EXPERIMENTAL STUDY

Combined and individual use of pancaspase inhibitor Q-VD-OPh and NMDA receptor antagonist riluzole in experimental spinal cord injury

Halil Can, M.D.,[1] Aydın Aydoseli, M.D.,[2] Cengiz Gömleksiz, M.D.,[1] Burcu Göker, M.D.,[3] Muhittin Emre Altunrende, M.D.,[4] Müge Dolgun, M.D.,[2] Altay Sencer, M.D.[2]

[1]Department of Neurosurgery, Medicine Hospital, Istanbul-*Turkey*
[2]Department of Neurosurgery, İstanbul University of İstanbul Faculty of Medicine, İstanbul-*Turkey*
[3]Department of Neurosurgery, Liv Hospital, İstanbul-*Turkey*
[4]Department of Neurosurgery, Gaziosmanpaşa Taksim Training and Research Hospital, İstanbul-*Turkey*

ABSTRACT

BACKGROUND: We investigated the effects of an N-methyl-D-aspartate receptor antagonist, riluzole, and a pancaspase inhibitor and basic apoptosis mediator, Q-VD-OPh, in combination or alone in posttraumatic spinal cord injury.

METHODS: In our study, 45 healthy male Sprague Dawley rats were used. Spinal trauma was induced by the clip compression technique via thoracal 7, 8, 9 laminectomies. After inducing the trauma, the drug was continuously administered intraperitoneally for 5 days. After inducing the trauma, the subjects were assessed using Tarlov's motor grading scale and inclined plane test. Five days after the trauma, the spinal cord specimens were harvested, and a histopathological examination was performed.

RESULTS: Compared with the other groups, a statistically significant difference with regard to better results for necrosis, inflammation, and apoptosis was observed in the riluzole only and combination groups. Statistically better motor function scores were observed in the Q-VD-OPh only group than in the other groups.

CONCLUSION: With regard to limiting secondary damage after trauma, statistically significant results were observed in the Q-VD-OPh only and Q-VD-OPh–riluzole combination groups. More extensive laboratory studies are required to limit and control the effects of secondary damage after spinal cord trauma.

Keywords: Apoptosis; caspases; necrosis; neuroprotection; NMDA receptor antagonist; pancaspase inhibitor; Q-VD-OPh; riluzole; spinal cord injury.

INTRODUCTION

Spinal cord injury (SCI) results in an irreversible primary traumatic damage, which is followed by a secondary damage that is mediated by different mechanisms. Researches mainly focused on preventing secondary damage mechanisms in SCI, which start at the exact moment of trauma and may continue for several weeks. Major cell death mechanisms, namely necrosis and apoptosis, are involved in secondary damage. Therefore, many experimental studies have been conducted to inhibit necrosis or apoptosis to prevent secondary damage.

Until recent years, necrosis has been accepted as the only mechanism that plays a role in secondary damage, and excitotoxicity has been indicated as the main factor in the necrosis pathway that leads to posttraumatic neural degeneration.[1,2] Glutamate is the key excitatory neurotransmitter in the central nervous system. In case of ischemia or hypoxia, the cellular energy levels decrease, causing glutamate to induce neurotoxicity by activating N-methyl-D-aspartate (NMDA) receptors.[3] At the same time, trauma-induced activation of voltage-sensitive sodium channels causes intracellular ion influx, particularly increased sodium and calcium levels; this leads to cytotoxic edema.[4] NMDA receptor antagonists in

Address for correspondence: Burcu Göker, M.D.
Ahmet Adnan Saygun Caddesi, Canan Sokak, No: 5, Ulus, Beşiktaş, İstanbul, Turkey
Tel: +90 212 - 999 84 19 E-mail: burcugoker79@yahoo.com

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experimental SCI models have been well studied to reverse excitotoxicity.[5,6] Although many NMDA receptor antagonists usually have limited clinical use because of their adverse effects, memantine, a non-competitive NMDA receptor antagonist, is clinically used for a series of cerebral disorder with minimal adverse effects. Memantine also has neuroprotective effects, which have been demonstrated in several experimental SCI models.[7–9] Besides Memantine, a sodium channel-blocking benzothiazole anticonvulsant and an NMDA receptor antagonist, riluzole, plays a neuroprotective role in preclinical SCI models.[10] Riluzole inhibits glutamate excretion at the presynaptic level and modulates glutamate transfer from synapses. Furthermore, by blocking voltage-dependent sodium channels, riluzole inhibits the G protein signaling guanylyl cyclase cascade and protects the cell from the excitotoxic effects of glutamic acid that is secreted after cell death.[11] Recent researches indicate that riluzole has a neuroprotective effect against the neurodegenerative disorder called amyotrophic lateral sclerosis (ALS). Riluzole has only minor adverse effects such as affecting serum alanine transaminase levels and has made a significant delay in the timing of tracheostomy, therefore it has been approved by FDA in the treatment of amniotrophic lateral sclerosis (ALS).[12]

Current studies have underlined the role and importance of apoptosis in secondary damage after SCI.[13–17] As defined "programmed cell death," apoptosis comprises cell autodigestion with enzymatic reactions and cell removal by phagocytes without inflammatory response. Caspases, which are cysteine proteases, play a crucial role in regulating apoptosis.[13,18,19] Q-VD-OPh is an irreversible pancaspase inhibitor whose neuroprotective effects have been demonstrated in experimental ischemia–hypoxia, stroke, and SCI models.[3,20] Q-VD-OPh acts as an inhibitor of caspase9/3, caspase 8/10, and caspase 12, which are major caspase pathways for apoptosis.[21,22] Various experimental studies have investigated the efficacy of caspase inhibitors in neuroprotection after SCI, and positive results have been reported.[15,23–25]

Since 1996, preclinical studies have demonstrated that riluzole alone improves SCI outcomes such as reduced tissue cavitation, better preservation of white matter and motor neurons, better mitochondrial function, better somatosensory evoked potentials, and locomotor scores.[26] This study aimed to investigate the efficacy of NMDA receptor antagonists/sodium channel blockers and pancaspases inhibitors alone and in combination for preventing apoptosis that occurs after a primary injury.

MATERIALS AND METHODS

In total, 45 healthy male Sprague Dawley rats obtained from the Experimental Research Center of Medical School of Istanbul, Istanbul University were used. The rats weighed 250–300 g and aged 10–12 months. They were housed under diurnal light conditions, i.e., 12 h of darkness and 12 h of light, and they were fed a standard diet during the study. All experimental protocols were approved by the institutional ethical committees and local institutional animal care and use committee of İstanbul University.

Q-VD-OPh was obtained from Calbiochem (Kimeks-İstanbul). In brief, 0.4 mg/kg Q-VD-OPH, a solid form of wide-spectrum caspase inhibitors (dissolved in DMSA solution:1 mg Q-VD-OPh/10 ml DMSA) and 5 mg/kg riluzole (Rilutek Sanofi/Avantis), a NMDA antagonist, were intraperitoneally administered to every rat at 1 h after the trauma.[11]

1. Experimental Groups

Rats were randomly categorized into the following five groups, with each group comprising nine rats with properties as listed below (Table 1).

Group1: After the trauma following thorocal 7, 8, 9 laminectomy, only SCI was induced and no medication was administered.

Group2: After the trauma following thoracal 7, 8, 9 laminectomy, physiological serum was intraperitoneally administered (0.9% NaCl).

Group 3 (treatment group; Q-VD-O Phgroup): After the trauma following thorocal 7, 8, 9 laminectomy, only Q-VD-OPh was intraperitoneally administered.

Group 4 (treatment group; riluzole group): After the trauma following thoracal 7, 8, 9 laminectomy, only riluzole was intraperitoneally administered.

Group 5 (treatment group; riluzole–Q-VD-OPh group): After the trauma following thoracal 7, 8, 9 laminectomy, riluzole and Q-VD-OPh were intraperitoneally administered in combination.

Surgical Procedure

This study was conducted according to the principles of American National Society for Medical Research of National Academy of Sciences for the access and maintenance of laboratory animals. All the rats were prepared for surgery with

Table 1. Description of the experimental groups

	Physiological serum (%0.9 NaCl)	Q-VD-OPh	Riluzole
Group 1			
Group 2	+		
Group 3		+	
Group 4			+
Group 5		+	+

overnight starving. General anesthesia was performed using 60 mg/kg ketamine (Ketalar; Eczacıbaşı/İstanbul, Turkey) and 9 mg/kg xylazine (Rompun-Bayer/İstanbul, Turkey). After general anesthesia, the dorsal part of each rats was shaved, and after local antisepsis, with the reference of the interscapular distance, a midline incision was made. Paravertebral muscles were bluntly dissected. After identification of thoracal laminas 7-8-9, total laminectomies and bilateral facetectomies were performed. (T7-9) Transverse processes were also removed to have a wide opening of the spinal cord, enabling the vertical clipping of the spinal cord. The dura mater was left intact during all these processes. The spinal cord of the rats of each group was compressed for 30 s using a Yaşargil aneurysm clip, and after removing the clip, the layers were closed in a standard manner.

In Group 1 no drug was administered. In the Group 2, physiological serum; in Group 3, 0.4 mg/kg Q-VD-OPh; in Group 4, 5 mg/kg riluzole; in Group 5, 0.4 mg/kg Q-VD-OPh and 5 mg/kg riluzole was intraperitoneally administered immediately after the trauma and the following 5 days. After 5 days, the rats were re-anesthetized by intraperitoneally injecting Ketalar (65 mg/kg). The thorax wall was incised and was cranially lifted and fixed. The diaphragm and pericardium were also incised. After the intracardiac injection of 2-cc KCl solution in the right ventricle, cardiac contractions were monitored. When the contractions ended, the rats were placed in the prone position, and via the former dorsal incision, spinal cord specimens were harvested from the laminectomy field. The specimens were fixed in 0.1 mol phosphate-buffered (pH7.4) 2.5% glutaraldehyde solution. Paraffin blocks were prepared from the specimens that were fixed 2.5% glutaraldehyde solution.

Histological Analysis

Hematoxylin and eosin (H&E) staining and TUNEL staining were performed for histological assessment. Hematoxylin staining is the primary method for revealing necrosis and apoptosis in cells.Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick and labeling (TUNEL) staining is based on the principle of specific binding of TdTto the 3-OH groups of DNA. After exposing the nuclear DNA of the histological sections, TdT is used to add dUTP-biotin to the endings of the DNA fragments.

Xylene was added to the paraffin blocks according to H&E staining. After soaking the samplesin alcohol, they were placed in water and immersed in hematoxylin for 5 min. The samples were washed under running tap water and were allowed to turn purple. After incubation in eosin for 2-3 min, the samples were rinsed again under running tap water, fixated with alcohol, and mounted with xylene-based Canada balsam.

For TUNEL staining, the samples were incubated in an incubator overnight at 56°C. Then, the samples were immersed in xylene for 30 min, in 96% alcohol for 10 min, 80% alcohol for 5 min, 70% alcohol for 5 min, and rinsed with distilled water. The samples were taken in TBS and dried. After 20 min, the samples were washed with TBS. Then, 3% H_2O_2 with methanol was performed on the samples, and after 5 min, the samples were washed using TBS. The 10X Klenow equilibration buffer, which was diluted 1:10 with distilled water, was performed and incubated for 30 min. After washing, the residue was soaked up using a paper towel, and pre-prepared Klenow labeling reaction mix and Klenow enzyme solution were performed on the samples. The samples were then mounted with paraffin and incubated for 1.5 h at 37°C in the incubator. Next, they were rinsed with TBS, and STOP buffer was performed. At 5 min after washing, the blocking buffer was performed. After 10 min, without being washed up with TBS, diluted 50X conjugate 1:50 blocking buffer was dripped. After 30 min, samples were washed with TBS, and DAB buffer was performed. After 10 min, samples were washed with distilled water. Methyl green was performed. After 30 s, the color was adjusted using acetone. Then, samples were dried and mounted with xylene.

The specimens were examined using the Olympus BX50 microscope at a magnification of 20×, and 2-3 mm thick sections of specimens were evaluated. For evaluating apoptosis, the percentage of cell numbers were classified as 0–25,[27] 25–50,[28] 50–75,[29] and 75–100.[30]

Clinical Neurological Examination

Clinical motor examination was performed on the third and seventh day as previously described by Tarlov.[9] According to this description:

Grade 5: Complete recovery.

Grade 4: Ability to walk; but posterior limbs have slight spasticity and lack of coordination.

Grade 3: Ability to get up but not being able to walk.

Grade 2: Minimal voluntary motor function of the posterior limbs but not being able to stand up on the posterior limbs.

Grade 1: No movement.

Evaluation with Inclined Plane Test

In 1977, Rivlin et al.[31] described the inclined plane test as an objective testing of motor functions. In this test, after horizontally placing the animal on an inclined plane, the angle between the ground and plane was gradually augmented. The maximum angle at which the rat could stand for 5 s on this plane without being overthrown was noted to be the degree of inclined plane for the animal. This test was repeated on the first, third, and fifth day for all groups.

Statistical Analysis

Except for the inclined plane test, all variables were assessed using Kruskal–Wallis and Mann–Whitney two-way comparison. In the two-way comparison, Bonferroni correction (significance level/correction number) was used for the level of significance. ANOVA and Scheffe's two-way comparison were used for comparing the inclined plane test degrees.

RESULTS

Histological Findings

Lymphocyte

Mann–Whitney test results showed a statistically significant difference in lymphocyte counts of the riluzole alone and riluzole–Q-VD-OPh groups (Groups 4 and 5) compared with those of the other groups. No significant difference was found among the other groups (Groups 1, 2, and 3). Furthermore, no statistically significant difference was observed between Groups 4 and 5.

PNL

Mann–Whitney test results showed a statistically significant difference in PNL counts of the riluzole alone and riluzole–Q-VD-OPh groups (Groups 4 and 5) compared with those of the other groups. No significant difference was found among the other groups (Groups 1, 2, and 3).In addition, no statistically significant difference was observed between Groups 4 and 5.

Necrosis

Mann–Whitney test results showed a statistically significant difference in necrotic cell counts of the riluzole alone and riluzole–Q-VD-OPh groups (Groups 4 and 5) compared with those of the other groups. No significant difference was found among the other groups (Groups 1, 2, and 3). Moreover, no statistically significant difference was observed between Groups 4 and 5.

Apoptosis

Mann–Whitney test results showed a statistically significant difference in apoptotic cell counts of the Q-VD-OPh alone and riluzole–Q-VD-OPh groups (Groups 3 and 5) compared with those of the other groups. No significant difference was found among the other groups (Groups 1, 2, and 4). In addition, no statistically significant difference was observed between Groups 3 and 5 (Fig. 1a, b).

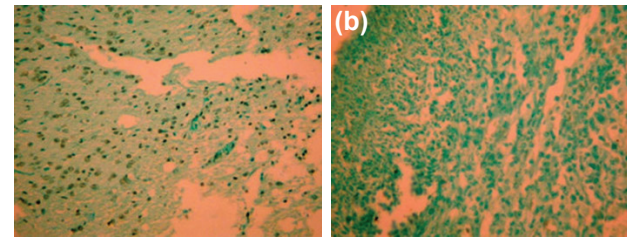

Figure 1. (a) A histopathological specimen prepared using TUNEL staining from the trauma only group showing an increased number of apoptotic cells. **(b)** A specimen prepared using TUNEL staining from the combination group showing a decrease in apoptotic cells and necrotic cavitation.

Functional Findings

Mann–Whitney U test, a two-way comparison method, results showed a statistically significant difference in better motor functions, assessed by the Tarlov motor grading scale, in Group 3 (Q-VD-OPh group) than inthe trauma only and placebo groups (Groups 1 and 2). Although Group 5 had better results with regard to better motor functions, no statistically significant difference was found between Group 5 and Groups 1, 2, and 4. No statistically significant difference was observed between Groups 3 and 5.

InclinedPlane Test Assessments

At the end of the fifth day, a statistically significant difference was observed regarding better inclined plane scores in Groups 3 and 5 compared with those in the other groups. There was no statistically significant difference between Groups 3 and 5, as well as that among Groups 1, 2, and 4 (Table 2).

DISCUSSION

In Turkey, the incidence of SCI is 500–600 new cases per year,

Table 2. Significance of the presence of lymphocyte, PNL, necrosis, apoptosis, and Tarlov scale/inclined plane scores for each group

	Group 1	Group 2	Group 3	Group 4	Group 5
Lymphocyte				p<0.001	p<0.001
PNL				p<0.001	p<0.001
Necrosis				p<0.001	p<0.001
Apoptosis			p<0.001		p<0.001
Tarlov scale			p<0.001		
Inclined plane scores			p<0.001		p<0.001

PNL: Polymorphonuclear leukocyte.

and the prevalence is 12.7/1000000 per year.[32] SCI is a serious health problemwhich generally occurs in individuals aged 16–30 years and causes loss of income and labor, thereby leading to an increase in treatment and care expenses.[33,34]

Although recent literature has basic current guidelines that propose surgery (decompression and stabilization) as a primary intervention for treating SCI, a basic guideline for medically treating SCI is required.[33,34] Nowadays, research is particularly based on the prevention or reduction of secondary damage after SCI.[35,36]

The primary problem of secondary damage is necrosis and apoptosis. Excitatory neurotransmitters such as glutamate play an important role in necrosis, and under pathological conditions, they may cause excitotoxicity. Excitotoxicity affects via two different pharmacologic and electrophysiological receptor groups: metabotropic and ionotropic receptors.[29,37–40] The best known ionotropic receptor is the NMDA receptor.[29,40,41] It has been stated that in cortical cell cultures where oxygen or glucose is absent, neurons primarily die via excitotoxic necrosis, but when this exitotoxicity is blocked by combined NMDA receptor antagonists, these neurons are dead because of apoptosis.[42,43]

Apoptosis is an active process that uses cellular protein and energy. It is a programmed cell death in which the autodigestion of cells through enzymatic reactions and macrophage phagocytation occur.[44] Caspase-dependent signaling pathways play a crucial role in inducing apoptosis.

To date, 14 mammalian caspases have been confirmed. There are extrinsic and intrinsic apoptopic pathways in which caspases function. Among the caspases, caspase-8 is primarily activated in the extrinsic pathway, whereascaspase-9 andcaspase-12 are basic mediators in the intrinsic pathway. A pancaspase inhibitor, Q-VD-OPh, prevents apoptosis via the following three basic mechanisms:[21,22,45]

1- By inhibiting the activation of the caspase-9 and caspase-3, which was initiated by cytochrome C secreted from the mitochondria.

2- By inhibiting the activation of caspase-8 and caspase-10, which is activated after binding to the TNF-alpha and Fas/CD95 death receptors.

3- By inhibiting the activation of caspase-12 that is located on the membrane of the endoplasmic reticulum and is a basic mediator for ER-mediated apoptosis.

Riluzole, a voltage-dependent sodium channel inhibitor, is also a mediator of the postsynaptic glutamate transfer and has a role in the G protein signaling guanylyl cyclase cascade. Riluzole is still being extensively used to treat ALS patients worldwide.[11,46–48] In vitro studies have shown that riluzole protects motor neurons from the exitotoxic effects of glutamatic acid, which is secreted after cell death owing to anoxia.[11,49]

In our study, statistically better resultsfor functional motor findings were obtained in Group 5 (Q-VD-OPh and riluzole) than in the trauma and riluzole only groups. There was no significant difference between Group 3 (Q-VD-OPh group) and 5. Regarding inclined plane test score findings on the third and fifth day, statistically better results were observed Groups 3 and 5 than in Groups 1, 2, and 4. No statistically significant difference was observed among Groups 1, 2, and 4. Furthermore, no statistically significant difference was observed when Groups 3 and 5 were compared. As indicators of secondary damage, histopathological findings such as necrosis, lymphocyte count, and PNL count were investigated. Regarding necrosis, PNL counts, and lymphocyte counts, statistically better results were observed in Groups 4 (riluzole group) and 5 than in Groups 1, 2, and 3. There was no statistically significant difference between Groups 4 and 5. Regarding the apoptosis rate, no statistically significant difference was observed between Groups 1, 2, and 4. However, we found statistically better results in Groups 3 and 5than in Groups 1, 2, and 4.

Conclusion

We found statistically better results in Group 5 (Q-VD-OPh andriluzole, an NMDA receptor antagonist) with regard to neurological findings, particularly the contribution of Q-VD-OPh was significant, although the use of riluzole appeared to be ineffective. We also noted statistically better results in the Q-VD-OPh alone and Q-VD-OPh–riluzole groups than in the other groups with regard to inclined plane score findings. We conclude that Q-VD-OPh significantly reduced the apoptosis rate but had no effect on PNL and lymphocyte counts, which are indicators of necrosis and inflammation. We also noted that riluzole significantly reduced PNL and lymphocyte counts, whereas it had no apparent effect on the apoptosis rate. Thus, we observed that a combination of Q-VD-OPh, a pancaspase inhibitor, and riluzole, an NMDA receptor antagonist, significantly reduced the apoptosis and necrosis, which are indicators of secondary damage.

Although the role of necrosis is more distinct in SCI, we conclude that therapies against the prevention of apoptosis may lead to better results for these injuries.

Conflict of interest: None declared.

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DENEYSEL ÇALIŞMA - *ÖZET*

Deneysel omurilik yaralanmasında genel kaspaz inhibitörü Q-VD-OPh ve NMDH reseptör antagonisti riluzole'ün izole ve birlikte kullanımı

Dr. Halil Can,[1] Dr. Aydın Aydoseli,[2] Dr. Cengiz Gömleksiz,[1] Dr. Burcu Göker,[3] Dr. Muhittin Emre Altunrende,[4] Dr. Müge Dolgun,[2] Dr. Altay Sencer[2]

[1]Medicine Hospital, Beyin ve Sinir Cerrahisi Kliniği, İstanbul
[2]İstanbul Üniversitesi İstanbul Tıp Fakültesi, Beyin ve Sinir Cerrahisi Anabilim Dalı, İstanbul
[3]Liv Hospital, Beyin ve Sinir Cerrahisi Kliniği, İstanbul
[4]Gaziosmanpaşa Taksim Eğitim ve Araştırma Hastanesi, Beyin ve Sinir Cerrahisi Kliniği, İstanbul

AMAÇ: Travma sonrası omurilik yaralanmalarında "ikincil hasar" olarak tanımlanan süreçte N-metil-D-aspartik asit reseptör antagonisti riluzole ve apoptozisin temel efektörü olan kaspazların genel inhibitörü Q-VD-OPh'nın ayrı ayrı ve birlikte kullanımlarının ikincil hasar gelişimi üzerine olan etkilerinin incelemesi amaçlandı.
GEREÇ VE YÖNTEM: Bu çalışmada Sprague-Dawley türünden sağlıklı 45 adet erkek sıçan kullanıldı. Omurilik travması dorsal 7, 8, 9 laminektomi sonrası klip kompresyon yöntemi kullanılarak gerçekleştirildi. İlaçlar travmadan hemen sonra başlamak üzere beş gün boyunca intraperitoneal olarak uygulandı. Travma sonrası denekler Tarlov skalası ve eğik düzlem testi ile değerlendirildi. Beş gün sonra alınan omurilik örnekler hemotoksilen - eozin ve TUNEL boyama yöntemi kullanılarak histolojik incelemesi yapıldı.
BULGULAR: Histolojik inceleme sonrası enflamatuvar yanıt, nekroz ve apoptoz riluzole ve kombine ilaç kullanılan grupta diğer gruplara göre istatiksel olarak anlamlı iyi sonuçlar elde edildi. Deneklerin klinik motor fonksiyon değerlendirilmesinde Q-VD-OPh kullanılan grupta diğer gruplara göre istatistiksel olarak anlamlı iyi sonuçlar elde edildi.
TARTIŞMA: Q-VD-OPh ve riluzole-Q-VD-OPh kombinasyonun travmada ikincil hasarın sınırlandırılmasında istatistiksel olarak iyi klinik ve histolojik sonuçlar elde edildi. Spinal travma sonrası ikincil hasarın etkisinin ortadan kaldırılması veya sınırlanması için laboratuvar çalışmaları daha kapsamlı yapılmalıdır.
Anahtar sözcükler: Apoptozis; kaspazlar; nekroz; NMDA reseptör antagonisti; omurilik yaralanması; pankaspaz inhibitörü; riluzol; sinir dokusunun korunması; Q-VD-OPh.

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