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SIRT2 inhibition: A novel approach to modulate TLR4/NF-κB and Nrf2 pathways in post-ICH neuroprotection

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Keywords: intracerebral hemorrhage; SIRT2; inflammation; oxidative stress; TLR4; NF-κB; Nrf2; HO-1; NQO1; stroke therapy

Abbreviations
ICH – intracerebral hemorrhage
LPS – lipopolysaccharide

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Abstract

Background and purpose: *Intracerebral hemorrhage (ICH) is a severe form of stroke associated with high morbidity and mortality. Secondary injury mechanisms, including neuroinflammation and oxidative stress, exacerbate neuronal damage. SIRT2, a NAD+-dependent deacetylase, is implicated in inflammatory and antioxidant responses. This study investigates the role of SIRT2 inhibition in modulating the TLR4/NF-κB inflammatory pathway and the Nrf2 antioxidant pathway in an in vitro post-ICH model.*

Materials and methods: *SH-SY5Y neuronal cells were exposed to lipopolysaccharide (LPS) to induce inflammation and hydrogen peroxide (H_2O_2) to simulate oxidative stress. AGK2, a selective SIRT2 inhibitor, was used to assess its effects on these pathways. Western blotting analyzed the expression of SIRT2, TLR4, NF-κB, Nrf2, HO-1, and NQO1, while real-time PCR quantified antioxidant (HO-1, NQO1) and pro-inflammatory cytokine (TNF-α, IL-6) gene expression.*

Results: *SIRT2 inhibition significantly reduced TLR4 and NF-κB expression while promoting Nrf2 activation and antioxidant gene upregulation. The combination of H_2O_2 and LPS increased pro-inflammatory cytokines, but SIRT2 inhibition mitigated this response, indicating its regulatory role in inflammation and oxidative stress.*

Conclusions: *SIRT2 inhibition effectively modulates inflammatory and antioxidant responses in an in vitro post-ICH model. Targeting SIRT2 may represent a potential therapeutic approach to mitigate secondary brain injury in ICH by balancing neuroinflammatory and oxidative stress pathways. Further studies are needed to explore its clinical relevance in hemorrhagic stroke.*

INTRODUCTION

Intracerebral hemorrhage (ICH) remains one of the most disturbing forms of stroke, affecting almost 10–15% of all stroke cases, yet it is responsible for a disproportionately high percentage of stroke-related illness and death worldwide ***(1)***. The primary insult in ICH results from the sudden rupture of cerebral blood vessels, leading to the accumulation of blood in the brain parenchyma *(2)*. However, it is the secondary injury mechanisms, which include inflammation, oxidative stress, and neuronal apoptosis, that play a pivotal role in determining the extent of neurological damage and patient outcomes post-ICH ***(3)***.

Among the molecular pathways driving secondary brain injury, inflammation and oxidative stress have emerged as key contributors to neuronal degeneration and functional deficits following ICH *(4, 5)*. These processes are primarily orchestrated by signaling pathways such as TLR4/NF-κB-mediated inflammatory responses and Nrf2-dependent antioxidant defenses *(6, 7)*. Toll-like receptor 4 (TLR4) has an important role in detecting damage-associated molecular patterns released from necrotic cells, triggering a flow of inflammatory reactions through triggering of the nuclear factor-kappa B (NF-κB) pathway. Pro-inflammatory cytokines TNF-α and IL-6 are produced as a result, which have role in worsen neuronal injury ***(8)***.

Conversely, nuclear factor erythroid 2-related factor 2 (Nrf2) functions as a key regulator of antioxidant pathways. Oxidative stress causes Nrf2 to migrates to the nucleus, where it triggers the transcription of genes for antioxidant enzymes heme oxygenase-1 (HO-1) and NAD(P)H quinone dehydrogenase 1 (NQO1). This pathway is very important as it lessen oxidative damage and encourages cell survival *(9)*.

Given the interconnected nature of inflammation and oxidative stress in the pathogenesis of ICH, therapeutic strategies that can modulate both processes are highly desirable. Recent research has increasingly focused on sirtuins, a family of nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases, which are involved in various cellular processes, including metabolism, stress resistance, and inflammation *(10)*. Among the seven mammalian sirtuins, SIRT2**,** which predominantly resides in the cytoplasm, has attracted significant attention for its role in regulating inflammation and oxidative stress in the central nervous system (CNS) ***(11)***. SIRT2 is known to deacetylate and inactivate NF-κB components, thereby suppressing inflammation. Additionally, it deacetylates FOXO3a, promoting the expression of antioxidant enzymes, highlighting its dual regulatory role in balancing inflammation and oxidative stress *(12, 13)*.

While SIRT2's role has been explored in multiple models of CNS inflammation—including Parkinson's disease, Alzheimer's disease, and ischemic stroke *(14–16)* – its specific role in the context of post-ICH neuroinflammation remains underexplored. Previous studies have demonstrated that SIRT2 is upregulated in numerous neurodegenerative conditions such as Parkinson's disease, Alzheimer's disease, and stroke ***(17)***. These discoveries propose that targeting SIRT2 may represent a favorable therapeutic strategy for reducing inflammation and oxidative stress in neuroinflammatory conditions such as ICH.

While the role of SIRT2 in modulating these pathways has been explored in other neuroinflammatory models, its specific function in the context of post-ICH neuroinflammation has not been fully elucidated. Furthermore, the potential cross-talk between the TLR4/NF-κB and Nrf2 pathways in response to SIRT2 inhibition has not been extensively studied, particularly in models of hemorrhagic stroke. Understanding this cross-regulation could provide important insights into how inflammation and oxidative stress are interconnected in post-ICH pathophysiology and offer novel therapeutic targets for mitigating secondary brain injury.

The objective of this study was to investigate the effects of SIRT2 inhibition on the interplay between inflammatory and oxidative stress pathways in an in vitro model of post-ICH neuroinflammation**.** Using LPS (to mimic inflammation) and H_2O_2 (to simulate oxidative stress) in neuronal cells, we assessed the impact of SIRT2 inhibition on the expression of key inflammatory markers (TLR4, NF-κB) and antioxidant proteins (Nrf2, HO-1, NQO1). By dissecting the dual role of SIRT2 in these pathways, we aim to determine whether SIRT2 inhibition could represent a viable strategy to mitigate secondary brain injury following ICH.

By elucidating the dual regulatory role of SIRT2 in these pathways, we aim to determine whether its inhibition offers a viable strategy to alleviate secondary injury after ICH. This investigation is crucial as it addresses a break in the literature by directing on the cross-regulation of inflammation and oxidative stress in the context of ICH and explores the potential of targeting SIRT2 as a therapeutic intervention for hemorrhagic stroke. Moreover, this study adds to the increasing research on the therapeutic side which involves targeting of sirtuins in neuroinflammatory conditions and offers a new possibility for the improvement of treatments aimed at reducing the long-term neurological deficits associated with ICH.

MATERIALS AND METHODS

Cell culture techniques for SH-SY5Y cells

SH-SY5Y human neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified incubator containing 5% CO_2.

Drug treatments

For experimental treatments, SH-SY5Y cells were seeded at a density of 2×10^5 cells per well in 6-well plates (for protein and RNA extraction) and 5×10^3 cells per well in 96-well plates (for viability assays). After an initial 24-hour adherence period, cells were exposed to either 1 µg/mL lipopolysaccharide (LPS) (triggering inflammation), or 200 µM hydrogen peroxide (H_2O_2) (triggering oxidative stress). AGK2 (10 µM) was used as the SIRT2 inhibitor, administered 2 hours prior to LPS and H_2O_2

co-treatment. In the presence or absence of AGK2, cells underwent co-treatment with LPS (1 μg/mL) and H_2O_2 (200 μM) to mimic post-ICH conditions.

MTT assay for cell viability

Cell viability was assessed using the MTT assay. SH-SY5Y cells were plated in 96-well plates at a density of 1×10^4 cells per well and treated with different concentrations of AGK2 (1.562, 3.125, 12, 25, 50, and 100 μM), LPS (0.5, 1, 2, 5, 10, and 20 μg/mL), and H_2O_2 (50, 100, 200, 300, 500, and 1000 μM). For the co-treatment with LPS and H_2O_2, a combination of 1 μg/mL LPS and 200 μM H_2O_2 was used. After 24 hours of treatment, MTT assay was carried out.

SDS-PAGE and western blotting for protein expression

To detect the protein levels of SIRT2, TLR4, NF-κB, Nrf2, HO-1, NQO1, Keap1, and GAPDH, SH-SY5Y cells were harvested and lysed. Western blotting was then conducted as per previously established protocols. The following primary antibodies were used:

- SIRT2 (Rabbit polyclonal, Cat. No. ab67299, Abcam)
- TLR4 (Mouse monoclonal, Cat. No. sc-293072, Santa Cruz Biotechnology)
- NF-κB p65 (Rabbit polyclonal, Cat. No. 8242, Cell Signaling Technology)
- Nrf2 (Rabbit polyclonal, Cat. No. ab62352, Abcam)
- HO-1 (Mouse monoclonal, Cat. No. ab13243, Abcam)
- NQO1 (Rabbit polyclonal, Cat. No. ab2346, Abcam)
- Keap1 (Rabbit polyclonal, Cat. No. 10503-2-AP, Proteintech)
- GAPDH (Mouse monoclonal, Cat. No. MAB374, Millipore)

Proteins were visualized using an enhanced chemiluminescence (ECL) substrate (SuperSignal™ West Pico PLUS, Thermo Fisher Scientific, Cat. No. 34580). Protein levels were quantified relative to GAPDH and expressed as fold changes compared to control samples.

Real-time PCR for mRNA expression of antioxidant and inflammatory genes

Total RNA was extracted from SH-SY5Y cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using a reverse transcription kit. Quantitative real-time PCR was performed using (Applied Biosystems StepOnePlus). Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the reference gene. Results were presented as fold change relative to control.

Data analysis

All experiments were conducted in biological triplicate, with data expressed as mean ± standard deviation (SD). Statistical analyses were carried out using GraphPad Prism software version 9.0, employing one-way ANOVA followed by Tukey's post hoc test to determine significance. A p-value of less than 0.05 was regarded as statistically significant.

RESULTS

Effect of various drug treatments and their combination on cell viability

The effect of varying concentrations of AGK2 on SH-SY5Y cell viability was assessed using the MTT assay. Treatment with AGK2 alone demonstrated a dose-dependent reduction in cell viability, as shown in Figure 1A. At lower concentrations (1 μM and 5 μM), the cell viability remained relatively high, with minimal cytotoxic effects. However, as the concentration increased to 12 μM, a significant reduction in cell viability was observed, with approximately 89% of cells remaining viable compared to the control group ($p < 0.05$). Concentrations of AGK2 above 12 μM resulted in further reductions in cell viability, with 50 μM showing a marked decrease to 65% viability. Based on these results, 10 μM AGK2 was selected as the optimal concentration for further experiments due to its moderate effect on cell viability while providing sufficient inhibition of SIRT2.

In Figure 1B, the impact of different concentrations of H_2O_2 on SH-SY5Y cell viability was examined to simulate oxidative stress conditions. As expected, H_2O_2 caused a dose-dependent reduction in cell viability. Cells treated with 100 μM H_2O_2 exhibited a modest reduction in viability, maintaining approximately 91% viability relative to the untreated control. However, when the concentration was increased to 200 μM, a significant decrease in cell viability was observed, with viability dropping to around 87 % ($p < 0.05$). Concentrations of 500 μM and above resulted in severe cytotoxicity, with less than 50% of the cells remaining viable. Therefore, 200 μM H_2O_2 was chosen as the optimal concentration to induce oxidative stress in subsequent experiments, as it provided a balance between oxidative stress induction and cell viability.

The effect of LPS, a known inflammatory stimulus, on SH-SY5Y cell viability was investigated in Figure 1C. Similar to H_2O_2, LPS also induced a dose-dependent reduction in cell viability. Treatment with low concentrations of LPS (0.5 μg/mL) resulted in negligible effects on cell viability, with more than 90% of cells remaining viable. However, as the concentration was increased to 1 μg/mL, a significant reduction in cell viability to approximately 85% was observed ($p < 0.05$). Higher concentrations of LPS (5 μg/mL) caused a more pronounced cytotoxic effect, showing cell viability to around 50%. Thus,

1 μg/mL LPS was selected as the concentration for inducing inflammatory stress in subsequent experiments, as it allowed for inflammation induction with manageable levels of cytotoxicity.

Figure 1D explored the combined effect of oxidative stress (200 μM H_2O_2) and inflammatory stress (1 μg/mL LPS) on SH-SY5Y cell viability. When cells were co-treated with 200 μM H_2O_2 and 1 μg/mL LPS, a synergistic reduction in cell viability was observed, with viability dropping to nearly 70% compared to untreated controls ($p < 0.05$). This dramatic reduction in cell viability suggests that the combined oxidative and inflammatory stress exacerbates cellular damage in SH-SY5Y cells, simulating the neurotoxic environment present post-ICH (intracerebral hemorrhage). These conditions were thus selected to model the combined effects of oxidative stress and neuroinflammation in subsequent experiments.

Effect of SIRT2 inhibition on TLR4, NF-κB, and Nrf2 protein levels post-ICH

In order to investigate the effect of SIRT2 inhibition on key inflammatory and oxidative stress pathways in SH-SY5Y cells under conditions simulating post-ICH, we examined how SIRT2 inhibition influences the expression of TLR4, NF-κB, Nrf2, and HO-1, markers involved in the inflammatory and antioxidant response (Figure 2).

SH-SY5Y cells were subjected to five different conditions: mock (untreated), LPS alone (1 μg/mL), H_2O_2 alone (200 μM), combined LPS + H_2O_2, and LPS + H_2O_2 with AGK2 pre-treatment. Western blot analysis was used to measure the expression levels of SIRT2 and key proteins involved in inflammation (TLR4, NF-κB) and oxidative stress pathways (Nrf2, HO-1).

Treatment with LPS alone significantly upregulated TLR4 (~220%) and NF-κB (~210%) compared to mock, indicating a robust inflammatory response, while changes in Nrf2 and HO-1 were not significant. H_2O_2 alone induced a moderate increase in NF-κB (~130%) and a strong upregulation of Nrf2 (~180%) and HO-1 (~200%), reflecting oxidative stress activation, but the increase in TLR4 relative to mock was not significant. The combination of LPS + H_2O_2 resulted in maximal induction of SIRT2 (~200%), TLR4 (~230%), and NF-κB (~220%), while also moderately increasing Nrf2 (~160%) and HO-1 (~180%).

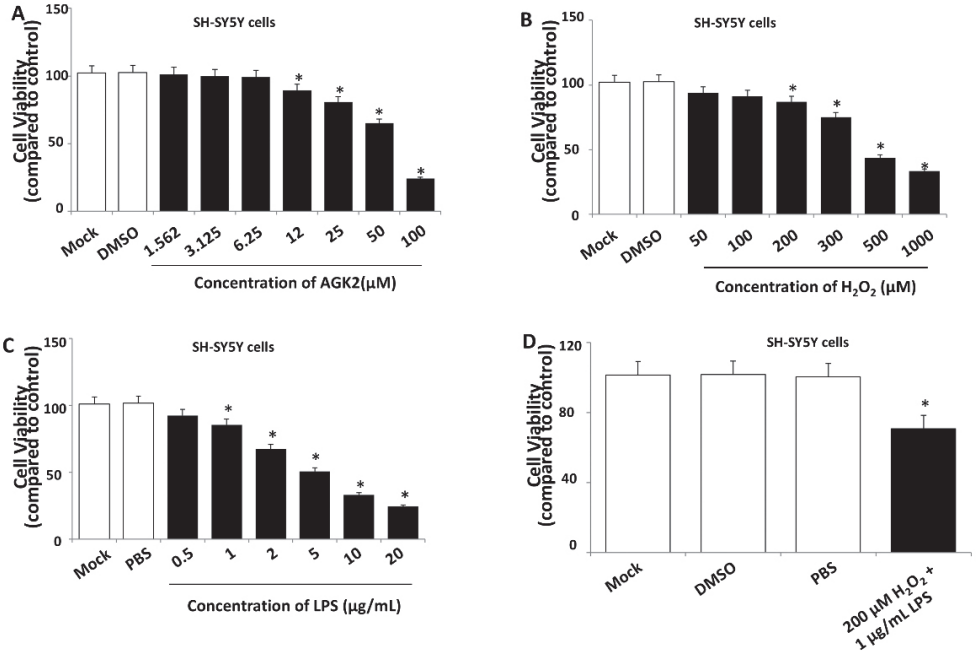

Figure 1. ***Effect of various drug treatments and their combination on SH-SY5Y cell viability.*** *(**A**) SH-SY5Y cells were treated with DMSO or increasing concentrations of AGK2 (1.562 μM, 3.125 μM, 6.25 μM, 25 μM, 50 μM, and 100 μM) for 24 hours. Cell viability was assessed using the MTT assay. The control group consisted of untreated SH-SY5Y cells. (**B**) To simulate oxidative stress, SH-SY5Y cells were exposed to various concentrations of H_2O_2 (50 μM, 100 μM, 200 μM, 500 μM, and 1 mM) for 24 hours. Viability was measured using the MTT assay, with untreated cells serving as the control. (**C**) SH-SY5Y cells were treated with LPS at different concentrations (0.5 μg/mL, 1 μg/mL, 2 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL) for 24 hours to induce inflammatory stress. Cell viability was evaluated using the MTT assay, and untreated cells were included as the control. (**D**) For the combined treatment, SH-SY5Y cells were co-treated with 200 μM H_2O_2 and 1 μg/mL LPS for 24 hours. Cell viability was assessed using the MTT assay. The untreated group served as the control. All experiments were conducted in biological triplicate (three independent experiments). Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test for multiple group comparisons. A p-value of less than 0.05 was considered statistically significant. Asterisks (*) indicate statistically significant differences ($p < 0.05$).*

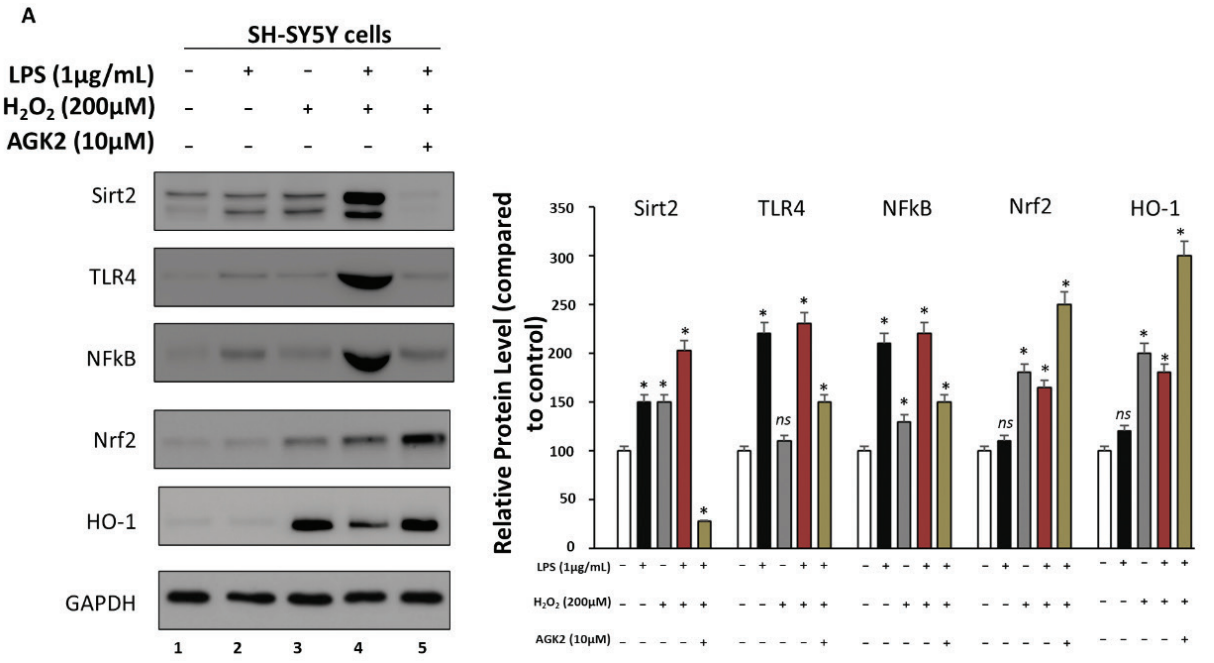

Figure 2. ***Effect of SIRT2 inhibition on TLR4, NF-κB, and Nrf2 pathways in SH-SY5Y cells under post-ICH conditions.*** *(**A**) Representative Western blot analysis showing the expression of SIRT2, TLR4, NF-κB, Nrf2, and HO-1 proteins in SH-SY5Y cells subjected to five experimental conditions: Mock (untreated), LPS alone (1 μg/mL), H_2O_2 alone (200 μM), LPS + H_2O_2, and LPS + H_2O_2 with AGK2 (10 μM) pre-treatment. GAPDH was used as the internal loading control. (**B**) Densitometric quantification of protein bands normalized to GAPDH and expressed as relative expression (%) compared to Mock control (set at 100%). Each bar represents mean ± SD from three independent biological replicates. Statistical analysis was performed using one-way ANOVA, followed by Tukey's post-hoc test for multiple group comparisons. A p-value of less than 0.05 was considered statistically significant. Asterisks (*) indicate $p < 0.05$ versus Mock; "ns" indicates non-significant differences.*

Importantly, SIRT2 inhibition with AGK2 markedly reversed these trends: SIRT2 protein levels dropped to ~40%, TLR4 and NF-κB were reduced to ~160% and ~150%, respectively, and antioxidant markers Nrf2 and HO-1 were significantly elevated to ~250% and ~300%, respectively, compared to mock.

In summary, these findings demonstrate that LPS and H_2O_2 individually activate distinct arms of the cellular stress response – inflammation and oxidative stress, respectively – while their combination simulates the dual stress of post-ICH conditions. SIRT2 inhibition by AGK2 exerts a protective effect by downregulating inflammatory mediators (TLR4, NF-κB) and enhancing antioxidant defenses (Nrf2, HO-1), suggesting its potential as a dual-target therapeutic strategy for mitigating post-ICH secondary brain injury.

Impact of SIRT2 inhibition on oxidative stress and inflammatory markers

Next, Real-time PCR was performed to quantify the mRNA expression levels of key antioxidant genes (HO-1, NQO1) and pro-inflammatory cytokines (TNF-α, IL-6) to assess the impact of SIRT2 inhibition on oxidative stress and inflammatory responses (Figure 3). This analysis provided insights into the therapeutic potential of SIRT2 inhibition under simulated post-ICH neuroinflammatory and oxidative stress conditions. The results demonstrated that SIRT2 inhibition via AGK2 significantly enhanced the expression of antioxidant genes, with HO-1 and NQO1 levels further upregulated compared to the LPS + H_2O_2-treated group. In contrast, SIRT2 inhibition led to a marked reduction in the mRNA levels of pro-inflammatory cytokines TNF-α and IL-6, which were elevated in response to LPS and H_2O_2 treatment. These findings provide strong evidence that SIRT2 plays a dual role in regulating both oxidative stress and inflammation in post-ICH conditions. By inhibiting SIRT2, we can significantly enhance antioxidant defenses while reducing harmful inflammation, which positions SIRT2 as a promising therapeutic target for managing neuroinflammation and oxidative damage post-ICH.

Modulation of NQO1 and Keap1 protein levels via SIRT2 inhibition in a post-intracerebral hemorrhage (ICH) oxidative stress model

Next, we aimed to further understand the role of SIRT2 inhibition in modulating oxidative stress responses, particularly through key markers such as NQO1 and Keap1 in a model mimicking post- ICH oxidative stress and inflammation. While Nrf2 and HO-1 are common-

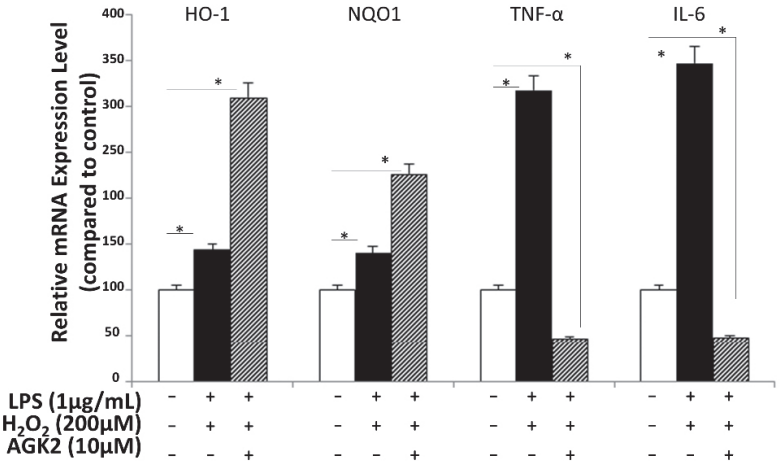

Figure 3. ***Impact of SIRT2 inhibition on oxidative stress and inflammatory markers.*** *Real-time PCR was conducted to assess the mRNA expression levels of key antioxidant genes (HO-1, NQO1) and pro-inflammatory cytokines (TNF-α, IL-6) in SH-SY5Y cells under three experimental conditions: mock treatment, LPS + H_2O_2 treatment, and treatment with the SIRT2 inhibitor AGK2 following LPS + H_2O_2 exposure. Cells were incubated for 24 hours before RNA extraction, and mRNA levels were normalized to GAPDH as a reference gene. Bar graph representation of the relative mRNA expression levels of HO-1, NQO1, TNF-α, and IL-6 under the three experimental conditions. mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH as a reference gene. Bar graph representation of the relative mRNA expression levels of HO-1, NQO1, TNF-α, and IL-6 under the three experimental conditions. Values are expressed as mean ± standard deviation (SD) from biological triplicates (three independent experiments).*

ly used to indicate antioxidant pathway activation (Figure 2), NQO1 was included to provide a more complete picture of the enzymatic defense mechanisms at play (Figure 4). Specifically, NQO1 plays a role in detoxifying reactive quinones, thus reflecting the functional output of Nrf2 activation and antioxidant response in the cells. Keap1,

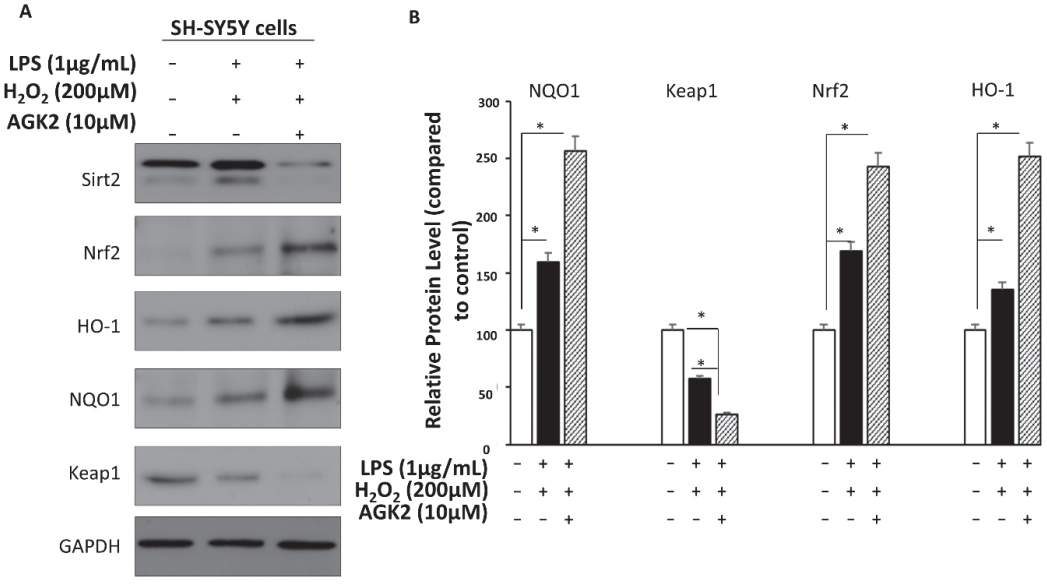

Figure 4. ***Modulation of NQO1 and Keap1 protein levels via SIRT2 inhibition in a post-intracerebral hemorrhage (ICH) oxidative stress model.*** ***(A)*** *Western blot analysis was used to assess the protein levels of NQO1 and Keap1 in SH-SY5Y cells treated under three experimental conditions: mock treatment (lane 1), co-treatment with LPS and H_2O_2 to simulate post-ICH oxidative stress (lane 2), and treatment with the SIRT2 inhibitor AGK2 following LPS + H_2O_2 exposure (lane 3). GAPDH was used as the loading control for normalization. Cells were incubated for 24 hours to mimic post-ICH neuroinflammation and oxidative stress before protein extraction.* ***(B)*** *Bar graph representation of the relative protein expression levels of NQO1, Keap1, Nrf2, and HO-1 under the three experimental conditions. Protein levels were normalized to GAPDH, and values are presented as mean ± standard deviation from three independent biological experiments. Statistical analysis was performed using one-way ANOVA, followed by Tukey's post-hoc test for multiple group comparisons. A p-value of less than 0.05 was considered statistically significant. Asterisks (*) indicate statistically significant differences ($p < 0.05$).*

on the other hand, acts as a regulator of Nrf2 by inhibiting its activation under normal conditions, and its degradation is crucial for Nrf2 pathway activation. The goal of this analysis was to determine whether inhibiting SIRT2 would potentiate the antioxidant defense by enhancing Nrf2 signaling, thereby providing cellular protection against oxidative stress in post-ICH conditions.

The results demonstrate that SIRT2 inhibition not only enhances the antioxidant defense by promoting Nrf2 pathway activation but also extends this protection by increasing NQO1 expression, an essential downstream antioxidant enzyme (Figure 4, lane 2 vs. 3). The further degradation of Keap1 suggests a stronger Nrf2 activation, allowing for a robust cellular response against oxidative stress in post-ICH conditions (Figure 4, lane 2 vs. 3). Therefore, inhibiting SIRT2 presents a dual benefit by increasing antioxidant capacity (through NQO1) and reducing inflammatory markers, positioning SIRT2 as a promising therapeutic target for managing oxidative stress and inflammation following intracerebral hemorrhage.

DISCUSSION

Neurological conditions pose a substantial global health burden, with intracerebral hemorrhage (ICH) representing one of the most severe forms of stroke due to its high morbidity and mortality. Oxidative stress and inflammation are well-established contributors to the secondary neuronal damage that follows ICH. Therefore, therapeutic strategies that can simultaneously attenuate these pathological processes are urgently needed *(18-19)*.

This study uncovers the dual regulatory role of SIRT2 inhibition in modulating both inflammatory and antioxidant signaling pathways under ICH-mimicking conditions. Using SH-SY5Y cells exposed to LPS and H_2O_2 to simulate the post-ICH microenvironment, we systematically evaluated the impact of SIRT2 inhibition via AGK2 on cell viability, key molecular mediators, and mechanistic pathways.

Initially, our viability assays (Figure 1) confirmed that 200 μM H_2O_2 and 1 μg/mL LPS together produce a synergistic cytotoxic effect, significantly reducing SH-SY5Y cell viability. This model effectively mimics the dual-stress environment of post-ICH neurons. AGK2 at 10 μM was identified as the optimal concentration to achieve SIRT2 inhibition without causing substantial cytotoxicity.

Western blotting revealed that SIRT2 inhibition under dual stress conditions markedly suppressed the expression of TLR4 and NF-κB while enhancing Nrf2 and HO-1 levels (Figure 2). These findings suggest that AGK2 not only mitigates the inflammatory cascade driven by TLR4/NF-κB signaling but also activates the antioxidant defense via the Nrf2 pathway. Densitometric analysis confirmed these changes were statistically significant.

Complementing these protein-level observations, qPCR analysis (Figure 3) demonstrated that AGK2 reduced mRNA levels of pro-inflammatory cytokines TNF-α and IL-6 while upregulating antioxidant transcripts HO-1 and NQO1. These data strongly support the transcriptional regulation of both pathways by SIRT2, in line with earlier reports suggesting that SIRT2 regulates inflammation through NF-κB deacetylation and modulates oxidative defense indirectly *(11, 13)*.

Further, analysis of NQO1 and Keap1 protein expression (Figure 4) provided additional mechanistic insight. SIRT2 inhibition not only increased NQO1, a downstream target of Nrf2, but also reduced Keap1 protein levels. As Keap1 normally sequesters Nrf2 in the cytoplasm, its degradation facilitates Nrf2 nuclear translocation and transcriptional activation *(20, 21)*. These findings suggest that SIRT2 may act upstream of Keap1-Nrf2 dynamics.

The schematic (Figure 5) shows how SIRT2 inhibition reprograms the cellular response under oxidative and inflammatory stress. H_2O_2 induces oxidative stress while LPS activates TLR4-driven inflammation. Their combination simulates a dual insult. With SIRT2 inhibition, Keap1 is degraded, permitting robust Nrf2 activation, and NF-κB signaling is suppressed, shifting the cell state toward cytoprotection.

Importantly, this model addresses the reviewer's concern regarding the phrase "oxidative stress activates TLR4." We clarify that H_2O_2 and LPS independently trigger oxidative and inflammatory stress, respectively, and their combined use simulates post-ICH pathology. This distinction is now appropriately reflected in both our results and mechanistic interpretation.

This dual modulation is clinically relevant. Current ICH therapies focus on symptomatic relief or surgical intervention but do not target the underlying molecular drivers of secondary injury. SIRT2 inhibition offers a targeted strategy to suppress inflammation while enhancing antioxidant defenses, potentially preserving neuronal function post-ICH *(6, 11, 19)*.

Previous studies have shown that SIRT2 deacetylates NF-κB p65, promoting pro-inflammatory gene transcription *(22)*, and that its inhibition can reduce neuroinflammatory burden in several models of CNS injury *(23)*. Likewise, Nrf2 activation has emerged as a key neuroprotective strategy due to its regulation of antioxidant genes *(20)*. Our data confirm that SIRT2 inhibition amplifies this antioxidant axis while attenuating inflammation.

Despite the robust in vitro data, this study has limitations. These include the absence of in vivo validation and the lack of longitudinal outcomes such as apoptosis, neu-

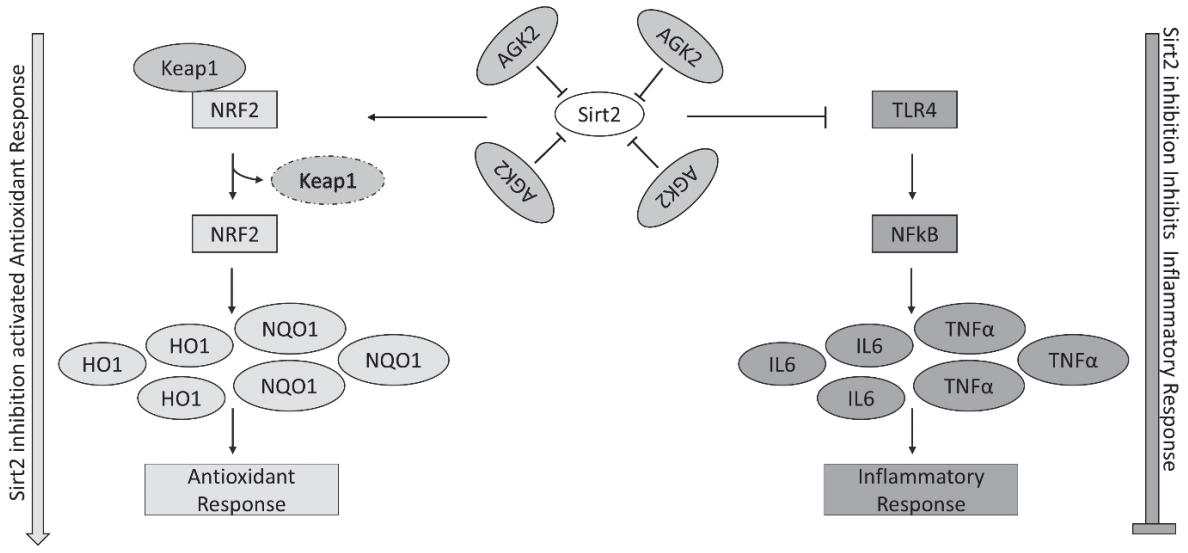

Figure 5. ***The regulation of inflammation and antioxidant defense by SIRT2 inhibition.***

ronal integrity, or behavioral recovery. Future studies should assess whether SIRT2 inhibition improves functional outcomes in rodent models of ICH or other neuroinflammatory disorders.

In conclusion, this study provides compelling evidence that SIRT2 inhibition modulates both inflammatory and oxidative stress responses in the context of ICH by regulating the TLR4/NF-κB and Nrf2 pathways. This mechanism, summarized in Figure 5, highlights the potential of targeting SIRT2 as a promising pharmacological approach for reducing neuroinflammation and oxidative injury following ICH.

This model illustrates the regulatory effects of SIRT2 inhibition on both the TLR4-NF-κB inflammatory pathway and the Nrf2 antioxidant pathway in post-intracerebral hemorrhage (ICH) conditions. Under oxidative stress induced by LPS + H_2O_2, TLR4 activation leads to NF-κB signaling and increased production of pro-inflammatory cytokines (TNF-α, IL-6). Concurrently, Keap1 inhibits Nrf2 nuclear translocation, limiting the expression of antioxidant genes (NQO1, HO-1). SIRT2 inhibition via AGK2 reduces Keap1 levels, facilitating Nrf2 translocation and enhancing antioxidant gene expression, while simultaneously suppressing NF-κB signaling and reducing pro-inflammatory cytokine levels.

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