

## Article

# Effect of a Localized Oxygen-Releasing Hydrogel Sheet on Early-Stage Infarct Evolution in a Rat Photothrombotic Stroke Model: A Preliminary Study

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## Abstract

Ischemic stroke triggers hypoxia, inflammation, and oxidative stress. Local oxygen delivery may prevent secondary injuries. Herein, we implanted a catalase-incorporated thiolated gelatin-based oxygen-releasing hydrogel sheet in a rat model of photothrombosis to evaluate early infarct attenuation and feasibility. Male Sprague–Dawley rats were allocated to four groups (n = 6/group): control at 24 h (G1), with hydrogel sheet at 24 h (G2), control at 72 h (G3), and with hydrogel sheet at 72 h (G4). Focal ischemia was induced with Rose Bengal and targeted illumination through a 6.0-mm cranial defect. A hydrogel sheet was applied to the cortex after surgery. The infarct burden was assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining and magnetic resonance imaging (MRI), while mRNA expression levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), brain-derived neurotrophic factor (BDNF), and superoxide dismutase (SOD) were measured by quantitative reverse transcription PCR. Body weight was monitored as a safety measure. At 24 h, TTC showed a significant infarct reduction in G2 compared with G1. At 72 h, infarct measures did not differ significantly between G4 and G3. MRI and gene expression analyses did not show statistically significant between-group differences and are presented as exploratory outcomes. Weight and perioperative status were similar across groups, indicating short-term tolerability. The hydrogel sheet was associated with reduced TTC-defined infarct burden at 24 h in this model; confirmatory studies will require larger, powered cohorts, longer follow-up with functional testing, and in vivo oxygen release profiling to optimize dose, placement, and exposure time.

**Keywords:** ischemia; infarct burden; rat model; photothrombosis; oxygen delivery; magnetic resonance imaging; tumor necrosis factor; brain-derived neurotrophic factor; superoxide dismutase



Academic Editor: Olga Kammona

Received: 18 January 2026

Revised: 1 February 2026

Accepted: 6 February 2026

Published: 12 February 2026

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## 1. Introduction

Ischemic stroke initiates a cascade of hypoxia, inflammation, oxidative stress, and cell death. These processes evolve within hours and extend tissue injury beyond the initial core [1,2]. Therefore, therapeutic strategies that restore or supplement oxygen at the lesion site may help limit the secondary damage.

Systemic hyperbaric oxygen therapy can increase tissue oxygenation and enhance vascular responses [3]. However, the treatment is intermittent, dosage is difficult to individualize, and exposure is whole-body rather than targeted [4]. A local and sustained oxygen delivery strategy may help address these limitations.

Implantable oxygen-releasing biomaterials have emerged as an approach for the localized delivery of supplemental oxygen [5,6]. Among these, a sheet-form hydrogel can be placed directly over the cortical surface without external devices and can be handled in a manner analogous to dural substitutes during neurosurgical procedures [5,6]. This configuration is conceptually relevant to settings such as decompressive craniectomy and duraplasty, where a material is routinely applied at the cortical surface.

The preclinical evaluation of these materials requires a focal and reproducible ischemic lesion with precise access to the cortical surface. The photothrombotic stroke model fulfills these conditions. It produces well-defined cortical infarcts by standardized illumination through a cranial defect and allows matched assessments using magnetic resonance imaging (MRI), histology, and molecular assays. In previous studies, thiolated gelatin (GtnSH)-based oxygen-releasing hydrogel sheets showed sustained oxygen release for up to 12 d *in vitro* while maintaining acceptable cytocompatibility [5,6]. This evidence provides a rationale for testing the same platform in an *in vivo* stroke model.

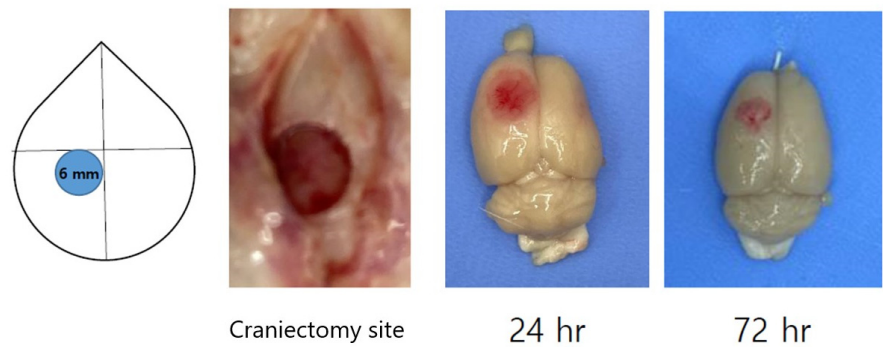
While prior work has established oxygen-generating hydrogel platforms and their characterization *in vitro* and *in vivo* [5,6], the present study evaluates the feasibility of applying an implantable oxygen-releasing hydrogel sheet directly to the cortical surface in an acute photothrombotic stroke setting, focusing on early time points (24 h and 72 h). This design emphasizes early infarct attenuation and tolerability under conditions relevant to acute injury evolution.

Therefore, we investigated implantable oxygen-releasing hydrogel sheets in a rat model of photothrombotic stroke. We focused on early time points (24 and 72 h) to capture the acute injury phase. The primary goal was to quantify the infarct burden via 2,3,5-triphenyltetrazolium chloride (TTC) staining at 24 h and 72 h. MRI was performed for qualitative confirmation of lesion induction and anatomical location. The secondary goal was to determine the RNA expressions of the tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), brain-derived neurotrophic factor (BDNF), and superoxide dismutase (Sod2). We hypothesized that localized oxygen delivery would reduce the early infarct burden. TNF- $\alpha$ , BDNF, and Sod2 mRNA levels were assessed as exploratory secondary outcomes.

## 2. Results

### 2.1. Cohort and Procedures

Twenty-four rats completed the protocol and were included in the analysis (four groups,  $n = 6$  per group). The groups were pre-assigned for evaluation at 24 h (G1 and G2, without and with hydrogel sheet, respectively) or 72 h (G3 and G4, without and with hydrogel sheet, respectively). All planned measurements—MRI ( $n = 6$ /group), TTC ( $n = 3$ /group), and quantitative reverse transcription PCR (qRT-PCR;  $n = 3$ /group)—were performed according to the study design. Macroscopic inspection confirmed a reproducible superficial cortical infarct beneath the 6-mm craniectomy window at both 24 and 72 h (Figure 1). No intraoperative death or major surgical complications were observed.



**Figure 1.** Photothrombotic cortical infarction: craniectomy window, intraoperative view, and macroscopic verification at 24 and 72 h. **(Left):** schematic of the 6-mm craniectomy used for targeted illumination and sheet placement. Intraoperative photograph showing a circular bone defect with the exposed cortex before treatment. **(Right):** whole-brain dorsal views at 24 and 72 h after photothrombosis. Red discoloration marks the superficial cortical infarct beneath the window, demonstrating a stable focal lesion over time.

2.2. Tolerability and Body Weight

The animals maintained good perioperative status. Body weight changed only modestly from baseline to 24 and 72 h, and no significant differences were observed between the groups at any time point (Table 1). These findings support the short-term tolerability of both the oxygen-releasing hydrogel sheet and control dura.

**Table 1.** Body weight changes (in g) after photothrombosis in each experimental group <sup>1</sup>.

| Group | No.         | Times After Operation |                |                |
|-------|-------------|-----------------------|----------------|----------------|
|       |             | 0                     | 24             | 72             |
| G1    | 1           | 281.86                | 270.04         |                |
|       | 2           | 280.79                | 281.07         |                |
|       | 3           | 273.65                | 272.09         |                |
|       | 4           | 277.27                | 264.04         |                |
|       | 5           | 275.86                | 277.05         |                |
|       | 6           | 261.56                | 266.09         |                |
|       | Mean<br>SEM | 275.17<br>2.99        | 271.73<br>2.64 |                |
| G2    | 1           | 288.23                | 283.94         |                |
|       | 2           | 271.4                 | 269.49         |                |
|       | 3           | 271.36                | 273.12         |                |
|       | 4           | 279.51                | 273.11         |                |
|       | 5           | 275.59                | 279.22         |                |
|       | 6           | 265.06                | 267.59         |                |
|       | Mean<br>SEM | 275.19<br>3.27        | 274.41<br>2.5  |                |
| G3    | 1           | 276.63                | 263.03         | 280.92         |
|       | 2           | 275.04                | 265.22         | 280.49         |
|       | 3           | 268.54                | 264.02         | 271.42         |
|       | 4           | 288.5                 | 287.87         | 304.25         |
|       | 5           | 277.31                | 271.75         | 282.58         |
|       | 6           | 269.89                | 268.82         | 281.83         |
|       | Mean<br>SEM | 275.99<br>2.9         | 270.12<br>3.79 | 283.58<br>4.46 |

**Table 1.** *Cont.*

| Group | No.  | Times After Operation |        |        |
|-------|------|-----------------------|--------|--------|
|       |      | 0                     | 24     | 72     |
| G4    | 1    | 290.08                | 296.4  | 296.86 |
|       | 2    | 272.52                | 273.77 | 284.54 |
|       | 3    | 269.03                | 282.57 | 285.04 |
|       | 4    | 264.5                 | 270    | 277.78 |
|       | 5    | 278.06                | 273.28 | 272.82 |
|       | 6    | 276                   | 274.28 | 277.98 |
|       | Mean | 275.03                | 278.38 | 282.5  |
|       | SEM  | 3.6                   | 3.99   | 3.43   |

<sup>1</sup> Body weight (g) of individual rats in G1 (control, 24 h), G2 (with hydrogel sheet, 24 h), G3 (control, 72 h), and G4 (with hydrogel sheet, 72 h) at baseline (0 h) and at 24 h and 72 h after surgery. Means and standard errors of the means (SEM) are shown for each time point. There were no significant differences in body weights between the groups at any time point, indicating good short-term tolerability of the procedures and materials.

### 2.3. Neurological Assessment

Neurological deficits, assessed using the modified Neurological Severity Score, were mild in all groups. The mean scores did not differ significantly among the groups, and no consistent behavioral abnormalities were noted during the observation window (Table 2). Thus, the model produced reproducible focal injuries without severe systemic compromise.

**Table 2.** Neurological scores after photothrombosis in each experimental group <sup>2</sup>.

| Group | No.  | Score |
|-------|------|-------|
| G1    | 1    | 1.00  |
|       | 2    | 2.00  |
|       | 3    | 2.00  |
|       | 4    | 2.00  |
|       | 5    | 1.00  |
|       | 6    | 2.00  |
|       | Mean | 1.67  |
|       | SEM  | 0.21  |
| G2    | 1    | 1.00  |
|       | 2    | 2.00  |
|       | 3    | 2.00  |
|       | 4    | 2.00  |
|       | 5    | 2.00  |
|       | 6    | 0.00  |
|       | Mean | 1.50  |
|       | SEM  | 0.34  |
| G3    | 1    | 0.00  |
|       | 2    | 2.00  |
|       | 3    | 2.00  |
|       | 4    | 2.00  |
|       | 5    | 2.00  |
|       | 6    | 1.00  |
|       | Mean | 1.50  |
|       | SEM  | 0.34  |

Table 2. Cont.

| Group | No.  | Score |
|-------|------|-------|
| G4    | 1    | 0.00  |
|       | 2    | 0.00  |
|       | 3    | 0.00  |
|       | 4    | 2.00  |
|       | 5    | 2.00  |
|       | 6    | 2.00  |
|       | Mean | 1.00  |
|       | SEM  | 0.45  |

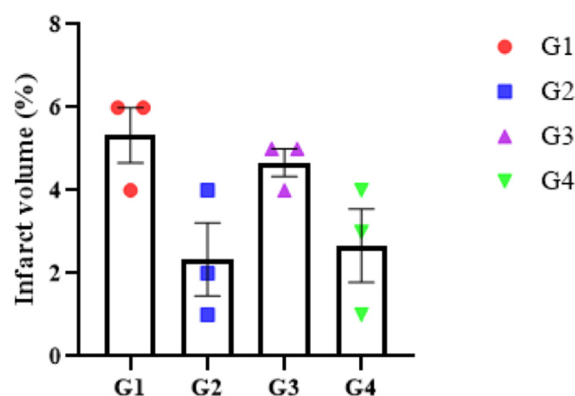
<sup>2</sup> Individual behavioral scores were assessed using the modified Neurological Severity Score (mNSS; higher values indicate more severe deficits) for G1 (control, 24 h), G2 (with hydrogel sheet, 24 h), G3 (control, 72 h), and G4 (with hydrogel sheet, 72 h). Means and standard errors of the means (SEM) are shown for each group. There were no significant differences in the mNSS between the groups, indicating that short-term neurological deficits were mild and comparable across treatment conditions.

#### 2.4. Infarct Burden by TTC

TTC staining showed an association between the oxygen-releasing hydrogel sheet and infarct burden (Figure 2). At 24 h, the infarct area was visibly smaller in the hydrogel sheet-treated group (G2) than in the corresponding control group (G1), with a significant reduction in the quantified infarct volume (Mann–Whitney U test,  $p < 0.05$ ; Figure 3). At 72 h, the infarct volume did not differ significantly between the hydrogel sheet group (G4) and the corresponding control group (G3) (Mann–Whitney U test; Figure 3).



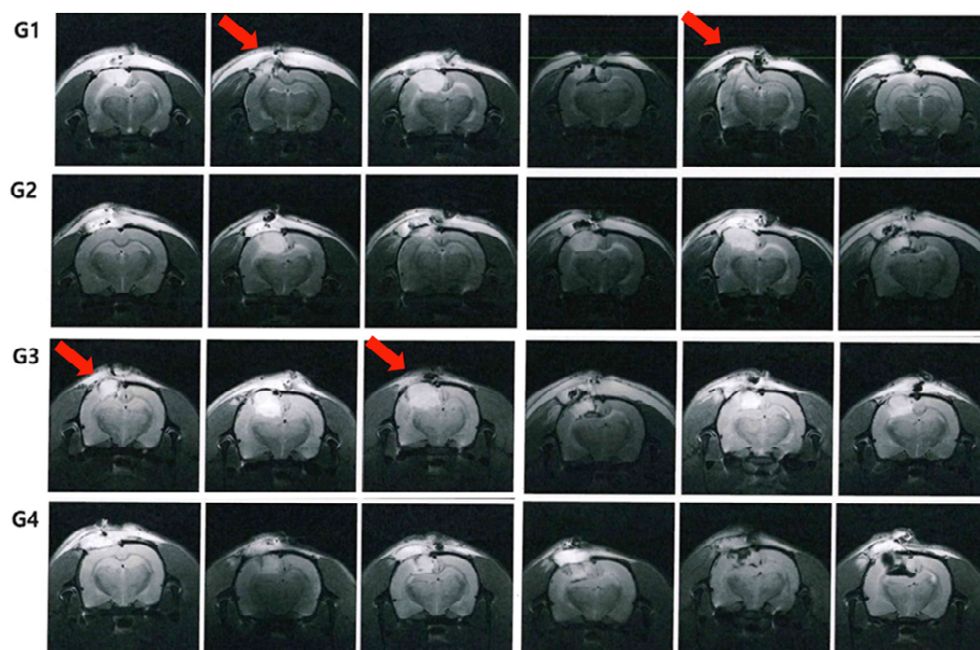
**Figure 2.** TTC staining of coronal brain sections showing the effect of an oxygen-releasing sheet on infarct burden at 24 and 72 h after photothrombosis. Representative 2-mm coronal sections stained with 1% TTC from four groups: G1 (control, 24 h), G2 (with hydrogel sheet, 24 h), G3 (control, 72 h), and G4 (with hydrogel sheet, 72 h). The viable tissue appeared red, whereas the infarcted tissue remained pale or unstained. Compared to the corresponding controls, the hydrogel sheet-treated group at 24 h (G2) showed a smaller pale area, indicating a significant reduction in infarct size, whereas at 72 h (G4), a decrease in infarct size was observed relative to G3.



**Figure 3.** Quantification of infarct volume by TTC staining at 24 h and 72 h after photothrombosis. Bar graph shows mean  $\pm$  SEM infarct volume (%) for G1 (control, 24 h), G2 (with hydrogel sheet, 24 h), G3 (control, 72 h), and G4 (with hydrogel sheet, 72 h). Colored symbols represent individual animals ( $n = 3$  per group). The sheet-treated group at 24 h (G2) exhibited a significant reduction compared to the corresponding control group (G1) ( $p < 0.05$ , Mann–Whitney U test). At 72 h, no statistically significant difference was detected between G4 and G3. Detailed methods for the TTC processing and analysis are provided in the text. Infarct volume was calculated as  $\Sigma(A \times t)$ , where  $A$  is the infarct area ( $\text{mm}^2$ ) in each TTC slice and  $t$  is the section thickness (mm).

### 2.5. MRI Lesion Volume

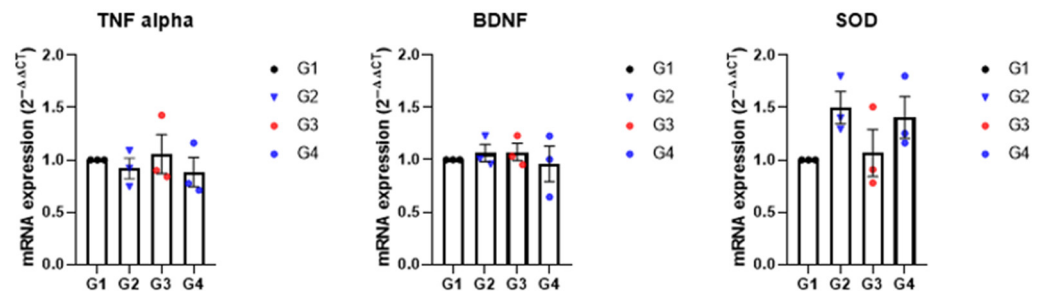
MRI was performed at 24 h (G1, G2) and 72 h (G3, G4) to qualitatively confirm successful stroke induction and to document the anatomical location of the photothrombotic lesion (Figure 4). Representative images demonstrate ipsilateral cortical signal changes consistent with the expected lesion location. Due to limitations of the MRI dataset (slice thickness of 0.8 mm and variable SNR), MRI lesion burden was not quantified, and no group-level comparisons of MRI lesion size are reported.



**Figure 4.** Representative MRI images after photothrombosis with or without an oxygen-releasing sheet. Representative axial T2 images at 24 h (G1, G2) and 72 h (G3, G4) show ipsilateral cortical signal changes consistent with the photothrombotic lesion location. Red arrows indicate examples of postoperative brain herniation/bulging at the craniotomy site observed in representative control groups (G1/G3). MRI was used for qualitative confirmation of stroke induction and lesion location only and was not quantified; therefore, no group-level MRI lesion burden comparisons are presented.

## 2.6. Gene Expression

The qRT-PCR analysis did not identify statistically significant between-group differences in TNF- $\alpha$ , BDNF, or Sod2 expression (Figure 5). Given that none of the comparisons reached significance and the sample size was limited ( $n = 3$  per group), these molecular findings are underpowered and should be interpreted as exploratory. Accordingly, the present gene expression data do not provide mechanistic evidence explaining the reduced TTC-defined infarct burden at 24 h.



**Figure 5.** Relative mRNA expression of TNF- $\alpha$ , BDNF, and Sod2 after photothrombosis with or without an oxygen-releasing sheet. Bar plots show mean  $\pm$  SEM qRT-PCR results expressed as  $2^{-\Delta\Delta CT}$ , normalized to GAPDH and calibrated to the 24 h control (G1 = 1.0). Groups: G1 (control, 24 h), G2 (with hydrogel sheet, 24 h), G3 (control, 72 h), and G4 (with hydrogel sheet, 72 h). Colored symbols denote individual animals ( $n = 3$ /group). No statistically significant differences were detected among groups for TNF- $\alpha$ , BDNF, or Sod2 (Kruskal–Wallis test with Dunn’s multiple-comparison correction).

The pre-specified primary endpoint, TTC-derived infarct burden at 24 h, showed a significant reduction in the sheet-treated group (Figures 2 and 3). In contrast, TTC at 72 h and MRI outcomes did not show statistically significant between-group differences and are therefore inconclusive with respect to treatment efficacy (Figures 4 and 5). Gene expression outcomes (TNF- $\alpha$ , BDNF, and Sod2) were assessed in a limited subset ( $n = 3$ /group) and did not show statistically significant differences; these molecular findings are therefore interpreted as exploratory (Figure 5). Safety outcomes, including body weight and neurological scores, were comparable across groups over the 72-h observation period (Tables 1 and 2).

## 3. Discussion

### 3.1. Summary of the Main Findings

In this exploratory study, we evaluated an implantable oxygen-releasing hydrogel sheet in a rat photothrombotic stroke model. The primary finding was a significant reduction in TTC-defined infarct volume at the acute 24 h time point in the sheet-treated group. However, this attenuation was not sustained at 72 h, and neither MRI lesion location nor gene expression profiles (TNF- $\alpha$ , BDNF, and Sod2) showed statistically significant differences between groups. These findings suggest that while the oxygen-releasing sheet provides a measurable early neuroprotective effect, its impact on the evolving infarct may be transient under the current experimental parameters. Body weight and perioperative status were comparable across groups, supporting short-term tolerability of the materials and procedures.

### 3.2. Mechanisms of Early Infarct Attenuation and Time-Dependent Effects

The significant reduction in TTC-defined infarct burden at 24 h provides preliminary evidence that a localized, implantable approach may influence the early phase of ischemic injury. The thin-sheet configuration is practically advantageous because it can be applied directly to the cortical surface—analogueous to a dural substitute—potentially enabling spatially confined delivery without the risks associated with systemic hyper-

oxia [5–9]. Nevertheless, given the exploratory nature of the dataset, these findings are best interpreted as preliminary evidence of early infarct attenuation rather than definitive proof of sustained neuroprotection.

Importantly, the TTC-defined difference observed at 24 h was not sustained at 72 h. Several non-mutually exclusive explanations warrant consideration. First, the effective oxygen-generating capacity of the sheet may decline over time in vivo. Because oxygen generation relies on peroxide-related intermediates and catalase activity, substrate depletion, diffusion limitations, and/or reduced enzymatic activity under physiological conditions—such as inflammation-associated microenvironmental changes and potential catalase degradation—could plausibly diminish net oxygen generation and transfer to peri-infarct tissue beyond the early post-implantation period [5,6,10,11]. This interpretation is consistent with the broader observation that in vivo performance can differ from in vitro release profiles [6,10]. Second, photothrombotic injury involves evolving secondary cascades—including neuroinflammation, oxidative stress, and edema—that may peak after the initial 24 h and may not be fully mitigated by oxygen supplementation alone [12–14]. Third, local tissue conditions (e.g., edema, protein adsorption, and inflammatory cell infiltration) may alter mass transport near the cortical surface and reduce effective oxygen diffusion at later time points [10,11,15,16]. Together, these considerations underscore the need for future studies designed to directly profile in vivo tissue oxygenation and oxygen-release kinetics under physiologic conditions and to optimize dose, placement, and exposure duration [10,11].

### 3.3. Interpretation of Secondary Endpoints: MRI and Molecular Markers

MRI results are interpreted cautiously. In this study, MRI was used for qualitative confirmation of lesion induction and anatomical location only (not quantified), and therefore it was not used to support efficacy or provide group-level comparisons of lesion burden. Future work should incorporate acquisition parameters optimized for quantitative lesion delineation and serial imaging to better characterize infarct evolution and enable longitudinal assessment [17,18].

Gene expression outcomes likewise require cautious interpretation. The apparent discrepancy between reduced TTC-defined infarct burden at 24 h and the absence of statistically significant differences in TNF- $\alpha$ , BDNF, and Sod2 mRNA may have several explanations. Transcriptional responses after photothrombosis are time-dependent, and a single terminal sampling point may miss peak or transient changes [12,19]. In addition, mRNA levels do not necessarily correlate with protein abundance or enzymatic activity at early time points [20]. Finally, tissue sampling and homogenization may dilute compartment-specific signals (e.g., peri-infarct versus core), and the small qRT-PCR subset ( $n = 3$  per group) further limits power in the presence of inter-animal variability [12,21]. Accordingly, these molecular endpoints are best viewed as exploratory and hypothesis-generating rather than mechanistic evidence.

Interpretation of between-group differences should also consider that material-related properties may influence local tissue responses. For example, thiolated gelatin (GtnSH) matrices have been reported to exhibit reactive oxygen species (ROS)-scavenging activity via pendant thiol groups, which could contribute to early histological differences independent of transcriptional changes in antioxidant genes [5,22]. The implications of these factors and other study limitations are addressed in the following Section 3.4.

### 3.4. Limitations

This was an exploratory study involving a small cohort ( $n = 6$ /group), and molecular endpoints were assessed in a subset (qRT-PCR,  $n = 3$ /group). Therefore, the study was

not powered to detect modest between-group differences in TNF- $\alpha$ , BDNF, or Sod2, and negative findings for these molecular endpoints should be considered inconclusive. The observation ended at 72 h; therefore, long-term effects and functional recovery were not assessed. We did not quantify oxygen release kinetics in vitro or measure brain tissue pO<sub>2</sub> in vivo, precluding dose–response inference. Neurological testing was brief and not powered by between-group differences. Only male rats and a single photothrombosis paradigm were used, limiting result generalizability. These constraints limit the strength of inference and define priorities for future confirmatory studies, including power-based sample expansion; longer follow-up with blinded behavioral batteries; oxygen release and tissue oxygenation profiling; dose/placement/exposure-time optimization; and validation across sexes and additional ischemia models (including reperfusion). In addition, baseline MRI was not performed before photothrombosis, and infarct evolution was assessed only at 24 and 72 h. Future studies should include baseline and serial MRI to allow more precise longitudinal comparisons. Moreover, MRI was not quantified in the present study and was used only for qualitative confirmation of stroke induction and lesion location. Therefore, MRI cannot be used to support treatment efficacy or to provide group-level comparisons of lesion burden. Future studies should incorporate an MRI acquisition protocol optimized for quantitative lesion delineation (improved resolution/SNR with access to full datasets) and serial imaging to evaluate infarct evolution. A key limitation is the absence of a material-matched blank gelatin hydrogel control that lacks the oxygen-generating system. Because the control condition used bovine-derived artificial dura, we cannot fully exclude the possibility that some observed differences are related to physico-chemical differences between materials (e.g., hydration, compliance, permeability, and host–material interactions) rather than to oxygen generation per se. Accordingly, our findings should be interpreted as preliminary feasibility data, and confirmatory studies should include a blank gelatin hydrogel sheet control matched in matrix composition and handling properties to isolate oxygen-specific effects.

#### 4. Conclusions

An implantable oxygen-releasing sheet was associated with a significant reduction in TTC-defined infarct burden at 24 h in a rat photothrombotic stroke model. At 72 h, and across MRI and gene expression endpoints, no statistically significant between-group differences were detected; therefore, these outcomes remain inconclusive regarding treatment efficacy. Short-term tolerability was acceptable. Confirmatory studies will require larger, powered cohorts, longer follow-up with blinded functional testing, and direct in vivo oxygen release and tissue oxygenation profiling.

#### 5. Materials and Methods

##### 5.1. Ethics Approval

All procedures complied with institutional and national guidelines for the care and use of laboratory animals. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee (Approval No. KMEDI-23081001-00). The animals were acclimatized before enrollment, and only healthy rats underwent surgery.

##### 5.2. Animals and Study Design

Oxygen-releasing hydrogel sheet. The oxygen-releasing sheet used in this study is a thiolated gelatin-based hydrogel matrix incorporating catalase and an oxygen-generating system based on peroxide-related intermediates. Upon hydration in situ, the oxygen-generating components produce oxygen, while catalase facilitates decomposition of hydrogen peroxide-related intermediates into oxygen and water, thereby improving oxygen

yield and reducing potential peroxide-related cytotoxicity. The material was provided in a pre-formed sheet configuration and was applied to cover the cortical surface over the craniotomy immediately after photothrombosis.

We conducted an exploratory controlled study in male Sprague–Dawley rats. Overall, 28 animals were planned to allow for dropouts; 24 rats were included and analyzed in four groups ( $n = 6$  per group): control at 24 h (G1), oxygen-releasing sheet at 24 h (G2), control at 72 h (G3), and sheet at 72 h (G4). MRI was performed at assigned time points (24 or 72 h). After imaging, three animals per group were used for histological analysis and three for gene expression analysis.

The primary outcome was the infarct burden, quantified by TTC staining and MRI. Secondary outcomes were mRNA expression levels of TNF- $\alpha$ , BDNF, and Sod2. Body weight was recorded as a general safety indicator. A bovine-derived artificial dura was selected as the control material to reflect a clinically relevant dural substitute in neurosurgical settings. However, because a material-matched blank gelatin hydrogel sheet control was not included in this exploratory in vivo study, the observed differences cannot be attributed exclusively to oxygen generation, and potential material-related effects cannot be fully excluded.

### 5.3. Surgical Preparation and Photothrombosis

Anesthesia was induced using 3% isoflurane and maintained at 1.5–2%. The scalps were shaved and sterilized with alcohol and povidone–iodine. A midline incision was made, and the periosteum was removed to expose the skull. A 6.0-mm circular cranial defect was created using a dental drill. Rose Bengal (20 mg/kg) was injected into the tail vein. Photothrombosis was induced by illuminating the exposed cortex for 20 min using a 532-nm fiber-coupled laser diode module (GL532T3-200FC, Shanghai Laser & Optics Century Co., Ltd., Shanghai, China), delivered through an FC/PC optical fiber (MM-200  $\mu$ m-DPC; 200- $\mu$ m core, Thorlabs, Newton, NJ, USA) and shaped using a fixed-focus collimator (F240FC-532, Thorlabs, Newton, NJ, USA) and a plano-convex lens (LA1027-ML, Thorlabs, Newton, NJ, USA). The output optical power at the target was set to 17 mW and verified using a photodetector (S305C, Thorlabs, Newton, NJ, USA) connected to a USB optical power meter (PM100USB) (Thorlabs, Newton, NJ, USA). After surgery, the wound was disinfected with povidone–iodine, and tramadol (10 mg/kg, s.c.) was administered for postoperative analgesia.

### 5.4. Oxygen-Releasing Sheet and Control Material

In the treatment groups (G2 and G4), a sterile oxygen-releasing sheet was placed gently over the exposed cortex immediately after illumination to cover the craniotomy defect. The sheet was custom-made (in-house) by the corresponding author's group and prepared according to previously published methods [5,6] (not commercially available). The sheet was used in a pre-formed configuration and trimmed to cover the 6.0-mm craniotomy defect, and was handled under aseptic conditions and kept sterile until implantation. Key physicochemical and biological characterization of the same GtnSH-based oxygen-generating hydrogel platform (including representative oxygen-release behavior and cytocompatibility) has been reported previously [5,6] and is summarized in Supplementary Table S1. In the present animal study, we did not re-quantify oxygen release kinetics for the batch used or directly measure in vivo tissue oxygenation; therefore, in vivo oxygen release and tissue oxygenation were not directly profiled.

In the control groups (G1 and G3), a commercially available bovine-derived dural substitute (DuraGen® Dural Graft Matrix, Integra LifeSciences, Plainsboro, NJ, USA) was

used. The periosteum was sutured, and the skin was closed with 4-0 black silk. All other perioperative procedures were identical between groups.

The oxygen-releasing sheet consisted of a thiolated gelatin-based hydrogel matrix incorporating an oxygen-generating system in which catalase facilitates decomposition of peroxide-derived intermediates into oxygen and water. The oxygen-releasing sheet and bovine-derived artificial dura may differ in physico-chemical properties (e.g., water content, stiffness, permeability, and degradation behavior), which could influence local tissue responses independent of oxygen release; therefore, material-related effects cannot be excluded and were considered when interpreting the results.

#### 5.5. MRI Acquisition and Analysis

MRI was performed at 24 h (G1, G2) or 72 h (G3, G4) according to group assignment to qualitatively confirm successful stroke induction and lesion location. Images were reviewed by an investigator blinded to group allocation. Quantitative lesion measurement (e.g., volumetric segmentation) was not performed because the available dataset (slice thickness of 0.8 mm and variable SNR in some subjects) did not allow stable lesion boundary definition across animals, and manual tracing carried a risk of inter-observer variability and potential overestimation of lesion boundaries.

#### 5.6. TTC Staining and Infarct Quantification

After MRI, the brains were removed and sliced into 2-mm-thick coronal sections. Sections were incubated in 1% TTC at room temperature for 15 min in the dark, then fixed in 10% neutral buffered formalin for approximately 24 h. Digital images of all slices were obtained. Infarct areas were measured using ImageJ software (version 1.54h; National Institutes of Health, Bethesda, MD, USA), and infarct volume was calculated as the sum of the infarct areas multiplied by the section thickness. Data are reported as mean  $\pm$  standard error of the mean.

#### 5.7. Neurological Assessment and Body Weight

Neurological deficits were assessed using the modified Neurological Severity Score (mNSS), which combines motor, sensory, reflex, and balance items on an ordinal scale, with higher scores indicating more severe deficits [23]. The mNSS protocol followed the established rat stroke models and included tail suspension, forelimb flexion, beam balance, and placing/proprioception tests. Assessments were performed by an investigator blinded to the group assignment. Body weight was measured at baseline and at the respective endpoints (24 or 72 h) to monitor perioperative status and tolerability.

#### 5.8. RNA Extraction and qRT-PCR

The ischemic brain tissue, including the lesion, was dissected and snap-frozen in liquid nitrogen. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time PCR was performed using the Power SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA). The target genes were TNF- $\alpha$ , BDNF, and Sod2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference gene. The 24-h control group (G1) was used as a calibrator. The annealing temperature was 60 °C for all the primer pairs. Primer sequences for TNF- $\alpha$ , BDNF, Sod2, and GAPDH are provided in Supplementary Table S2. The SOD target gene analyzed in this study was Sod2 (Mn-SOD). Relative expression levels were calculated using the  $2^{-\Delta\Delta CT}$  method. These markers were selected to capture the key axes of the ischemic response (inflammation, trophic support, and redox balance).

Due to small sample sizes and potential non-normality, nonparametric tests were used. Two-group comparisons at each time point were performed using the Mann–Whitney U test (G2 vs. G1 at 24 h; G4 vs. G3 at 72 h). For comparisons involving more than two groups, the Kruskal–Wallis test with Dunn’s multiple-comparison correction was used. All analyses were performed using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). Statistical significance was defined as  $p < 0.05$ . Data are presented as mean  $\pm$  standard error of the mean, unless otherwise specified.

Because the dissected specimen encompassed the lesion and adjacent ipsilateral tissue, it may include mixed compartments (core and peri-infarct), and homogenization could dilute localized transcriptional changes.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/gels12020159/s1>, Table S1: Summary of previously reported characterization of the GtnSH-based oxygen-generating hydrogel platforms related to the oxygen-releasing sheet used in this study (compiled from References [5,6]). Table S2: Primer information (qRT-PCR).

**Author Contributions:** K.H.: investigation, data curation, writing—original draft, and writing—review and editing. H.-J.Y.: methodology and data curation. H.-J.C.: methodology and data curation. M.K.N.: methodology and investigation. S.S.: methodology and data curation. K.M.P.: conceptualization and supervision. K.-S.C.: conceptualization, formal analysis, supervision, validation, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (RS-2022-NR070832).

**Institutional Review Board Statement:** The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC; Approval No. KMEDI-23081001-00).

**Data Availability Statement:** The data supporting the findings of this study are not publicly available due to institutional policy. Data are available from the corresponding authors upon reasonable request.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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