Clinical Research

AR-A014418 as a glycogen synthase kinase-3 inhibitor: Anti-apoptotic and therapeutic potential in experimental spinal cord injury

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ABSTRACT

Objectives: We aimed to investigate the effects of AR-A014418, a strong inhibitor specific to GSK-3beta, on neuronal apoptosis and neuroprotection in the traumatic SCI model.

Materials and methods: In this study, three groups were generated from 36 Wistar rats; (1) control, (2) spinal cord trauma group created by clip compression technique after laminectomy, and (3) AR-A014418 (4 mg/kg, i.p., DMSO) treatment group after laminectomy and spinal cord trauma. The TUNEL assay for apoptosis detection, immunohistochemical staining for bax and TGF-beta were applied in spinal cord tissues. For light microscopic examination, necrotic, and apoptotic cells were counted, and PMNL counting was applied to detect inflammation. Functional recovery was tested by field locomotor test in the 3rd and 7th days following surgery.

Results: In the trauma group, diffuse hemorrhage, cavitation, necrosis and edematous regions, degeneration in motor neurons and leukocyte infiltration were observed in gray matter. In the AR-A014418-treated groups, healthy cells were observed in more places compared to the trauma groups, however, cavitation, hemorrhagic, and edematous areas were seen in gray matter. In the AR-A014418-treatment groups, the number of apoptotic cells in the 3rd and 7th days (respectively; p < 0.05, p < 0.01), were significantly decreased compared to the trauma groups, as were the levels of bax (p < 0.01) and TGF-beta 1 immunoactivity. Results of the locomotor test were significantly increased in the treatment group (p < 0.001) as compared to the trauma group.

Abbreviations: SCI, spinal cord injury; GSK-3, glycogen synthase kinase-3; DMSO, dimethylsulfoxide; PMNL, polymorphonuclear leukocytes; H&E, hematoxylin and eosin; CV, cresyl violet; TUNEL, the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

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AR-A014418 como inhibidor de la glucógeno sintasa quinasa 3: potencial anti-apoptótico y terapéutico en lesión medular

Resumen

Objetivo: Este trabajo tuvo como objetivo investigar los efectos del AR-A014418, un potente inhibidor específico de la GSQ-3beta, en la apoptosis y neuroprotección neuronales en el modelo de lesión medular traumática.

Materiales y métodos: Se generaron tres grupos a partir de 36 ratas Wistar: (1) control, (2) grupo de traumatismo medular obtenido mediante técnica de pinzamiento post-laminectomía, y (3) grupo de tratamiento mediante AR-A014418 (4 mg/kg, i.v., DMSO) post-laminectomía y traumatismo medular. Se aplicaron el test TUNEL para la detección de la apoptosis, tinción inmunohistoquímica para bax y TGF-beta en los tejidos medulares. Se llevó a cabo un examen microscópico y recuento de células necróticas y apoptóticas, así como recuento de LPMN para detectar inflamación. La recuperación funcional fue verificada mediante la prueba de campo del aparato locomotor en los días 3 y 7 después de la cirugía.

Resultados: Se observó hemorragia difusa, cavitación, necrosis y regiones edematosas, degeneración de las neuronas motoras e infiltración leucocitaria en la materia gris en los grupos traumáticos. En los grupos con tratamiento AR-A014418 se observaron células sanas con mayor abundancia en comparación con los grupos traumáticos. Sin embargo, se observaron áreas de cavitación, hemorragia y edema en la materia gris. En los grupos con tratamiento AR-A014418, el número de células apoptóticas en los días 3 y 7 (p<0,05 y p<0,01, respectivamente), se redujo significativamente en comparación con los grupos traumáticos, así como los niveles de bax (p<0,01) y las células inmunopositivas para TGF-beta 1. Los resultados de la prueba del aparato locomotor se incrementaron significativamente en el grupo de tratamiento (p<0,001) en comparación con el grupo traumático.

Conclusiones: Mediante un estudio experimental de un modelo de traumatismo medular se observó como la apoptosis neuronal era desencadenada de manera significativa por daños secundarios postraumáticos. Sin embargo, la mejora neurológica se vio acelerada por la prevención de la apoptosis mitocondrial y reducción de la inflamación causadas por el inhibidor de GSQ-3beta, AR-A014418.

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Palabras clave:
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Introduction

Spinal cord injury (SCI) can be caused by a number of injuries to the spine that result in a loss of function such as mobility or feeling. Frequent causes of damage are trauma such as car accidents, falls or gunshot, or diseases such as polio or spina bifida. Following mechanical injury to the spinal cord, primary and secondary pathological changes occur. Primary damage leads to irreversible tissue injury that is almost impossible to treat. Secondary damage amplifies the extent of initial damage and apoptosis is critical for triggering collateral damage following primary injury to the spinal cord. Depending on the trauma grade, a variety of sensory and motor neurologic deficits are seen in SCI. Total spinal cord injury is seen neurologically in almost half of the patients, however, on the other hand, 54% of patients are quadriplegic and 46% of them suffer from paraplegia. In 1911, Allen defined secondary spinal cord damage, which resulted in a change to the classical approach. Experimental and clinical studies show that neurological deficits after SCI are the result of the secondary damage due to apoptotic cascade activation after primary damage. Primary damage was shown to appear as a result of both tissue injury during trauma and neuronal necrotic cell death. On the other hand, secondary damage occurs after a chain of events as a result of activation of a variety of cell death pathways. The aim of the clinical and experimental research on neuron trauma is to prevent or to reduce the secondary damage. Although a lot of research has been done on SCI, providing a full recovery treatment on spinal cord damage has not been found yet. Apoptosis is carried out by death activators binding to cell surface receptors and triggering the mitochondria pathway. Cytochrome-c is released into the cytoplasm by the
effect of pro-apoptotic members on the mitochondria and through the formation of a complex by binding the latent cytosolic Apaf-1 protein and activation of the inactive precursor of caspase-9. The apoptotic process is carried on by the activation of caspase-9 and caspase effectors (caspase-3).\textsuperscript{7,12,14} Apoptosis plays an active part in secondary damage, and is seen as a result of central nervous system ischemia, degenerations, inflammations, and traumatic injuries.\textsuperscript{15} In the recent years, the existence of neuronal apoptosis after traumatic human SCI was reported. Apoptosis can be triggered by a variety of insults including free radical damage, releasing of cytokines, inflammatory injury, and excitotoxicity.\textsuperscript{16} The apoptosis seen on microglia deteriorates the inflammatory secondary damage.\textsuperscript{17} In experimental studies, apoptosis in oligodendrocytes is shown to aggravate the occurrence of demyelination after the first few weeks of spinal cord damage, and serious consequences are caused by increasing the loss of neural cells.\textsuperscript{5,18} In studies that looked at apoptotic cell death after spinal cord trauma, TNF\textsuperscript{19} and Fas\textsuperscript{20} were found to stimulate the receptor pathway and activate caspase-3 and -8.\textsuperscript{1,21} Release of cytochrome-c also causes caspase activation via the mitochondrial pathway by the increase of neuronal Ca\textsuperscript{2+} concentration.\textsuperscript{22}

TGF-beta is a regulatory cytokine and plays a part in brain damage, angiogenesis, inflammation, anti-inflammation, neuroprotection, and scar accumulation.\textsuperscript{23} TGF-beta is involved in wound healing after SCI and reduces the probability of secondary damage by reducing the mononuclear phagocyte accumulation around the wound.\textsuperscript{24} Glycogen synthase kinase-3 (GSK-3) is a serine-threonine protein kinase. It plays an important role in the pathophysiology of a lot of diseases. In addition, it is involved in the mechanism of neural cell death related with the disease.\textsuperscript{25,26} GSK-3 has two major isoforms; alpha and beta. GSK-3beta (tau phosphorylation kinase-1) is a serine-threonine kinase that is primarily localized at neurons, and found in brain at very high levels. Throughout growth it is present at very high levels, but its expression is reduced in adults. Active GSK-3beta cell death stimulates the signal transduction involving in neuron loss and loss of synaptic plasticity.\textsuperscript{25,26} An abnormal increase in GSK-3beta expression and activity in the adult brain is directly related with the key neuropathologic mechanism of Alzheimer’s disease.\textsuperscript{26} GSK-3 induces cell death by the intrinsic apoptotic mitochondrial pathway but inhibits the death receptor-mediated extrinsic apoptotic signaling pathways. There may be differences in the effects of each GSK-3 isoform in different types of cells, so the balance between GSK-3 isoforms may be important in regulating the extrinsic apoptosis signaling pathway. Intrinsc apoptotic signals that are activated by cell damage are induced with GSK-3. These signals cause abnormal control mechanisms of expression in anti-apoptotic or pro-apoptotic proteins during the mitochondrial pathway.\textsuperscript{27} GSK-3 is known to regulate the activity of a large number of transcription factors that control gene expression of proteins participating in the mitochondrial pathway of the apoptotic cascade. Therefore, mitochondrial function is also influenced by GSK-3 mediated regulation during intrinsic apoptotic signaling. Some of these proteins are regulated by nuclear GSK-3 where a small, but dynamic, portion of cellular GSK-3beta is located. Nuclear GSK-3beta is in a relatively greater activation state than cytosolic GSK-3beta, and apoptotic conditions can increase the level and/or activity of GSK-3beta in the nucleus.\textsuperscript{28,29} GSK-3beta inhibitors are used as key regulators in apoptosis.\textsuperscript{27} Disorders of neuro-behavioral functions are reported to be overcome by the neuroprotective effects of GSK-3beta inhibition.\textsuperscript{30}

AR-A014418 is a new member of a family of particularly effective and selective inhibitors of GSK-3beta enzyme activity. It has an ability to reduce tau phosphorylation and cell death. AR-A014418 can pass the blood brain barrier and has a powerful effect.\textsuperscript{28,31} It has been shown that treatment with TZD-8 (4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione), a potent and selective GSK-3beta inhibitor, is able to reduce tissue damage, inflammation, and apoptosis in the spinal cord injury, thereby significantly ameliorating the recovery of limb function.\textsuperscript{25} AR-A014418 is much more effective than other GSK-3beta inhibitors in the cerebral ischemia model.\textsuperscript{28,32}

In the literature, AR-A014418 has not yet been used in spinal cord injury, either clinically or experimentally. In this study, we aimed to investigate the effects of AR-A014418 on neuronal apoptosis and evaluate its neuroprotective effects in the experimental spinal cord trauma model.

**Materials and methods**

All experiments were approved by the Ethics Committee for Animal Experiments of Istanbul University and followed the NIH Guide for the Care and Use of Laboratory Animals.

**Anesthesia**

All the rats used in the study were starved a day before the operation. Anesthesia was induced by the intramuscular application of 60 mg/kg ketamine (Ketalar, Parke-Davis, Eczacbasi, Istanbul, Turkey) and 9 mg/kg Xylazine (Rompun, Bayer, Istanbul, Turkey). In case of need 20% of the initial dose was repeated intermittently.

**Surgical procedure**

The backs of the animals were shaved after anesthesia and they were placed on the operation table in the prone position. Body temperature was, as far as possible, maintained at 37°C with heating pads throughout the operation until the end of the anesthesia. After local antisepsis was applied by povidone iodine (Poviodeks, KIM-PA, Istanbul, Turkey), a skin incision was made using interscapular distance as a reference point. Subdermal tissues and the paravertebral muscle fascia were advanced. Paravertebral muscles were swabbed by blunt dissection. The thoracic 6th, 7th and 8th vertebral laminae were set forth and a total laminectomy was done. Through this procedure the dura mater was left intact. Different procedures were applied to each group through further steps of operation.
Spinal cord injury

Spinal cord trauma was performed by the vascular clip compression technique developed by Rivlin and Tator. This method can be applicable to small animals and has been reported as much more reliable than balloon compression and weight reducing methods. In this study, the thoracic 6, 7 and 8 total laminectomy was applied to set front the diameter. Afterwards, FE 781 K coded Yasargil aneurysm clips with 70 g closing power were used to constitute spinal cord trauma with 60’s application.

Experimental groups

Animals (Wistar type albino male rats) were separately divided into 3 randomized groups such that each group had 12 animals. Animals whose weights lay between 240 and 320 g were used in this study.

Group I (n = 12, sham-operated control group)

After skin incision and dissection of paravertebral muscles; the laminae were set front and total laminectomy was applied at thoracic 6–8 levels. A 3.0 silk suture was used to sew the muscles and the skin. In 6 of the rats, intracardiac perfusion was used at the third day with 10% formaldehyde. In the remaining 6 rats the same procedure was conducted on the seventh day. After the intracardiac perfusion with 10% formaldehyde was complete, the surgical zone was opened again and approximately 2 cm spinal cord was removed under microscope.

Group II (n = 12, trauma control group)

The laminectomy was applied to the animals as described in first (I) group and then the clip compression technique was used to constitute spinal cord damage. Dimethylsulfoxide (DMSO) in an equivalent dose to that of AR-A014418 was administered intra-peritoneally (i.p.) just after the trauma. As described in the first group, spinal cord samples were taken at the third and the seventh days.

Group III (n = 12, AR-A014418-treated group)

The same surgical and trauma procedures were applied to each animal in the second (II) group. A single dose of 4 mg/kg AR-A014418 (Sigma, A3230) dissolved in DMSO solution was given i.p. just after the trauma. Spinal cord samples were taken at third and seventh days as described for the first (I) group.

Neurological evaluation of functional healing of all the rats in each group at the third and the seventh days after surgical operation was done before sacrificing the animals.

Light microscopy—histopathology

Spinal cord tissues were defined by neutral formalin solution and embedded in paraffin by following the conventional light microscopy techniques. 5 µm paraffin cross-sections were taken and put on microscope slides covered with poly-L-lysine (PLL, Sigma P-8920, St. Louis, MO). The cross-sections of spinal cord tissue were stained with hematoxylin and eosin (H+E), and cresyl violet (CV) dyes for histopathological evaluation.

In order to make the statistical evaluation, polymorphonuclear leukocytes (PMNL) of 10 different areas were counted on the H+E stained tissue cross-section of each animal in every group. The average value of the PMNL number was calculated by an independent researcher to evaluate the inflammatory response in trauma groups.

Immunohistochemistry

Histostain-Plus Bulk Kit (Zymed LAB-SA DetEction System, 85-9043), rabbit polyclonal Bax (Delta Biolabs, DB005, 1:50 dilution), and rabbit polyclonal TGF-beta 1 (Santa Cruz Biotech., sc-146, 1:100 dilution) antibodies were used with the Streptavidin-Biotin Peroxidase method in order to stain the paraffin cross-sections by the immunohistochemistry stain procedure offered in the kit.

To determine the specificity of immunostaining, sections were incubated following the above procedure, except for omission of incubation with the primary antibody. Instead of primary antibody, control serum was also used as control.

Apoptosis assay (TUNEL method)

The in situ DNA end marking method was used to assess apoptosis and was conducted according to the protocol provided in the kit (ApopTag Plus, In Situ Apoptosis Detection Peroxidase kit, S7101-KIT, Chemicon). For staining specificity controls the thymus tissue of dexamethasone-treated rat was used as positive control. Distilled water was used instead of Tdt enzyme as a negative control.

Apoptotic cell count

Marked apoptotic cell counting (TUNEL) for all groups of spinal cord tissue was performed under high-power fields (original magnification 40×) with a light microscope. Apoptotic cells in 12 different unit areas were counted on the cross-sections by two independent researchers. These 12 fields were randomly selected from slides without discrimination between white and gray matter. Average cell per unit area number was found by taking the average of the results.

Evaluation of the immunohistochemical stain signals

TGF-beta 1 immunoperoxidase staining; under a microscope at 40× magnification, 15 areas were selected randomly and immunostained cells were evaluated semi-quantitatively. The results were scored as −: no staining, +: weak staining, ++: moderate staining, +++: strong staining.

Bax immunoperoxidase staining; 15 randomized areas were chosen from all gray and white substances of the spinal cord tissue, and were evaluated under a microscope at 40× magnification.
magnification by counting of the positive staining cells. Statistical comparison was conducted for each.

**Locomotor evaluation**

The locomotor test was applied to the animals according to the open field BBB scoring. The animals were observed for scoring in a 75 cm × 120 cm field at the third and the seventh days before euthanasia was carried out by two researchers.

**Statistical evaluation**

In order to evaluate the collected data statistically, the non-parametric Kruskal–Wallis test was used for comparison among the groups. If the results of Kruskal–Wallis test were significant with \( p < 0.05 \), “post hoc” analysis between two groups was performed by the Dunn test. Data are expressed as means ± SD where applicable.

**Results**

**Histopathologic findings**

In the spinal cord tissue sections stained with HE and CV, edema, hemorrhage, leukocyte infiltration, and necrosis were evaluated through histological examination. The neural and vascular structure of the spinal cord was obtained normally in control groups (Figs. 1A and 2A). Cross-sections from the third and the seventh days of trauma groups showed hemorrhage, cavitation, necrotic, and edematous areas in gray matter and degeneration in motor neurons and leukocyte infiltration (Fig. 1B and C). The third day trauma group exhibited areas containing abundant necrotic (●) and apoptotic cells (→) (Fig. 2B). The seventh day trauma group showed areas containing necrotic cells (Fig. 2C). On the third day AR-A014418-treated trauma group showed moderate cavitation areas, hemorrhagic and edematous regions, although the healthy cell populations occupied more space (Fig. 1D). In the seventh day of the AR-A014418-treated group, there were fewer hemorrhagic areas and decreased cavitation degeneration, accompanied by an increased number of healthy cells, as compared to the trauma groups (Figs. 1E and 2E).

**Evaluation of inflammation (PMNL count)**

In the third day trauma groups PMNL counts 48.86 ± 3.07 in both gray and white matter were statistically significantly higher than in other groups (\( p < 0.001 \)). In the trauma seventh day group, the PMNL count was found to be 28.74 ± 2.20. In AR-A014418 third day and in AR-A014418 seventh day the PMNL count was found to be 15.5 ± 2.36 and 5.98 ± 0.58, respectively, and average PMNL count was shown to be decreased compared to that of the trauma groups (\( p < 0.001 \)) (Fig. 1F).

**TGF-beta 1 immunohistochemical staining**

TGF-beta 1 immunopositivity was evaluated in all tissue cross-sections at all gray and white matter. Very weak (−) TGF-beta 1 immunopositivity was observed in the control group (Fig. 3A). Significant TGF-beta 1 immunopositivity (+) was observed in the trauma third day group (Fig. 3B). In the trauma seventh day group, significantly more TGF-beta 1 immunopositivity (++) was seen compared to all other groups (Fig. 3C). In the third day and seventh day of the AR-A014418-treated trauma
groups, decreased TGF-beta 1 immunopositivity was observed compared to the trauma groups (Fig. 3D and E).

**Apoptotic cell count**

In the TUNEL staining method, cell nuclei were evaluated as brown TUNEL (+). Positive apoptotic cell number marked with TUNEL per unit area was found to be 0.11 ± 0.04 in control groups (Fig. 4A and F). In trauma groups, apoptosis was commonly observed among oligodendrocytes and other glial cells at the lesion surrounding tissues (Fig. 4B and C). In the trauma third day group apoptotic cells per unit area was found to be 2.15 ± 0.45. In the trauma seventh day group, positive cell number marked with TUNEL per unit area was found to be 3.86 ± 0.58. Apoptotic cell number was found to be 1.21 ± 0.22 in the third day group of AR-A014418 and 0.81 ± 0.07 in the seventh day group that were prominently decreased compared to the trauma groups (p < 0.05, p < 0.001, respectively) (Fig. 4D–F).
**TUNEL**

![TUNEL images](image)

**Fig. 4** - TUNEL method. Control (A), in the trauma 3rd day (B) and trauma 7th day (C), a marked appearance of dark brown apoptotic cells (arrows) was demonstrated. AR-A014418-treated 3rd day (D), AR-A014418-treated 7th day (F) groups. Graphs showing the mean values of apoptotic cell numbers in the trauma groups (F); *p* < 0.05 vs. trauma 3rd days, **b** *p* < 0.05 vs. trauma 3rd days, **c** *p* < 0.01 vs. trauma 7th days.

**Bax immunohistochemical staining**

In all tissue cross-sections, Bax immunopositivity was counted by scanning of the gray and white matter. Weak Bax immunopositivity was observed in the control group, but in a very small proportion of cells (Fig. 5A). In the trauma third day group, a large number of Bax immunopositive cells were seen (94.6 ± 5.03) (Fig. 5 F). An increase in Bax immunopositive cell number was observed in trauma seventh day compared to the trauma third day (106.6 ± 7.47) (Fig. 5 F). An increase in Bax immunopositive cell number was observed in trauma seventh day compared to the trauma third day (106.6 ± 7.47) (Fig. 5 F). In the third day of the AR-A014418-treated trauma group, decreased Bax immunopositivity was observed (48.4 ± 7.76) (Fig. 5D). In the seventh day of the AR-A014418 application trauma group (31.4 ± 9.01), a significant decrease in Bax immunopositive cell number was observed compared to the control groups and AR-A014418-treated third day groups (p < 0.01, p < 0.05, respectively) (Fig. 5E and F).

**Results of functional neurological improvement**

In neurological evaluation of the subjects, average BBB scores were found to be 0.83 ± 0.75 and 0.83 ± 0.75 for the trauma third day group and trauma seventh day group, respectively. The AR-A014418-treated third day and seventh day trauma groups were found to be 1.33 ± 0.81 and 4.50 ± 1.22, respectively. A significant increase was observed in the BBB values of the AR-A014418-treated seventh day group compared to the other trauma groups (p < 0.001). An increase in locomotor abilities of both AR-A014418 groups was observed compared to the control group (Fig. 6).

**Discussion**

There are currently no available treatment options for the recovery of primary injury after SCI. Studies of acute SCI damage treatment have largely focused on preventing secondary neural damage by pharmacological treatment protocols aimed to minimize the neurological sequela.22

An increase or decrease in cell death by apoptosis is reported to play a part in the pathogenesis of a lot of diseases like cancer, autoimmune disorders, viral infections, and neurodegenerative diseases.37,38 Emery et al. was able to detect the existence of apoptosis in traumatic SCI in humans. In 14 patients out of a total of 15 who died between 3 h and 2 months after spinal cord damage, apoptotic cells were shown near viable cells that were located around the lesion center and within the adjacent white matter.39 When clip compression7 and weight reducing14 methods were used in animal models, neuronal and glial apoptosis were developed in both. Yong et al. reported apoptosis in neurons, astrocytes, oligodendroglia, and microglia of rats after SCI.40 Liu et al. determined the apoptosis in the neurons and glia within 4 h in the lesion region after the trauma. Neuronal apoptosis and glial apoptosis were significantly increased in the 8th and 24th hours, respectively. On the other hand, on the seventh day more cell death was observed with progressive enlargement of the initial lesion and its cavitation. A second wave of apoptosis was found to have occurred in white matter at the seventh day, related to axon degeneration in oligodendrocytes.5,7 In the trauma group of the SCI model, common hemorrhage, cavitation, necrosis and edema regions, degeneration in motor neurons and leukocyte infiltration were observed in the gray
Fig. 5 – Immunoreactivity of bax. Control (A); Bax immunoreactivity is stronger in the trauma 3rd day (B), trauma 7th day (C) groups compared to control group. In the AR-A014418-treated 3rd day (D) and AR-A014418-treated 7th day (E) groups immunoreactivity of bax is seen weaker compared to untreated trauma groups. Evaluation of Bax immunopositive cell numbers in the trauma groups (F); a p < 0.01 vs. Trauma 3rd and 7th days, b p < 0.05 vs. AR-A014418 3rd days.

Fig. 6 – Changes in the mean BBB scores of trauma groups (mean ± SD). * p < 0.001 vs. all groups.

matter. Some regions of necrotic and apoptotic cells were found among those. Apoptotic cells were commonly observed in cells which were located near tissues around the lesion. Apoptotic cells marked positively with TUNEL were found to be significantly increased in trauma group compared to the control group. This finding showed that apoptotic cell death plays an important part in the progress of secondary trauma.

Some of the studies have used small molecule inhibitors of GSK-3 such as arylindolemaleimide SB-216763, anilino-maleimide SB-415286 and anilino-4-arylmaleimide glycogen synthase kinase-3. However, almost all of these inhibitors are not selective for GSK-3 and affect cdk-2 and cdk-5, which share 33% amino acid homology with GSK-3. In this study, AR-A014418 (N-(4 methoxy benzyl)-N²-(5-nitro 1,3 thiazol-2yl) urea) was used after spinal cord trauma for therapeutic reasons and it is a selective and potent thiazol inhibitor to GSK-3beta. Additionally, AR-A014418 is the first member of a specific inhibitor family of GSK-3beta which does not cause inhibition of cdk-2 and cdk-5. It has been reported that the AR-A014418 has a protective effect on the N2A neuroblastom cells against the cell death via phosphatidylinositol 3 kinase and protein kinase beta. In order to find out the most effective dose of AR-A014418, comparative studies were carried out. From these studies, it was determined that a dose of 4 mg/kg provided optimum inhibition. The optimal dose of GSK-3 was shown to prevent apoptosis.

In this study, the 4 mg/kg dose was used for AR-A014418 according to the cerebral ischemia studies that were carried out before. Inhibition of GSK-3beta significantly reduced spinal cord inflammation, and tissue damage, neutrophil infiltration, NO syntheses, nitrotyrosine and COX-2 expression,
and additionally it was shown to improve motor healing scores.\textsuperscript{26} In this study, PMNL number was found to be significantly increased in the lesion region at the third day after trauma. After the use of the GSK-3beta inhibitor AR-A014418 in the trauma group, it was determined that PMNL number was significantly reduced in both trauma groups and facilitated the neurological healing.

GSK-3 can control the expression of anti-apoptotic and pro-apoptotic proteins at the intrinsic apoptotic signaling pathway. In general, it was reported that GSK-3 prevents the expression of anti-apoptotic proteins and increases the expression of pro-apoptotic proteins.\textsuperscript{27} The Bax as the proapoptotic protein plays an important role in the cell death and involves to neurodegeneration in central nervous system. Dong et al. studied the lateral cord cross-section model of spinal cord damage and reported that apoptotic death of the oligodendrocytes can be dependent on Bax activation and axonal degeneration.\textsuperscript{28} Cuzzocrea et al. reported that the apoptotic cell death was reduced with GSK-3beta inhibitor (TDZD-8) treatment after SCI. They showed that Bax expression diminished after application of the GSK-3beta inhibitor (TDZD-8) in the trauma group; however, Bcl-2 expression was shown to be increased.\textsuperscript{29} In tissue cross-sections of the AR-A014418-treated groups, healthy cells were found to be in greater number, but significantly there were areas displaying edema, hemorrhage, and cavitation. In the AR-A014418-treated third and seventh day groups, the numbers of apoptotic cells per unit area were found to be reduced compared to the trauma groups. This finding indicated that apoptotic cell death was significantly prevented by the GSK-3beta inhibitor. In this study, Bax immunopositive cells were significantly increased in the third day trauma group compared to the control group. Bax immunopositive cell numbers were increased in trauma seventh day group compared to those of the third day group. In the AR-A014418-treated seventh day trauma group, Bax immunopositive cell numbers were found to be significantly reduced compared to the third day of the AR-A014418-treated and the trauma groups. These findings support the hypothesis that use of AR-A014418 as a GSK-3beta inhibitor is able to prevent the expression of pro-apoptotic proteins, which could develop in the early stages of the mitochondrial apoptosis pathway.

TGF-beta is a powerful regulatory cytokine that is involved in a number of biological processes.\textsuperscript{30} TGF-beta is very important for inflammatory gene synthesis, which plays a role in healing after SCI, and mediates the eventual termination of inflammation. A reduction in secondary damage progress was reported by a reduction in PMNL accumulation around the lesion.\textsuperscript{31} In the SCI treated rats, the increased TGF-beta accumulation has been observed in neurons after just 24 h and moreover, this accumulation persisted even in 7th day of the trauma despite a gradual reduction. In the erythropoietin treated animals with SCI, this accumulation was determined to be significantly reduced after seven days.\textsuperscript{32} Tyor et al. determined that there was a 50\% reduction in lesion size after 48 h of spinal cord trauma compared to the untreated group as a result of TGF-beta treatment. Additionally, they reported a relative reduction in neurotoxin production with significant reduction in mononuclear phagocyte number.\textsuperscript{33} In this study, an increase in TGF-beta 1 immunopositivity was observed in the trauma third day group. It was more intensive in the seventh day of trauma compared to the all other groups. TGF-beta 1 expression in the AR-A014418-treated third and seventh day trauma groups was determined to be reduced compared to the trauma groups. In this SCI model, our findings showed that treatment with AR-A014418 controls tissue damage, reduces the pathological effects of decreasing of TGF-beta 1 expression, and contributes to the healing process in spinal cord tissues.

In this study, after the evaluation of subject neurological functions, the average BBB values obtained showed a significant increase in seventh day results of the AR-A014418 group compared to all the trauma groups. The locomotor abilities of the subjects of the AR-A014418-treated trauma group were found to be increased compared to the subjects of the trauma groups. This finding showed that administration of AR-A014418 to trauma groups was able to contribute to the healing of neurological functions after spinal cord injury.

In this experimental spinal cord trauma model, neural apoptosis was significantly triggered in secondary damage developed after trauma. However, neurological healing was expedited by treatment with AR-A014418, a GSK-3beta selective inhibitor, which acted to prevent apoptosis via the mitochondrial pathway and reduce inflammation. Our results showed that the GSK-3beta inhibitor AR-A014418 can be used to prevent the progress of trauma and reduce inflammation in the treatment of secondary spinal cord damage.

Author’s contribution

Matem Tunçdemir contributed in laboratory methods, microscopic examination, data collection, and article writing. Aziz Yıldırım, Alper Karaoğlan and Osman Akdemir contributed in surgical methods, data collection, and article writing. Melekg Öztürk contributed in laboratory methods, microscopic examination, data collection, and article writing.

Conflict of interest

The authors have no conflict of interest to declare.

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