Reviewer 1

Comments and Suggestions for Authors

The present article "Protective Effects of Velvet Antler Methanol Extracts on Hypoxia-Induced Damage in Caenorhabditis elegans through HIF-1 and ECH-8 Mediated Lipid Accumulation" elucidates how MEs counteract hypoxia-induced damage in C. elegans, particularly through the role of ech-8 in reprogramming lipid metabolism via the hif-1 pathway. It also highlights the importance of integrating traditional herbal medicines with modern scientific research and demonstrates that methanol extracts from velvet antler (MEs) enhance the survival of C. elegans under hypoxic conditions.

Reply: We are very grateful to the reviewer for the insightful and constructive feedback and giving us such a valuable opportunity revise our manuscript. According to the reviewers' comments, we made corresponding modifications to the original manuscript.

1 – Introduction, 2nd paragraph: Velvet Antler is a type of cartilaginous tissue that covers the growing antlers of deer species such as red deer, elk, and moose, put this information in the paragraph.

Reply: We appreciate the reviewer's suggestion. We have revised the introduction to include information that Velvet Antler is a type of cartilaginous tissue that covers the growing antlers of deer species such as red deer, elk, and moose. The detail is as follows:

"*Velvet antler, a revered traditional Chinese medicinal ingredient, comprises a distinctive type of cartilaginous tissue enveloping the developing antlers of several deer species, such as red deer, elk, and moose. This dietary supplement holds esteemed status in Asian cultures due to its purported health benefits and anti-aging properties, attracting considerable scientific interest and serving as a focal point for numerous research efforts.*"

2 – Introduction, 3rd paragraph: In addition to the gene ech-8, please include:

- **Gene Function: Description of the gene's biological functions.**
- **Gene Structure: Information about the gene's structure, including the number of exons, introns, and relevant structural characteristics.**
- **Gene Expression Details: Details on the gene's expression in different developmental stages, physiological, and pathological conditions.**
- **Known Gene Variants: Information on known gene variants, including mutations and polymorphisms.**

Reply: We appreciate the reviewer's suggestion. We have expanded the description of the gene *ech-8* to include its biological functions, structure (number of exons and introns), expression details in various developmental stages and conditions, and information on known gene variants, including mutations and polymorphisms. The detail is as follows:

"*The gene ech-8, an ortholog of human EHHADH (enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase), plays a crucial role in fatty acid metabolism, particularly in the catabolism of fatty acids to meet energy demands during fasting or*

increased metabolic activity (Eur J Hum Genet. 2023, 31, 265-272). It is located on chromosome IV(10236626-10239176) and comprises 8 exons. The expression of this gene varies across different developmental stages and in response to nutritional status. Study has indicated knock down the ech-8 gene can result in metabolic disorders affecting fat content (Plos One. 2009 4, e6279.).”

3 – Materials and Methods, 2.1. Strains: C. elegans were grown on Nematode Growth Medium (NGM) with Escherichia coli bacteria. It is known that culturing media like NGM can be contaminated with Mycoplasma due to their small size and lack of a cell wall, potentially affecting biological experiments. It is crucial to detect and eliminate Mycoplasma to ensure research integrity. How was the quality control conducted to ensure there were no contaminants like Mycoplasma in this medium? Was detection done using commercial kits or PCR targeting constitutive Mycoplasma genes?

Reply: We appreciate the reviewer very much for the comment. We did not detect Mycoplasma by using commercial kits or PCR targeting constitutive Mycoplasma genes. Mycoplasma typically infects vertebrate cells and tissue cultures, particularly mammalian cells. However, it seems there is no evidence to suggest that *C. elegans* can be contaminated with Mycoplasma. In our study, to ovoid contaminate by microorganism, the NGM and reagents required in this article were sterilized in an autoclave. In addition, aseptic operation is ensured during the experimental operation to avoid the production of contaminants.

4 – Materials and Methods, ATP Measurement: The article states, "The resulting supernatant was collected after centrifugation (12,000 rpm, 5 min, 4°C), and ATP concentrations were determined using an enhanced luciferase-based ATP assay kit (Beyotime Biotechnology, S0027, Shanghai, China), following the manufacturer's instructions." Please specify the wavelength used for measurement in a spectrophotometer. Also, provide the wavelength used for protein concentration measurements.

Reply: We appreciate the reviewer's suggestion. We have specified the wavelength used for ATP concentration measurements as well as the wavelength used for protein concentration measurements in the spectrophotometer. The detail is as follows:

“*The resulting supernatant was collected following centrifugation (12,000 r/min, 5 min, 4 °C), and the ATP concentrations were determined at 560 nm using a luciferase-based enhanced ATP assay kit (Beyotime Biotechnology, S0027, Shanghai, China) as per the manufacturer’s instructions. Additionally, the protein concentration of the samples was assessed at 562 nm using a Bicinchoninic Acid (BCA) Assay kit (Tiangen, PA115-01, Beijing, China). The measured ATP levels were then normalized to the protein concentration. This process was repeated independently three times with three replicates at each time point.”*

5 – Materials and Methods, 2.7. RNA Isolation and Quantitative PCR (qPCR):

Please specify how RNA quality control was performed (including the type of spectrophotometer used), and the RNA quality ratio 260/280. Also, describe the method used for RNA concentration measurement.

Reply: We thank the reviewer for the valuable feedback. We have clarified the RNA quality control methods, including the type of spectrophotometer used and the RNA quality ratio 260/280. Additionally, we described the method used for measuring RNA concentration in the updated manuscript. The detail is as follows:

"*To ensure RNA quality, we evaluated RNA quality and integrity by comparing the brightness and clarity of the three strips (28S, 18S, 5S) by agarose gel electrophoresis. In addition, the absorbance of RNA at 260 nm and 280 nm was measured using a microplate to obtain the ratio 260/280. and quantify the concentration of RNA.*"

6 – Results, Table 1: Sequences of qPCR primers: Please provide the amplicon size in base pairs for each primer pair.

Reply: We appreciate the reviewer's suggestion. We have revised it in Table 1 and marked it in the updated manuscript. The detail is as follows:

Table 1. Sequences of qPCR primers

Gene	Forward primer sequence	Reverse primer sequence	Product length
cdc-42	*ctgctggacaggaagattacg*	*ctcggacattctcgaatgaag*	111
y45f10d.4	*gtcgcttcaaatcagttcagc*	*gttcttgtcaagtgatccgaca*	139
hif-1	*ttaacagtcccccgagttgc*	*gcttccgatgactgggttga*	173
ech-8	*ttgaacgatcaggatgccgt*	*ctcatcggaccaccagtagc*	155

7 – Materials and Methods, 2.8. Statistical analysis lines 120-134: It was reported that continuous variables, including parasitemia, age, weight, and height, were compared between the two sites using a t-test for normally distributed data or the Wilcoxon rank sum test for non-normally distributed data. Include the dilution of the antibodies. Specify which test was used to check the normality of the data. Remember that you can only use parametric tests if your data is normally distributed.

Reply: We appreciate the reviewer for the comment. We did not use antibodies in this study. In addition, we confirm the correctness of using the test method in the analysis method based on your suggestions. The detail is as follows:

"*2.10. Statistical analysis*

Statistical analysis was performed using Gr.aphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). Data were presented as the mean ± SEM. Prior to performing statistical tests, normality of the data was confirmed using the Shapiro-Wilk test, provided there were a minimum of 3 independent replicates. For survival assay, locomotion assay, pharyngeal pumping assay, ATP levels assay, qPCR assays, and Oil Red O based lipid staining assay, the student's t-test was utilized to analyze the

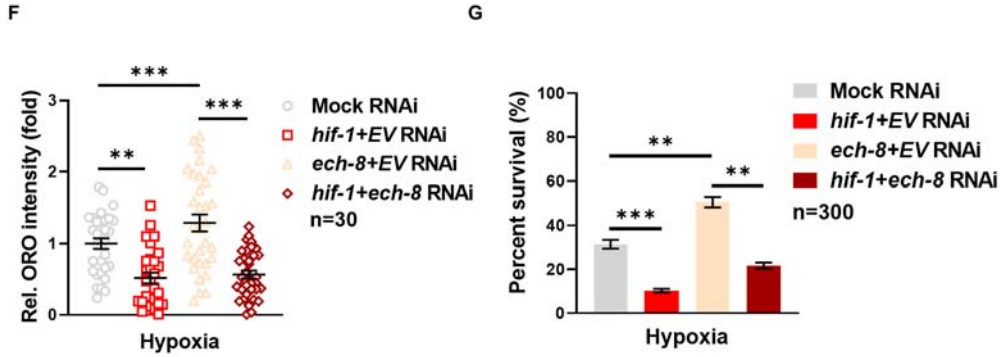
difference between the two groups, and the one-way ANOVA method was applied to analyze the differences among groups. For lifespan assay, comparison was calculated using the Log-rank (Mantel-Cox) test compared with the control group. For mitochondrial morphology, p values were determined by the chi-square test. The above experiment was repeated no less than three times and $p<0.05$ was considered a significant difference."

8 – Materials and Methods, 2.9. RNA Interference (RNAi) Assay: In addition to E. coli HT115 with an empty L4440 plasmid used as a negative control in the RNAi experiment, other types of controls such as dsMock (double-stranded Mock), dsControl (double-stranded Control), and dsGFP (double-stranded Green Fluorescent Protein) should be used as nonspecific controls to verify whether observed effects are due to specific gene silencing or nonspecific RNAi effects.

Reply: We appreciate the reviewer's suggestion and apologize for any confusion caused by our previous description.

In the single-gene RNAi experiment, *E. coli* HT115 with an empty L4440 plasmid was used as negative control in either *hif-1* or *ech-8* RNAi experiments.

For the combined RNAi assay targeting both *hif-1* and *ech-8*, *E. coli* HT115 (DE3) bacteria containing RNAi clones for *hif-1* and *ech-8* were mixed in a 1:1 ratio based on the absorbance of the bacterial cultures. Additionally, for the *hif-1* or *ech-8* RNAi alone groups, *E. coli* HT115 (DE3) bacteria containing RNAi clones for *hif-1* or *ech-8* were combined with *E. coli* HT115 containing an empty L4440 plasmid in a 1:1 ratio based on the absorbance of the bacterial cultures. This combination served as the control for the *hif-1* and *ech-8* combined RNAi group. Furthermore, *E. coli* HT115 with an empty L4440 plasmid alone was used as a negative control. These controls were generated using standard cloning techniques and were administered to *C. elegans* using the same feeding method as the experimental RNAi clones. Observations from these controls help distinguish specific gene silencing effects from nonspecific RNAi responses. We have revised these descriptions and modified the **Figure 4F, 4G** in the updated manuscript. EV indicated the empty vector. The details are as follows:



9 – Materials and Methods, Statistical Analysis: The paragraph incorrectly suggests conducting ANOVA analysis without subjecting the data to normality tests (Shapiro-Wilk Test; Kolmogorov-Smirnov Test). Normality testing can only

be performed with a minimum of 3 to 5 replicates (test rigor). Please revise this paragraph accordingly.

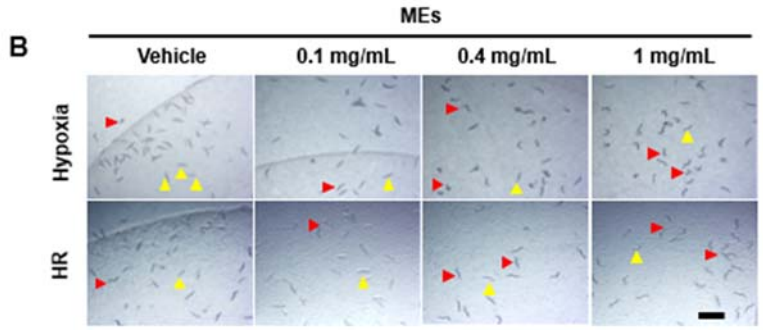
Reply: Thank you for your careful review of our manuscript. We have made corresponding changes in the updated manuscript. The detail is as follows:

"*2.10. Statistical analysis*

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). Data were presented as the mean ± SEM. Prior to performing statistical tests, normality of the data was confirmed using the Shapiro-Wilk test, provided there were a minimum of 3 independent replicates. For survival assay, locomotion assay, pharyngeal pumping assay, ATP levels assay, qPCR assays, and Oil Red O based lipid staining assay, the student's t-test was utilized to analyze the difference between the two groups, and the one-way ANOVA method was applied to analyze the differences among groups. For lifespan assay, comparison was calculated using the Log-rank (Mantel-Cox) test compared with the control group. For mitochondrial morphology, p values were determined by the chi-square test. The above experiment was repeated no less than three times and $p<0.05$ was considered a significant difference."

10 – Results, Figure 1: Include a scale bar on Figure 1B.

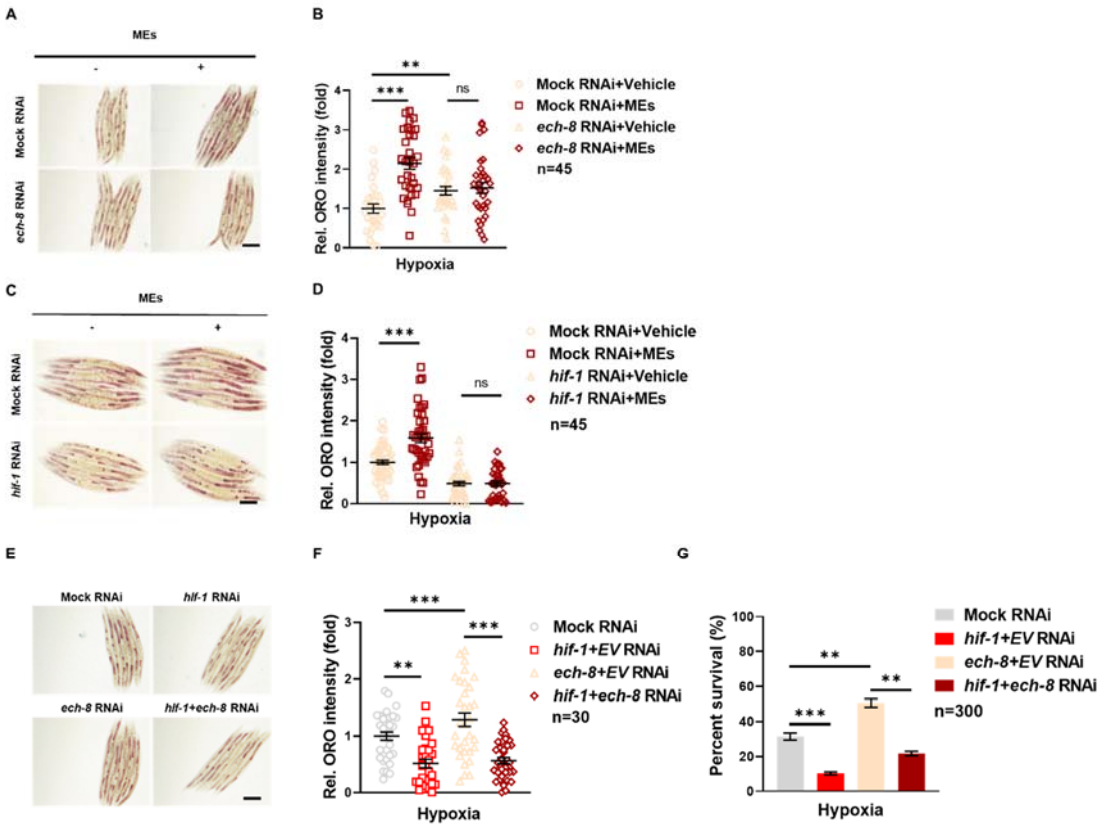
Reply: We appreciate the reviewer's suggestion. A scale bar has been added to Figure 1B for better clarity. The detail is as follows:



scale bar represents 2 mm.

11 – Results, Figure 4: Increase the size of Figure 4 as the figures are currently too small to clearly see the details.

Reply: We appreciate the reviewer's suggestion. The size of Figure 4 has been changed to improve visibility and clarity of the details. The detail is as follows:



12 – Discussion: Enhance the Discussion by relating findings to other studies. Remember that Discussion is not merely a repetition of results. The current discussion lacks...

Reply: We thank the reviewer's constructive feedback. We have enriched the Discussion section by relating our findings to other relevant studies in the updated manuscript. The details are as follows:

(1) "*In prior research, HIF-1 has been identified as a lipid-binding protein whose functionality critically relies on lipid reserves under hypoxic conditions (Cell Rep. 2014, 9, 349-365.). Furthermore, HIF-1 modulates hypoxia-induced lipolysis in C. elegans by inhibiting PKA and ATGL proteins, emphasizing its central role in nematode lipid metabolism during oxygen deprivation (Mol Cell Biol. 2019, 39, e00390-18). In our study, RNAi-mediated suppression of HIF-1 during hypoxia markedly decreased lipid content, accompanied by a corresponding reduction in in vivo ATP metabolism.*"

(2) "*Lipid accumulation appears to be a critical adaptive response to hypoxia facilitated by MEs. This process appears to be regulated through sophisticated modulation of lipid storage, particularly via the action of the enzyme ECH-8. Lipid metabolism significantly influences the body's nutritional balance during processes such as fasting and hypoxia. In C. elegans, lipid storage metabolism critically impacts*

survival (Front Cell Dev Biol. 2023, 27, 1104725; Nat Commun. 2020, 20, 6018.). Disruption of the lipid oxidation pathway was observed to affect lipid droplet formation, which is crucial for lipid storage and membrane fluidity.”