

Exploratory Research on Standardization of Key Parameters in In Vitro and In Vivo Models of Ischemic Stroke

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Abstract:

Standardizing stroke models is crucial for experimental reproducibility. This study optimized key parameters in common models. In HT22 cells, oxidative stress (H₂O₂), ischemia-reperfusion (OGD/R), and chemical hypoxia (CoCl₂) models were established, defining optimal injury conditions. In C57BL/6J mice, the filament-induced tMCAO model was employed to compare effects of ischemia durations (45, 60, 90 min). Results demonstrated that all cellular injuries exhibited concentration/time-dependent patterns. In mice, prolonged ischemia exacerbated neurological deficits and infarct volume. A 60-minute occlusion achieved an optimal balance between stable infarction and survival. This study establishes key parameters for these models, emphasizing the necessity of precise induction conditions to ensure reproducibility. The findings provide a standardized foundation for mechanistic and therapeutic research.

Keywords:

Ischemic Stroke, Model Standardization, Oxygen-Glucose Deprivation, Thread-Trombolysis Method

1. Introduction

Stroke is one of the leading causes of disability and death worldwide, with ischemic stroke accounting for over 80% of cases [1]. In-depth research into its pathophysiological mechanisms and the identification of effective therapeutic targets heavily rely on reliable experimental models capable of simulating clinical pathological processes [2]. Currently, stroke research models primarily include in vitro cellular models and in vivo animal models.

Among in vitro models, hydrogen peroxide (H₂O₂) induction is commonly used to mimic secondary oxidative stress damage following stroke [3]; oxygen-glucose deprivation/reoxygenation (OGD/R) models effectively replicate energy metabolism disorders in the ischemic core and penumbra, along with reperfusion injury [4]; cobalt

chloride (CoCl₂) induction simulates a chemically hypoxic environment by stabilizing hypoxia-inducible factor (HIF-1 α) [5]. However, reported induction concentrations and durations for these models vary significantly across literature, hindering comparative analysis and reproducibility.

Among in vivo models, the thread-occlusion-induced transient middle cerebral artery occlusion (tMCAO) model has become the most widely used animal model for ischemic stroke due to its advantages: no craniotomy required, feasibility of ischemia-reperfusion, and similarity to clinical thrombolysis and thrombectomy scenarios [6]. However, this model exhibits substantial result variability, with one key influencing factor being the duration of ischemia. Insufficient duration may result in unstable or undersized infarcts, while excessive duration dramatically increases mortality and may introduce confounding factors such as extensive hemorrhage [7].

Therefore, standardizing model construction is a critical issue requiring urgent resolution in stroke research. This study aims to explore and optimize key parameters for three cellular injury models-H₂O₂, OGD/R, and CoCl₂-through systematic concentration/time gradient experiments. It further clarifies the impact of varying ischemia durations on model stability and severity in the online-thrombosis tMCAO model. The objective is to provide direct experimental data support for establishing a reproducible, standardized protocol for constructing stroke models.

2. Experimental Section

2.1. Construction of the H₂O₂ model

After 6 hours of cell attachment, the medium was replaced with fresh medium containing different final concentrations of H₂O₂ (0, 25, 50, 100, 200, 400 μ M). Cells were treated for 1, 2, 4, 6, 8, 10 and 12 hours, respectively.

2.2. Construction of the OGD/R model

To induce OGD/R injury, cells were first seeded in culture dishes and cultured under standard conditions for 24 hours to ensure complete confluence. Subsequently, the medium was replaced with glucose-free minimal essential medium (MEM), and cells were transferred to an anaerobic chamber (Mitsubishi, Japan) for a 1, 2, 4, 6, 8, 10, 12-hour OGD) treatment to establish ischemic conditions. Following OGD, reperfusion was achieved by switching to complete MEM and returning cells to a normoxic incubator (5% CO₂, 37°C) for 24 hours [8].

2.3. CoCl₂ Model Establishment

Cells were treated with complete medium containing different concentrations of CoCl₂ (0, 100, 200, 400, 600, 800 μ M) for 1, 2, 4, 6, 8, 10, and 12 hours, followed by 6 hours of reoxygenation. Each group consisted of 3 replicate wells, and the experiment was independently repeated three times.

2.4. Cell Viability Assay

The CCK-8 assay was employed to systematically evaluate cytotoxicity in HT22 cells (a mouse hippocampal neuronal cell line). Specifically, HT22 cells were seeded at a density of 5×10^3 cells/well in a 96-well plate and cultured in complete medium (Dulcamara 1640 medium supplemented with 10% FBS) at 37°C and 5% CO₂ for 24 hours to allow complete attachment. Following attachment, cells were treated with various agents. After treatment, 10 μ L of CCK-8 reagent was added to each well and incubated for 0.5 hours. Measure the absorbance at 450 nm using a microplate reader

and calculate cell viability using the formula: Cell viability (%) = (Asample – Ablank) / (Acontrol – Ablank) × 100. Here, Asample, Acontrol, and Ablank represent the absorbance values of the model group, blank control group, and blank well, respectively [9].

2.5. Mice model establishment

This study employed the filament ligation method to establish a tMCAO model in adult male C57/B6 mice (6-8 weeks old, 20-24 g), with procedures strictly adhering to animal ethics and experimental requirements. Briefly, a midline cervical incision was made to dissect and expose the common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA). The ECA and distal CCA were permanently ligated, and a vascular clamp was placed on the proximal ICA. Following a “V”-shaped incision on the CCA, a silicone-coated suture plug (Beijing Bio-Tech Co., Ltd.) was inserted through this incision. After releasing the clamp, the suture plug was gently advanced into the ICA until resistance was encountered, marking the onset of ischemia. The suture plug was removed after 45 min, 1 h, or 1.5 h of ischemia to achieve reperfusion, which was maintained for 24 hours. The sham group underwent only CCA exposure followed by suture closure without occlusion. The treatment group received immediate pharmacological intervention after reperfusion. All mice were placed on a temperature-controlled pad during surgery to prevent hypothermia [10].

2.6. Infarct volume assessment by TTC staining

After euthanasia, brains were removed, rinsed with PBS, and flash-frozen at -80°C for 3 min. Coronal sections (five 1-mm slices) were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution at 37°C in the dark for 30 min. Infarct volume was quantified using ImageJ software and expressed as a percentage: Infarct volume (%) = (infarcted ipsilateral hemisphere volume/total ipsilateral hemisphere volume) × 100 [11].

2.7. Neurological scores

Neurological function was assessed using the Longa 5-point scale: 0, no deficit; 1, failure to fully extend the contralateral forelimb; 2, circling to the contralateral side; 3, leaning to the contralateral side; 4, no spontaneous walking with depressed consciousness; 5, death [12].

3. Results and Discussion

3.1. Exploration of Standardized Parameters for Cell Models

To establish a stable and reliable in vitro stroke model, this study systematically evaluated the effects of three commonly used injury methods H₂O₂-induced oxidative stress, oxygen-glucose OGD/R-simulated ischemia-reperfusion injury, and CoCl₂-induced chemical hypoxia-on HT22 cell viability. The aim was to determine the optimal injury parameters for each model (Figure 1a).

3.1.1. H₂O₂-Induced Oxidative Damage

H₂O₂ treatment causes concentration- and time-dependent decreases in HT22 cell viability. CCK-8 assay results indicate that treatment with 200 μM H₂O₂ for 4 hours or 100 μM for 10 hours reduces cell viability to approximately 50% of the control level, representing the half-maximal inhibitory concentration (IC₅₀). Concentrations below 50 μM or short exposure times (e.g., 6 hours) did not cause significant cellular

damage, while concentrations exceeding 400 μM resulted in massive rapid necrosis with poor model reproducibility (Figure 1b). Therefore, treatment with 100-200 μM H_2O_2 for 4-10 hours represents the optimal parameter range for inducing oxidative damage in HT22 cells.

3.1.2. OGD/R-Induced Ischemia-Reperfusion Injury

The OGD/R model successfully mimics energy metabolism disorders and secondary damage during ischemia-reperfusion. Experiments revealed that after 6 hours of OGD followed by 12 hours of reoxygenation, HT22 cell viability significantly decreased to approximately 60% of the control group, accompanied by increased LDH release, indicating clear ischemia-reperfusion injury (Figure 1c and 1d). When OGD duration was extended to 8-12 hours, although cell viability further decreased to below 40%, cell mortality rates rose sharply, model variability increased, and stability deteriorated. In contrast, the injury induced by 4 hours of OGD was relatively mild, making it less suitable for observing pronounced intervention effects. Comprehensive comparison indicates that 6 hours of OGD followed by 12 hours of reperfusion produces a stable, moderate, and reproducible ischemia-reperfusion injury phenotype in HT22 cells, establishing an optimal time window for subsequent mechanistic studies.

3.1.3. CoCl_2 -Induced Chemically Induced Hypoxia Damage

CoCl_2 induces cellular damage by mimicking hypoxic conditions. Within the concentration range of 200-600 μM , CoCl_2 exhibits a pronounced concentration-dependent inhibition of HT22 cell viability. Specifically, 12-hour treatment with 400 μM CoCl_2 induces significant cellular damage, reducing cell viability to approximately 55% of the control group (Figure 1e). At this concentration, the degree of cellular damage is moderate, making it suitable for studying hypoxia-related signaling pathways. When concentrations are increased to 600 μM or higher, the cytotoxicity becomes excessive, leading to extensive non-specific cell death and rendering it unsuitable for simulating pathological chronic hypoxia conditions.

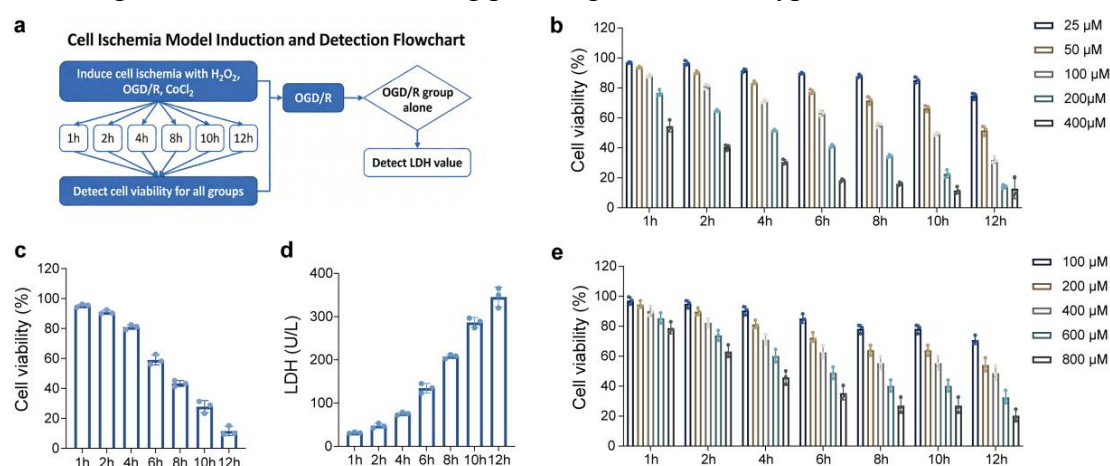


Figure 1. Cell viability of different cell models under different treatments. (a) Experimental flowchart. (b) Changes in cell viability after exposure to different concentrations of H_2O_2 for different durations. (c) Changes in cell viability during OGD at different times. (d) Changes in LDH after OGD at different times. (e) Changes in cell viability after intervention with different concentrations of CoCl_2 for different durations.

3.2. Optimization of Ischemic Duration in the tMCAO Model

To determine the critical parameters ensuring stability and reproducibility in the thread-occlusion tMCAO model, this study systematically evaluated the impact of varying ischemic durations (30, 45, 60, 90 minutes) on model outcomes. Comprehensive assessments were conducted across multiple dimensions, including neurological deficit, structural damage, and survival rates.

Brain infarction volume was quantified via TTC staining. In the sham-operated group, all brain tissue stained bright red with no infarct lesions observed. All tMCAO groups exhibited typical pale infarct areas, with infarct volume significantly increasing with prolonged ischemia duration ($P < 0.001$) (Figure 2a). Ischemia lasting 45 minutes produced relatively small and variable infarct volumes, accounting for approximately $15\% \pm 5\%$ of the ipsilateral cerebral hemisphere (Figure 2b). Ischemia for 60 minutes increased infarct volume to $30\% \pm 3\%$, with more distinct and consistent infarct foci. Ischemia for 90 minutes resulted in stable and significant infarcts, reaching $40\% \pm 3\%$ of the hemisphere with well-defined borders. However, approximately 30% of samples in this group showed evident intracranial hemorrhage on TTC staining, suggesting that excessive ischemia may have triggered hemorrhagic transformation, complicating the assessment of pure ischemic infarction.

Concurrently, neurological deficit assessment revealed varying degrees of neurological impairment (Longa score > 0) in all tMCAO groups (Figure 2c). Neurological scores significantly increased with prolonged ischemia duration: the 45-minute group exhibited mild to moderate deficits, the 60-minute group showed significantly worsened impairment, while the 90-minute group exhibited severe neurological damage, with considerable inter-individual variability. Postoperative 24-hour survival rates demonstrated clear ischemia-time dependence: 90% in the 45-minute group, 80% in the 60-minute group, and less than 60% in the 90-minute group (Figure 2d). Based on these findings, a 60-minute ischemia duration represents the optimal balance between inducing stable, significant cerebral infarction (approximately 35% volume) and maintaining an acceptable mortality rate (80%). This duration is recommended as the standard parameter for establishing a moderate-to-severe tMCAO model.

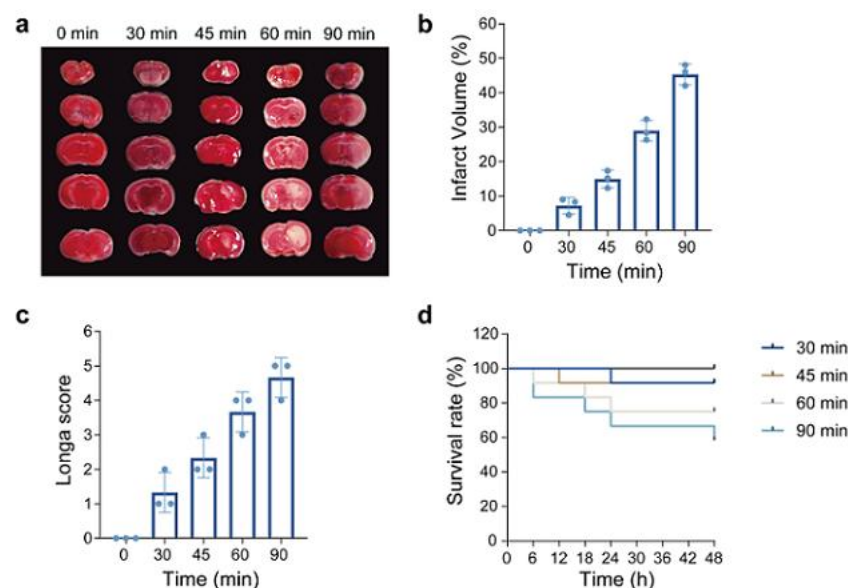


Figure 2. Exploration of the time in the tMCAO model. (a) Images of brain tissue after different treatments with TTC. (b) Quantification of the infarct area ($n = 3$). (c) Neurological scores of mice after different treatment times ($n = 3$). (d) Survival rate of mice ($n = 12$).

4. Discussion and Conclusion

This study systematically investigated the establishment parameters of commonly used stroke models, providing a reference for model standardization. At the cellular level, optimal conditions were identified for three models: the H₂O₂ model (100-200 μ M, 4-10 h) induces apoptotic injury; the OGD/R model is best established with 4 h OGD/12 h reperfusion, which activates death pathways while preserving observable reperfusion processes; while the CoCl₂ model (400 μ M, 12 h) effectively mimics hypoxia signaling. At the animal level, ischemic duration is pivotal for determining the tMCAO model phenotype. Results indicate that 45-minute ischemia causes mild and unstable damage; 60 minutes produces stable infarction suitable for drug efficacy evaluation; 90 minutes yields large infarct volume but carries high mortality and hemorrhage risks. Thus, 60-minute ischemia achieves optimal balance among injury severity, model stability, and animal welfare. Limitations include: use of a single cell line requiring future validation in primary cells; observations confined to the acute phase; and operator variability as a significant source of variation necessitating standardized training.

This study systematically screened and proposed standardized protocols for constructing stroke models: at the cellular level, it is recommended to use 100-200 μ M H₂O₂ treatment for 4-10 hours, 4 hours of OGD combined with 24 hours of reoxygenation, or 400 μ M CoCl₂ treatment for 12 hours; at the animal level, it is recommended to induce 60 minutes of ischemia in C57BL/6J mice to establish a stable tMCAO model. Standardizing these critical parameters reduces experimental variability, enhances comparability and reproducibility across studies, thereby establishing a reliable methodological foundation for stroke mechanism and therapeutic research.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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References

- [1] Wu, Y.; Wang, X.; Zhou, X.; Cheng, B.; Li, G.; Bai, B. Temporal Expression of Apelin/Apelin Receptor in Ischemic Stroke and its Therapeutic Potential. *Front Mol Neurosci*, 2017, 10, 1.
- [2] Chen, X.; Li, C.; Li, J.; Sheng, L.; Liu, X. Upregulation of miR-1306-5p decreases cerebral ischemia/reperfusion injury in vitro by targeting BIK. *Biosci Biotechnol Biochem*, 2019, 83, 2230-2237.

- [3] S. W. Seto, D. Chang, W. M. Ko, X. Zhou, H. Kiat, A. Bensoussan, S. M. Lee, M. P. Hoi, G. Z. Steiner and J. Liu. Sailuotong Prevents Hydrogen Peroxide (H₂O₂)-Induced Injury in EA.hy926 Cells. *Int J Mol Sci*, 2017, 18.
- [4] Li, L.; Wang, M.; Ma, Y.M.; Yang, L.; Zhang, D.H.; Guo, F.Y.; Jing, L.; Zhang, J.Z. Selenium inhibits ferroptosis in hyperglycemic cerebral ischemia/reperfusion injury by stimulating the Hippo pathway. *PLoS One*, 2023, 18, e0291192.
- [5] B. Kaur, B. Miglioranza Scavuzzi, F. A. S and N. Z. D. A simplified protocol to induce hypoxia in a standard incubator: A focus on retinal cells. *Exp Eye Res*, 2023, 236, 109653.
- [6] Geng, J.; Zhang, Y.; Li, S.; Li, S.; Wang, J.; Wang, H.; Aa, J.; Wang, G. Metabolomic Profiling Reveals That Reprogramming of Cerebral Glucose Metabolism Is Involved in Ischemic Preconditioning-Induced Neuroprotection in a Rodent Model of Ischemic Stroke. *J Proteome Res*, 2019, 18, 57-68.
- [7] Jin, R.; Wang, M.; Zhong, W.; C. R. Kissinger, J. E. Villafranca and G. Li. J147 Reduces tPA-Induced Brain Hemorrhage in Acute Experimental Stroke in Rats. *Front Neurol*, 2022, 13, 821082.
- [8] Z. Zhou, L. Shang, Q. Zhang, X. Hu, J. F. Huang and K. Xiong. DTX3L induced NLRP3 ubiquitination inhibit R28 cell pyroptosis in OGD/R injury. *Biochim Biophys Acta Mol Cell Res*, 2023, 1870, 119433.
- [9] Hu, J.; Zheng, Z.; Li, X.; Li, B.; Lai, X.; Li, N.; Lei, S. Metformin Attenuates Hypoxia-induced Endothelial Cell Injury by Activating the AMP-Activated Protein Kinase Pathway. *J Cardiovasc Pharmacol*, 2021, 77, 862-874.
- [10] Deng, M.; Zhong, X.; Gao, Z.; Jiang, W.; Peng, L.; Cao, Y.; Zhou, Z.; Huang, L. Dynamic changes in Beclin-1, LC3B and p62 at various time points in mice with temporary middle cerebral artery occlusion and reperfusion (tMCAO). *Brain Res Bull*, 2021, 173, 124-131.
- [11] J. Tanaka, K. Kiyoshi, T. Kadokura, K. I. Suzuki.; S. Nakayama. Elucidation of the enzyme involved in 2,3,5-triphenyl tetrazolium chloride (TTC) staining activity and the relationship between TTC staining activity and fermentation profiles in *Saccharomyces cerevisiae*. *J Biosci Bioeng*, 2021, 131, 396-404.
- [12] G. P. Morris, A. L. Wright, R. P. Tan, A. Gladbach, L. M. Ittner.; B. Vissel. A Comparative Study of Variables Influencing Ischemic Injury in the Longa and Koizumi Methods of Intraluminal Filament Middle Cerebral Artery Occlusion in Mice. *PLoS One*, 2016, 11, e0148503.



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