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α7-Nicotinic Acetylcholine Receptor Activation Mitigates Neuroinflammation Associated with Hypoxia-Reoxygenation Injury in Zebrafish Model

(Pengaktifan Reseptor Asetilkolina α7 Nikotinik Mengurangkan Keradangan Neuro yang Berkaitan dengan Kecederaan Hipoksia-Pengoksigen Semula dalam Model Ikan Zebra)

MOHAMMAD YUSUF HASAN[1,†], AZIM HAIKAL MD ROSLAN[1,†], NORAZRINA AZMI[1], NORLINAH MOHAMED IBRAHIM[2], ALINA ARULSAMY[3], VANESSA LIN LIN LEE[3], ROSFAIIZAH SIRAN[4] & MOHD KAISAN MAHADI[1,*]

[1]Centre for Drug Herbal and Development, Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia
[2]Department of Medicine, Faculty of Medicine, Universiti Kebangsaan Malaysia, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia
[3]Neuropharmacology Research Laboratory, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, 47500 Bandar Sunway, Selangor, Malaysia
[4]Neuroscience Research Group (NRG), Faculty of Medicine, Jalan Hospital, Universiti Teknologi MARA, Sungai Buloh Campus, 47000 Sungai Buloh, Selangor, Malaysia

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†both authors contributed equally to this work

ABSTRACT

Ischemic stroke is a leading cause of death worldwide, where reduced blood flow to brain tissues can cause potential permanent neurological damage. Current treatments, such as intravenous thrombolysis with tissue plasminogen activator and mechanical thrombectomy, aim to restore cerebral blood flow within hours of stroke onset, often associated with ischemic reperfusion injury. Emerging strategies target the α7-nicotinic acetylcholine receptor (α7nAChR) to resolve neuroinflammation in various pathological conditions; however, the therapeutic effects of these strategies in ischemic reperfusion injury remain unknown. This study investigates the neuroprotective and anti-inflammatory effects of α7nAChR activation in zebrafish following ischemia-reperfusion injury. The hypoxia/reoxygenation model was established by perfusing pure nitrogen gas in a hypoxia chamber for 10 min, followed by a 1-h recovery/reoxygenation in the beaker. Gene expression markers for proinflammatory and anti-inflammatory factors were examined using qRT-PCR from the surviving brain tissues. Mitochondrial dehydrogenase activity was measured to investigate the level of brain damage. A six-minute open tank test assessed behaviour, precisely the turning angle, distance travelled, maximum acceleration, and meandering. Hypoxia/reoxygenation significantly increased the expression of proinflammatory markers, such as TNF-α and IL-6, whereas an α7nAChR agonist reduced the expression of these markers. However, there was no discernible improvement in locomotor activity or brain damage in the agonist group, implying that the neurological impairment was not fully reversed following PNU 282987 pre-treatments.

Keywords: Inflammation; ischemic reperfusion injury; ischemic stroke; α7nAChR

ABSTRAK

Strok iskemia merupakan salah satu punca utama kematian di seluruh dunia apabila pengurangan aliran darah ke tisu otak boleh menyebabkan kerosakan neurologi kekal. Rawatan semasa seperti trombolisis intravena dengan aktivator plasminogen tisu dan trombektomi mekanikal bertujuan untuk mengembalikan aliran darah serebrum dalam beberapa jam selepas bermulanya strok, yang sering dikaitkan dengan kecederaan reperfusi iskemia. Strategi baharu yang menyasarkan α7-Nikotinik Reseptor Asetilkolina (α7nAChR) untuk menyelesaikan neuroinflamasi dalam pelbagai keadaan patologi sedang diterokai, namun kesan terapeutik dalam kecederaan reperfusi iskemia masih belum diketahui. Penyelidikan ini mengkaji kesan neuropelindung dan anti-radang daripada pengaktifan α7nAChR pada ikan zebra selepas kecederaan iskemia-reperfusi. Model hipoksia/pemulihan oksigen semula diwujudkan dengan menggunakan gas nitrogen tulen yang disalurkan ke dalam ruang hipoksia selama 10 minit, diikuti dengan pemulihan/pemulihan oksigen semula selama 1 jam di dalam bekas. Penanda ekspresi gen bagi faktor pro-radang dan anti-radang dikaji menggunakan qRT-PCR daripada tisu otak yang masih hidup. Aktiviti dehidrogenase mitokondria diukur untuk mengkaji tahap kerosakan otak. Ujian tangki terbuka selama enam minit menilai tingkah laku, khususnya sudut pusingan, jarak perjalanan, pecutan maksimum dan pergerakan berliku. Hipoksia/pemulihan oksigen semula secara signifikan meningkatkan ekspresi penanda pro-radang

seperti TNF-α dan IL-6, manakala agonis α7nAChR mengurangkan penanda ini. Namun, tiada peningkatan ketara dalam aktiviti lokomotor dan kerosakan otak pada kumpulan agonis, menunjukkan bahawa kecacatan neurologi tidak dipulihkan sepenuhnya selepas pra-rawatan PNU 282987.

Kata kunci: Inflamasi; kecederaan reperfusi iskemia; strok iskemia; α7nAChR

INTRODUCTION

Stroke remains one of the leading causes of death worldwide. In 2021, 7.44 million people died from strokes worldwide, with ischaemic stroke (IS) accounting for 3.71 million of those deaths (Martin et al. 2024). Insufficient blood supply to brain tissue leads to the development of ischaemic stroke, which affects the normal central nervous system function. This blockage is associated with blood clots or atherosclerosis in the blood vessels that supply the brain. The brain begins to undergo apoptosis without sufficient nutrients and oxygen, potentially causing permanent damage to brain functions (Wu et al. 2024).

The primary goal of therapeutic approaches in ischaemic stroke is to restore cerebral blood flow as soon as possible, preventing further deterioration of the brain injury. Intravenous thrombolysis is the gold standard treatment for ischaemic stroke, in which tissue plasminogen activator (tPA) is administered within 4.5 h of the stroke to dissolve clots impeding blood flow in the arteries (Virani et al. 2020). Clinicians use mechanical thrombectomy for larger clots or when tPA is ineffective. Evidence suggests that thrombectomy performed within 6 to 24 h of the onset of a stroke improves clinical outcomes (Al-Mufti et al. 2024; Saver et al. 2016). In addition, antiplatelet therapy, such as aspirin, is immediately administered to prevent further clot formation, especially if thrombolysis and thrombectomy are not recommended (Mokin et al. 2019).

Reintroducing blood flow to ischaemic brain tissues is essential for preventing severe neurological damage and death. However, accumulating evidence showed worsening IS symptoms, such as neurological deficits and aggravated neuronal cell death post-reperfusion. This condition is known as ischemic reperfusion (IR) injury. Various pathophysiological mechanisms are associated with IR injury, including mitochondrial damage, oxidative stress, increased intracellular calcium levels, the initiation of inflammatory responses, and metabolic energy failure (Wu et al. 2024). Experimental studies targeting cerebral ischaemic reperfusion injury (CIRI) have garnered interest, but the results are still preliminary (Hou & Brenner 2024; Zeng et al. 2022). Hence, there is an urgent need for adjunctive strategies to reperfusion treatment to limit and prevent cerebral ischemic reperfusion injuries (Hasan, Siran & Mahadi 2023).

Activating the α7nAChR may aid in managing CIRI. α7nAChR is present in neurons and non-neuronal cells (Xu et al. 2021). In the brain, α7nAChR mediates neurotransmitter release (Cheng & Yakel 2015), modulates synaptic plasticity (Townsend et al. 2016), and provides neuroprotection against excitotoxicity (Zhou et al. 2017). α7nAChR activation is also a vital component of the cholinergic anti-inflammatory pathway (CAP), regulating inflammatory responses (Wu et al. 2021). Research suggests that activating α7nAChR through vagus nerve stimulation or selective agonists can reduce inflammation in various disease models (Xu et al. 2021; Zhou et al. 2021). In addition, α7AChR activation has neuroprotective effects in ischaemic stroke models (Han et al. 2014; Hasan, Siran & Mahadi 2023). Nonetheless, the anti-inflammatory potential of α7nAChR agonists in preventing cerebral ischemic reperfusion injury remains uncertain.

This study aimed to investigate the neuroprotective effects of α7nAChR activation on brain impairment and behaviour in zebrafish following ischemia-reoxygenation (I/R) injury. Additionally, we examined the impact of α7nAChR activation on pro- and anti-inflammatory markers, as well as the involvement of signalling pathways in the zebrafish brain post-ischemia. We hypothesise that pre-treatment with PNU 282987, an α7nAChR agonist, may provide neuroprotective and anti-inflammatory benefits, potentially mitigating cerebral ischemia-reperfusion injury in the zebrafish model.

MATERIALS AND METHODS

ANIMALS

Heterogeneous wild-type adult zebrafish (*Danio rerio*), approximately four to six months old, were purchased from a local aquatics shop (Bangi Aquatics, Malaysia). To reduce variability of the fish obtained, all fish used were from the same batch of purchases (Lee et al. 2022). The zebrafish were housed and maintained under husbandry conditions at Monash University Malaysia. The zebrafish were housed in aquatic tanks (36 cm × 26 cm × 22 cm) connected to a water circulation system that ensured proper aeration, temperature control (26-28 °C), and pH regulation (6.8-7.1). The tanks were exposed to a light intensity of 250 lux with an auto-regulated 14:10-h light-to-dark cycle. The fish were fed TetraMin® Tropical Flakes *ad libitum* twice daily, with a live brine shrimp (Artemia) supplement provided every alternate day. A 7-day period was allowed before the commencement of experiments to minimise stress (Lee et al. 2022). All experimental procedures were approved by the Monash University Malaysia Animal Ethics Committee (Project ID:37007). Zebrafish were treated with 30 mg/L benzocaine before any invasive procedures, and all efforts were made to minimise distress (Lee et al. 2022).

HYPOXIA-REOXYGENATION (H/R) INDUCTION

Adult zebrafish were subjected to hypoxia-reoxygenation treatment to simulate cerebral ischemia/reperfusion (I/R) injury pathophysiology. The I/R induction was adapted from Cao et al. (2010) and Yu and Li (2011). In brief, the hypoxia chamber was constructed from thick, transparent acrylic with dimensions of 20 cm × 20 cm × 20 cm. A thick black rubber seal was placed around the lid's bottom edge to ensure an airtight environment. The chamber featured two small ports located 5 cm from the top on each side; the left port was connected to the outside air, allowing oxygen exchange, while the right port was attached to a purified nitrogen gas tank (99.999% N_2).

Before inducing systemic hypoxia, the chamber was filled with normal water (26-28 °C) to about 60% capacity, leaving 40% open for oxygen exchange. After flushing purified N_2 into the water at a constant flow rate of 15 mg/L for 10 min, the chamber was sealed to initiate hypoxia. The water's dissolved oxygen (DO_2) concentration was measured at 2 mg/L or less, meeting the hypoxic threshold set by the Committee on Environment and Natural Resources (Eby & Crowder 2002). Later, a zebrafish from the tank was transferred to the hypoxic chamber, and the water was continuously circulated with purified N_2 at a constant flow rate of 15 mL/min. The duration a zebrafish remained in the hypoxic chamber was defined as either a) lying motionless except for occasional opercular movements for 60 s or b) lying on one side or with the abdomen up for 60 s (Yu & Li 2011). When hypoxia symptoms were identified, the zebrafish was immediately removed from the chamber and placed in the recovery beaker for 1 h.

ZEBRAFISH HYPOXIA STAGES

The zebrafish hypoxia stages were identified based on previous research (Braga et al. 2013). Stage 1 features regular fish movements for 2-3 min. During stage 2, the fish loses its posture for another 3-4 min. During the third stage, the fish lie at the bottom of the tank, making short movements and opercular beats. The fish will die if left for another 2-3 min at stage 3. In stage 3, the fish is removed from the hypoxia chamber after 1 min (60 s) and placed in a recovery beaker.

DRUG TREATMENT AND GROUPS

The zebrafish were divided into three groups (control, injured, and agonist), with six zebrafish per group. Firstly, the zebrafish was individually immersed in 30 mg/L benzocaine until it stopped moving. The fish was removed from the beaker, weighed, and intraperitoneally administered PNU 282987 (Sigma, USA) at 10 mg/mL using a Hamilton syringe (MICROLITER, Hamilton Co., USA). Upon completion of the treatment, the zebrafish were kept in the beaker for 40 min before beginning hypoxia induction (Kinkel et al. 2010; Kundap et al. 2017).

LOCOMOTOR ACTIVITY ANALYSIS

The behaviour and locomotor activities of the zebrafish were captured using a Sony Digital Handycam video camera (HDR-PJ340E) and analysed with the EthoVision XT (Noldus) program (Noldus, Netherlands). After 1 h in the recovery beaker, the locomotor activities of all the groups were examined using a novel tank, as detailed in a study (Braga et al. 2013). The tank, measuring 23.9 cm × 28.9 cm × 15.1 cm in height, was constructed from plastic and divided into three horizontal sections (bottom, middle, and top). The tank was filled with 10 L of normal fish water and positioned on a stable benchtop to minimise environmental disturbances. All the fish were kept in the behaviour tank for 24 h before the experiment to reduce the novel tank effect. After the experiment, each fish from the different groups (control, injured, and agonist) was individually transferred to the tank, and its behaviour was captured and recorded for 6 min (n = 9). The locomotor activities of different zebrafish groups were assessed using a Sony Digital camera, and the analysis was performed using the EthoVision XT (Noldus) program. The total distance travelled, maximum acceleration, meandering, and turn angle were analysed.

BRAIN EXTRACTION

All zebrafish were euthanised in benzocaine at 30 mg/L., and the skin and skull bones covering the zebrafish head were removed (Makhija & Jagtap 2014). Then, the dissection was performed by cutting at the junctional level between the spinal cord and the brain stem. The brain stem was gently lifted with an insect pin, and the ventral roots of the cranial nerves were cut. The optic nerve, olfactory lobe, and cranial nerve were sectioned with small scissors from the whole brain. The brain was carefully extracted using micro-dissecting tweezers and the tips of insect pins. For gene expression analysis, the zebrafish brains were quickly transferred to a pre-chilled 2 mL microtube containing 200 µL of ice-cold TRIzol and then stored at -80 °C for further investigation.

MITOCHONDRIAL DEHYDROGENASE ASSAY

TTC (2,3,5-triphenyltetrazolium chloride) staining quantifies the amount of formazan produced by mitochondrial dehydrogenase activity in living tissues, indicating the presence of viable tissue (Makhija & Jagtap 2014). The absorbance per tissue dry weight (g) was normalised as a percentage compared to the control. The brain damage of fish that survived the hypoxia treatment and recovered within 1 h was investigated. Freshly dissected brains were transferred to a 2 mL tube containing 1 mL of PBS (pH7.4) (VWR Life Science AMRESCO® USA) containing 2% tetrazolium chloride (TTC) dissolved solution (Sigma-Aldrich, St. Louis, MO, USA). The brain was incubated with TTC at 37 °C for 40 min in a dark environment (Makhija & Jagtap 2014). The TTC solution

was then removed and replaced with 10% formalin (pH7.4) to stop the reaction. The brains were dried at 40 °C for 2 h and weighed individually on a scale. Each brain was transferred to a 96-well plate with 200 µL DMSO, shielded from light. The plates were shaken continuously for 4 h to dissolve the formazan produced by TTC. The pink-to-red formazan eluate was measured at 490 nm using a microplate reader and reported as absorbance per tissue dry weight (g).

EXTRACTION AND cDNA SYNTHESIS FOR THE EXAMINATION OF INFLAMMATORY MARKERS EXAMINATION

The mRNA isolation was carried out using the protocol provided by the kit's manufacturer (Qiagen, United States), as described in another study (Lee et al. 2022). The zebrafish brain was first homogenised in TRIzol® to extract mRNA. The obtained mRNA was then quantified using a Nanodrop Spectrophotometer and converted into cDNA with the Qiagen Reverse Transcription Kit (USA).

REAL-TIME PCR

The gene expression levels were determined using real-time quantitative RT-PCR (Applied Biosystems, USA). The QuantiNova SYBR® Green PCR Kit and appropriate Qiagen primer sets were used for each gene, as described by Choo et al. (2018). The genes studied included TNF-α, IL-10, IL-6, NFκB, and STAT3. Eukaryotic translation elongation factor 1 alpha 1b (eef1a1b) was the reference gene. The following are the pre-designed and optimised primers used in the qRT-PCR analysis.

TNF-α: Dr_tnfa_1_SG QuantiTect Primer Assay (Cat no. QT02097655).

IL-6: Dr_il6_1_SG QuantiTect Primer Assay (Cat no. QT02096990).

IL-10: Dr_il10_1_SG QuantiTect Primer Assay (Catno. QT02063922).

NF-κB: Dr_nfkb1_2_SG QuantiTect Primer Assay (Cat no. QT02498762).

STAT3: Dr_stat3_1_SG QuantiTect Primer Assay (Cat no. QT02177126).

eef1a1b: Dr_eef1a1b_2_SG QuantiTect Primer Assay (Cat no. QT02042684).

The PCR began with a 2-min incubation at 95 °C, followed by the thermal cycling phase. The thermal cycling parameters for the PCR were 40 cycles, with each cycle consisting of 95 °C for 5 s and 60 °C for 15 s, as per the manufacturer's instructions. To calculate the relative expression level (fold change) of the genes of interest, the threshold cycle (Ct) values from these genes were normalised against the Ct value of the housekeeping gene eef1a1b. The formula for the calculation of gene expression is as follows:

$$Gene\ expression\ level = .2^{(Ct\ eef1a1b - Ct\ Gene\ of\ interest)}$$

STATISTICAL ANALYSIS

Statistical analysis for the behavioural study was conducted using Microsoft Excel and GraphPad Prism. The data were presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was employed, followed by Tukey's post-hoc test, with significance levels denoted as $*p < 0.05$ and $**p < 0.01$ compared to the control group.

RESULTS

GENE EXPRESSION OF INFLAMMATORY MARKERS

Zebrafish exposed to hypoxia can develop a variety of inflammatory molecular changes in their brains. Hypoxia increased mRNA levels of proinflammatory cytokines ($p < 0.05$), including TNF-α, IL-6, and NF-κB (Figure 1(A)-1(C)), but did not affect anti-inflammatory cytokines, specifically IL-10 (Figure 1(D)). Pre-treatment with PNU 282987 at a 120 mg/kg concentration before hypoxia reduced TNF-α gene expression ($p<0.05$), indicating that it reduces brain inflammation. PNU 282987 significantly reduced proinflammatory cytokines, such as TNF-α, IL-6, and NF-κB ($p < 0.05$), compared to the hypoxia group, but did not affect the anti-inflammatory marker IL-10. Interestingly, STAT3, which has a dual function, either pro-inflammatory or anti-inflammatory, is significantly increased ($p<0.05$) by hypoxia-reperfusion but is not substantially affected by PNU 282987 (Figure 1(E)).

BRAIN DAMAGE AND SURVIVAL RATE

As shown in Figure 2(A), hypoxia-reoxygenation significantly reduced cerebral mitochondrial activity by 25% compared to the control ($P<0.05$). PNU 282987 treatment did not substantially improve mitochondrial dehydrogenase activity. Interestingly, PNU 282987 increased zebrafish survival rates after hypoxia ($P<0.05$), as shown in Figure 2(B), but did not improve brain damage. The survival rate is calculated as the percentage of zebrafish that survived the hypoxia treatment. The fish survival rate increased from 52% in the hypoxia-alone group to 79% in the agonist group.

BEHAVIOR ANALYSIS

Following a 1-h recovery period, a 6-min open tank test was used to assess behaviour. The zebrafish's swimming patterns in the tank showed a significant decrease ($P<0.05$) in turned angle (Figure 3(A)) and distance travelled (Figure 3(B)) in the hypoxia group compared to the control group, indicating that hypoxia is detrimental to their locomotion. However, no significant difference was found in maximum acceleration (Figure 3(C)) in response to hypoxia. Pre-treatment with α7nAChR agonist did not reduce the impact

of hypoxia on the distance travelled or turn angle. A similar trend was observed for meandering (a movement with no fixed direction or course), which increased significantly after 1 h of recovery ($P < 0.05$) (Figure 3(D)). However, there was no significant difference between the hypoxia-reoxygenation and agonist-treated groups.

DISCUSSION

The study used zebrafish to investigate the neuroprotective and anti-inflammatory effects of α7nAChR activation as a pre-treatment before ischemia-reoxygenation (I/R) injury. Parameters examined include changes in inflammatory markers, viable brain tissue, and locomotor analysis. PNU 282987 treatment significantly reduced the elevated expression of proinflammatory markers, including TNF-α, IL-6, and NF-κB. STAT3, which has anti-inflammatory and proinflammatory roles, was dramatically increased by I/R injury but not by PNU 282987 pre-treatment. IL-10, an anti-inflammatory marker, was not significantly affected across groups. The survival rate of zebrafish increased in

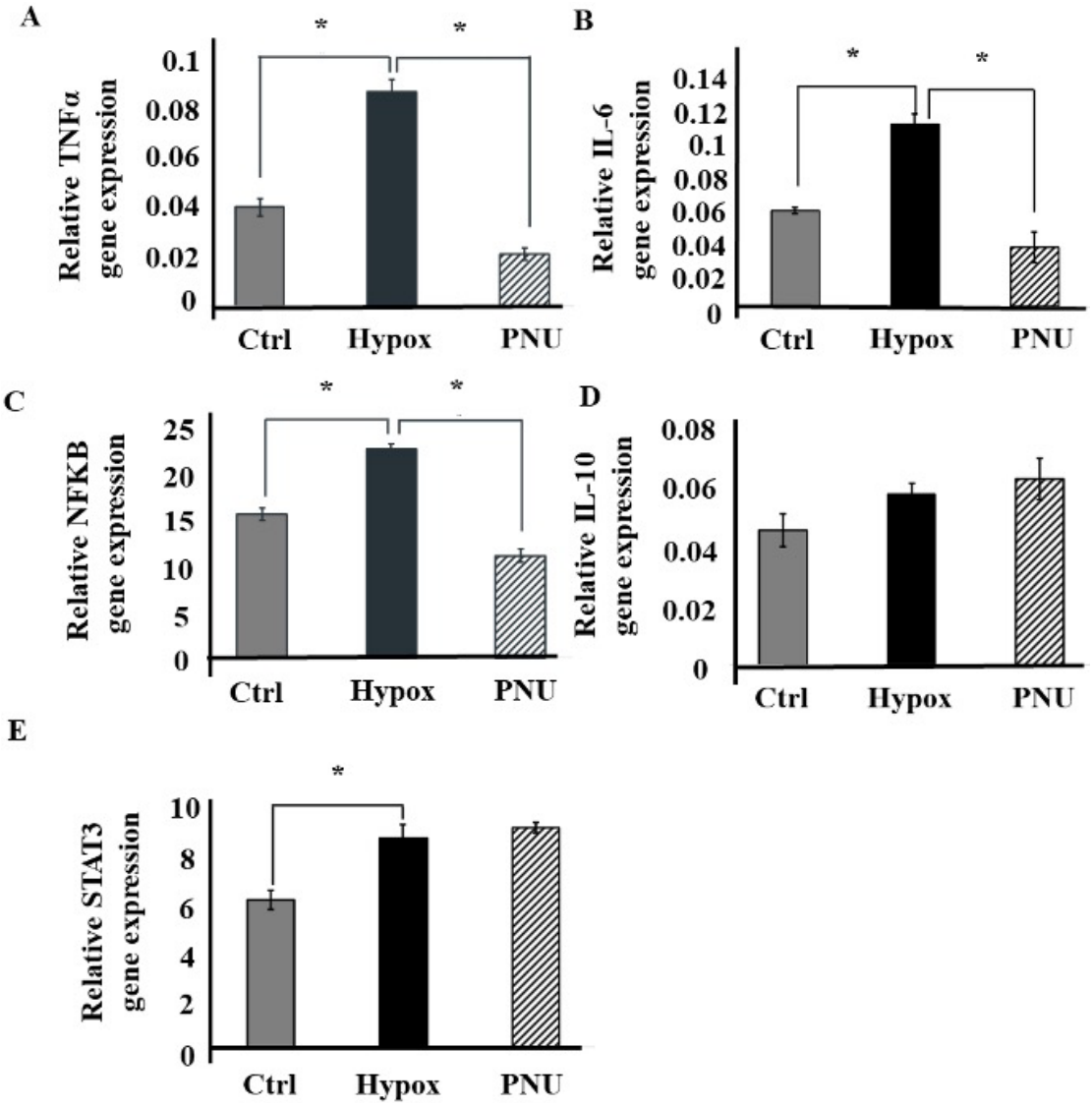

FIGURE 1. Gene expression of inflammatory and anti-inflammatory markers using q-RT-PCR. A) TNF-α B) IL-6 C) NFκB mRNA expression increased in response to hypoxia-reoxygenation compared to control, significantly reduced gene expression levels when pre-treated with 120 mg/kg of PNU 282987. D) The IL-10 mRNA level showed no difference in response to hypoxia and agonists compared to the control. E) STAT3 mRNA expression increased in response to hypoxia and showed no significant difference in response to the agonist. Statistical significance was measured by one-way ANOVA; Values are presented as mean ± S.E.M. (n=6),* for $p < 0.05$

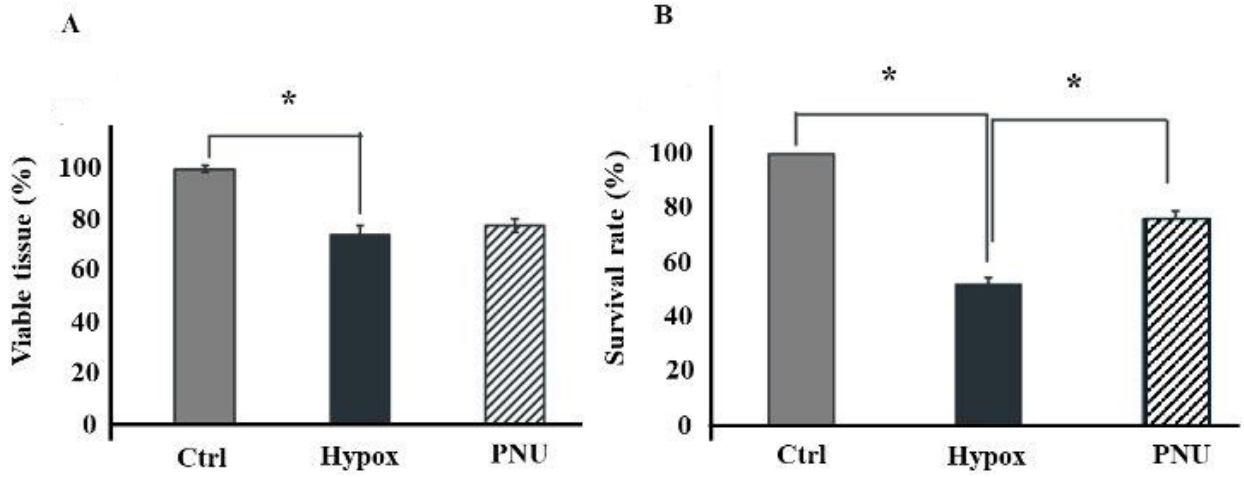

FIGURE 2. A) Brain damage (mitochondrial dehydrogenase activity) measured by absorbance/ brain weight (g%) % in zebrafish in response to hypoxia and agonist, along with the hypoxia group as compared to the control post-1-h recovery. Data represent means ± S.E.M (n = 6). Statistical differences are represented by * $p < 0.05$ level (one-way ANOVA followed by Tukey's post hoc test). B) Survival rate of zebrafish in response to hypoxia and pre-treatment with agonist and hypoxia. Values are presented as mean ± SEM (n=23), * for $p < 0.05$

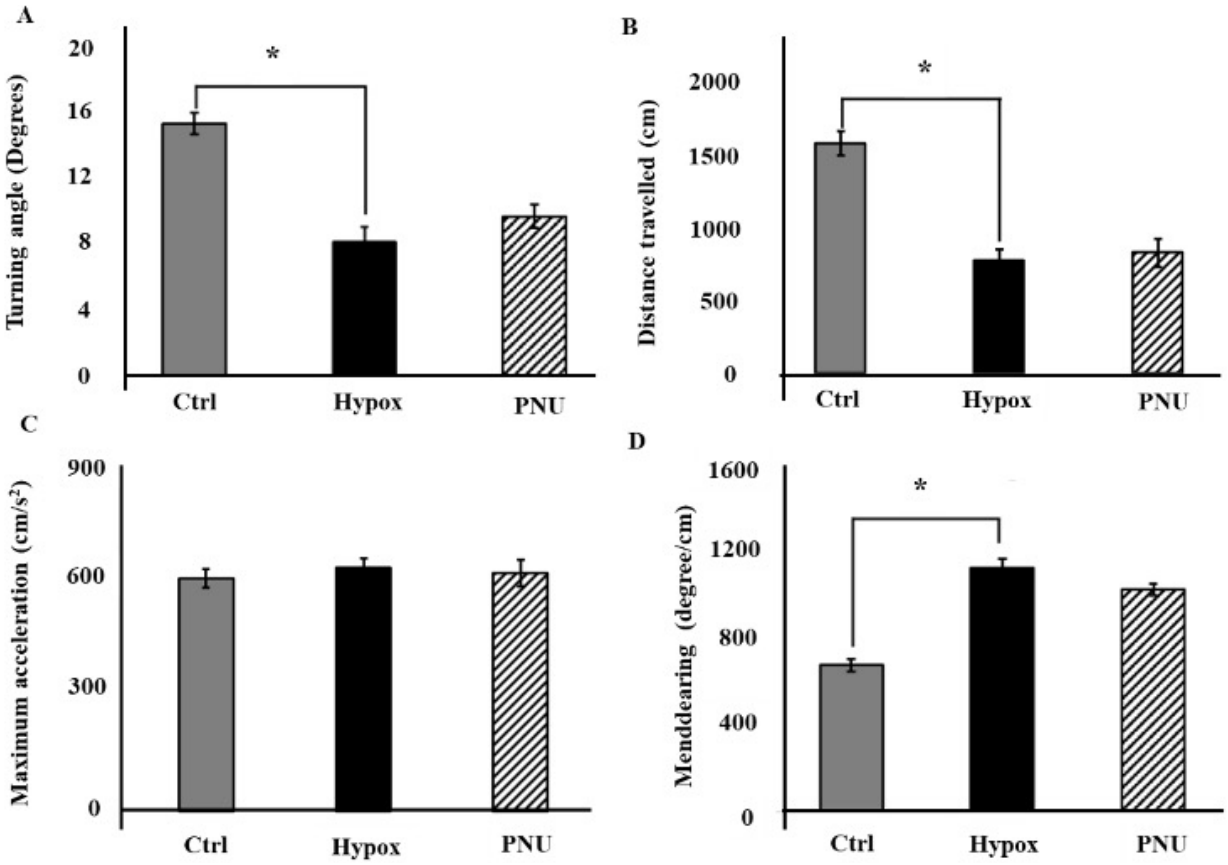

FIGURE 3. Open tank test (6 min) following a 1-h recovery interval to assess basic locomotor parameters showing the effects on A) Turn angle, B) Distance travelled, C) Maximum acceleration, and D) Meandering using EthnoVision XT - Video tracking software (n=9). Data represent means ± S.E.M. statistical differences at $p < 0.05$ (one-way ANOVA followed by Tukey's post hoc test)

the agonist-treated group. Nevertheless, viable brain tissue and locomotor analysis in the surviving zebrafish showed no significant effects.

NEUROPROTECTIVE AND ANTI-INFLAMMATORY POTENTIAL OF α7nAChR ACTIVATION IN PREVENTING CEREBRAL ISCHEMIC REPERFUSION INJURY

The primary goal of thrombolytic therapy is to limit cerebral ischemic injury, with a primary focus on safeguarding the penumbra region (Jia et al. 2024). Preserving the neurological function of the penumbra region holds excellent significance for rehabilitating ischemic stroke. Nevertheless, previous studies in animal models with cerebral ischemic reperfusion injury showed elevated neuroinflammation (Guo et al. 2019; Yang et al. 2024). Inhibition of inflammatory cascades plays a significant role in reducing cerebral ischemia/reperfusion (I/R) injury and improving recovery outcomes (Yang et al. 2024). This study performed pharmacological examinations exploring the role of α7nAChR activation in zebrafish with cerebral I/R injury, as this receptor has been recognised to possess an anti-inflammatory property in I/R conditions (Hasan et al. 2024). We investigated the impact of PNU 282987 on the inflammatory profiles and behaviour of zebrafish post-hypoxia injury.

Inflammation plays a crucial role in cerebral ischemic reperfusion injury. Initially, we investigated the anti-inflammatory potential of α7nAChR activation in the zebrafish brain under hypoxia-reoxygenation conditions. Exposure to hypoxia (dissolved oxygen levels < 0.8-2 mg/L) for 8-10 min resulted in a survival rate of 52% among the zebrafish. The mortality rate of zebrafish in response to hypoxia varies across different studies, depending on the genetic makeup (Almeida, Bianchini & Marins 2013), developmental stage (Barrionuevo, Fernandes & Rocha 2010), and the presence of additional stressors, such as heat (Lim & Bernier 2023). After a recovery period of 1 h, we analysed only the brains of the surviving zebrafish for inflammatory markers. Our results indicated that mRNA levels of TNF-α and IL-6 were significantly elevated in the hypoxia group, while treatment with PNU 282987 reduced these inflammatory markers. However, the anti-inflammatory marker IL-10 showed no effects in response to hypoxia or PNU 282987 treatment. Given that α7nAChR is a critical modulator of cytokine release, we employed its selective agonist, PNU-282987, to mitigate the potential exacerbation of inflammation caused by hypoxia-reoxygenation (Hasan et al. 2024). Our findings demonstrate that PNU 282987 reversed the expression of IL-6 and TNF-α in the zebrafish brain following the I/R injury. In line with these results, we also observed that PNU 282987 reduced levels of NFκB, a critical transcriptional regulator of these genes (Yuan et al. 2020). Previous studies have similarly reported that the activation of α7nAChR negatively regulates NFκB activation and reduces the production of inflammatory cytokines in various central nervous system cells, including microglia (Hasan et al. 2024).

We further investigated the behaviour and brain injury to explore the neuroprotective effect of PNU 282987 treatment. We examined turn angle and mendearing to quantify the disorientation and erratic movement patterns associated with stroke-induced neurological damage (Chavda et al. 2021; Lee et al. 2015). In addition, we assessed the maximum acceleration as stroke injury will notably impair the ability to perform rapid movements (Chavda et al. 2021). The general locomotor activity and endurance were examined by measuring the total distance travelled, which was diminished in the stroke-induced zebrafish (Chavda et al. 2021). Our findings indicated a significant increase in the survival rate of zebrafish subjected to hypoxia in the PNU 282987 pre-treated group compared to the hypoxia-only group, suggesting a neuroprotective role for PNU 282987 against cerebral ischemic reperfusion injury. Interestingly, PNU282987 did not mitigate the deficits in locomotion or the loss of viable brain tissue resulting from I/R injury. This finding contrasts with previous evidence, where activation of α7nAChR receptors effectively reduced infarct size, neuronal death and improved motor skills in ischemic stroke models in mice (Aguado et al. 2023; Wang et al. 2023). Variations in the timing of agonist treatment, the duration of the recovery phase, the specific disease model employed, and interspecies differences potentially contribute to the discrepancies observed. While this study represents the first examination of cerebral reoxygenation injury in a zebrafish model, we suggest that future research investigate locomotor activity after extending the reoxygenation phase to more than 1 h.

ROLE OF STAT3 AND NFκB IN α7nAChR-ACTIVATED ANTI-INFLAMMATORY PATHWAY

NFκB is a crucial signalling molecule activated rapidly in response to cerebral ischemia-reperfusion (I/R) injury. NFκB plays a significant role in modulating inflammatory processes upon activation, creating a positive feedback loop that intensifies inflammation and ultimately leads to organ damage. Notably, a study indicated that NFκB protein levels were elevated in the ischemic penumbra 24 h following reperfusion. Previous investigations have established that the activation of NFκB p65 exacerbates brain injury after both permanent focal and global ischemia (Clemens et al. 1997; Gabriel et al. 1999; Nurmi et al. 2004). Our research demonstrated that the upregulation of NFκB gene expression in response to hypoxia-reperfusion injury was significantly inhibited by pre-treatment with PNU 282987, as illustrated in Figure 1(B). A corresponding decrease in the expression levels of proinflammatory cytokines, including TNF-α and IL-6, accompanied this inhibition. These findings suggest that PNU 282987 treatment exerts anti-inflammatory effects, potentially inhibiting the NF-κB signalling pathway, which indicates a mechanism for α7nAChR-mediated anti-inflammation.

A previous study demonstrated that activation of the α7 nicotinic acetylcholine receptor (α7nAChR) initiated the cholinergic anti-inflammatory pathway (CAP) via STAT3 signalling, a negative regulator of inflammatory responses (Zhu et al. 2018). Investigations conducted in macrophages showed that activating the α7nAChR receptor leads to an upregulation of STAT3 phosphorylation and an increased interaction with the p65 subunit of the NF-κB complex (Báez-Pagán, Delgado-Vélez & Lasalde-Dominicci 2015). In our study, we aimed to explore the role of STAT3 in α7nAChR activation in adult zebrafish brain tissue. Despite using a different model, contrary to prior findings, we observed a significant increase in the mRNA levels of STAT3 in response to hypoxia. At the same time, pre-treatment with an α7nAChR agonist did not alter STAT3 levels compared to hypoxia alone.

Conflicting evidence exists regarding the role of STAT3 in cardioprotection, with some studies indicating that activation of the STAT3 signalling pathway does not consistently correlate with cardiovascular protection (Harhous et al. 2019). During the initiation of inflammatory cascades associated with atherosclerotic plaque formation, STAT3 activation promotes the expression of chemoattractant proteins that recruit macrophages within human vascular endothelial cells (Zegeye et al. 2018). However, in a hypertension model, which predisposes individuals to stroke, inhibition of STAT3 activity *in vitro* was shown to prevent the formation of proinflammatory macrophages in response to increased endothelial stretch (Loperena et al. 2018). Additionally, there are divergent findings regarding the direct regulation of TNF-α production in macrophages following lipopolysaccharide (LPS) stimulation by STAT3 and its phosphorylation (Agbanoma et al. 2012; Wall et al. 2009). This inconsistency may be partially attributed to the production of various cytokines that can interact with one another and influence complex signalling pathways (Lee et al. 2009; Samavati et al. 2009). Therefore, it is crucial to elucidate the multifaceted role of the STAT3 pathway in mediating the anti-inflammatory effects associated with α7nAChR activation during cerebral ischemia-reperfusion injury.

LIMITATIONS AND FUTURE STUDY

While this study has offered insights into the mechanisms associated with α7nAChR activation in response to ischemic reperfusion injury and the impact of hypoxia on inflammatory responses in zebrafish, certain limitations remain in the experimental outcomes. A key question concerns the use of the zebrafish model in cerebral ischemia-reoxygenation injury. The application of adult zebrafish to study brain damage caused by ischemia-reperfusion injury remains in its infancy, with no standardised model established to date. For instance, some studies suggest that zebrafish exhibit resistance to hypoxic conditions, whereas others have successfully induced hypoxic injury in this species (Barrionuevo, Fernandes & Rocha 2010; Braga et al. 2013; Zhang et al. 2024). Nonetheless, the zebrafish model for ischemia-reperfusion (I/R) injury remains valuable due to its advantages in high-throughput screening, reproducibility, and ease of induction. These features contrast with more complex, higher-order models, such as rodents, in which cerebral I/R injury is well-established through middle cerebral artery occlusion. Although rodent brain anatomy more closely resembles that of humans, enabling comprehensive behavioural assessments including locomotor, sensorimotor, and cognitive functions, the zebrafish model offers distinct practical benefits for screening experimental applications (Yu & Li 2011).

Additionally, the lack of specific antibodies for zebrafish necessitated numerous experiments conducted at the mRNA level exclusively. Further validation through experiments at the protein level is essential, particularly regarding cytokine expression. Furthermore, we based our observations on whole brain tissue; thus, future studies should focus on the responses of specific cell types involved in cerebral ischemic injury, such as macrophages and microglia. Investigating the role of α7nAChR activation within these brain cells may enhance our understanding of the anti-inflammatory potential of these agonists more effectively.

A pertinent question is whether zebrafish's behaviour or locomotor activities are independent of molecular changes in the brain. Our findings indicate that locomotor deficits persist despite improving brain inflammation following pre-treatment with PNU 282987. Thus, it suggests that locomotor activity and inflammation may not be directly correlated, implying that this drug could be a therapeutic strategy to reduce inflammation. Similar observations were reported by Lee et al. (2018), where behavioural changes did not improve following the administration of glucosamine, despite notable molecular improvements and neuroprotective effects in hypoxia-induced neuroinflammation.

Future research should involve the administration of various agonists of α7nAChR at different time points to further explore the anti-inflammatory mechanisms involved. Prolonged exposure to these agonists may elicit additional neuroprotective effects. Such investigations would provide a valuable foundation for the development of therapeutic agents aimed at mitigating hypoxia-induced brain damage. Further research is warranted to elucidate the molecular mechanisms underlying α7nAChR activation in zebrafish in response to ischemic injury. In conclusion, pre-treatment with PNU 282987 effectively reduced inflammation in the zebrafish brain following ischemia-reperfusion injury. The anti-inflammatory effect can potentially be mediated by inhibiting the NFκB pathway.

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*Corresponding author; email: kaisanmahadi@ukm.edu.my