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RESPONSE TO LETTER

Response to "Emodin in Skeletal Muscle Ischemia-Reperfusion: Model Validity and Analytical Concerns" [Response to Letter]

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Dear editor

We thank Gao et al for their interest in our study and for raising important methodological considerations regarding experimental skeletal muscle ischemia-reperfusion (I/R) research. We welcome the opportunity to clarify the scope and interpretation of our findings. We agree that experimental models, biochemical assays, and semiquantitative histopathological assessments should be interpreted within their methodological limitations. However, these limitations should be distinguished from the question of whether the experimental intervention produced measurable I/R injury and whether emodin treatment was associated with differences in the prespecified biochemical and histopathological outcomes reported in our study.[1]

Validity of the Ischemia-Reperfusion Model

Gao et al correctly emphasize the importance of collateral circulation in the rat hindlimb. Rosero et al demonstrated that infrarenal aortic occlusion produces less severe lower-limb ischemic injury than tourniquet-induced ischemia and highlighted the importance of the collateral vascular network in this experimental setting.[2] This is an important physiological characteristic of the rat model and should be considered when interpreting the magnitude of ischemic injury.

However, the findings of Rosero et al do not establish that infrarenal aortic occlusion fails to produce ischemic injury. Rather, they indicate that collateral perfusion may mitigate the severity of ischemia compared with tourniquet-induced ischemia.[2] Therefore, the presence of collateral circulation qualifies the severity and nature of the model but does not, by itself, invalidate its use for studying I/R-associated tissue injury.

In our study, the presence of an experimental injury phenotype was supported by the observed biological response. The untreated I/R group demonstrated significantly greater muscle-fiber disorganization and degeneration, neutrophil infiltration, interstitial edema, and total muscle injury compared with the control groups.[1] The median total muscle injury score was 6.50 (4.50–8.25) in the I/R group compared with 0.00 (0–2.25) in the Sham group. In parallel, TBARS-derived MDA values increased from 203.31 ± 24.80 nmol/g tissue in the Sham group to 587.05 ± 175.54 nmol/g tissue in the I/R group, while CAT activity decreased from 1.12 ± 0.35 to 0.41 ± 0.28 U/mg protein.[1] Collectively, these findings demonstrate that the experimental protocol generated a clear biochemical and morphological I/R injury phenotype under the conditions studied.

https://doi.org/10.2147/DDDT.S659972
Received: 28 September 2026
Accepted: 12 October 2026
Published: 5 November 2026

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We nevertheless agree that direct assessment of tissue perfusion using laser Doppler flowmetry, laser Doppler perfusion imaging, or another validated perfusion method would have provided additional quantitative information regarding the magnitude and inter-animal variability of ischemia. Such measurements were not performed in the present study, and this should be recognized as a limitation.

The suggestion that individual differences in collateral perfusion could contribute to variability in ischemic burden is physiologically plausible. However, because collateral perfusion was not directly measured, its magnitude and variability cannot be retrospectively quantified in our animals. Accordingly, it would also be speculative to attribute the observed treatment-associated differences to differential collateral flow. Animals were randomly allocated to the four experimental groups, and each animal constituted an independent biological replicate.[1] Future studies incorporating direct perfusion monitoring would help address this issue more comprehensively.

Interpretation of TBARS-Derived MDA Measurements

We agree with Gao et al that the conventional thiobarbituric acid reactive substances (TBARS) assay is not chemically specific for MDA. Marrocco et al reviewed important methodological limitations affecting oxidative-stress biomarkers, including methods used to assess lipid oxidative damage, and emphasized the need for careful interpretation of such biomarkers.[3] Thus, methods with greater analytical specificity are preferable when molecularly specific quantification of MDA is required.

An important methodological detail of our protocol is that butylated hydroxytoluene (BHT) was added to the tissue supernatant before the TBA reaction and heating step.[1] Marrocco et al specifically note that additional MDA may be generated during the analytical procedure and that this artifact may be reduced by adding an antioxidant such as BHT and by limiting the heating time.[3] Nevertheless, the use of BHT does not render the TBARS assay specific for MDA and therefore does not eliminate the fundamental analytical limitations of the assay. Accordingly, TBARS-derived measurements should not be interpreted as molecularly specific MDA quantification equivalent to chromatographic or mass-spectrometric determination.

A more analytically precise interpretation of our finding is therefore that emodin treatment was associated with a significant reduction in TBARS-detectable lipid oxidation products compared with untreated I/R animals.

Importantly, the interpretation of a protective effect in our study was not based on this biochemical endpoint alone. Compared with untreated I/R animals, the IR+E group also demonstrated significantly lower scores for muscle-fiber disorganization and degeneration, neutrophil infiltration, interstitial edema, and total muscle injury.[1] Thus, the biochemical finding was accompanied by directionally concordant changes in an independent histopathological outcome domain.

We also emphasize that our biochemical findings were selective rather than uniform. Although CAT activity was significantly reduced by I/R relative to the control groups, the increase observed in the IR+E group compared with the untreated I/R group did not reach statistical significance. Likewise, neither SOD activity nor GSH levels differed significantly among groups.[1] Accordingly, the findings should not be interpreted as evidence that emodin globally restores endogenous antioxidant defenses.

We therefore acknowledge that wording such as "inhibiting lipid peroxidation," when based on a TBARS assay alone, may imply greater analytical specificity than the method permits. The data more conservatively support the conclusion that emodin was associated with reduced TBARS-detectable lipid oxidation products, accompanied by attenuation of histopathological injury.

Histopathological Scoring and Observer Reliability

We agree that formal assessment and reporting of inter-observer and intra-observer reliability can strengthen semiquantitative histopathological studies. Such reliability coefficients were not reported in our study, and we acknowledge this as a methodological limitation.

However, the study by Sanz-Nogués et al cited in the Letter should be considered within the context of its specific objective and experimental design. Their study was specifically designed to develop and validate a new multiparametric semiquantitative histopathological scoring system. Ischemia was surgically induced in mice by femoral artery ligation and excision, and the scoring system assessed inflammation, fibrosis, necrosis, adipocyte infiltration, and muscle-fiber

degeneration/regeneration.[4] The investigators evaluated inter-rater reliability among three appraisers and intra-rater reliability after repeat assessment. Kendall's W values for the individual histopathological features ranged from 0.77 to 0.94, with intra-rater agreement of 0.94 or greater.[4]

Our study addressed a different experimental question and employed an acute I/R model in Wistar rats using infrarenal aortic clamping followed by reperfusion.[1] Histopathological assessment included hemorrhage, neutrophil infiltration, muscle-fiber disorganization/degeneration, and interstitial edema, each scored on a 0–3 ordinal scale, together with a composite total muscle injury score. Therefore, the species, ischemia model, histopathological parameters, and experimental objective differ from those of Sanz-Nogués et al.[4]

The study by Sanz-Nogués et al importantly demonstrates the value and feasibility of formal reproducibility assessment in semiquantitative skeletal-muscle histopathology.[4] However, it does not establish that the absence of a reported reliability coefficient necessarily invalidates findings obtained using a different histopathological scoring approach. Rather, the absence of formal reliability assessment in our study represents a limitation regarding the precision and reproducibility of the semiquantitative assessment and should be considered when interpreting the histopathological results.

In our study, the histopathological findings showed a coherent group-wise pattern. Compared with untreated I/R animals, the IR+E group had significantly lower scores for muscle-fiber disorganization/degeneration ($p = 0.024$), neutrophil infiltration ($p = 0.026$), interstitial edema ($p = 0.002$), and total muscle injury ($p = 0.002$). Conversely, hemorrhage, for which no significant group difference was demonstrated, was reported as nonsignificant ($p = 0.540$).[1] This internally coherent pattern is consistent with the observed treatment-associated histopathological differences, although it does not substitute for formal inter- and intra-observer reliability assessment.

Future studies would benefit from prespecified and fully described scoring criteria, blinded assessment by multiple independent observers, formal inter- and intra-rater reliability analyses, and, where feasible, complementary quantitative morphometric approaches.

Conclusion

We appreciate the methodological considerations raised by Gao et al. Direct perfusion monitoring would have strengthened characterization of the ischemic insult; more analytically specific methods would have provided greater specificity for the assessment of lipid peroxidation products; and formal observer-reliability analysis would have strengthened the histopathological assessment. These are legitimate considerations for the interpretation and design of future studies.

Nevertheless, these methodological considerations appropriately qualify the interpretation of our findings but do not, in themselves, invalidate the observed between-group differences in biochemical and histopathological outcomes. The infrarenal aortic clamping protocol produced measurable skeletal-muscle injury, and emodin treatment was associated with lower TBARS-derived values and attenuation of several histopathological indices compared with untreated I/R animals.[1]

Accordingly, our findings are most appropriately interpreted as evidence of partial protection under the specific conditions of this experimental model, rather than as evidence of complete hindlimb ischemia, molecularly specific inhibition of lipid peroxidation, global restoration of antioxidant defenses, or definitive therapeutic efficacy. This interpretation is consistent with the original data, in which emodin did not completely prevent histopathological injury and did not significantly restore CAT activity, SOD activity, or GSH levels relative to untreated I/R animals.[1]

The study should therefore be viewed as initial in-vivo evidence supporting further investigation of emodin in skeletal muscle I/R, with future studies incorporating direct perfusion measurements, analytically more specific oxidative-stress biomarkers, formal histopathological reliability assessment, and mechanistic analyses.

Disclosure

The authors report no conflicts of interest in this communication.

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