenoproteins synthesis, given that

both forms require to be metabolised into selenide before their metabolisation into selenophosphate for incorporation into SEPSECS?

Results

Fig 4 (and the in-text corresponding results) have no P-values – please include P-values for a clearer information on statistical significance.

Fig 4g: the mRNA expression of some selenoproteins peak at 12h but decrease (and some return to zero expression level) at 24h (e.g. Gpx1, Gpx4, TrxR2, TrxR3, SelP). Could the authors explain such effect? Also, could the authors be clear that not all the selenoproteins were assessed (and provide an explanation)?

Fig 4h: Could the authors explain why only a few selenoproteins were measured at the protein level (fewer than those measured at mRNA level)?

Fig 4i: a brief caption should be added to explain the figure.

Fig 5: as seen for Fig 4, there are no P-values for the statistical analysis, so it's unclear if the results are significant. If they are not, the authors must consider rewriting the results to not oversell them.

Fig 6: please indicate the P-values corresponding to * and ** in Fig c,d and e. Please provide P-values for Figs f, g and h. It's unclear if the results are significant. If they are not, the authors must consider rewriting the results to not oversell them.

Fig 6a: please add brief explanation for the hypothesis displayed in this figure.

Discussion

The authors could explore the potential applications, effects and limitations of the use of this compound in clinical studies, shedding light into future studies.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors' response was satisfactory and there were no other issues.

Reviewer #2

(Remarks to the Author)

The authors have significant improvements in the manuscript, including the assessment of ferroptosis to examine the effects of the Or/In-Se NPs. However, ferroptosis is a complex cell death pathway that involves different aspects and molecules, and defining the effects of the studied molecule in this pathway requires more than measuring the expression of 2 genes (Gpx4 and Alox15). That said, I recommend the authors include other specific markers of ferroptosis (e.g. TfR1 expression and other ferroptosis reaction products) in order to distinguish ferroptosis from other stress conditions (PMID: 38187349, PMID: 36828775, PMID: 37556320).

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have addressed my concerns.

Version 3:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

I am writing to review specifically the western blots for NRF2 and Keap1.

The use of CST antibody 8882S is curious, as it is a discontinued antibody. In examining the Western blots, I am not convinced that we are seeing the same thing in the three replicates. If you look at the 97-100kDa bands, replicate 2 and replicate 3 do not show a convincing upregulation of these bands over the time course. In fact, the bands are quite static at 97-100kDa. Although the upper bands may be post-translationally modified – the lack of such bands in replicate 1 means that there is significant variation in exposure of the film. Altogether the quality of the data is not quite convincing that Or/In-Se increases Nrf2. If the method of quantification shown in Supp Fig 17b is to quantify the NRF2 97-100kDa bands and all additional heavier bands – then this must be explicitly stated in the methods. In order to improve the experiments, I would suggest repeating the experiment with CST 12721 antibody. One could also IP with one NRF2 antibody and blot with another, controlling of course for total protein.

The Keap1 blots do not show a clear increase in Keap1 over time – rather static, but this is congruent with the quantification

results.

Version 5:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

I am satisfied with the experimental replicates with the new antibody. I have no other concerns regarding the antibodies.

I have also been asked to review the manuscript as to whether Supplementary Figure 18 adequately supports the relevant claims (Line 380-387) in the manuscript particularly regarding TrxR1.

The authors state in the manuscript that - As shown in Fig. 4h and Supplementary Fig. 18, after incubated with Or/In-Se NPs for 8-24 h, the expression level of two important classes of antioxidant selenoenzymes, including glutathione peroxidase2, 4 (Gpx2, Gpx4), and thioredoxin reductase1 (TrxR1), were significantly improved, and the antioxidant selenoproteins, such as selenoprotein O, P, T, W, (SelO, SelP, SelT and SelW), also upregulated obviously.

In Fig 4h, I am not sure I see a marked difference between control vs treated SH-SY5Y cells cultured with Or/In-Se NPs at any of the time points for TrxR1. In fact, in the supplementary figures, there is no statistical difference for TrxR1. There is also no differences for SelS, SelK, and SelT. This should be noted.

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Comments and Response

Response to Reviewer #1:

This manuscript reported a unique Se atom reprogramming strategy to treat ischemic stroke. The authors elucidated in detail the chemical structure and ROS-scavenging activity of Or/In-Se NPs. This design is novel and the physicochemical characterization and cellular level experiments are detailed. However, the mechanisms of Or/In-Se NPs are unattractive and data at the rodent experimental level are insufficient. There are some important issues that need to be addressed as follows.

Our response:

Thanks a lot for the reviews' comments and kindly suggestions, which greatly helped us to improve the quality of our manuscript. We have tried our best to supplement the experiments according to the suggestions to further demonstrate our conclusions, and carefully revised the manuscript to make it clearer and more convincing.

1. The action mechanisms and therapeutic efficacy of Or/In-Se NPs are unattractive. The evidence for *in vivo* therapeutic efficacy is very weak and data presentation is not standardized.

Our response:

According to the reviewer's comments, we have repeated ischemic reperfusion injury model and increased the number of MCAO mice to further evaluate the therapeutic efficacy of Or/In-Se NPs *in vivo*. We further monitored the progression of cerebral infarction by MRI imaging and performed the behavioral analysis after treated by Or/In-Se NPs at different time points. The obtained data was also conducted the statistical analysis and quantification to standardize these results.

1) The data of Figure 5 indicated there were not statistically different.

Our response:

Thanks for the reviewer's comment. We further increased the number of ischemic

reperfusion injury mice in each treatment group and performed the behavioral analysis every day to evaluate the therapeutic efficacy of Or/In-Se NPs. The results showed that the treatment of Or/In-Se NPs was able to significantly improve the elapsed time of the mice on the rotarod, remarkably decrease the proportion of MCAO mice veering to the right and ameliorate hemiplegia (**Fig. 5c, d**). Furthermore, based on behavioral scores, we can infer that Or/In-Se NPs treatment effectively mitigates mobility impairment associated with brain injury in MCAO mice, especially treated with Or/In-Se NPs at 0.8 mg/kg for 3 days (**Fig. 5e**). Ultimately, the TTC staining of 3 days-treated MCAO mice also showed that Or/In-Se NPs reduced the infarct area and significantly improved the treatment effect with increasing dose of Or/In-Se NPs (**Fig. 5f** and **Supplementary Fig. S21**). The obtained data was conducted by statistical analysis and further indicated that Or/In-Se NPs can effectively attenuate ischemia reperfusion-induced cerebral infarction and motor dysfunction *in vivo*. These results and discussion have been added into the revised manuscript.

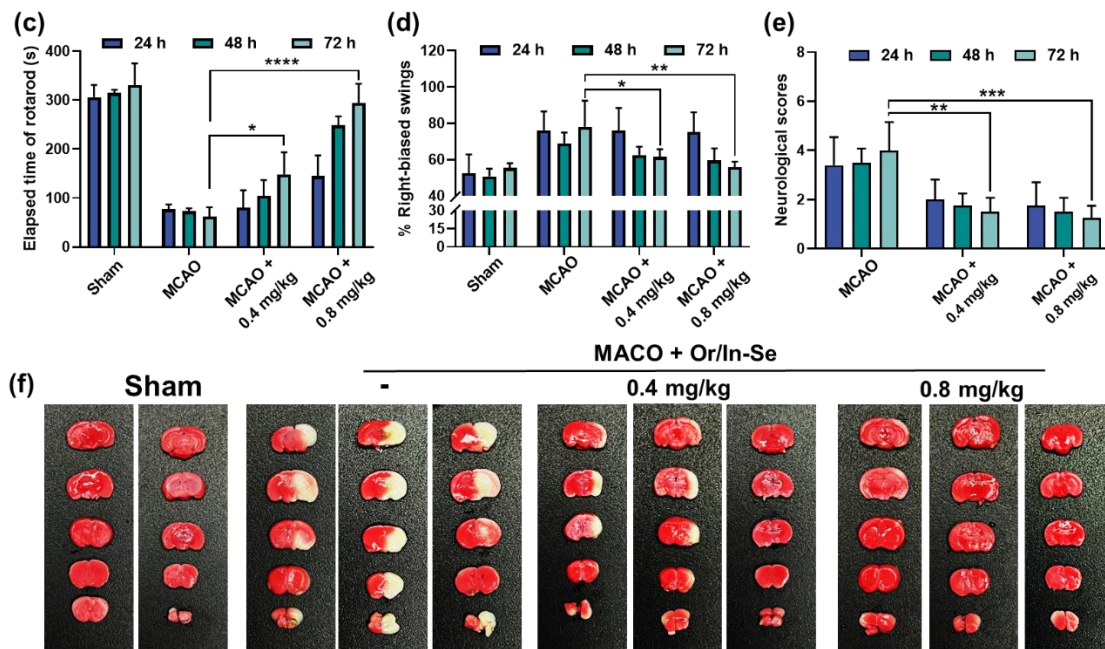
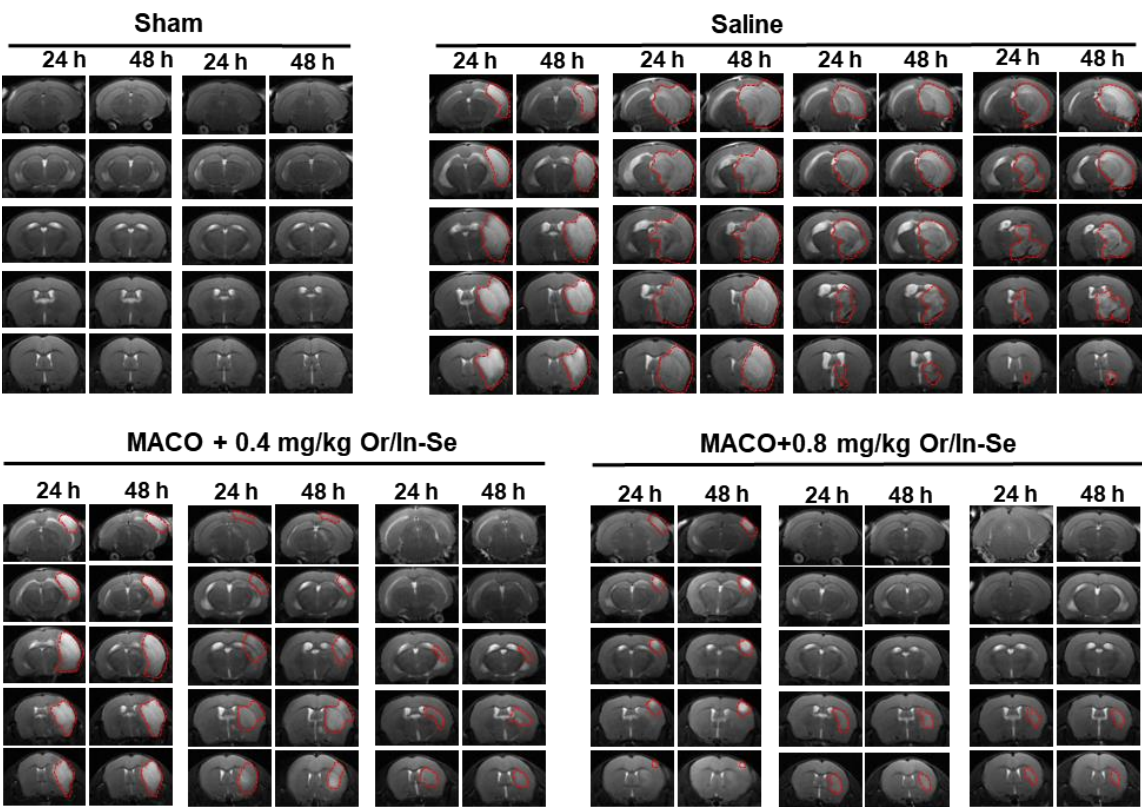


Fig. 5. Neuronal function evaluation of the treated MCAO mice with behavioral tests, including (c) tail suspension test, (d) Rota-Rod test and (e) neurological scores at different time points ($*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$). (f) Representative brain tissue images of TTC staining in ischemic stroke mice with different treatment for 72 h (n=3).

2) The authors should provide MRI results on progression of cerebral infarction.

Our response:

According to the reviewer’s suggestion, we further monitored the progression of cerebral infarction by MRI imaging with MCAO mice after treated by Or/In-Se NPs at different time points. As shown in **Supplementary Fig. S22** and **Fig. 5g**, the MRI *T2* images exhibited that the infarct area of MCAO group mice was slightly increased with the extension of untreated time. While after treated with Or/In-Se NPs, the infarct area of brain tissue was significantly reduced with the increase of the concentration and treated time. Furthermore, we quantified these MRI data and also demonstrated that Or/In-Se NPs administration effectively reduced the value of infarct area, which was reduced almost ~ 40% after treated with 0.8 mg/kg of Or/In-Se NPs (**Supplementary Fig. 23**). These results and discussion have been added into the revised manuscript.



Supplementary Fig. S22. MRI *T2* images for monitoring the progression of infarct areas in the treated MCAO mice at time points of 24 h and 48 h (n=4).

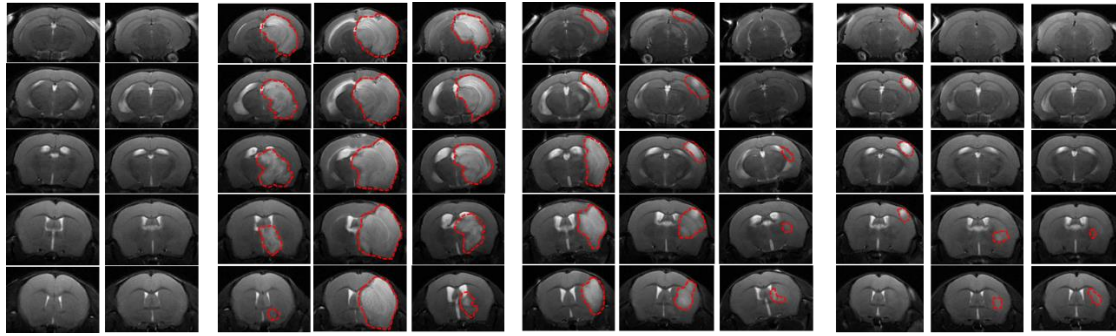
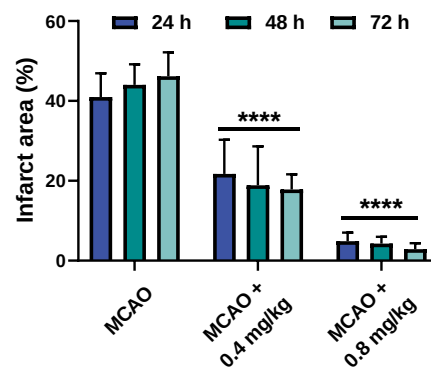


Fig. 5. (g) MRI *T2* images of MCAO mice at different brain layers with different treatment for 72 h (Red cycle: Infarct area, n=3).

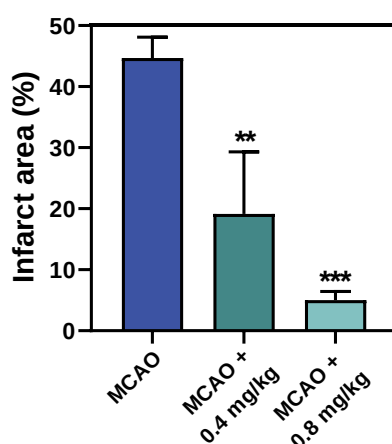


Supplementary Figure S23. Corresponding infarct areas of different groups quantified the maximum cross section of MRI *T2* images at different treated time points (n = 3).

3) The authors must provide the quantitative results of TTC (Figure 5).

Our response:

According to the reviewer's suggestion, we further quantified the TTC staining to evaluate the therapeutic efficacy of Or/In-Se NPs. The results showed that the brain infarct area significantly decreased after treated with Or/In-Se NPs in a dose-dependent manner (**Supplementary Fig. S21**). These results and discussion have been added into the revised manuscript.



Supplementary Fig. S21. Infarct areas of the treated MCAO mice quantified the TTC staining images by ImageJ (n=3).

4) The results of the MRI and the TTC were not well match.

Our response:

We have revised this issue according to reviewer's good suggestion. Actually, the results of the MRI and TTC in Fig. 5c and 5e didn't match well, which could be due to the fact that the MCAO mice in these two experiments were not from the same animal. In addition, the scan planes of MRI imaging and the cut planes of TTC staining are inconsistent. Based on these questions, we have repeated ischemic reperfusion injury model and performed the MRI imaging and TTC staining on the same MCAO mice to ensure the consistency of experimental results and accurately evaluate the therapeutic effects of Or/In-Se NPs *in vivo*. As shown in **Fig. 5f**, the TTC staining of 3 days-treated MCAO mice also showed that Or/In-Se NPs reduced the infarct area and significantly improved the treatment effect with increasing dose of Or/In-Se NPs. The results of MRI *T2* images at different treated time points also demonstrated that Or/In-Se NPs significantly inhibited the progression of cerebral infarction, which is consistent with the TTC staining results of MCAO mice after 3 days-treatment (**Fig. 5g**). Furthermore, the corresponding infarct areas quantified by the MRI *T2* images and TTC staining were also consistent to indicate the effective therapeutic effects of Or/In-Se NPs *in vivo*.

(Supplementary Fig. S21 and S23). All these results and discussion have been added into the revised manuscript.

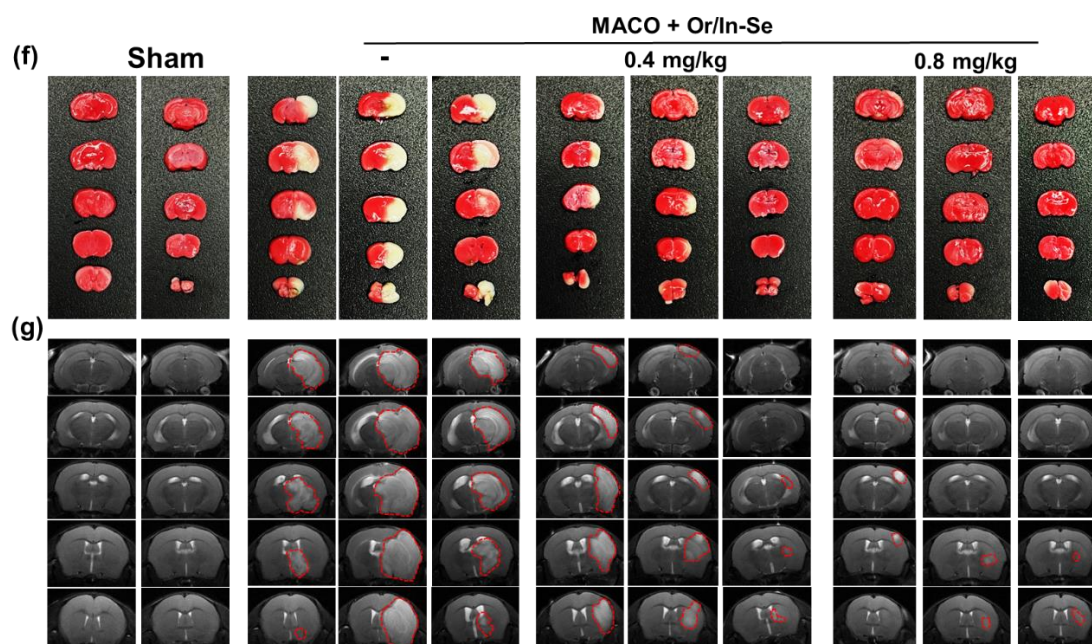


Fig. 5. (f) Representative brain tissue images of TTC staining in ischemic stroke mice with different treatment for 72 h (n=3). (g) MRI T2 images of MCAO mice at different brain layers with different treatment for 72 h (Red cycle: Infarct area, n=3).

5) The In-Se NP treatment should be as control group in vivo and in vitro experiments.

Our response:

Thanks for the reviewer's comments. In this study, we used the triclinic crystal Se (t-Se) and amorphous elemental Se (α -Se) as the control groups of inorganic selenium nanoparticles (In-Se NP) to evaluate the effects of antioxidant therapy. Firstly, for the detection of antioxidant activity, both t-Se and α -Se did not show obvious free radical scavenging capability even at a concentration of 10 μ g/mL (**Fig. 3a**). The results of ESR spectra (**Fig. 3e**) also demonstrated that Or/In-Se NPs could effectively catalyze H_2O_2 into $\cdot OH$, following by further decomposition, while t-Se and α -Se could not catalyze the decomposition of H_2O_2 , which suggesting the catalytic ability of Or/In-Se NPs is much stronger than that of other Se species. Furthermore, these results also could be further proofed in OGD cell model, where t-Se and α -Se failed to clear the ROS in SH-SY5Y cells under OGD condition, thereby did not significantly improve cell survival

(Fig 4a, b). All these results suggest that In-Se NP doesn't exhibit significant *in vitro* free radical scavenging potential to realize the antioxidant therapy, and further indicated that the antioxidant activity of Or/In-Se NPs is derived from the organic Se-C component. These discussions were further emphasized in the revised manuscript.

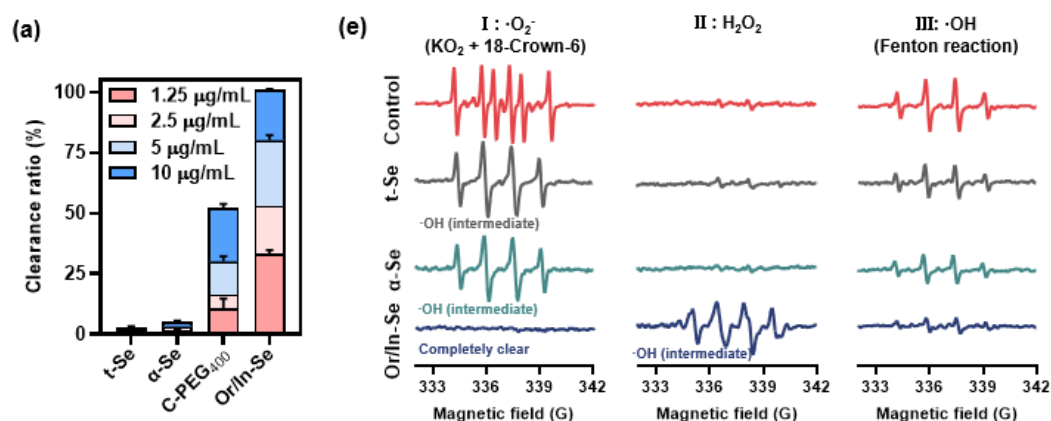


Fig. 3 (a) Clearance ratio of ABTS free radical in different Se species by ABTS assay. (e) EPR signals of $\cdot\text{O}_2^-$, H₂O₂ and $\cdot\text{OH}$ scavenging ability of different Se species (20 µg/mL).

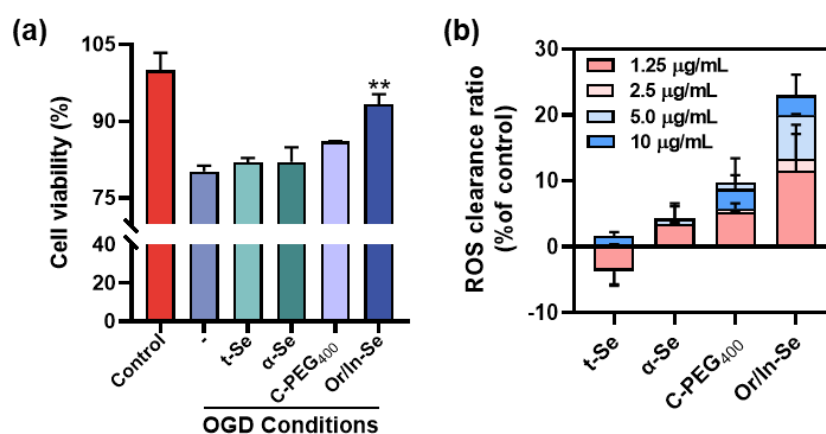


Fig. 4 (a) Cell viability of SH-SY5Y in OGD model after treated with 5 µg/mL t-Se, α-Se, C-PEG₄₀₀ and Or/In-Se NPs for 24 h. (b) The intercellular ROS level of SH-SY5Y with different treatment in OGD model detected by DCFH-DA.

2. The MCAO mouse model damage is insufficient because time to block the middle cerebral artery is too short.

Our response:

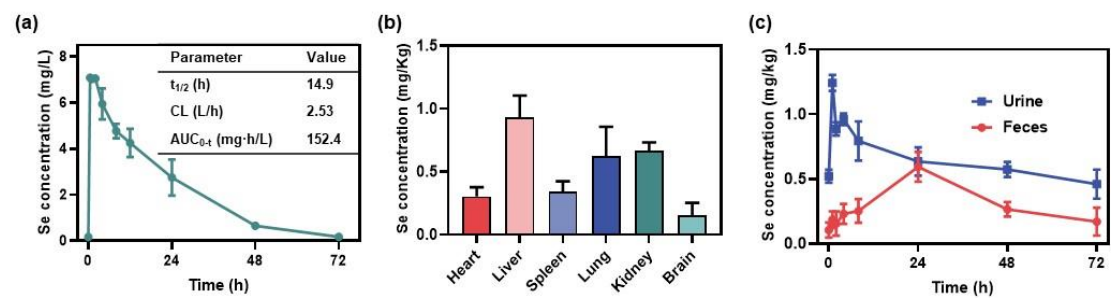
Thanks for the reviewer's comments and kindly suggestion. In this study, we constructed the MCAO reperfusion model by inserting the filament with a silicone tip into the middle cerebral artery of the rat for 45 min according to the references (Am. Heart. J. 1990, 119(2 Pt 1):272-6). To enhance the brain damage of MCAO mice, we prolonged the occlusion duration to 1 hour in repetitive ischemia-reperfusion injury model, and also detected the effective antioxidant therapy of Or/In-Se NPs for ischemic stroke (as shown in **Fig. 5c-f**). These results and discussion have been added in the revised manuscript.

3. The data about the process of blood circulation, the distribution of the tissues and potential toxicity of Or/In-Se NP *in vivo* should be provided.

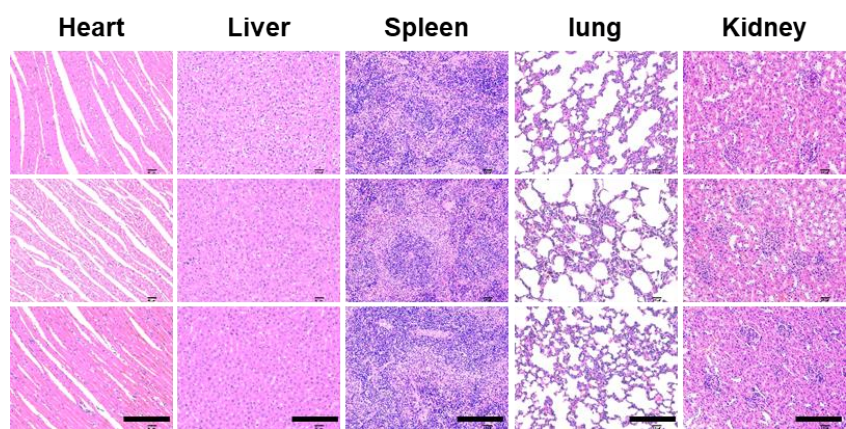
Our response:

Thank the referee for the goodwill suggestions to improve the quality of the manuscript. The pharmacokinetic analysis and biosafety evaluation of nanomedicine are the basis and prerequisite for its application *in vivo*. Therefore, according to review's suggestion, we have systematically examined the pharmacokinetics, distribution and metabolism of Or/In-Se NPs *in vivo*. The fitted blood concentration curve of Or/In-Se NPs quantified by Se revealed that the half-life ($t_{1/2}$) and the area under curve (AUC_{0-t}) of Or/In-Se NPs are 14.9 h and 152.4 mg/L*h, respectively (**Supplementary Fig. S19a**), which are much higher than that of the clinically used edaravone ($t_{1/2}$: 4.47 h, AUC_{0-t} : 2.3 mg/L*h) (Clin. Ther. 2023, 45, 1251–1258), suggesting the high blood circulation and adequate drug exposure of Or/In-Se NPs *in vivo*. Furthermore, the distribution of Or/In-Se NPs in the main organs showed that the nanoparticles mainly accumulated in the liver and kidney (**Supplementary Fig. S19b**), thereby metabolized by the kidney and excreted mainly in the urine (**Supplementary Fig. S19c**). Subsequently, we further evaluate the biosafety of Or/In-Se NPs by histopathology and hematology. The results of H&E staining showed that Or/In-Se NPs didn't cause the obvious pathological changes in the

main organs (**Supplementary Fig. S20**). Importantly, the hematological data also showed that the blood routine and blood biochemical indexes of the Or/In-Se NPs treated rats were sustained in normal range (**Supplementary Table S3**). These results indicate that Or/In-Se NPs exhibit high biocompatibility and biosafety *in vivo*. These results and discussion have been added in the revised manuscript.



Supplementary Fig. S19. The concentration of Se in blood serum (a), main organs (b), and urine and feces (c) of rats at different time points after injection of Or/In-Se NPs (2 mg/kg).



Supplementary Fig. S20. The H&E staining of the main organs in Or/In-Se NPs treated rats.

Supplementary Table S3. The blood biochemical index for rats after 72 h injection of Or/In-Se NPs.

Parameters	Unit	Or/In-Se NPs	Reference
ALT	U/L	50.25±8.15	21.53-61.75
AST	U/L	154.31±26.99	41.47-195.65
γ-GT	U/L	0.84±0.04	0.58-6.81
BUN	mg/DL	22.97±3.44	9.75-22.71
UA	μmol/L	61.18±13.82	58.38-122.65
CR	μmol/L	31.89±6.41	10.90-118.07
CK	U/L	480.98±29.87	58.24-530.38
T-CHO	mmol/L	2.39±0.35	1.13-3.48
TG	mmol/L	0.67±0.13	0.34-2.19
HDL-C	mmol/L	0.93±0.17	0.31-0.73
LDL-C	mmol/L	0.26±0.03	0.09-0.24
GLU	mmol/L	8.76±0.76	4.98-15.95

Response to Reviewer #2:

This study presents a new selenium-based compound that combines inorganic and organic selenium for a more efficient delivery and use by the cells. They tested the effects of the compound in vitro and in vivo in a ischemic stroke model. This work is of great significance to the field, as it opens avenues for a selenium-based therapy for cases of extreme oxidative stress production. However, the authors must be clearer when presenting their results, providing P-values and writing the results according to the statistical findings. Further, I suggest exploring two other mechanisms that can be involved in the benefits of the compound, and highlight the importance of discussing the clinical relevance and limitations of the findings.

Our response:

Thanks a lot for the reviews' comments and kindly suggestions, which greatly helped us to improve the quality of our manuscript. We have carefully analyzed all data and

conducted the statistical analysis to make the results statement clearer and more convincing. We also further emphasize the importance of selenium-based antioxidant therapy, and further discuss their clinical applications and limitations in the revised manuscript.

Overall comments

Although the role of selenoproteins in the redox balance, research has pointed out the effects of selenoproteins in neurogenesis (PMID: 35120590), which would also be relevant for stroke. The authors could explore this effect in the animal model. Further, I'd also suggest exploring the effects of the treatment on ferroptosis – this is of particular importance given that the authors reported upregulation of Gpx4 after the treatment.

Our response:

Thanks a lot for the reviewer's kindly suggestion. It is a good idea to further explore the effects of this NPs on stroke-triggered ferroptosis. Generally, Se has widely been reported to show neuroprotection function and to promote neurogenesis in disease like Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and neurological disorders, and especially free radical related disease of ischemic reperfusion injury (Cell 2018, 172, 409, J. Inorg. Biochem. 153, 1, J. Neurochem. 2003, 86(1),1). Meanwhile, as the executor for function of Se, selenoproteins like Gpx4, has been reported to be a regulator of ferroptosis, and to play a crucial role in stroke treatment (Cell 2019, 177, 1262). At the same time, as the referee mentioned, our results indicated that Or/In-Se NPs could effectively upregulated selenoproteins, especially Gpx4 expression (**Fig. 4h**). Therefore, in order to explore the function of Or/In-Se NPs in neuroprotection through blocking ferroptosis, we also performed immunofluorescence to assess the expression of Gpx4 and arachidonate 15-lipoxygenase (ALOX15, a marker for lipid peroxidation) in brain tissue of the Or/In-Se NPs treated MCAO mice. As shown in **Fig. 6g and h**, the administration of Or/In-Se NPs obviously enhance the expression of Gpx4 and down regulate the expression of ALOX15, suggesting that supplementation of this NPs will effectively suppress the lipid peroxidation through

upregulating Gpx4, thereby reverse neuron ferroptosis caused by stroke. We also detected the increased expression of Neuronal nuclei antigen (NeuN) in brain tissues, which further indicate the effective neuroprotection of Or/In-Se NPs (Fig. 6k). Taken together, all these results suggested that Or/In-Se NPs exhibit the potential to inhibit ferroptosis and promote neuroprotection in ischemic stroke. These results and discussion have been added in the revised manuscript.

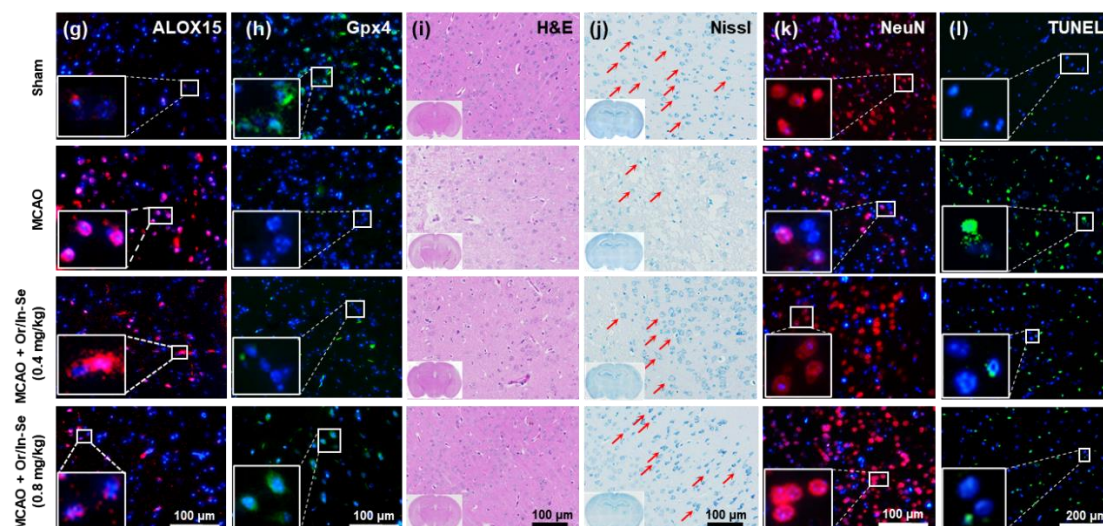


Fig. 6 Immunofluorescence images of (g) ALOX15 and (h) Gpx4 expression in the damaged brain of MCAO mice with different treatment. (i) H&E staining, (j) Nissl staining, (k) NeuN expression and (l) TUNEL staining images of damaged brain tissues in MCAO mice with different treatment.

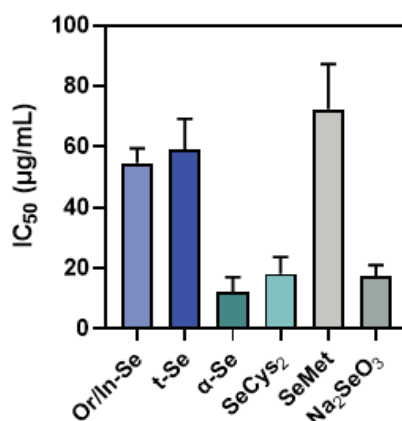
Introduction

Overall, I recommend the introduction to mention the narrow safe therapeutic window of selenium, with both too little and too much exposure having detrimental health effects. Some papers to consider: PMID: 31388899, PMID: 34262926

Our response:

Thank you for the reviewer's rigorous consideration. We have added more introduction and discussion on the safe therapeutic window of Se in clinical application with the citation of the above-mentioned references. Furthermore, we also detected the toxicity of Se, and interestingly found that different Se species exhibited different safety range to neuron. As shown in **Supplementary Fig. S13**, Or/In-Se NPs, t-Se and

selenomethionine (SeMet) exhibited much higher IC₅₀ value than that of other Se species, further indicating the high efficiency and low toxicity of Or/In-Se NPs as Se-based nanomedicine to neuron. These results and discussion have been added into the revised manuscript.



Supplementary Fig. S13. The IC₅₀ value of different Se species to SH-SY5Y cells.

L28-30: Could the authors be more specific regarding the confusion around the clinical use of selenium?

Our response:

We are sorry for the misuse of the word leading to the misunderstanding. Therefore, we have revised the sentence to “*The high toxicity of inorganic Se and the low stability of organic Se have greatly limited their successful clinical application in disease treatment*” to make it clear in the revised manuscript.

L44-49: Even though the explanation given for how organic x inorganic selenium would be driven to different roles is clear, it sounds to me that it lacks rationale. Could the authors provide background information to justify the notion that organic selenium would scavenge free radicals while inorganic selenium would be used for selenoproteins synthesis, given that both forms require to be metabolised into selenide before their metabolisation into selenophosphate for incorporation into SEPSECS?

Our response:

Thanks a lot for the kindly consideration on the role of different Se species. Actually, it is really important to discriminate the contribution of each Se species in the biological action of Or/In-Se NPs nanosystem. Firstly, from the structure characteristics, the organic and inorganic parts of Or/In-Se NPs was linked with a weak Se-Se bond with bond length of 3.1 Å, which endows the nanosystem to decompose to organic and inorganic Se under physiological stimuli. As the bond energy of Se-Se bond (172 kcal mol⁻¹) in inorganic Se is much lower than Se-C (244 kcal mol⁻¹) in organic, the inorganic part of the Or/In-Se NPs tend to be transformed more quickly. For inorganic Se, the electrode potentials of Se(IV)-(0) (H₂SeO₃/Se(s)) is -0.40 V, lower than that of O(0)-(-II) (O₂/H₂O), implying the possibility to be oxidated and further transformation (Angew. Chem. Int. Ed. Engl. 2022, 61(43), e202210313, Adv. Healthc. Mater. 2021, 10(16), e2100598). On the other hand, the Se atom was anchored on a hierarchical porous carbon membrane to be a selenabenzene structure (Angew. Chem. Int. Ed. Engl. 2019, 58(38), 13466-13471, Org. Lett. 2023, 25(36),6720-6724). The single-disperse Se atom in organic part of Or/In-Se NPs has two pair of unbounded electrons, which may bind to unoccupied orbital of atoms from free radicals during scavenging process, thus exhibiting the catalytic activity. Such a process could be regarded as exogenous antioxidant activity. These discussions have been added and addressed in the revised manuscript.

Results

Fig 4 (and the in-text corresponding results) have no P-values – please include P-values for a clearer information on statistical significance.

Our response:

We thank the reviewer's carefulness. We have added statistical analysis and difference to **Fig 4**.

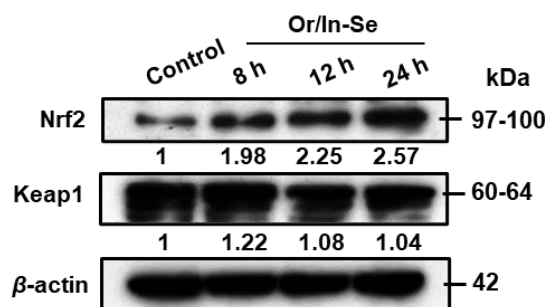
Fig 4g: the mRNA expression of some selenoproteins peak at 12h but decrease (and some return to zero expression level) at 24h (e.g. Gpx1, Gpx4, TrxR2, TrxR3, SelP).

Could the authors explain such effect? Also, could the authors be clear that not all the selenoproteins were assessed (and provide an explanation)?

Our response:

Thanks for the very good suggestion to discuss about the dynamic expression of selenoprotein mRNA. Actually, as we know, the protein synthesis is a rapid process influenced by selenium sources. With the consumption of selenoprotein, Or/In-Se NPs treatment can promote the synthesis of selenoprotein by rapidly increasing the content of hydrogen selenide and selenophosphate inside the cells, thus promoting the synthesis of intracellular mRNA template. When the demand for selenoprotein reached saturation, mRNA expression decreased accordingly (Int.J.Mol.Sci. 2018, 19(9), 2665). For instance, in our study, comparing with 12 hours, the mRNA expressions of Gpx1, Gpx4, TrxR2, TrxR3 and SelP decreased at 24 hours, while the mRNA expressions of Gpx3 and SelH increased at 24 hours. These differences in selenoprotein mRNA expression may be due to hierarchical differences in tissue requirements for selenoproteins (J. Neurochem. 2009, 110, 133-142), leading to different mRNA template requirements and different expression levels.

In addition, the synthesis of selenoprotein mRNA is also regulated by some other factors. For example, cells can regulate the expression of antioxidant enzyme genes through Nrf2/Keap1 under oxidative stimulation conditions (Sci.Adv. 2021, 7, 1-14, Cell.Rep. 2019, 28, 746-758). Our previous studies also found that selenium can bind to Keap1 as an electrophilic reagent, promote Nrf2 dissociation and nuclear transfer, and thus activate this signaling pathway to up-regulate the expression of selenoprotein-related genes (ACS Nano, 2023, 17, 14053-14068). Therefore, we further found that Or/In-Se NPs can significantly up-regulate the expression of Nrf2, and the activation of this signaling pathway may be a key factor for promotion of selenoprotein gene expression by Or/In-Se NPs (**Supplementary Fig. S17**). These results and discussion have been added into the revised manuscript.



Supplementary Fig. S17. Expression level of Nrf2 and Keap1 in SH-SY5Y cells after cultured with Or/In-Se NPs (10 $\mu\text{g/mL}$) for 8, 12 and 24 h.

Fig 4h: Could the authors explain why only a few selenoproteins were measured at the protein level (fewer than those measured at mRNA level)?

Our response:

We thank the reviewer's comments. Actually, the main functions of selenoproteins have been found including selenium transport (SelP), redox balance (Gpx1-4, Gpx6, SelK, SelR, SelW and TrxR1-3) and protein folding (Sep15, SelN, SelM and SelS et al) (Free Radical Biology and Medicine 190, 2022, 105-115). Among them, Gpx4, SelK, SelW and SPS2 are considered to play an important role in the growth and development of brain tissue and the maintenance of function (Biol Trace Elem Res. 2014, 161(3), 231, Free Radic. Bio. Med. 2022, 105). Therefore, we focused on these proteins like Gpx, SelS, SelK and SelW in this study. At the same time, redox-related selenoproteins are closely related to oxidative stress caused by ischemia-reperfusion injury, including TrxR, SelO, SelT, SelP and so on (Antioxid. Redox Signal. 2007, 9(7),775). Therefore, in this study, we have further detected the expression levels of the above selenoproteins by Western blot analysis. As shown in **Fig. 4h** and **Supplementary Fig. S18**, Or/In-Se NPs could effectively increase the expression level of these selenoproteins, which was consistent with the results of qPCR (**Fig. 4g**). However, we also found that, some selenoproteins could not be detected by Western blotting, which is possibly due to the hierarchical differences in tissue requirements for selenoproteins (EMBO J. 2000, 19(24), 6882-6890), thus the expression levels of them in brain tissue is too low to be

detected by Western blotting. These results and discussion have been added into the revised manuscript.

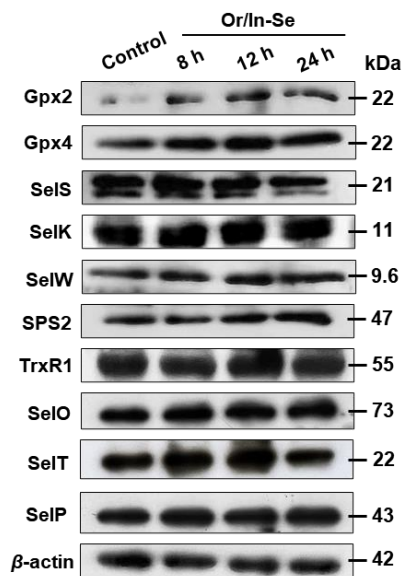
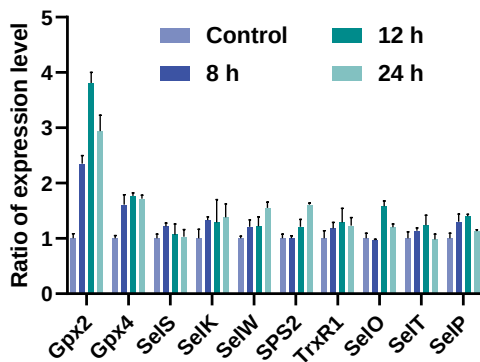


Figure 4. (h) Expression level of main selenoproteins in SH-SY5Y cells cultured with Or/In-Se NPs (10 μ g/mL) for 8, 12 and 24 h.



Supplementary Fig. S18. Quantification for selenoproteins in SH-SY5Y cells after cultured with Or/In-Se NPs (10 μ g/mL) for 8, 12 and 24 h.

Fig 4i: a brief caption should be added to explain the figure.

Our response:

We thank the reviewer's advisement. We have made caption explanation for **Fig 4i**.

Fig 5: as seen for Fig 4, there are no P-values for the statistical analysis, so it's unclear if the results are significant. If they are not, the authors must consider rewriting the results to not oversell them.

Our response:

We gratefully appreciate the reviewer's comments. We have repeated ischemic reperfusion injury model and added statistical analysis and difference to **Fig 5**.

Fig 6a: please add brief explanation for the hypothesis displayed in this figure.

Our response:

Thanks for the reviewer's suggestions. We have added explanation for the scheme illustration of **Fig 6a**.

Discussion

The authors could explore the potential applications, effects and limitations of the use of this compound in clinical studies, shedding light into future studies.

Our response:

We thank the reviewer for focusing on this question. We have added more discussion about the drawbacks, potential applications, effects, limitations and prospect of Or/In-Se NPs for further clinical studies in the revised manuscript.

Comments and Response

Response to Reviewer #1:

The authors' response was satisfactory and there were no other issues.

Our response:

Thanks for the reviewer's positive comment, which encourages us a lot.

Response to Reviewer #2:

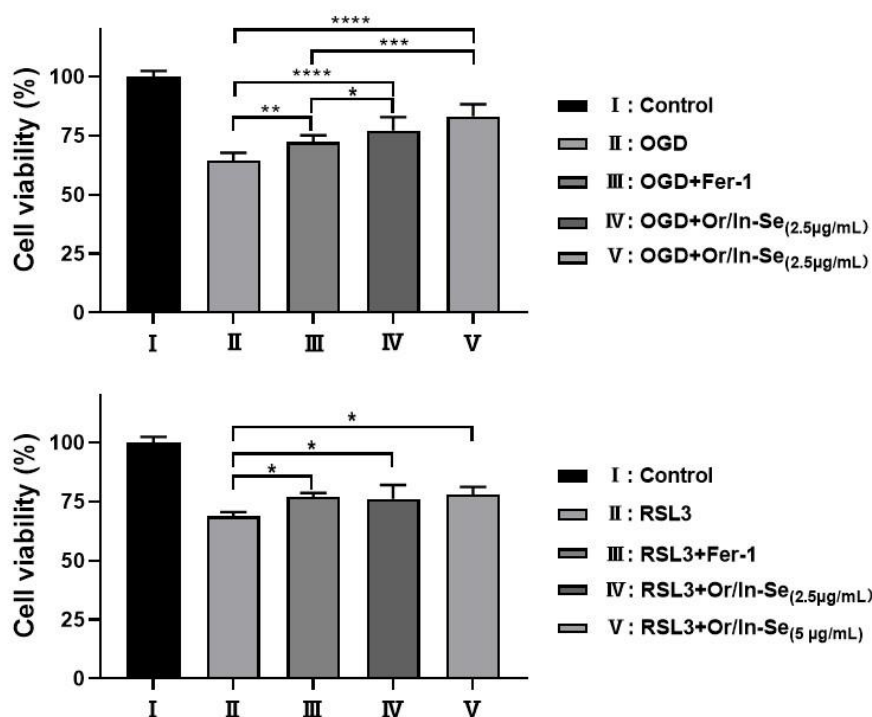
The authors have significant improvements in the manuscript, including the assessment of ferroptosis to examine the effects of the Or/In-Se NPs. However, ferroptosis is a complex cell death pathway that involves different aspects and molecules, and defining the effects of the studied molecule in this pathway requires more than measuring the expression of 2 genes (Gpx4 and Alox15). That said, I recommend the authors include other specific markers of ferroptosis (e.g. TfR1 expression and other ferroptosis reaction products) in order to distinguish ferroptosis from other stress conditions (PMID: 38187349, PMID: 36828775, PMID: 37556320)

Our response:

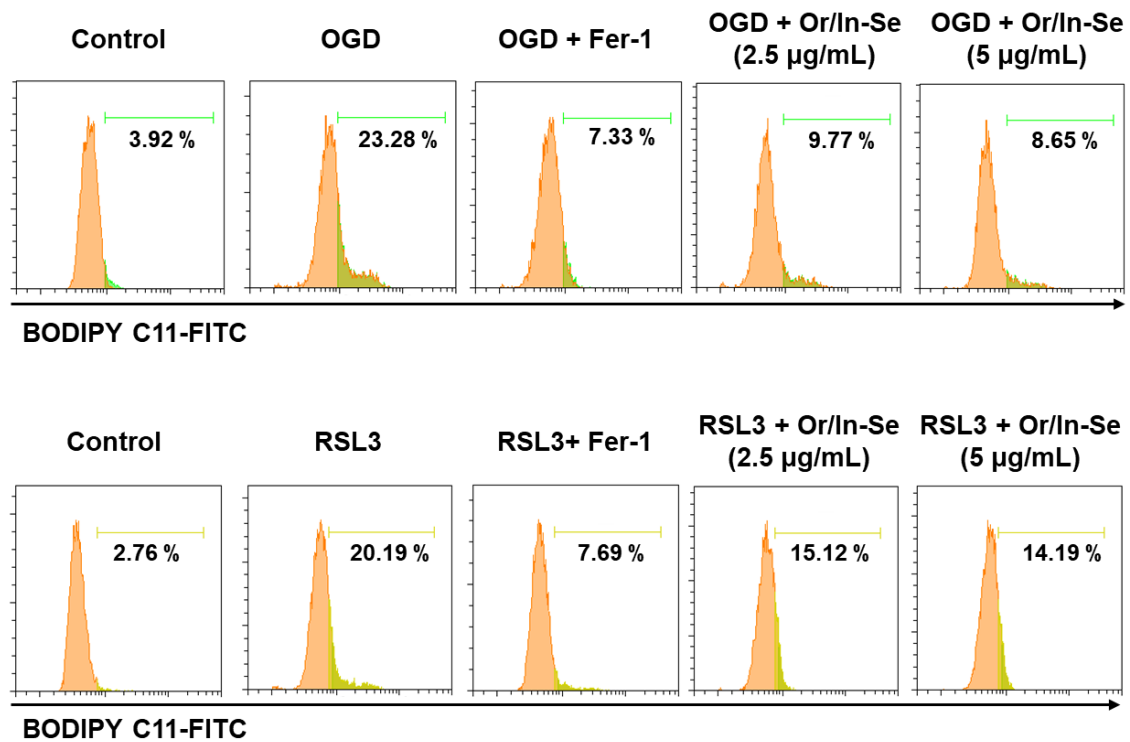
Thanks a lot for the reviewer's kindly suggestion on the action mechanisms of ferroptosis. According to reviewer's comments, in order to further explore the role of ferroptosis in neuroprotection of Or/In-Se NPs, we have also evaluated the cell viability with the treatment of Or/In-Se NPs and ferroptosis inhibitor ferrostatin-1 (Fer-1) in both OGD cell model and ferroptosis cell model induced by ferroptosis activator (RSL3). As shown in **Supplementary Fig. S27**, we observed that Fer-1 could effectively reverse the OGD-induced cell death, implying that ferroptosis plays an important role in OGD-induced neuronal cell damage, which is consistent with the finding reported previously (*Cell* **2019**, 177, 1262–1279, *Chem. Sci.* **2022**, 13(10), 2992–300, *Neural. Regen. Res.* **2024**, 19(3), 611–618, *Brain Res. Bull.* **2023**, 193, 146–157). Moreover, the treatment of Or/In-Se NPs could also significantly inhibit OGD-induced cells death, which is comparable with Fer-1. Furthermore, in RSL3-induced ferroptosis model, Or/In-Se NPs demonstrates similar protection effects with Fer-1, further indicating that Or/In-Se NPs

could obviously inhibit ferroptosis (**Supplementary Fig. S27**).

In addition, we further examined lipid peroxidation level with the probe of BODIPY 581/591 C11 to assess the inhibition effects of Or/In-Se NPs on ferroptosis. As shown in **Supplementary Fig. S28**, both Or/In-Se NPs and Fer-1 can effectively decrease the lipid peroxidation in OGD cells and RSL3-induced ferroptosis. In addition, we further assessed the expression of TfR1 (a specific ferroptosis marker) in the brain tissues of MCAO mice by immunofluorescence. The results reveal that the administration of Or/In-Se NPs significantly decreased the expression level of TfR1 in MCAO-induced damaged brain tissue (**Fig. 6i**). This change is consistent with the findings on the other two specific markers of ferroptosis (ALOX15 and Gpx4) in **Fig. 6g, h**. Taken together, these results confirm that, the neuroprotective effects of Or/In-Se NPs involve inhibition of MCAO-induced ferroptosis. All these results and discussion have been added in the revised manuscript.



Supplementary Fig. S27. Cell viability of SH-SY5Y cells with treatment of Fer-1 (5 μ M) and Or/In Se NPs (2.5 and 5 μ g/mL) under OGD (6 h) model and RSL3 (10 μ M) model. (n.s. $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).



Supplementary Fig. S28. Lipid peroxidation level of cells treated with Fer-1 (5 μM) and Or/In Se NPs (2.5 and 5 $\mu\text{g/mL}$) under OGD model (6 h) and RSL3 (10 μM) model.

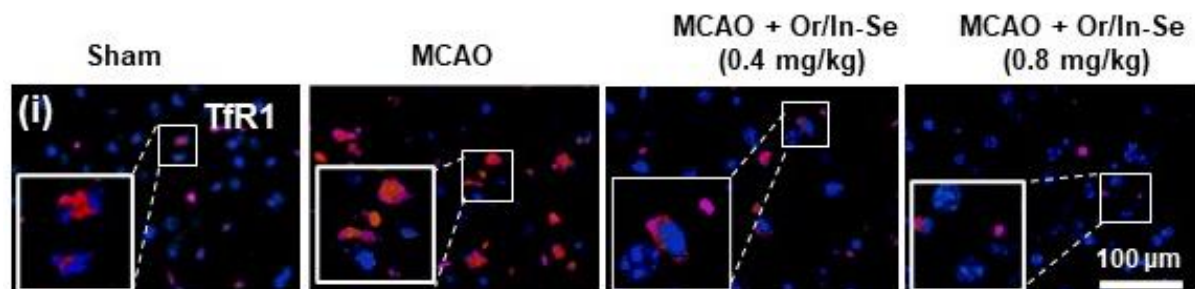


Fig. 6 | (i) TfR1 expression in the damaged brain of MCAO mice with different treatment.

Comments and Response

Response to Reviewer #2:

Thanks for the reviewer' s positive comment, which encourages us a lot.

Comments and Response

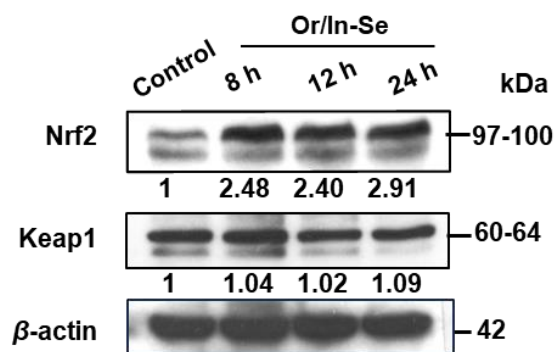
Response to Reviewer #3:

I am writing to review specifically the western blots for NRF2 and Keap1.

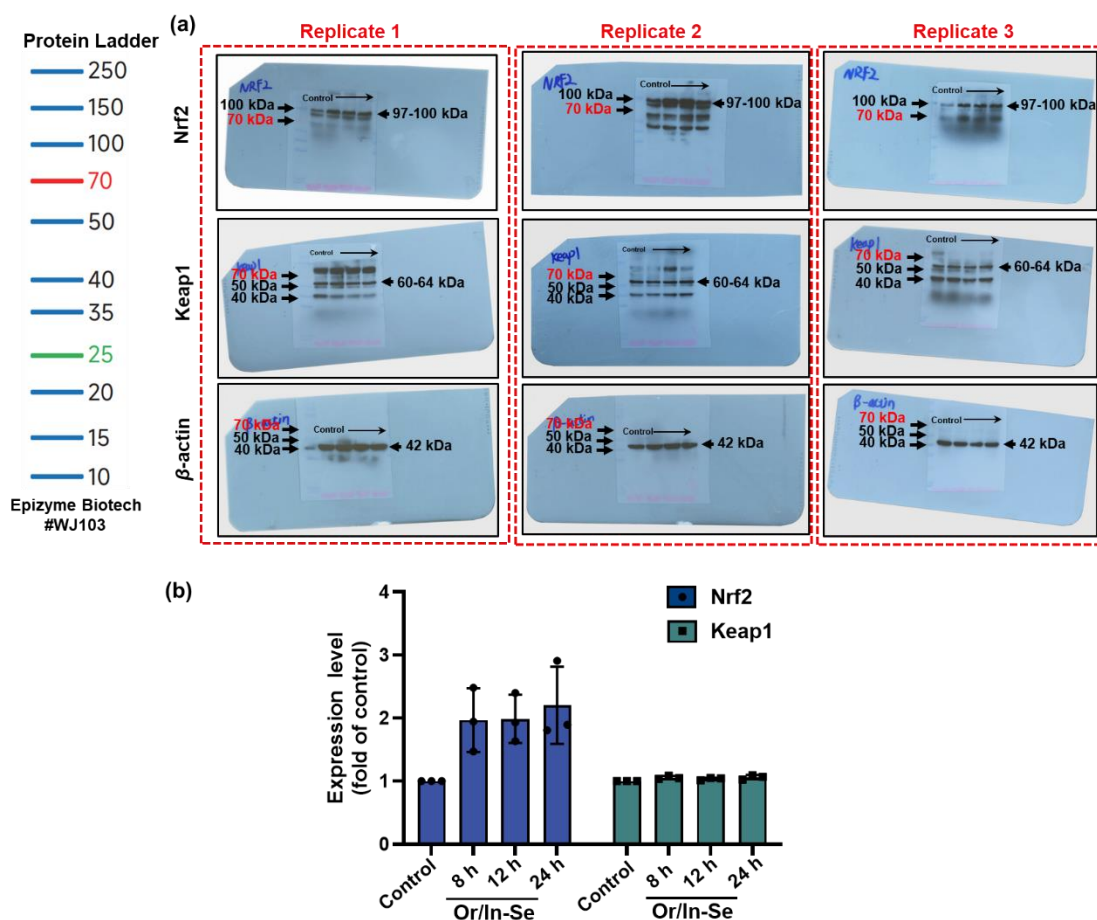
The use of CST antibody 88882S is curious, as it is a discontinued antibody. In examining the Western blots, I am not convinced that we are seeing the same thing in the three replicates. If you look at the 97-100kDa bands, replicate 2 and replicate 3 do not show a convincing upregulation of these bands over the time course. In fact, the bands are quite static at 97-100kDa. Although the upper bands may be post-translationally modified – the lack of such bands in replicate 1 means that there is significant variation in exposure of the film. Altogether the quality of the data is not quite convincing that Or/In-Se increases Nrf2. If the method of quantification shown in Supp Fig 17b is to quantify the NRF2 97-100kDa bands and all additional heavier bands – then this must be explicitly stated in the methods. In order to improve the experiments, I would suggest repeating the experiment with CST 12721 antibody. One could also IP with one NRF2 antibody and blot with another, controlling of course for total protein.

Our response:

Thanks a lot for the reviews' comments and kindly suggestions, which greatly help us to improve the quality of our manuscript. In our previous experiment, we have used Nrf2 antibody (#8882S) to detect the expression level of Nrf2 with antibody from CST. This issue has been explained and declared in our previous revision with version of NCOMMS- 23-43913C at 20240629. And now, according to the suggestion of reviewer, we have repeated and conducted the experiment with the new purchased antibody from CST (#12721), and we found that the results were well consistent and found obvious increase of Nrf2 expression at 97-100kDa, which confirms that Or/In-Se NPs can up-regulate the expression of Nrf2. Therefore, we updated these results in the **Supplementary Figure 33**, and chose the representative results to show in **Supplementary Figure 17**. Meanwhile, the relative expression level of all proteins is quantified correspondingly based on the bands of specific molecular weight, and we have also added the description in the methods section of the manuscript.



Supplementary Figure 17. Expression level of Nrf2 and Keap1 in SH-SY5Y cells after incubation with Or/In-Se NPs (10 μg/mL) for 8, 12 and 24 h.



Supplementary Figure 33. (a) Uncropped Western Blotting images with protein ladders and (b) quantification of Nrf2 and Keap1. The replicate 1 was used as representative results in Supplementary Figure 17.

The Keap1 blots do not show a clear increase in Keap1 over time – rather static, but this is congruent with the quantification results.

Our response:

Thanks for the reviewer's comment. In order to confirm the credibility of the expression of Keap1 provided previously. We have also re-run the Western blot of Keap1 and Nrf2

in the same membrane to further explore the effects of Or/In-Se NPs on Keap1 expression. The repeated bands and quantified relative expression level of Keap1 slightly but not significantly increased, which was consistent with the previously provided results. These results also have been updated as **Supplementary Figure 17** and **Supplementary Figure 33** in the revised manuscript.

Comments and Response to reviewer-3

Response to Reviewer #3:

I am satisfied with the experimental replicates with the new antibody. I have no other concerns regarding the antibodies.

Our response:

Thanks for the reviewer's positive comment, which encourages us a lot.

I have also been asked to review the manuscript as to whether Supplementary Figure 18 adequately supports the relevant claims (Line 380-387) in the manuscript particularly regarding TrxR1.

The authors state in the manuscript that - As shown in Fig. 4h and Supplementary Fig. 18, after incubated with Or/In-Se NPs for 8-24 h, the expression level of two important classes of antioxidant selenoenzymes, including glutathione peroxidase2, 4 (Gpx2, Gpx4), and thioredoxin reductase1 (TrxR1), were significantly improved, and the antioxidant selenoproteins, such as selenoprotein O, P, T, W, (SelO, SelP, SelT and SelW), also upregulated obviously.

In Fig 4h, I am not sure I see a marked difference between control vs treated SH-SY5Y cells cultured with Or/In-Se NPs at any of the time points for TrxR1. In fact, in the supplementary figures, there is no statistical difference for TrxR1. There is also no differences for SelS, SelK, and SelT. This should be noted.

Our response:

According to the reviewer's suggestion, we have revised the discussion and conclusion of the selenoproteins expression in the revised manuscript as following:

As shown in Fig. 4h and Supplementary Fig. 18, after incubation with Or/In-Se NPs for 8-24 h, the expression level of two important antioxidant selenoproteins, glutathione peroxidase 2 and 4 (Gpx2, Gpx4), were significantly increased. Meanwhile, the expression of the antioxidant selenoproteins, such as selenoprotein O, P and W (SelO, SelP and SelW), were also slightly upregulated. Furthermore, selenophosphate synthetase 2 (SPS2), related to selenoprotein synthesis, also increased to a certain extent after being incubated with Or/In-Se NPs. The expression of other selenoproteins, such as thioredoxin reductase1 (TrxR1) and selenoproteins S, K, T (SelS, SelK and SelT), demonstrated no significant difference after incubation with Or/In-Se NPs. These results imply that Gpx2 and Gpx4 may play more important role in reversing neuronal oxidative damage by Or/In-Se NPs.