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Effects of combined and individual use of N-methyl-D aspartate receptor antagonist magnesium sulphate and caspase-9 inhibitor z-LEDH-fmk in experimental spinal cord injury

Sıçanlarda oluşturulan deneysel omurilik yaralanmasında N-metil D-aspartat reseptör antagonisti olan magnezyum sülfat ve kaspaz-9 inhibitörü olan z-LEDH-fmk'nın tek başına ve kombine kullanımlarındaki etkinliklerinin karşılaştırılması

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BACKGROUND

We investigated the individual and combined effects of magnesium sulphate, which is an N-Methyl-D aspartate receptor antagonist (NMDA), and z-LEHD-FMK, which is a caspase 9 inhibitor, on the genesis of secondary injury in a rat spinal cord injury model. We aimed to minimize the effects of secondary injury in spinal cord trauma by choosing these two agents which served to block the two major mechanisms of cell loss, apoptosis and necrosis.

METHODS

The drugs were given to the subjects according to their groups, either in singular or combined fashion. For motor examination, the subjects were kept under close clinical evaluation for five days. Histopathological examination and the emerging spinal cord samples were prepared with haematoxylene-eosin and Tunel techniques.

RESULTS

A statistically significant difference in favor of the treatment groups has been found between the treatment and control groups in terms of histological data. However, there was no difference in the evaluation of motor examination between trauma and treatment groups.

CONCLUSION

We have found no difference between the individual and combined uses of $MgSO_4$ and z-LEHD-FMK in the prevention of secondary injury; however, there were better histological results in the treatment groups compared to trauma and control groups which gives us hope for future investigations.

Key Words: Apoptosis; magnesium sulphate; necrosis; neuroprotection; spinal cord injury; z-LEDH-FMK.

AMAÇ

Sıçanlarda oluşturulan omurilik travması sonrasında bir N-metil D-aspartat (NMDA) reseptör antagonisti olan magnezyum sülfat'ın ve kaspaz-9 inhibitörü olan z-LEHD-FMK'nın ikincil hasar gelişimi üzerine olan etkileri karşılaştırıldı. Apoptozis ve nekrozun omurilik travması sonrası hasar görmüş hücrelerin kaybedilmesindeki iki ana yolu teşkil etmelerini göz önüne alarak kullandığımız bu iki ajan ile ikincil hasarın iki ana mekanizmasını birlikte engelleyerek ikincil hasarı en aza indirmeyi hedefledik.

GEREÇ VE YÖNTEM

Omurilik travması sonrası deneklere gruplarına göre, ayrı ayrı ve kombine olarak, ilaç tedavisi uygulandı. Denekler beş gün boyunca klinik olarak gözlendi ve nörolojik fonksiyonları kaydedildi. Histopatolojik değerlendirme için beşinci gün sonunda alınan omurilik örnekleri hematoksilen eozin ve Tunel yöntemi ile boyanarak mikroskobik olarak incelendi.

BULGULAR

Elde edilen verilerin karşılaştırılmasında, tedavi grupları ile kontrol grupları arasında histopatolojik açıdan tedavi grupları lehine istatistiksel olarak anlamlı fark bulundu, ancak motor inceleme bulgularının değerlendirmesinde travma ve tedavi grupları arasında anlamlı bir farklılık yoktu.

SONUÇ

İkincil hasar gelişiminin önlenmesinde $MgSO_4$ ve z-LEHD-FMK'nın kombine kullanımı ile izole kullanımları arasında istatistiksel olarak anlamlı bir fark bulunmamış, ancak tedavi grupları travma ve kontrol gruplarından daha iyi sonuçlara sahip olduğu görülmüş ve bu da omurilik travmasının gelecekteki tedavisi açısından umut verici bulunmuştur.

Anahtar Sözcükler: Apoptozis; magnezyum sülfat; nekroz; omurilik yaralanması; sinir dokusunun korunması; z-LEDH-FMK.

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Until the last decade, necrosis has been accepted as the only mechanism for cell death in tissue damage following spinal cord injury (SCI). However, recent publications have also indicated the importance of apoptotic cell death after SCI.[1-4] Unlike necrosis, apoptosis is a "programmed cell death" mechanism, where the cells are autodigested by enzymatic reactions and then removed by phagocytes without an inflammatory response. Caspases, formerly known as cysteine proteases, are the key regulators of apoptosis.[4-7] Various caspases and apoptotic cascades induced by caspase-dependent signaling have been described. There are several studies demonstrating promising results for preventing secondary injury in SCI with inhibition of apoptosis.[3,8-13] Caspase-9 is a key initiator of apoptosis, which activates the mitochondria-mediated pathway (intrinsic pathway).[14] z-LEDH-fmk is a selective, irreversible caspase-9 inhibitor that has been found to be effective as an antiapoptotic agent in animal models of cerebral ischemia and SCI.[15,16]

Various reports demonstrated the effects of antiapoptotic caspase inhibitors and magnesium treatment in SCI separately. However, there is no research on the combined inhibition of these two major pathways of secondary injury. In this study we investigated the individual and combined effects of $MgSO_4$, which is a NMDA receptor antagonist, and z-LEDH-fmk, which is a caspase-9 inhibitor, on the trauma-induced secondary injury in a rat SCI model acquired by static compression technique. The aim was to demonstrate the inhibitory effects on secondary injury of these two agents that block the two major mechanisms of cell loss: apoptosis and necrosis.

MATERIALS AND METHODS

In the study, 54 male Sprague-Dawley rats obtained from the Research Center for Experimental Medicine were used. The animals, weighing 280-340 g and aged 10-12 months were fed a normal diet during the study period and housed under diurnal light conditions. All experimental protocols were approved by the local institutional animal care and use committee and institutional ethical committees of Istanbul University.

Traumatic injury model

The SCI was produced by acute spinal cord compression technique described by Rivlin and Tator.[17] The rats were injured using Yaşargil FE 716 K (with a closing force of 110 g, Aesculap/Germany) aneurysm clips which produce a compression force of 110 g with 30 seconds of compression duration.

Surgical procedure

The surgical procedure was performed under general anesthesia after intraperitoneal injection of 9 mg/kg xylasine (Bayer, Istanbul, Turkey) and 60 mg/kg ketamine hydrochloride (Parke-Dewis, Eczacıbası, Istanbul, Turkey). The rats were placed in prone position. Body temperature of the rats was kept constant using a heating lamp at 37 ºC monitored with a rectal temperature probe. After shaving the dorsal region of each rat and scrubbing with povidine iodine solution (Adeka, Samsun, Turkey) a dorsal midline skin incision was performed (Figure 2). Following dissection of paravertebral muscles (Figure 3), middorsal two level (approximately T7-9) laminectomies and bilateral facetectomies were performed (Figure 4). The transverse processes were also removed in order to apply the aneurysm clip vertically to the spinal cord axis (Figure 5). The dura mater was left intact. After surgical interventions the wound was closed in layers with 3/0 atraumatic silk sutures.

Treatment groups

The rats were randomly and blindly divided into the following six groups each consisting of 9 animals:

• *Group 1 (Sham-Operated Controls):* Only laminectomies (n=9).

• *Group 2 (Trauma-Only Controls):* Laminectomy and SCI (n=9).

• *Group 3 (Placebo Controls):* After laminectomy and SCI rats were treated with intraperitoneally-injected physiological serum (0.9% NaCl) immediately after procedure and daily for the following five days (n=9).

• *Group 4 (Trauma and $MgSO_4$ Treatment Group):* Laminectomy and SCI performed. A single dose of 100 mg/kg of $MgSO_4$ (Biofarma, Istanbul, Turkey) was administered intraperitoneally immediately after the procedure and daily for the following five days (n=9).

• *Group 5 (Trauma and Combined Treatment Group):* Following laminectomy and SCI, the rats were treated with combination therapy of intraperitoneally-injected 100 mg/kg of $MgSO_4$ (Biofarma, Istanbul, Turkey) and 0.6 umol/kg of z-LEHD-fmk (Calbiochem GmGH, Kimeks, Istanbul, Turkey) immediately after the procedure and daily for the following five days. The dry form of z-LEHD-fmk was diluted in physiological serum.

• *Group 6 (Trauma and z-LEHD-fmk Treatment Group):* Following laminectomy and SCI, the rats were treated with intraperitoneally-injected 0.6 µmol/kg of z-LEHD-fmk (Calbiochem GmGH, Kimeks, Istanbul, Turkey) immediately after the procedure and daily for the following five days.

The animals were followed-up for five days after the surgical procedure. Functional assessments were performed on the first, third, and fifth days postoperatively. The animals were reanesthetized on the fifth day as described above and then killed with intracar-

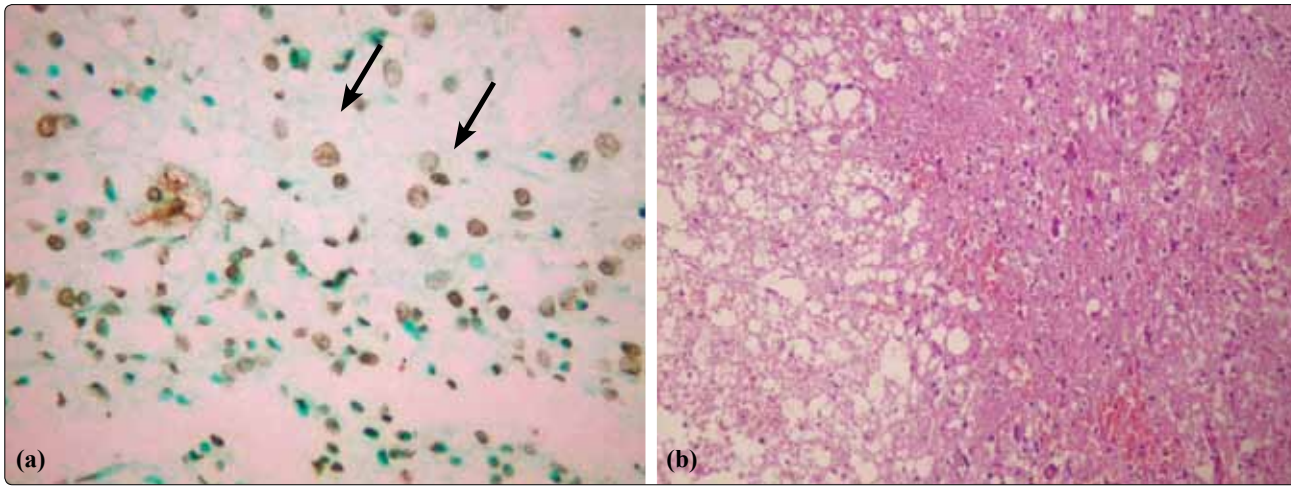

Fig. 1. **(a)** Photomicrograph shows a specimen from trauma-only group prepared with TUNEL technique, indicating the increased number of brown coloured apoptotic cells. **(b)** H&E staining of the same specimen shows increased number of cavitation areas. *(Color figur can be viewed in the online issue, which is available at www.tjtes.org).*

diac injection of 2cc KCl solution. The animals were then placed in prone position. The skin was reopened and samples from the laminectomized spinal cord were taken using a longitudinal dural incision. The specimens were fixed in 0.1 mol phosphate-buffered 2.5% glutaraldehyde solution.

Functional assessment

Neurological function of each rat was evaluated with the objective "inclined-plane technique" on the first, third, and fifth days after the surgical procedure.[18] The subjective "Tarlov motor grading scale" was also used to assess functional recovery.[19] The assessments were performed by investigators who were blind to procedures.

Histological studies

The specimens obtained on the fifth day postoperatively were prepared for histological investigation. Hematoxylin and eosin (H&E) staining and TUNEL staining were used for histological assessment. For H&E staining, samples were fixed in 0.1 mol phosphate-buffered 2.5% glutaraldehyde solution and embedded in parafin. 5 µm-thick sections were cut and stained with H&E. For TUNEL staining, samples were prepared according to the TUNEL technique,[20] which enables counting of apoptotic cells. The specimens were obtained as close as possible from the center of the lesion site. 5 µm-thick sections were cut and stained with the TUNEL technique. The samples were examined with Olympus Bx50 light microscope at magnification level of 20x.

A blinded investigator not involved in the procedures counted the number of the apoptotic cells in these sections. The percentage of the apoptotic cells among all cells were recorded and classified as follows: 0-25%, 25-50%, 50-75% and 75-100%. The number of the lymphocytes and polymorpho nuclear leukocytes (PNL) and their percentage among the inflammatory cells in these sections were recorded as an indicator of the inflammatory response. Cavitation areas in the sections were assessed as a sign of necrosis; the number of cavitation areas was also recorded and quantified as a percentage.

Statistical analysis

All the data were analyzed with Kruskal-Wallis variance analysis and Mann-Whitney U-test except the parametric data from the motor evaluation with inclined-plane technique. Parametric data were analyzed with ANOVA variance analysis and Scheffe test for multiple comparisons. All results are based on two-sided tests for p=0.05.

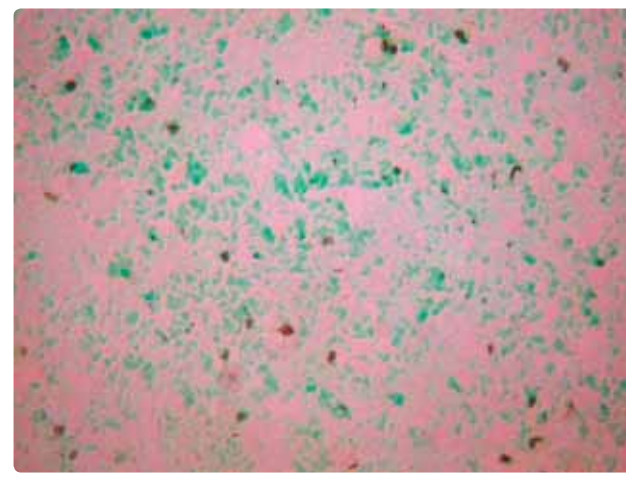

Fig. 2. Photomicrograph shows a specimen from combined treatment group prepared with TUNEL technique. A significant decrease is observed in the number of apoptotic cells.

(Color figur can be viewed in the online issue, which is available at www.tjtes.org).

RESULTS

Functional findings

There was a statistically significant difference between the sham-operated control and trauma groups in the means of inclined-plane scores ($p<0.001$). Trauma caused a significant decrease in inclined-plane scores as expected. The results obtained in the trauma-only and placebo groups were significantly different from those in the treatment groups, but there were no significant differences between these two groups. All three treatment modalities caused increases in inclined-plane scores. The z-LEDH-fmk and combined treatment groups showed a significant increase in inclined-plane scores compared to $MgSO_4$ group. There was no statistically significant difference between z-LEDH-fmk and combined treatment groups.

Functional assessment using "Tarlov motor grading scale" resulted in no statistically significant difference among all the trauma groups, except significantly better results for sham-operated control group.

The results for functional assessment are summarized in Table 1 and Table 2.

Histological findings

There was a significant difference in the means of lymphocytes and PNL counts and percentages in sham-operated control group compared to trauma-only and placebo groups. As expected there were no lymphocytes or PNL in the control groups. Trauma-only and placebo groups showed significantly worse results than the combined and z-LEDH-fmk treatment groups. There was no significant difference between the three treatment groups, although the percentage of lymphocyte and PNL was lower in the combined treatment group compared to the other two treatment groups ($p<0.001$). The presence of necrosis was assessed and no significant difference was found between all groups ($p<0.002$).

The results for TUNEL staining and apoptotic cell counts revealed a significant difference in the sham-operated group compared to trauma-only and placebo groups. These two groups showed significantly worse results compared to the combined and z-LEDH-fmk groups. There was no significant difference between the treatment groups ($p<0.001$).

The results for histological assessment are summarized in Table 3, Table 4, and Table 5.

DISCUSSION

Despite many efforts, SCI still has no definite cure and continues to be a serious medical threat to the patient as well as a burden on the patient's family and the state economy. The fact that 61% of patients are between 16-30 further contributes to the graveness of the problem.[21]

Table 1. Distribution of Tarlov Motor Grading scores in six experiment groups

Tarlov Motor Grading	Day 1	Day 3	Day 5
Group 1	5	5	5
Group 2	1	1	1
Group 3	1	1	1
Group 4	1	1	1
Group 5	1	1	1
Group 6	1	1	1
KW-χ^2	53	49	40.48

Table 2. Summary of inclined plane results for the six groups of rats

Inclined plane	Day 1	Day 3	Day 5
(degree)	Mean±SD	Mean±SD	Mean±SD
Group 1	42.33±0.50	42.55±0.53	43.00±0.00
Group 2	28.77±1.20	28.33±1.32	28.78±1.20
Group 3	27.77±0.97	27.89±0.33	28.00±0.86
Group 4	30.89±1.61	30.67±1.58	30.77±1.20
Group 5	33.00±0.71	34.00±0.71	34.33±0.71
Group 6	32.77±0.66	33.33±0.50	33.55±0.72

Table 3. PNL and Lymphocytes counts as percentage

	PNL (%)	Lymphocyte (%)
Group 1	0	0
Group 2	100	100
Group 3	100	100
Group 4	55.6	55.6
Group 5	11.1	11.1
Group 6	22.2	22.2
KW-χ^2	34.65	34.65

PNL: Polymorpho nuclear leukocytes.

Primary and secondary injuries following the trauma result in tissue damage. Direct mechanical damage to neurovascular structures is called primary injury, which has no treatment by primary health care measures other than prevention. After the primary injury, the initially-intact, surrounding neural tissue is also attacked by various molecular pathways. This delayed tissue damage is called secondary injury. Preventing secondary injury by modulating different molecular mechanisms has become the focus of numerous investigations.[22-25] Currently there is no definitive treatment for secondary injury in SCI, despite the common use of corticosteroids, which are expected to prevent secondary injury through several mechanisms.[26]

Traumatic SCI results in acute mechanical damage and ischemia and leads to neural tissue degeneration. Necrosis is accepted as the main mechanism causing cell death.[27] Excitotoxicity has been assumed to be the

Table 4. The presence of necrosis in six experimental groups as percentage

	Necrosis	
	– (%)	+ (%)
Group 1	100	0
Group 2	44.4	55.6
Group 3	33.3	66.7
Group 4	55.6	44.4
Group 5	88.9	11.1
Group 6	100	0
KW-χ^2	18.48	–

Table 5. The presence apoptosis in six groups of rats

	Apoptosis (%)			
	0-25	25-50	50-75	75-100
Group 1	100	0	0	0
Group 2	0	33.3	44.4	22.2
Group 3	0	33.3	55.6	11.1
Group 4	66.7	11.1	22.2	0
Group 5	88.9	11.1	0	0
Group 6	77.8	22.2	0	0
KW-χ^2	37.02			

key factor in necrosis and neuronal degeneration.[23,28] Excitatory neurotransmitters, especially glutamate, the primary neurotransmitter in the spinal cord, play a crucial role by causing neurotoxicity in conditions like spinal cord ischemia or SCI where the cellular energy levels are decreased. The prevention of secondary injury after SCI with the use of N-methyl-D-aspartate (NMDA) receptor antagonists is well studied. There is limited but promising research on the use of magnesium sulphate ($MgSO_4$) in SCI.[29-34] $MgSO_4$ is an NMDA receptor antagonist, which has been used clinically as a neuroprotective agent for treating acute stroke and other conditions like preeclampsia, atrial fibrillation, myocardial infarction 29 and experimentally in animal models of brain injury.[35-37]

Recent studies have demonstrated that necrosis is not the only mechanism in cell death; apoptosis also has a major role in this process.[38-40] Because apoptosis needs energy, heavily injured cells die via necrosis, but the surrounding mildly damaged neural tissue can lose cells via apoptosis. Investigations focused on regulating apoptosis with new drug therapies in order to protect neural tissue and motor function.[41]

$MgSO_4$ is a well-known NMDA receptor blocker that has a neuroproctective effect in neural tissue through glutamate antagonism and reduction of excitotoxicity.[24,36,42] Additionally, its antiapoptotic effect through caspase 3 inhibition has been shown in an animal model of hipoxic-ischemic brain injury.[43] This agent is still widely used in clinical practice for the treatment of conditions like preeclampsia, atrial fibrillation, stroke, myocardial infarction and vasospasm following subarachnoid hemorrhage (SAH). In a controlled clinical trial done by van den Bergh et al., $MgSO_4$ was shown to reduce delayed cerebral ischemia following SAH significantly.[44] Wong et al. demonstrated the reduction of symptomatic vasospasm after SAH in a prospective randomized trial.[45] Solaroglu et al. have shown the inhibitor effect of $MgSO_4$ on necrosis and apoptosis through caspase 3 blockage in SCI.[33]

However, experimental and clinical studies on the effects of $MgSO_4$ on secondary injury in SCI are limited.[24,30] Our study revealed that with $MgSO_4$ use, better histological results were achieved, such as reduced number of inflammatory cells and reduced necrosis and apoptosis, which may indicate effective inhibition of secondary injury. Although $MgSO_4$ has the ability to block both major pathways of secondary injury, namely necrosis through NMDA receptor inhibition and apoptosis through caspase 3 inhibition, no statistically significant difference was found among $MgSO_4$, z-LEDH-fmk and combined treatment groups in the current study. Despite the lack of statistical significance, histological results was found to be better in z-LEDH-fmk and combined treatment groups with less inflammatory cells and reduced necrosis and apoptosis compared to $MgSO_4$ treatment group. We believe further research is necessary to assess the therapeutic effect of $MgSO_4$ after SCI.

Caspases play an important role in apoptosis. Caspase 9 is the key initiator of the cytochrome-c dependent apoptosis. Brown et al.[46] demonstrated that caspase inhibition in cytokine deprived hematopoetic cells and blocks cell death. Kaptanoglu et al investigated inhibition of caspase 3 activation with mexiletine treatment and achieved better neurological results compared to metilprednisolon treatment.[47] z-LEDH-fmk is a selective, irreversible, potent caspase 9 inhibitor with known antiapoptotic effect.[15,16] Mouw et al. demonstrated better neurological results with the use of z-LEDH-fmk in a reversible focal cerebral ischemia model.[7] In a different investigation z-LEDH-fmk is proven to reduce lymphocyte apoptosis in a polimicrobial sepsis model in rats.[48] Colak et al. reported the effective inhibition of apoptosis with z-LEDHfmk in an animal model of SCI.[9]

The combined use of $MgSO_4$ and z-LEDH-fmk was evaluated in the literature and there were no obvious complications or toxicity recorded due to combined treatment. Histological findings were significantly better in all treatment groups when comparing trauma to placebo groups. There were no significant

differences between the treatment groups. The percentage of lymphocytes and PNL was the lowest in the combined treatment group followed by z-LEDH-fmk and $MgSO_4$ treatment groups. The percentage of apoptotic cells was found to be the lowest in the combined treatment group. However necrosis was found to be the lowest in z-LEDH-fmk treatment group, followed by the combined and $MgSO_4$ treatment groups. Though there was no significant difference, the acute inflammatory response and apoptosis seemed to be reduced with the combined use of these two agents. The percentage of acute inflammatory and apoptotic cells was the lowest in this group, followed by z-LEDH-fmk and $MgSO_4$ alone.

Inclined-plane test revealed better results for the comparison of treatment groups to the trauma and placebo groups. The combined and z-LEDH-fmk groups showed significantly better results compared to $MgSO_4$ group. Among the three treatment modalities, the most unfavorable results were achieved in $MgSO_4$ group. However, no statistically significant difference was found between the combined and z-LEDH-fmk groups. There were no significant differences in the means of motor evaluation between all groups. Compared to similar studies in the literature, our study revealed no success regarding neurological function. Maybe there is an association with the selected trauma model. In most of the recent studies the weight-drop technique was used, whereas in our study clip-compression method was used. Weight-drop method was criticized because of the difficulty to standardize the trauma.[15,49] The motor grading scales were low for all groups (1.0) except the sham-operated group and remained the same for the rest of the study, although there were progress in inclined-plane scores. Tail movement lasted for 3-4 seconds 13 seconds after the clip was applied, then the tail remained atonic indicating paraplegia. These relatively subjective test results were better in most other studies, even in trauma groups without treatment. The reason for the unfavorable motor grading scale results may be caused by the short follow-up period of this study.

In this study, we demonstrated that the individual and combined use of $MgSO_4$ and z-LEDH-fmk could achieve better histological and functional results. Although there was no statistically significant difference found between the treatment groups in histological evaluation, combined use showed better histological results compared to the individual use, which gives us hope for future investigations. This combination blocks necrosis and apoptosis, two main pathways leading to secondary injury following SCI. Further in-vivo and in-vitro investigations should be planned in order to create a new potential combined therapy for patients with SCI.

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