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Suppression of Aquaporin-4 by Antisense Oligonucleotides Reduces Brain Edema in Experimental Traumatic Brain Injury

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ABSTRACT

AIM: To investigate the suppression of aquaporin-4 (AQP4) synthesis through intracerebroventricular (i.c.v.) injection of antisense oligonucleotide after focal cortical contusion injury in mice.

MATERIAL and METHODS: This study used 12-week-old female Swiss albino mice (weight, 20–25 g) to create a focal cortical contusion model by the weight-drop method (35 g blunt weight, 70 cm height) onto the parietal cortex after craniectomy. The sham group underwent craniectomy without trauma. In the control group, weight was dropped onto the parietal cortex immediately after i.c.v. injection of Dulbecco's Modified Eagle Medium after craniectomy. In addition, 1 nM of aquaporin-4 (AQP4) antisense oligonucleotide (ASO) was injected via the i.c.v. route immediately after trauma (0 hour) and 4 hours after trauma. All animals underwent magnetic resonance (MR) imaging and were sacrificed at 24 hours. The brain–water content was determined using the wet/dry weight method.

RESULTS: In the sham group, the average percentage of the brain–water content was 77.75% compared with the control group with 79.87%, and the difference was significant (p=0.017). The average was 78.81% and significantly reduced in the therapy group compared with the control group (p=0.026) at 0 hour. In the 4-hour treatment group, the average of 79.11% was not significant (p=0.39). MR imaging findings also showed a substantial reduction in brain edema in the 0-h treatment group. However, the 4-h treatment results, when compared with the control trauma group, did not show a significant difference.

CONCLUSION: This study demonstrated that AQP4-ASO therapy, when administered early after diffuse traumatic brain injury, leads to a significant reduction in brain edema.

KEYWORDS: Aquaporin-4, Brain edema, Traumatic brain injury, Antisense oligonucleotide, MRI, Mice

ABBREVIATIONS: DMEM: Dulbecco's modified eagle medium, **MR:** Magnetic resonance, **TBI:** Traumatic brain injury

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INTRODUCTION

Severe traumatic brain injury (TBI) and secondary brain edema have incredibly high morbidity and mortality rates (6,22). Primary traumatic events involve skull fractures, contusion, hematoma, laceration, and diffuse axonal damage. By contrast, post-traumatic secondary damage is defined as interrelated pathophysiological processes with long-lasting results that occur in the subsequent period because of cellular and molecular mechanisms. Significant phenomena contributing to secondary damage include the release of various neurochemical mediators, increased intracranial pressure, brain edema, hydrocephalus, and herniations. Brain edema is one of the well-known pathophysiological phenomena. Cell death, release of mediators, increased intracranial pressure, impaired blood–brain barrier, cerebrovascular autoregulation, and several other cellular processes during primary brain damage contribute to brain edema.

By contrast, brain edema further increases the intracranial pressure causing herniations that even results in death. In this respect, the prevention and recovery of post-traumatic brain damage are significant treatment targets (4,26,28,29,32). Cytotoxic and vasogenic brain edema are two primary forms characterized by increased intracellular and extracellular water volumes, respectively. Cytotoxic edema occurs secondary to cellular damage, while vasogenic edema develops because of increased permeability of the blood–brain barrier. Nevertheless, both edema types contribute to traumatic brain damage (4,5,12,26,29). Recent studies have indicated that cytotoxic edema has a more prominent role than vasogenic edema in the pathophysiology of diffuse TBI. The direct proportion of brain edema, increased intracranial pressure, mortality in diffuse TBI, and increased expression of aquaporins located in the cell membrane, especially aquaporin-4 (AQP4), is proven in the literature (13). This study aimed to suppress transcription product, which enables binding of AQP4 to the cell membrane, by using antisense oligonucleotide (ASO), to observe beneficial effects on edema since cytotoxic edema has a primary role in traumatic brain edema. It is consequently proposed that AQP4-ASO may become a new treatment strategy for the improvement of brain edema during traumatic brain damage.

MATERIAL and METHODS

A focal cortical contusion model was induced in 23 12-week-old female *Swiss albino* mice, weighing 20–25 g, by performing craniectomy of the parietal cortex in the ipsilateral hemisphere, followed by free-falling of a 35-g blunt-end weight from 70 cm height using Feeney's weight-drop method. In this model, 2 µl of 1 nM AQP4-ASO (dissolved in Dulbecco's Modified Eagle Medium [DMEM] without serum) was administered to the ventricular space via i.c.v injection (0.1 mm posterior and a 0.9 mm lateral from Bregma 3.1 mm depth). AQP4-ASO was designed as previously described (15). In summary, we used an AQP4-ASO (5/56- FAM/T*C*A*T*A*C*G*G*A*A*G*A*C*A*A* T*A*C*C*T *C-3), which was purchased from IDT-DNA (USA). It specifically inhibits AQP4 channel function by suppressing its membrane binding efficacy.

It was administered at 0 hour after the trauma in the first experiment group (n=6) and at 4 hours following trauma in the second experiment group (n=6). The control group received DMEM without serum (n=6). The animals underwent craniectomy without applying trauma, and the skin was sutured in the sham group (n=5).

The animals were anesthetized with isoflurane at doses of 5 mL/min and 2–3 mL/min for induction and maintenance, respectively, and subsequently underwent surgical procedures and the weight-drop protocol. The site of the burr-hole to be performed with the micro drill was marked on the Bregma for ASO injection inside the lateral ventricle (Figure 1). Extreme care was applied so that the drill tip is not in contact with the brain tissue and the formed iatrogenic edema. Physiological saline was applied to the bone at regular intervals to cool and reverse the thermal effect caused by the drill, and the bone was lifted. The tube of the weight-drop apparatus was placed precisely above the craniectomy site by an investigator who ensured that the weight fell precisely on the exposed area. Oxygen support (2 L/min) was administered to prevent brain swelling because of post-traumatic respiratory depression by isoflurane. The flap was folded back and sutured before awakening. Oxygen was administered until the mouse awakened and vital signs reached optimal values; the animals were monitored with a pulse oximeter during the procedure. This method decreased the mortality ratio, and only two

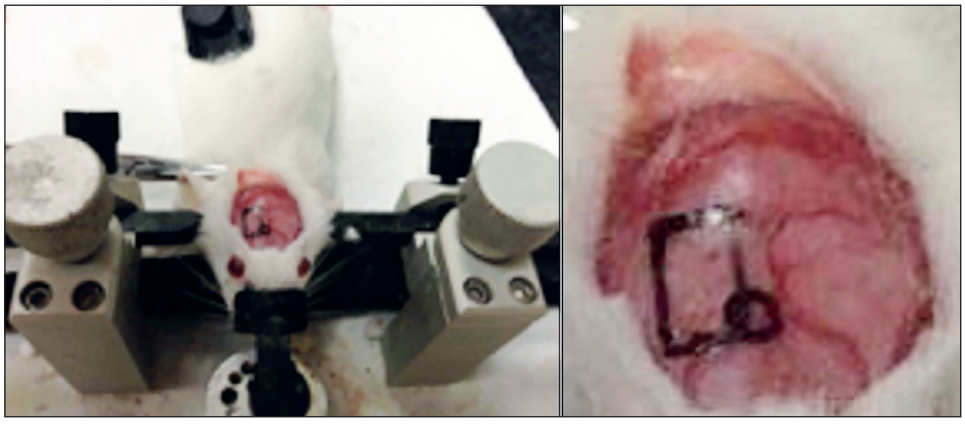


Figure 1: Determination of craniectomy borders.

animals died. Body temperatures were monitored by a rectal probe and stabilized at 37.0 ± 1°C with the homeothermic blanket control unit (Harvard Apparatus). Pulse rate and oxygen saturation (V3304 Digital Table-Top Pulse Oximeter, Surgivet) were monitored from the lower extremity.

Animals remained in cages (containing food and water) for 24 hours under standard conditions, and vital signs were measured at certain time intervals. Analgesia was administered to restless and moaning animals. Naproxen 0.4 µg (10 nM) was injected intraperitoneally for pain management.

All mice underwent MRI, followed by euthanasia, in Bilkent University National Magnetic Resonance Research Center. The animals received intraperitoneal injection of 10 mg/kg xylazine and 100 mg/kg ketamine as anesthetics, respectively, and they were placed in the MRI device. The depth of anesthesia was assessed with control of reflexes by toe pinch. The mice were placed in an MR scanner with 3-Tesla magnetic field strength (Magnetom Trio, Siemens Healthcare, Erlangen, Germany) using appropriate head holders. By using a handmade 8-channel receive-only phase-array surface coil with circular loop elements of 3.5 cm diameter, T2 turbo spin-echo images (repetition time/echo time, 4420/94 ms; field of view, 78 × 78 mm; slice thickness, 2 mm; interslice gap, 0; and number of excitations, 5) were obtained in all three orthogonal planes. The study time was 10 min for each mouse. An experienced radiologist who was blinded to the treatment groups assessed images in terms of the existence and size of the parenchymal damage. The maximum length of the parenchymal damage was measured in its most extensive sections as transverse and anteroposterior diameters. The animals were placed on hyperthermic blankets at 37°C degrees to avoid hypothermia, and vital signs were monitored with a pulse oximeter.

Calculation of the Brain–Water Ratio

The cortical regions of the mouse brains were isolated from the white matter by scraping and accepted as the "traumatic brain region" and "region containing post-traumatic changes" in groups subject to weight falling. Cortices were placed on papers that did not absorb water (weighed before the procedure) and immediately weighed on sensitive scales, not allowing time for the tissue to lose water. The measured weight was recorded as the wet brain weight. Ipsilateral cortices were placed in a 99°C oven for 24 hours. Dried cortices were re-weighed on sensitive scales, and the measured weight was recorded as the dry weight. The ratio of the brain–water content was calculated using the following formula: brain–water content % = ((wet weight – dry weight) / wet weight)) × 100.

Statistical Methods

Data are presented as mean ± standard error. The brain–water contents of the groups were compared with the Kruskal–Wallis test; in case of significance, the Mann–Whitney U test was used for the evaluation, and p≤0.005 value was accepted as significant. Statistical evaluations were performed with IBM SPSS Statistics version 20 (IBM Corp., NY, USA).

RESULTS

Statistical Evaluation of the Difference Between Wet/Dry Weight Ratios

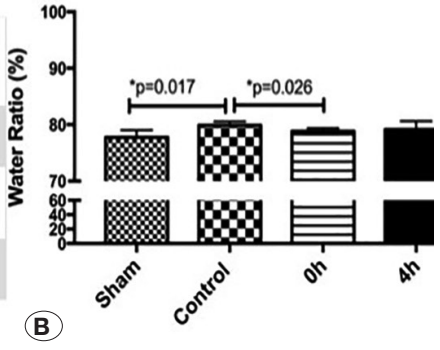
The wet/dry ratio of the trauma group increased relative to that of the sham group. The average water ratio of 77.75% in the sham group increased up to 79.87% in the trauma group, and the difference was significant (p=0.017). The administration of AQP4-ASO at 0 hour decreased the brain–water content in comparison with the trauma group. While the average percentage of the wet/dry weight in the trauma group was 79.87%, it reduced at 0 hour of the AQP4-ASO therapy group (p=0.026). By contrast, no significant difference was found between the AQP4-ASO group at 4 hours, and the trauma group with respect to the brain–water ratios, calculated by measuring the wet and dry weights of the cortex at the craniectomy site. The average brain–water content was 79.11% (p=0.39) (Figure 2).

MRI

Edema signs were identified in the cortical and subcortical white matter areas of the trauma groups. Hemorrhagic areas and hematoma were also identified, along with edema in some animals. A midline shift was noted in one mouse. Findings were noticeable immediately under the craniectomy

Water ratio (%)	Sham (n=5)	Control (n=6)	AQP4-ASO (0h) (n=6)	AQP4-ASO (4h) (n=6)
Mean	77.75	79.87	78.81	79.11
Std deviation	1.29	0.69	0.59	1.62
Std error	0.57	0.31	0.26	0.72

A



B

Figure 2: Statistical evaluation of the differences between the wet/dry weight ratios in the experimental groups **(A)** and comparisons between groups **(B)**.

site. "Coup" and "contrecoup" contusions were also observed (Figure 3). In the AQP4-ASO 0-h therapy group, edema was identified just in only one mouse. The reduction of brain edema was remarkable in most animals. The decrease in edema was remarkable and comparable with that of the sham group. In addition, images showed the same characteristics as those of the sham group (Figure 3).

The results of the AQP4-ASO 4-hour therapy group compared with the trauma group did not show a significant difference, and edema was identified in all animals. In addition, no difference was found in edema size in comparison with the trauma group (Figure 3). Moreover, intracerebral hemorrhage was associated with edema in two mice in the AQP4-ASO 4-h therapy group.

DISCUSSION

Despite the emergence of modern diagnostic and treatment methods, the morbidity and mortality rates are still very high in this patient group. Therefore, recent studies have mainly addressed the prevention of secondary pathophysiological events after diffuse traumatic brain damage and the administration of efficient medical treatment (11,15).

Marmarou's weight-drop method is the most common trauma model in experimental TBI. This model enables the formation of biomechanical changes and clinical characteristics of diffuse axonal damage and follow-up at the initial hours (6–24 hours) of acute post-traumatic brain edema. Even if the Marmarou model has not mimicked all pathophysiological changes in patients with head trauma, it is accepted as the standard model for experimental treatments of post-traumatic brain damage (21). Our pilot studies with this model have led to very high mortality rates in the early post-traumatic period, and no evidence of edema in the sham and control groups can be visualized within MRI study limits. Moreover, no significant difference in brain–water content was noted when wet and dry brain weights were measured. Thus, Feeney's weight-drop method was implemented. This model is not the gold standard among the experimental severe head trauma models; however, it is an easily applicable, low-cost, and controllable model. Monitoring of animals during experiments, maintenance of optimum vital signs in all experimental groups, exclusion of unstable animals and those that received nonstandard protocol, and prevention of another trauma became a standardized treatment algorithm.

Enhancement in TBI model mechanics and increased utilization of MRI has confirmed that cytotoxic edema is more significant than vasogenic edema in the pathophysiology of TBI. Furthermore, cytotoxic edema initiates earlier during the post-traumatic period than vasogenic edema and lasts longer (3,7,8,19,20,21).

MRI studies performed during the first 24-h period after the trauma revealed leakage in the blood–brain permeability. Experimental MRI studies on rats showed the onset of cellular

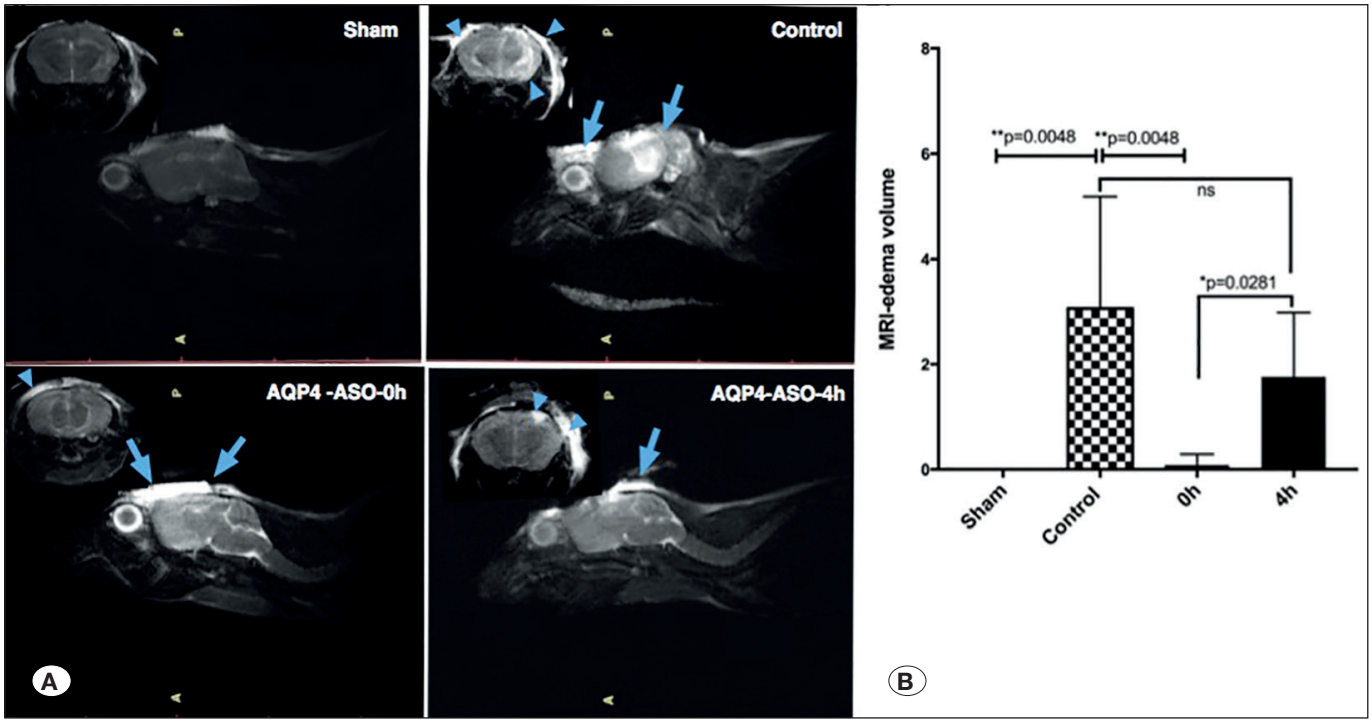


Figure 3: A) Magnetic resonance (MR) imaging did not detect evidence of edema in the sham group. T2 images reveal diffuse edema in the control group. In the AQP4-ASO 0-h group, T2-weighted MR images reveal insignificant hyperintensity because of edema, and the 4-h treatment group images reveal edema just inferior to the craniectomy region. The blue arrows show the position of the edema in coronal and sagittal images of the brain. **B)** MR imaging findings of the experimental groups are summarized. Data are presented as the mean ± standard deviation from at least five independent experiments.

swelling in the periphery of the contusion area during the first 6 h in the early post-traumatic period with T2, diffusion, and ADC mapping (14,18,25,30). Our study differed from previous studies because MRI was performed within 24 hours following trauma, which is the most intense period of edema, and this was not previously performed on mice. However, this technique may provide additional data for future studies after developing coils that can be applied to mice.

Cerebral AQP4, which is the recently identified and extensively studied AQPs, is a bidirectional transmembrane water channel located in the end foot of astrocytes. AQPs, especially AQP4, have a significant role in cytotoxic edema (4,23,29). Studies with AQP4 knockout mice with dominant cytotoxic edema have reported less edema and favorable clinical outcomes. However, models like brain edema, in which vasogenic edema is more prominent, provided transgenic mice unfavorable results. These results show that AQP4, acting as a bidirectional water channel, increases intracellular water accumulation during cytotoxic edema and facilitates the disposal of excessive water during vasogenic and interstitial edema (19,31).

Nonetheless, an increase in AQP4 expression is reported at the trauma region, while a decrease is reported around the site of trauma. These findings indicate the complex control of balance in the role of AQP4 in post-traumatic brain edema (26). Thus, the aforementioned studies have provided information about the role of AQP4 in brain edema, which is unfortunately not straightforward.

Recent studies have investigated the effects of experimental drugs on astrocytes, AQP4, and brain–water content (13). Experimental studies have been increasingly performed for the development of new treatment strategies. However, none of them gave optimum results. Insights from latest research have emerged about the role of AQPs in trauma pathophysiology, and this has been more clearly understood.

Recent evidence suggests that the regulatory role of arginine vasopressin (AVP) in the expression of AQP4 in various tissues (10,17,27). In addition, AVP V1 receptor antagonism upregulates AQP4, causing a decrease in brain–water content and thereby brain edema in the ischemic stroke mouse model (17). Similar results have been observed in experimental traumatic brain damage models with AVP V1 receptor antagonism (27). Similar treatment models have utilized antibody-driven direct or indirect inhibition of membrane AQP4 oligonucleotides, therefore targeting not only the pathological region but the receptor of the whole brain tissue and membrane AQP4 oligonucleotides (13). The inhibition of membrane AQP4 oligonucleotides led to the dysfunction of otherwise normal tissues; however, the inhibition of AQP4, which acts in the pathophysiology of vasogenic edema during the process of diffuse traumatic brain damage, may accelerate these pathophysiological processes, resulting in the deteriorated condition of the patients (7,30). Our study was based on the inhibition of AQP4 expression at the trauma region in the brain tissue, causing suppression of functional channel protein to the cell membrane (26). This treatment does not affect functional membrane AQP4 in healthy brain tissues or impair their functions. Our treatment did not change the function or number of available AQP4; however, it impaired the nucleotide sequence of newly synthesized functional AQP4, providing an important treatment option in the physiopathology of cytotoxic edema, emphasizing the superiority of our study. The brain tissue is rich in AQP4, as observed in immunohistochemical staining, and no evidence of a decrease in AQP4 quantity before or after treatment supports this notion.

Our study aimed to suppress the connection of functional channel proteins, synthesized as a result of AQP4 expression, to the cell membrane via ASOs. ASOs connect to the mRNA according to Watson–Crick's double-stranded principle and form a steric blockage and prevent translation. The ASO–mRNA hybrid is enzymatically degraded with RNAaseH activity, that is, the RNAaseH mechanism. ASOs contain phosphodiester structures. Oligonucleotides with non-modified phosphodiester bonds are quickly degraded in biological fluids by endo- and exonucleases. Several modifications have been developed to provide more extended *in vivo* stability and prevent degradation (24).

ASOs may prevent transcription of the connection site of AQP4 to the cell membrane during AQP4 mRNA transcription. Thus, it may suppress the formation of AQP4 functional AQP4 combined with a membrane receptor but does not decrease AQP4 expression, leading to diminished channel function and improved edema. Treatment with ASO reduced edema in the earliest phase of trauma at 0 hour, and significant values were obtained ($p<0.01$). However, no effective results were obtained 4 hours after the treatment. Early intervention could be more effective before the onset of cytotoxic edema at a very early period within hours after the trauma (1,2,3,8,20,21,29). Therefore, the modification of the antisense dose or a change in time intervals of administration may be considered. The difference in outcomes can also be monitored when treatment is administered by a different delivery route, such as intranasal or intravenous rather than i.c.v.

Administration of antisense at 24 hours, the peak hour of edema, or later, may worsen the clinical outcomes. This is caused by aquaporin's anti-edema properties in the late hours of trauma, especially at 24 hours when AQP4 aids in the flushing out of the water from the cell, which decreases the swelling caused by the edema. Inhibiting AQP4 expression by administering ASO at 24 hours suppresses the anti-edema properties of AQP4, which can worsen edema formation in the tissue and swelling within cells (14,18,25). Antisense administration after day 7 or later is predicted to be ineffective (14,29). Since AQP4 has a bidirectional mechanism and its anti-edema effect occurs in the later stages, the admission of aquaporin inhibitory agents will help decrease edema in the early stages of trauma; however, as is known, it activates anti-edema mechanisms in the late period (9,16,33).

As study limitations, we were unable to observe the possible positive outcomes of reduced edema since the animals were sacrificed at 24th hour, which is the peak of the edema. However, we were able to see the reduction of edema in mice that were given ASO at 0 hour by thorough inspection and

comparison of their MR images with the control group. Future investigations could look at the more long-lasting effects of reduced edema by sacrificing animals in the later stages of edema.

CONCLUSION

Consequently, aquaporin inhibition given late after trauma will not be effective and will block clearance mechanisms of intracellular edema. In this respect, if AQP4 becomes the target molecule in diffuse traumatic brain damage, this treatment should be administered as soon as possible, even in the trauma site or during transportation to an emergency room.

This study showed improved functional outcomes, such as reduced edema formation by early AQP4-ASO administration at 24 hours after TBI in mice. The 24-hour time point was picked for the termination of the experiments since the edema formation reached its peak level during this time. For future studies, examining the long-term effects of AQP4-ASO on brain edema formation should be addressed with longitudinal MRI studies. In addition, long-term functional outcomes should be investigated further by neurobehavioral tests, such as the Morris water maze test and rotarod. We showed that the suppression of AQP4's function at the early stages of TBI reduced the formation of brain edema. The cellular and molecular mechanisms of this effect in TBI pathology should also be addressed with downstream analysis in future studies.

AUTHORSHIP CONTRIBUTION

Study conception and design: MH

Data collection: MH, SL, CCA, HO

Analysis and interpretation of results: SL, MH

Draft manuscript preparation: MH, SL

Critical revision of the article: MH, KKO, MM

All authors (MH, SL, HO, CCA, KKO, MM) reviewed the results and approved the final version of the manuscript.

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