Investigation of γ-secretase Inhibitor DAPT as a Potential Regulator of Notch Pathway in Rat's Neural Stem Cells after Spinal Cord Injury

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Abstract

Aim: Investigation of γ-secretase Inhibitor DAPT as a Potential Regulator of Notch Pathway in Rat's Neural Stem Cells after Spinal Cord Injury. **Materials and Methods:** A total of 120 male mice, aged between 3 and 4 months and weighing 30-35 g, were procured from the Experimental Animal Center of Southwest Medical University. The mice were kept in a controlled environment with a humidity range of 50-70% and a temperature range of 22-24°C, following a 12-hour light/dark cycle. The mice were allocated into three groups: Group 1, also known as the sham group, consisted of 40 mice; Group II, referred to as the I/R group, consisted of 40 mice that underwent I/R alone; and the DAPT group, which included 40 mice receiving DAPT therapy. The participants were organised into four distinct subgroups based on the duration of their respective periods: 1, 5, 10, and 15 days. **Results:** No discernible TUNEL-positive cells were seen in the spinal cord tissues of the sham group. In comparison to the sham group, a statistically significant increase in the number of TUNEL-positive cells was seen in mice subjected to spinal ischemia/reperfusion injury ($P < 0.01$). The results of the quantitative analysis revealed a statistically significant decrease in the number of TUNEL-positive neurons in the DAPT group compared to the I/R group ($P < 0.05$). The Notch1-positive signal exhibited cytoplasmic localization, whilst the nuclei were stained blue using 4′,6-diamidino-2-phenylin-dole (DAPI). In comparison to the sham group, a higher number of cells expressing Notch1 were seen in the spinal cord of the I/R group at each designated time point ($P < 0.01$). Following DAPT treatment, there was a significant reduction in the number of Notch1-positive cells as compared to the I/R group at various time intervals ($P < 0.05$). The western blot technique was used to quantify the amounts of Hes1 and Hes5 proteins in the cells of the spinal cord. In comparison to the sham group, the protein expression levels of Hes1 and Hes5 were shown to be significantly elevated in the I/R group ($P < 0.01$). The levels of IL-6 and TNF-α in the I/R group exhibited a progressive increase during the first 10-day period, followed by a subsequent drop from day 10 to day 15. Nevertheless, the levels of IL-6 and TNF-α were considerably suppressed after administration of DAPT ($P < 0.05$). **Conclusion:** In summary, DAPT demonstrates a beneficial effect on the improvement of neuronal structural damage in the spinal cord. The administration of DAPT has the potential to induce anti-inflammatory and anti-apoptotic effects via the inhibition of GFAP expression. The results of our study provide further theoretical support for the use of DAPT as a possible pharmacological intervention for spinal damage.

Keywords: DAPT, Notch Pathway, Neural Stem Cells, Spinal Cord Injury

Introduction

The elevated mortality rate and neurological impairments linked to brain trauma contribute to a significant economic burden experienced by both families and society.[1] Presently, the management techniques employed for traumatic brain injury encompass several measures, including the prevention of cerebral edoema, the mitigation of intracranial pressure, and the utilisation of conservative therapy modalities such as sub-hibernation. Nevertheless, it is important to

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acknowledge that the effectiveness of various treatment techniques has not produced satisfactory outcomes.[2] The preservation and restoration of neuronal integrity and functionality play a critical role in the therapeutic approach to brain damage. Therefore, the development of innovative neuroprotective drugs holds importance in the treatment of cerebral damage. The Notch signalling system is of significant importance in various physiological and pathological processes due to its ability to regulate intercellular contact-dependent communication, cell differentiation, proliferation, apoptosis, and cell fate determination.[3]

The binding contact between the extracellular domain of the Notch receptor and the Notch ligand, which is situated on the surface of an adjacent cell, serves as the initial step in the activation of signal transduction. The activation of the Notch receptor occurs through its binding with a specific ligand, which then induces the γ-secretase complex to initiate the enzymatic cleavage of proteins located in the transmembrane region of the Notch receptor. This enzymatic process leads to the liberation of the Notch intracellular domain (NICD) from the intracellular side of the cellular membrane. The NICD molecule demonstrates a direct process of nuclear entry upon its release, during which it interacts with the transcription factor RBP-J. This interaction results in the activation of downstream target genes, such as Hes.[4] Therefore, the γ-secretase inhibitor demonstrates the ability to specifically hinder the activation of the Notch signalling pathway.[5]

The Notch pathway is a highly conserved signalling pathway that plays a crucial role in intercellular communication and is involved in various cellular processes, such as development, tissue homeostasis, and immune response. The operating mechanisms exhibited by rats (Rattus norvegicus) are analogous to those observed in other mammalian species, including humans. The subsequent exposition provides a concise overview of the Notch signalling system in rats, as elucidated in the scholarly source denoted as reference.[6]

In the realm of rats, akin to other mammalian species, the activation of the Notch pathway entails the existence of transmembrane receptor proteins recognised as Notch receptors. It has been acknowledged that mammals possess four different Notch receptors, specifically Notch1, Notch2, Notch3, and Notch4. The Notch receptors participate in interactions with ligands that are bound to the cellular membrane of adjacent cells. The primary ligands involved in the Notch signalling pathway are Delta-like ligands (DLL1, DLL3, and DLL4) and Jagged ligands. When a Notch receptor, situated on a certain cell, interacts with its corresponding ligand, located on an adjacent cell, it triggers a series of proteolytic cleavage events. Enzymes, including γ-secretase, have a crucial function in aiding a highly critical cleavage process, wherein the Notch intracellular domain (NICD) is released from the cellular membrane. The Notch intracellular domain (NICD) participates in many interactions with transcription factors, including as CSL (CBF1/RBP-Jκ in mammals), Mastermind-like (MAML), and co-activators, within the cellular nucleus. Consequently, this complex system triggers the process of transcriptional activation in specific genes. The range of genes that are influenced by the Notch signalling pathway is subject to variation depending on the particular environment and cellular state. However, these genes frequently involve the control of cell fate, differentiation, and proliferation. Previous investigations have demonstrated that Hes (Hairy and Enhancer of Split) and Hey are among the notable target genes.[7-10]

The activation of the Notch pathway is responsible for modulating target genes, which plays a pivotal role in the determination of cell fate, encompassing differentiation and the preservation of a stem cell state.

The Notch signalling pathway is of significant importance in the process of embryonic development as it contributes to the specification of cell fate in various tissues and organs. In adult rats, as shown in other animal species, the Notch pathway continues to play a role in the regulation of tissue homeostasis, particularly in tissues marked by high rates of cell renewal, such as the gastrointestinal tract and the integumentary system. The Notch pathway is a complex and highly regulated signalling mechanism that impacts a wide range of biological processes in rodents and other mammalian species. Understanding the operational mechanisms of the aforementioned pathway holds great significance in the domains of fundamental scientific inquiry and the advancement of potential therapeutic interventions for illnesses marked by the disturbance of the Notch pathway.[11]

DAPT, scientifically defined as N-[N-(3,5-Difluorophenacetyl)-L-alanyl], is commonly utilised as a γ-secretase inhibitor in many research and experimental settings. Additionally, the compound referred to as S-phenylglycine t-butyl ester is a small molecular drug. The subsequent discourse presents an all-encompassing overview of the pharmaceutical compound known as DAPT, elucidating its role as a γ-secretase inhibitor. The γ-secretase enzyme complex is responsible for the final proteolytic cleavage of Notch receptors, which is a crucial step in the Notch signalling pathway. Inhibition of γ-secretase activity results in the prevention of the release of the Notch intracellular domain (NICD) from the cellular membrane, hence blocking future Notch signalling pathways. The utilisation of this inhibitory methodology is frequently utilised in laboratory investigations to scrutinise the implication of the Notch pathway in various biological phenomena and pathological conditions. DAPT is a chemically synthesised compound that has been purposefully designed to efficiently inhibit the activity of γ-secretase. The compound under consideration is classified as a member of the nonsteroidal anti-inflammatory medication (NSAID) group and functions as a γ-secretase inhibitor.[12-14] DAPT is a compound that acts as a competitive inhibitor of the enzyme γ-secretase. The compound demonstrates a strong attraction to the active site of the enzyme complex, thereby impeding its capacity to facilitate the cleavage of numerous substrates, including the Notch receptor.

By participating in this activity, it effectively hinders the activation of the Notch signalling pathway. Researchers

frequently employ DAPT in both in vitro cell culture and in vivo animal trials to examine the role of Notch signalling in various cellular phenomena, encompassing but not restricted to cell fate determination, developmental processes, tissue homeostasis, and pathological conditions. The evaluation of the impact of inhibiting the Notch pathway on these processes can be accomplished by employing DAPT, a compound that functions as a γ-secretase inhibitor. DAPT is frequently utilised in the investigation of the functional aspects of the Notch pathway in various medical scenarios, including cancer, neurodegenerative disorders, and other pathological conditions that may involve aberrant regulation of Notch signalling.[15-17] Moreover, this technology exhibits the capacity to manipulate the process of cellular differentiation and ascertain the fate of stem cells within an artificial laboratory environment. DAPT is frequently administered to cellular or animal specimens within a culture medium or as an essential element of experimental interventions.

It is imperative to recognise that dual antiplatelet therapy (DAPT) may manifest unintended effects, thus requiring researchers to exercise prudence and incorporate control studies in their implementation of this therapeutic modality. The compound known as DAPT, which functions as a γ-secretase inhibitor, is frequently employed in scientific research to investigate the Notch signalling pathway and its implications in various biological processes and pathological states. The mechanism by which this intervention operates involves the inhibition of cleavage of the Notch receptor, thereby hindering subsequent signalling processes. This allows researchers to investigate the impacts of inhibiting the Notch pathway in animal models.

Materials and Methods

Animals

The investigation and sample collection were conducted at the Experimental Animal Centre of Southwest Medical University. A cohort of 120 male mice, ranging in age from 3 to 4 months and weighing between 30 and 35 g, were obtained from the Experimental Animal Centre of Southwest Medical University. The mice were housed in a controlled environment, maintaining a humidity range of 50-70% and a temperature range of 22-24°C, while adhering to a 12-hour light/dark cycle. The research investigations were conducted in accordance with the regulations set forth by the Ministry of Health of China pertaining to the ethical treatment and utilisation of animals. The research protocol has been granted permission by the Experimental Animal Research Committee. During every phase of the trial, a sufficient quantity of food and beverages were readily available. The mice were divided into three groups based on allocation. Group 1, denoted as the sham group, had a total of 40 mice. Group II, referred to as the I/R group, consisted of 40 mice who exclusively experienced I/R. Lastly, the DAPT group encompassed 40 mice who were administered DAPT therapy. The individuals were categorised into four discrete subgroups according to the length of their individual time intervals: 1, 5, 10, and 15 days.

Mouse Models of Spinal Cord Injury

Prior to receiving surgery, the mice were provided with a one-week acclimatisation period at the facility. The I/R mice models were established using the approach previously described by Longa *et al*.[18]. In brief, the mice were subjected to intraperitoneal anaesthesia using a 10% (w/v) chloral hydrate solution at a dosage of 5 mL/kg and thereafter rendered immobile on the surgical table. The bilateral common carotid arteries were identified and surgically separated from the carotid sheath and vagus nerve via a midline neck incision. The surgical procedure involved the exposure and subsequent isolation of the right common carotid artery, internal carotid artery, and external carotid artery. A microvascular clamp was utilised to temporarily occlude the internal carotid artery. A minor surgical procedure was conducted in the vicinity of the bifurcation of the common carotid artery, with a length of approximately 3-4 mm. A nylon suture measuring 2.1 cm in length and with a diameter of 0.15 ±0.01 mm was meticulously introduced into the internal carotid artery. The suture's tip was rounded with the use of heat, and subsequently moved forward until it achieved total occlusion of the origin of the middle cerebral artery. The mice in the sham group had a surgical technique that closely resembled the procedures performed on the other groups, except for the omission of the suture placement. Throughout the entirety of the preoperative and postoperative procedures, strict measures were taken to ensure that the body temperature of the mice remained consistently at 37°C until the animals regained consciousness. After the mice had fully recovered from anaesthesia, they were afterwards placed in the authorised animal facilities, where they were given free availability of both food and water.

Drug Intervention

The DAPT solution (20 mg/mL; MCE Co., Monmouth Junction, NJ) was produced by the dissolution of DAPT powder in dimethyl sulfoxide. The DAPT group received an intraperitoneal injection of DAPT solution (5 mL/kg) three hours prior to the induction of middle cerebral artery blockage. The mice in the other two groups were administered a 5 mL/kg solution of dimethyl sulfoxide, as described by Wang *et al*.[19].

Neurobehavioral Assessment

As per the Longa scoring technique[18], an impartial observer assessed neurobehavioral impairments at a time point 2 hours before to sampling. The observer assigned ratings on a scale ranging from 0 to 4, while remaining unaware of the group identities. The individual has no discernible behavioural impairment.

Score 1: The left forelimb had a discernible neurobehavioral impairment of a minor kind and was unable to achieve a straightened position.

Score 2: The observed behaviour in mice exhibited a consistent inclination towards leftward circling.

Score 3: The mice exhibited spontaneous locomotion in an anticlockwise direction.

Score 4: The mice exhibited an inability to ambulate voluntarily and had a loss of awareness.

The research included mice with scores ranging from 1 to 3, whereas mice who did not exhibit behavioural abnormalities at the four designated time periods were eliminated from the study.

The Process of Preparing Paraffin Slices

In each grouping, mice were administered anaesthesia using 5% (8 mL/kg) chloral hydrate and thereafter subjected to perfusion with physiological saline via an aortic root catheter until the liver exhibited a pale appearance. This was followed by perfusion with a cooled 4% paraformaldehyde solution at a temperature of 4°C. The spinal cord was extracted and afterwards immersed in a 4% paraformaldehyde solution for a duration of one night at a temperature of 4°C. Following the process of dehydration and embedding in paraffin, 7-μm coronal slices of the spinal cord were used for immunofluorescence, Nissl staining, and TdT-mediated dUTP-biotin nick-end labelling (TUNEL) staining.

The Spinal Cord was Subjected to Nissl and TUNEL Staining Techniques.

The paraffin sections were subjected to Nissl staining and subsequently imaged using an Olympus light microscope, manufactured in Tokyo, Japan, at a magnification of 400×. The process of detecting apoptotic cells in brain tissue sections was carried out by employing the TUNEL labelling kit, in accordance with the established experimental procedure. The study conducted by Wang *et al*.[19] revealed that... The paraffin sections were subjected to a deparaffinization process, subsequently treated with a 3% hydrogen peroxide solution and TdT enzyme. Following this, the sections were treated with antibodies that were conjugated with digoxigenin. The sections were visualised using a light microscope. The quantification of cells positive for Nissl staining and TUNEL labelling was conducted using Image Pro Plus 6.0 software.

The Technique of Immunofluorescence Staining is Employed in Various Scientific Disciplines to Visualise and Detect Specific Molecules or Antigens Within Biological

In order to identify cells expressing Notch1 and GFAP in the spinal cord sections, a 30-minute incubation in 2.0 M HCl was performed to induce DNA denaturation. Subsequently, the reaction was neutralised by incubating the sections in 0.1 M boric acid for a duration of 10 minutes. Subsequently, sections of the spinal cord were subjected to a rinsing process using phosphate-buffered saline (PBS) solution containing 0.3% Triton for a duration of 30 minutes. Following this, the sections were pre-incubated in a solution of 10% normal goat serum for a period of 2 hours at room temperature. Subsequently, the sections were incubated overnight at a temperature of 4°C with polyclonal rabbit anti-Notch1 antibody (diluted at a ratio of 1:150) and monoclonal rabbit anti-GFAP antibody (a marker for astrocytes, diluted at a ratio of 1:200). Finally, the sections were incubated for 1 hour at a temperature of 37°C with Cy3-conjugated affinity purified goat anti-rabbit IgG (diluted at a ratio of 1:100) in a chamber with controlled humidity. In each spinal cord segment, cell-type specific markers, namely anti-Notch1 and anti-GFAP, were used. The quantification of cells expressing Notch1 and GFAP was performed using laser scanning confocal microscopy.

The Western Blot Test is a Widely Used Laboratory Technique in Molecular Biology and Biochemistry.

Mice belonging to each subgroup were administered anaesthesia using a 5% (8 mL/kg) solution of chloral hydrate. Subsequently, they were subjected to fast perfusion with physiological saline using an aortic root catheter. The spinal cord was expeditiously extracted and then cryopreserved at a temperature of -80°C. The spinal cord tissue was homogenised using a glass homogenizer by adding protein extraction buffer at a ratio of 1:5 (volume of buffer to weight of tissue). The protein concentrations were measured by using a bicinchoninic acid test kit. The Hes1 and Hes5 proteins were subjected to electrophoresis using sodium dodecyl sulfate-polyacrylamide gels with a stacking gel concentration of 5% and a separating gel concentration of 10%. Subsequently, the proteins were deposited onto polyvinylidene difluoride membranes. Following the application of a 5% skimmed milk powder blocking solution, the membranes were subjected to an overnight incubation at 4°C with rabbit polyclonal antibodies against Hes1 and Hes5 (diluted at a ratio of 1:200), as well as rabbit polyclonal anti-β-actin (diluted at a ratio of 1:4000). Following a 15-minute wash with phosphate-buffered saline (PBS), the membranes were subjected to an incubation period of 2 hours at room temperature with a horseradish peroxidase-conjugated secondary antibody derived from goats and anti-rabbit (1:6000) in concentration. The visualisation of proteins was conducted by using a chemiluminescence solution (Millipore) that had been improved for sensitivity. The quantification of blot intensity was performed utilising a Multi Functional Imaging System. The protein levels of Hes1 and Hes5 were determined by calculating their ratios to β-actin.

Transmission Electron Microscopy (TEM) is a Powerful Imaging Technique that Utilises a Beam of Electrons to Examine the Internal Structure and Morphology of a Sample at a High Resolution.

The use of transmission electron microscopy was employed to assess the alterations in neuronal ultrastructure inside the spinal cord in each specific subgroup. The spinal cord tissues were dissected into sections measuring 1 mm3 and promptly immersed in a 2.5% glutaraldehyde solution at a temperature of 4°C for the duration of one night. Following the washing step using a 0.1 M phosphate-buffered saline (PBS) solution at a pH of 7.4, the sections were then treated with a 1% osmic acid solution for an estimated duration of 1 hour. After subjecting the spinal cord tissues to dehydration in ethyl alcohol, they

were afterwards embedded in Epon Araldite resin. The embedded tissues were then sliced into ultrathin sections, measuring around 50 nm in thickness. These sections were then subjected to examination using transmission electron microscopy, and images were captured.

The Enzyme-Linked Immunosorbent Assay (ELISA) is a Widely Used Laboratory Technique that is Used to Detect and Quantify Certain Proteins or Antibodies in Biological Samples.

Mice from each group were slaughtered at various time intervals to obtain residual specimens. To create 10% fresh spinal cord tissue homogenates, the spinal cord was separated and cleaned using cold saline. The enzyme-linked immunosorbent assay (ELISA) was used, as per the manufacturer's guidelines, to quantify the levels of interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) produced in the spinal cord.

Statistical Analysis

The data are presented in the form of the mean value plus or minus the standard deviation. The data were analysed using SPSS 25.0 software (SPSS, Chicago, IL, USA) by an observer who was unaware of the experimental groups. A unidirectional analysis of variance (ANOVA) followed by the Student-Newman post hoc test. The Keuls test was used to evaluate the statistical significance. A significance level of $P < 0.05$ was deemed to be statistically significant.

Results

DAPT Improves Neurobehavioral Deficits

Table 1 and Figure 1 displays the neurobehavioral scores obtained from the study. The mice in the sham group did not exhibit any apparent neurological impairment. The experimental group of mice that underwent I/R exhibited a greater degree of neurological function impairment in comparison to the sham ($P < 0.01$). Following the administration of DAPT, there was a notable enhancement in neurobehavioral impairments, as shown by statistically significant results ($P < 0.05$).

Table 1. Neurobehavioral scores

	Group 1	Group II	Group III	P value
1 days	0	2.45	1.55	
5 days	0	3.63	2.14	0.003
10 days	0	3.98	2.54	
15 days	0	1.88	1.11	

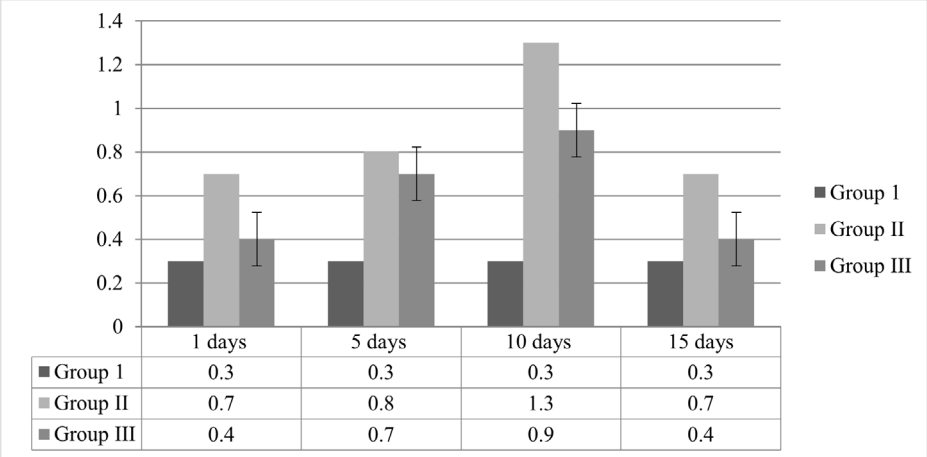


Figure 1. Neurobehavioral scores

DAPT Alleviates Pathological Injury of Neurons

The control group had neuronal structures that were within normal parameters, characterised by distinct cell boundaries, centrally located nuclei, and a plentiful presence of Nissl bodies in the cytoplasm (Table 2 and Figure 2). In contrast, the neural cells in the ischemia/reperfusion (I/R) group exhibited evident damage, characterised by the presence of reduced cell bodies, pyknosis, and fragmentation and disintegration of nuclei. In the subgroup subjected to a 1-day I/R protocol, the neurons exhibited little signs of damage. In the subgroup subjected to a 5-day I/R protocol, there was observed disorganisation of neurons and the occurrence of nuclear pyknosis. The grouping subjected to a 10-day I/R protocol had the most severe neuronal damage. The phenomenon of cellular kernel pyknosis is characterised by the presence of triangular-shaped cells. The subgroup subjected to 15-day I/R exhibited an elevation in the quantity of neurons, resulting in a modest amelioration of the inflicted damage as compared to the subgroup exposed to 10-day I/R. In contrast to the I/R subgroup, the administration of DAPT had a mitigating effect on neuronal damage in the DAPT group at various time intervals.

Table 2. Number of neurons (/field)

	Group 1	Group II	Group III	P value
1 days	5.0	3.5	4.0	
5 days	5.0	3.0	2.0	0.004
10 days	5.0	2.0	2.0	
15 days	5.0	3.0	4.0	

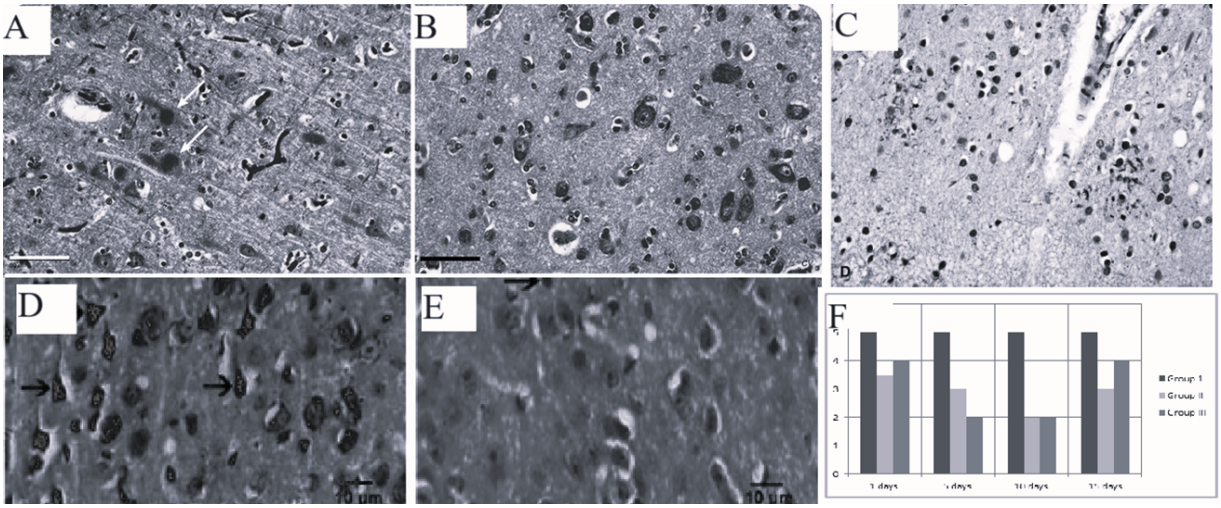


Figure 2: *Optical microscopy analysis of DAPT-treated neurons in the right prefrontal cortex of cerebral I/R mice. (A) A mouse's right cerebral cortex, with the prefrontal cortex shown by a box. (B) Con artists: Nissl bodies were plentiful in the cytoplasm, and the neurons were organised in a typical pattern. (C) subgroup with I/R lasting 1 day; (D) subgroup with I/R lasting 3 days; (E) subgroup with I/R lasting 7 days; (F) uantitation of Nissl-positive cells at 400x. Information is presented as a mean SD for the DAPT subgroup.*

DAPT Decreases Cell Apoptosis

No discernible TUNEL-positive cells were seen in the spinal cord tissues of the sham group. In comparison to the sham group, a statistically significant increase in the number of TUNEL-positive cells was seen in mice subjected to spinal ischemia/reperfusion injury ($P < 0.01$). The results of the quantitative analysis revealed a statistically significant decrease in the number of TUNEL-positive neurons in the DAPT group compared to the I/R group ($P < 0.05$). (Table 3, Figure 3 and 4) presents the data.

Table 3. Number of apoptotic cells (/field)

	Group 1	Group II	Group III	P value
1 days	0	7	4	
5 days	0	10	6	0.003
10 days	0	18	12	
15 days	0	5	4	

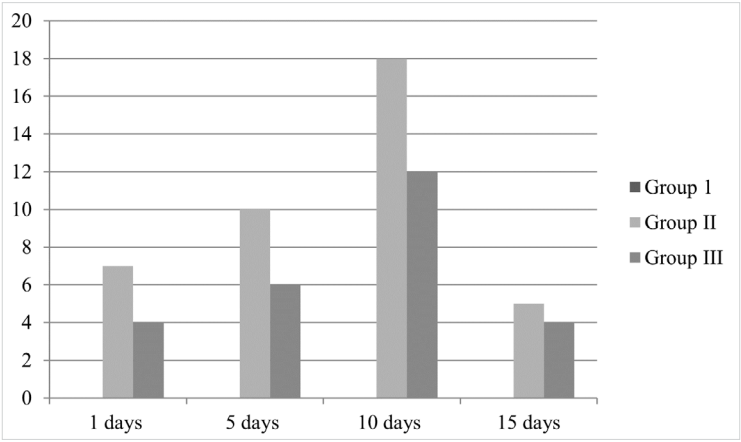


Figure 3. Number of apoptotic cells (/field)

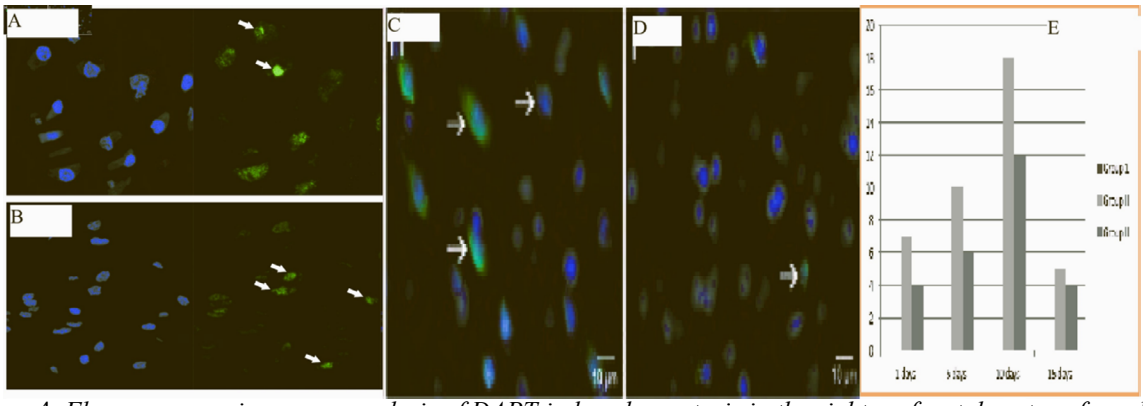


Figure 4: *Fluorescence microscopy analysis of DAPT-induced apoptosis in the right prefrontal cortex of cerebral I/R mice. TUNEL staining examples at 400x magnification (A-D). Cells that had died from apoptosis were stained green, and their DAPI-stained nuclei were stained blue. Bars represent 10 micrometres of scale.*

DAPT Decreases GFAP-Positive Cells

A small number of cells expressing GFAP were seen in the sham group. In the subgroup subjected to 1-day I/R, there was a significant increase in the number of GFAP-positive cells as compared to the sham group. In the subgroup subjected to 5 days of I/R, cells expressing GFAP had a characteristic star-shaped morphology and were found to be considerably increased compared to the control group that underwent a sham procedure. However, in the subgroup treated with DAPT, there was a significant reduction in the number of GFAP-positive cells ($P < 0.01$). Furthermore, across the four subgroups of the I/R group, there was a notable surge in the presence of GFAP-positive cells at the 10-day mark. These cells exhibited observable changes in their morphology, characterised by both thickened and shorter processes. In comparison to the subgroup subjected to a 15-day I/R protocol, the DAPT group exhibited a significant reduction in the quantity of GFAP-positive cells and a shallower intensity of cell staining ($P < 0.01$). The findings of this study demonstrated that spinal I/R damage resulted in an upregulation of GFAP-positive cells and triggered the activation of gliosis. Nevertheless, it is obvious that the administration of DAPT therapy effectively inhibited the activation of astrocytes characterised by tiny cell bodies and thin processes at four specific time periods. (Table 4 and Figure 5) is shown in the next section.

Table 4. Number of GFAP-positive cells (/field)

	Group 1	Group II	Group III	P value
1 days	2	18	14	
5 days	2	24	18	0.001
10 days	2	27	22	
15 days	2	12	10	

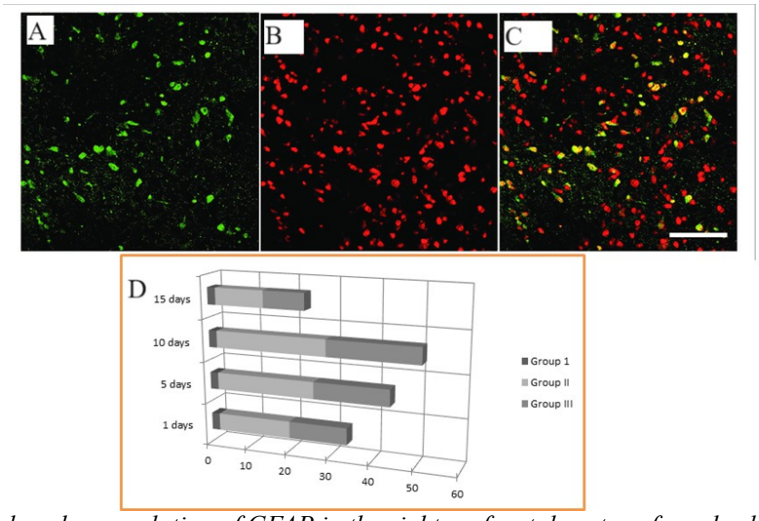


Figure 5: *DAPT-induced upregulation of GFAP in the right prefrontal cortex of cerebral I/R mice as shown by immunofluorescence microscopy. Immunofluorescence labelling of GFAP-immunoreactive cells (arrows) in the prefrontal cortex (A-I) at 400x magnification. Cells positive for GFAP were stained red, with staining concentrated in the cytoplasm and Number of GFAP-positive cells (/field)*

DAPT Reduces Notch1-Positive Cells

The Notch1-positive signal exhibited cytoplasmic localization, whilst the nuclei were stained blue using 4′,6-diamidino-2-phenylin-dole (DAPI). According to the data shown in Table 5 and Figure 6, a small number of cells expressing Notch1 were detected in the sham group. In comparison to the sham group, a higher number of cells expressing Notch1 were seen in the spinal cord of the I/R group at each designated time point ($P < 0.01$). Following DAPT treatment, there was a significant reduction in the number of Notch1-positive cells as compared to the I/R group at various time intervals ($P < 0.05$). Among the four subgroups of DAPT treatment, the quantity of cells expressing Notch1 remained consistently elevated at the 10-day mark.

Table 5. Number of Notch1-positive cells (/field)

	Group 1	Group II	Group III	P value
1 days	4	14	8	
5 days	4	16	10	0.003
10 days	4	18	13	
15 days	4	10	6	

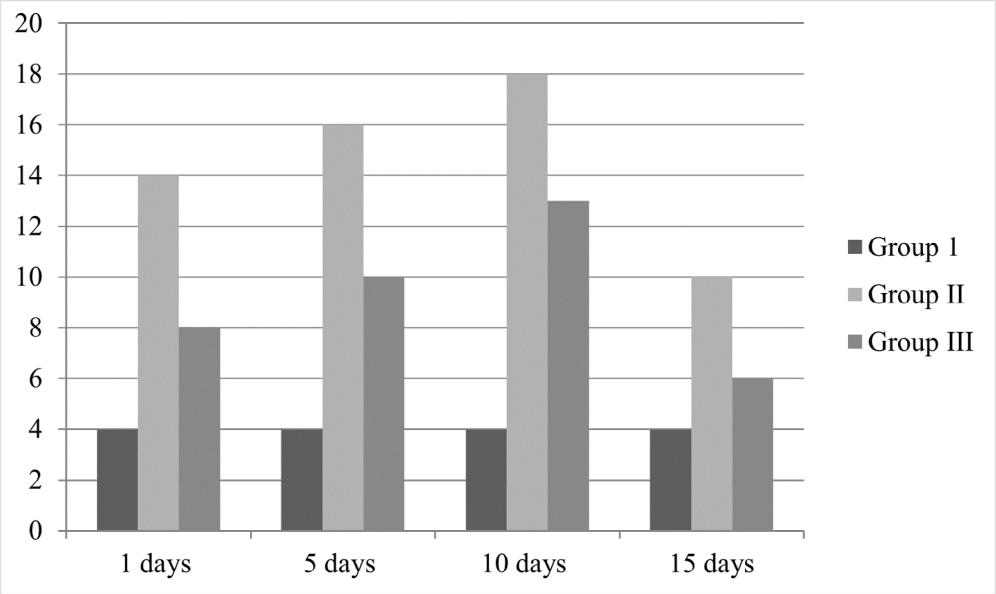


Figure 6. Number of Notch1-positive cells (/field)

DAPT Inhibits Expression of Hes1 and Hes5

The western blot technique was used to quantify the amounts of Hes1 and Hes5 proteins in the cells of the spinal cord. In comparison to the sham group, the protein expression levels of Hes1 and Hes5 were shown to be significantly elevated in the I/R group ($P < 0.01$). Nevertheless, the levels of the two proteins exhibited a significant drop in the DAPT group as compared to the I/R group at various time intervals ($P < 0.05$) (Table 6,7 and Figure 7).

Table 6. Relative protein expression of Hes1 (/β-actin)

	Group 1	Group II	Group III	P value
1 days	0.3	0.7	0.4	
5 days	0.3	0.8	0.7	0.001
10 days	0.3	1.3	0.9	
15 days	0.3	0.7	0.4	

Table 7. Relative protein expression of Hes5 (/β-actin)

	Group 1	Group II	Group III	P value
1 days	0.3	0.7	0.4	
5 days	0.3	1.1	0.7	
10 days	0.3	1.4	1.1	0.002
15 days	0.3	0.8	0.5	

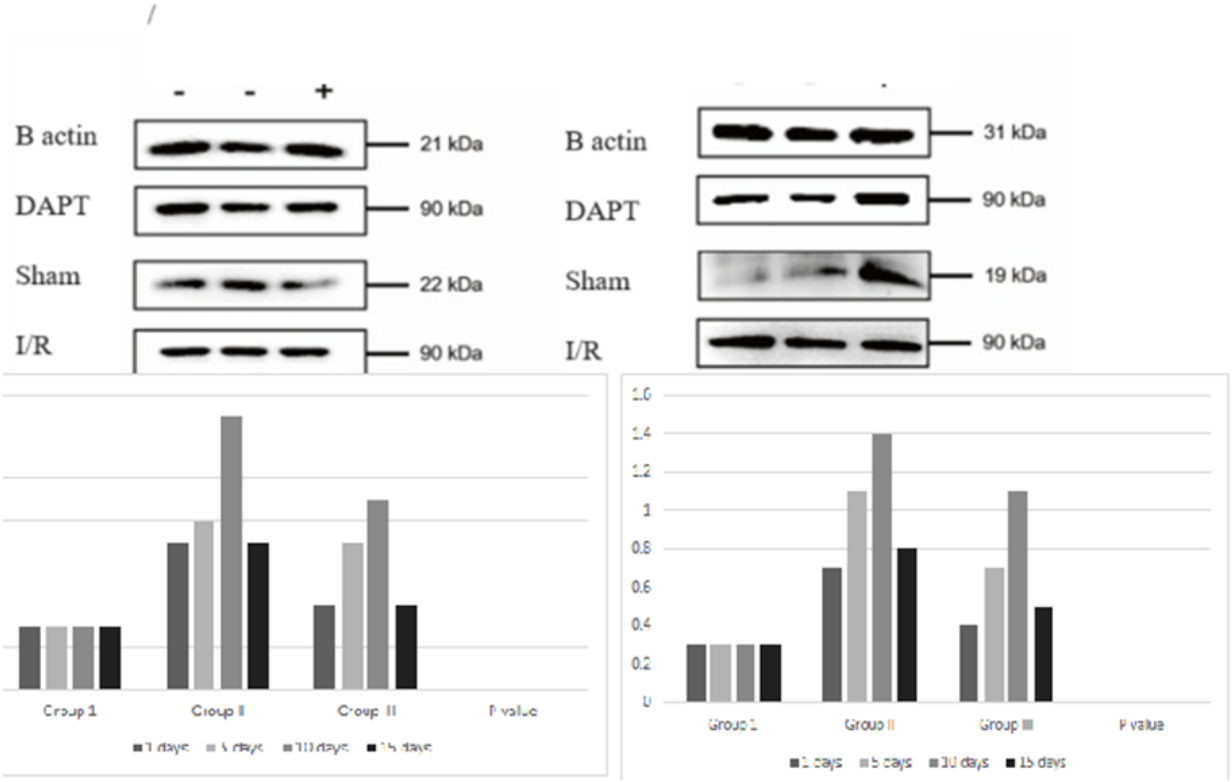


Figure 7: *Expression of Hes1 (A) and Hes5 (B) proteins in the cerebral I/R mice's right prefrontal cortex after treatment with DAPT was examined using Western blotting. The ratio of the optical density of the target protein to that of -actin was used to determine the level of expression. (n = 5 per group at each time point)*

DAPT Alleviates Ultrastructural Damage of Neurons

Inside the control group, the neurons had a substantial presence of organelles inside the cytoplasm. Nevertheless, the neurons in the spinal cord injury of the I/R group exhibited heterogeneous levels of damage at distinct time intervals. In the subgroup subjected to a 1-day I/R protocol, observations revealed disorganisation of organelles, enlargement of intracellular gaps, and a small swelling of the mitochondria. Within the subgroup subjected to a 5-day I/R protocol, the nuclei exhibited pyknosis, whereas the mitochondria displayed significant swelling. Within the subgroup subjected to a 10-day I/R protocol, the extent of neuronal ultrastructural impairment was found to be particularly pronounced, characterised by the presence of incomplete nuclear envelopes and the dissolution of organelles. In the subgroup subjected to a 15-day I/R protocol, it was observed that the cristae of some mitochondria exhibited fracturing, although the overall quantity of organelles shown an increase when compared to the subgroup subjected to a 10-day protocol.

DAPT Inhibits Production of IL-6 and TNF-α

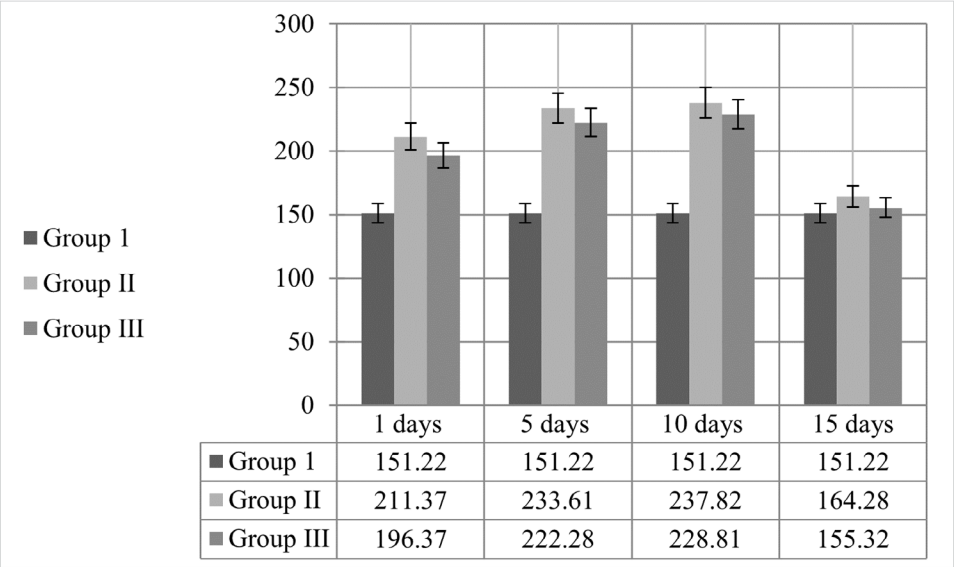
According to the data shown in Tables 8,9 and Figure 8 there was a substantial rise in the levels of IL-6 and TNF-α in each subgroup subjected to I/R, as compared to the sham group ($P < 0.01$). The levels of IL-6 and TNF-α in the I/R group exhibited a progressive increase during the first 10-day period, followed by a subsequent drop from day 10 to day 15. Nevertheless, the levels of IL-6 and TNF-α were considerably suppressed after administration of DAPT ($P < 0.05$).

Table 8. TNF-α concentration (ng/L)

	Group 1	Group II	Group III	P value
1 days	145.85	177.98	166.79	
5 days	145.85	206.74	194.58	0.001
10 days	145.85	211.63	206.61	
15 days	145.85	161.33	150.39	

Table 9. IL-6 concentration (ng/L)

	Group 1	Group II	Group III	P value
1 days	151.22	211.37	196.37	
5 days	151.22	233.61	222.28	0.002
10 days	151.22	237.82	228.81	
15 days	151.22	164.28	155.32	



	1 days	5 days	10 days	15 days
Group 1	151.22	151.22	151.22	151.22
Group II	211.37	233.61	237.82	164.28
Group III	196.37	222.28	228.81	155.32

Figure 8. IL-6 concentration (ng/L)

Discussion

A spinal cord injury (SCI) is a traumatic event of significant severity that leads to long-lasting paralysis, deficits in sensory function, neuropathic pain, and dysfunction of the bowel and bladder. From a demographic perspective, the chronic phase of spinal cord injury (SCI) is where the majority of individuals are situated, highlighting the urgent necessity for breakthroughs in treatment during this specific period. Extensive research has yielded substantial data supporting the efficacy of neural stem/progenitor cell (NS/PC) transplantation for the treatment of spinal cord injury (SCI). Nevertheless, it is commonly recognised that the optimal time frame for conducting such transplantation is during the subacute phase following the onset of the injury. The therapy of chronic spinal cord injury (SCI) presents considerable difficulties due to the existence of phase-dependent changes in the intramedullary environment, such as the formation of glial scars and cavities. Unfortunately, there is a lack of scholarly literature showcasing positive outcomes when explicitly targeting the chronic period. Indeed, numerous studies have demonstrated ambiguous findings on the effectiveness of cell transplantation in promoting significant motor function recovery among individuals with chronic spinal cord injuries. The available literature has offered limited but valuable findings regarding the intricate matter under consideration. These findings suggest that the concurrent application of neural precursor cell transplantation and chondroitinase ABC therapy, which promotes the degradation of chondroitin sulphate proteoglycans, has the potential to augment the recovery of locomotor function in individuals with chronic spinal cord injury.[18]

However, these procedures are distinguished by their intricate technical nature and limited therapeutic relevance, principally due to the requirement of prior placement of an intrathecal catheter before cell transplantation can be performed. The findings of a recent study demonstrated that the application of a gamma-secretase inhibitor (GSI), known for its ability to inhibit the Notch signalling pathway, resulted in a reduction in the proportion of actively dividing cells and an augmentation of neuronal maturation in human induced pluripotent stem cell-derived neural stem/progenitor cells (hiPSC-NS/PCs) when cultivated in vitro. The transplantation of these cells has been observed to yield substantial axonal regeneration and promote the restoration of motor function in animal models exhibiting subacute spinal cord damage. The results obtained from this study have prompted the implementation of GSI therapy as a treatment option for individuals with chronic spinal cord injury (SCI). The favourable effects on functional recovery have been demonstrated as a result of the maturation of neuronal cells derived from grafts. More precisely, it facilitates the restoration of neuronal pathways in the injured spinal cord, even when the transplantation procedure is performed during the later stages of the injury.[19-21]

The Nissl staining technique is commonly employed in the field of histology for the purpose of assessing changes in cellular morphology. Based on the results obtained from our light microscopy and transmission electron microscopy examinations, it was seen that the brain structure in the sham group appeared to be within typical ranges. Furthermore, there was a notable abundance of organelles exhibiting well-defined and distinguishable anatomical features. Anomalous distribution and organisation of neural cells were seen within the I/R cohort, along with conspicuous cellular enlargement, vacuolization, and mitochondrial degradation. Apoptosis is a notable pathogenic phenomenon that plays a significant role in the development of cerebral ischemia/reperfusion (I/R) injury. The possible benefits of preventing neuronal apoptosis include mitigating the loss of neuronal cells, reducing spinal injury resulting from ischemia/reperfusion (I/R) damage, and delaying the progression of spinal necrosis. The present study documented that ischemia/reperfusion (I/R) surgery elicited a notable augmentation in the quantity of terminal

deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-positive cells within the spinal cord. Consistent with previous studies, it has been demonstrated that spinal ischemia/reperfusion (I/R) injury can lead to substantial spinal dysfunction, increased programmed cell death, and disturbance of the typical anatomical structure.[21-23] The role of proinflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 alpha (IL-1α), in the pathogenesis of spinal cord dysfunction has been established. Tumour necrosis factor-alpha (TNF-α) is recognised as the principal pro-inflammatory cytokine among a diverse range of inflammatory factors. It demonstrates multiple mechanisms that effectively support the initiation and progression of inflammatory responses. Interleukin-6 (IL-6), a glycoprotein cytokine, is synthesised by monocytes, macrophages, and endothelial cells. The multifunctional nature of this entity is responsible for its manifestation of several biological functions. The initial phase of spinal cord ischemia/reperfusion (I/R) injury is distinguished by a significant increase in the expression of several molecules, including TNF-α and IL-1β. The current investigation documented a notable augmentation in the expression of interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) in the ischemia/reperfusion (I/R) group, aligning with previous research findings. Furthermore, in situations of spinal cord injury, there is an increased release of IL-1β, TNF-α, IL-6, and other inflammatory agents. This heightened release leads to an amplified inflammatory response, which ultimately causes permanent neuronal damage. In severe instances, apoptosis or necrosis may occur.[24-27] The Notch pathway has been recognised as a crucial modulator of homeostasis and embryonic development in various organs, such as the lung, liver, kidney, and heart.[28-30] Moreover, the Notch pathway plays a crucial role in the maintenance of immunological homeostasis by utilising its ability to enhance intercellular communication. Within the domain of mammalian cells, a comprehensive identification has been made of four distinct Notch receptors (Notch1–4) and five distinct Notch ligands. DAPT, a chemical known for its ability to block γ-secretase, has been widely acknowledged as an inhibitor of the Notch signalling pathway. In contemporary times, Dual Antiplatelet Therapy (DAPT) has garnered substantial attention in the scholarly exploration of Alzheimer's disease, atherosclerosis, and neuroblastoma. The administration of dipyridamole plus aspirin (DAPT) has demonstrated efficacy in suppressing the release of interleukin-6 (IL-6) and mitigating the activation of inflammatory responses in the context of rheumatoid arthritis. Arumugam *et al*.[7] reported that dual antiplatelet therapy (DAPT) demonstrated the capacity to attenuate neuronal injury in the localised ischemic stroke model by suppressing the activation of microglial cells.[7] Hes1 and Hes5 are essential downstream target genes in the Notch signalling pathway, performing critical functions. The co-expression of Hes1 and Hes5 has been observed to be closely linked with the expression of Notch1, a pivotal constituent of the Notch signalling cascade. Previous investigations have demonstrated that the modification of the Notch signalling pathway exerts a substantial influence on the expression levels of Notch1, Hes1, and Hes5.[31-34] The findings of our experiment offer substantiation for the efficacy of DAPT as an inhibitor, demonstrating notable attenuation of the expression of Notch1, Hes1, and Hes5. The application of Nissl staining and transmission electron microscopy methods demonstrated that the administration of DAPT led to a significant increase in the number of intact neurons, while simultaneously reducing cellular edoema and mitochondrial swelling. The experimental group that received dual antiplatelet therapy (DAPT) exhibited a statistically significant reduction in the number of apoptotic cells, as well as a drop in the concentrations of interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α). According to previous research, it has been proposed that Dual Antiplatelet Therapy (DAPT) could potentially have a positive effect on the protection of spinal tissue by suppressing the inflammatory response and decreasing apoptosis.[35]

Astrocytes, the major kind of glial cells found in the brain, are distinguished by their expression of GFAP, a significant marker protein observed in fully developed astrocytes. In addition, astrocytes play a vital role in providing comprehensive structural, metabolic, and trophic support to neurons within the brain tissue. The findings of the study indicated that following a stroke, there was a notable augmentation in the proliferation and activation of astrocytes. The cell soma displayed an increase in size, whilst the processes saw a reduction in length and an increase in thickness. Astrocytes has the capacity to promote the restoration of neural function through their capability to sequester the infarct, restore the integrity of the blood-brain barrier, and facilitate the repair of compromised neurons.[17,36,37] Additionally, the treatment of dual antiplatelet therapy (DAPT) led to a decrease in the expression of glial fibrillary acidic protein (GFAP) in neonatal rats that were exposed to hypoxic-ischemic brain injury. Hence, a growing body of evidence suggests a substantial contribution of astrocytes to the operation of the nervous system. The results of our study suggest that the administration of dual antiplatelet therapy (DAPT) led to a decrease in the expression of glial fibrillary acidic protein (GFAP) in comparison to the group that underwent ischemia/reperfusion (I/R). The administration of DAPT has the ability to hinder the gliosis process and consequently facilitate the restoration of spinal cord injury.

Conclusion

In summary, DAPT demonstrates a beneficial effect on the improvement of neuronal structural damage in the spinal cord. The administration of DAPT has the potential to induce anti-inflammatory and anti-apoptotic effects via the inhibition of GFAP expression. The results of our study provide further theoretical support for the use of DAPT as a possible pharmacological intervention for spinal damage. Nevertheless, more

research is needed in our next investigations to explore the impact of DAPT on other pathways associated with spinal damage, including reactive oxygen species and leukocyte infiltration.

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