Research Article

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Effects of Rifampicin on Experimental Spinal Cord Ischemia/Reperfusion Injury in Rats

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Abstract

Objective: The effect of rifampisin was examined using a spinal cord ischemia/reperfusion (I/R) injury model in rats.

Materials and Methods: 25 Wistar Albino rats weighing 200-250 g were used for the study. Rats were divided in 5 groups. After laparatomy, aorta was clamped 45 minutes below the left renal artery in all groups except sham-operated group. 1 cc saline was injected to vehicle group and rifampicin (20 mg/kg) was administered to treatment group intraperitoneally. In group 5, 20 mg/kg of rifampicin applied intraperitoneally before laparotomy. 2 hours after application of rifampicin, the animals underwent clip compression for 45 minutes after exposure of the abdominal aorta. At 1 h and 24 h, all groups were examined for neurologic outcome according to Tarlov scale. At 24 h, rats were sacrificed. The spinal cord was excised by laminectomy between the T8-12 levels and tissue MDA levels were studied.

Results: At 1 h, difference between motor scores of sham-operated group and other groups was statistically significant (P=0.008). At 24 h, difference between trauma and treatment or p-treatment group was statistically significant (P<0.05). When MDA levels of the groups were compared by using Kruskal Wallis variance analysis, the result was statistically significant (P=0.001). When trauma and vehicle group were compared with treatment group by Mann Whitney U test, the results were statistically significant (P=0.008).

Conclusions: To our knowledge, this is the first study that shows the effects of rifampicin on spinal cord ischemia/reperfusion injury. Rifampicin was found to be effective on spinal cord ischemia/reperfusion injury, but further investigations are mandatory.

Keywords: Ischemia/Reperfusion; Malondialdehyte; Neuroprotective effect; Rifampisin; Spinal cord

Introduction

High mortality and morbidity resulting from spinal cord injury is still one of the most important and devastating issues of modern medicine. Despite various experimental studies that have tried to find the required answers regarding spinal cord injury, unfortunately no clinically valuable achievement has been made to date. Literature provides different methods for the "spinal cord ischemia model". The present study uses the model described by Zivin et al., due to its significant reproducibility [1]. Rifampicin (RIF) is a macrocyclic antibiotic used as an antituberculosis agents. The main role of rifampicin,(3-[[(4-methyl-1-piperazinyl)imino]methyl]-5,6,9,17,19,21-Hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-[N-(4-Methyl-1piperazinyl)formimidoyl]-2,7-poxypentadeca[1,11,13] trienimino) naphtha [2,1-b] furan-1,11-(2H)-dione21-acetate[13292-46-1]), a semisynthetic antibiotic produced from *Streptomyces mediterranei*, is a free radical scavenger.

Numerous studies have shown the radical scavenger effects of rifampisin [2,3]. The neuroprotective effect of rifampicin, through its glucocorticoid receptor activation was also shown [4]. In this study, we aimed to examine the effect of rifampicin on a spinal cord ischemia/reperfusion (I/R) model.

Materials and Methods

Animal Handling

Twenty-five Wistar Albino rats weighing between 200 and 250 g were used in this study. The animals were kept under constant laboratory conditions of 18 to 21 room temperature, a 12-hour light-dark cycle, and were allowed free access to food and water. All experiments were approved by the Institutional Review Board of Karadeniz Technical Universtiy, Faculty of Medicine (09.02.2005/02), and were treated according to the research guidelines.

Anesthesia and Surgical Procedure

The rats were fasted for 24 hours with free access to water before the surgical procedure. Anesthesia was induced by intramuscular

administration of 50 mg/kg ketamine hydrochloride (Ketalar, Pfizer, Istanbul). The rats were numbered with ear tags. Their abdomens were shaved and cleaned with 10% polyvinylpyrolidone-iodine.

All of the animals underwent laparotomy under aseptic conditions. No further intervention was applied to the rats in the Sham Group. The surgical incisions of the rats were closed in layers. The abdominal aortas of the animals in other groups were exposed after opening the retroperitoneum. An aneurysm clip with 50 g closing force (Yasargil FE 69, Aeusculab, Germany) was applied to the abdominal aorta below the renal artery orifices for 45 minutes. No further intervention was performed on the animals in Group 2 and the surgical incisions were closed in layers. Immediately after the clip compression, the animals in Group 3 and 4 received 1 cc of normal saline and 20 mg/kg rifampicin intraperitoneally, respectively. The animal in Group 5 received 20 mg/kg rifampicin intraperitoneally before laparotomy. After 2 hours of application of rifampicin, laparotomy were performed. The surgical incisions of the animals were closed in layers.

Description of Groups

Animals were divided into five equal groups including 5 rats in each group.

Group 1 (Sham-operated): The rats in this group underwent surgical laparotomy. No further intervention was performed in this group.

Group 2 (Trauma): After laparotomy, we applied aneurysmal clip to the abdominal aorta of the rats in this group for 45 minutes. No further intervention was performed in this group.

Group 3 (Vehicle): The animals in this group underwent clip compression for 45 minutes after exposure of the abdominal aorta. Before closure of the peritoneum, the animals received 1 cc of normal saline intraperitoneally.

Group 4 (Treatment): The animals in this group underwent clip compression for 45 minutes after exposure of the abdominal aorta. Before closure of the peritoneum, the animals received 20 mg/kg of Rifampicin (Rif, Koçak Farma, Turkey) intraperitoneally.

Group 5 (profilactic-treatment=p-treatment): 20 mg/kg of Rifampicin applied intraperitoneally before laparotomy. 2 hours after application of rifampicin, the animals underwent clip compression for 45 minutes after exposure of the abdominal aorta.

Motor Function Examination

The animals were examined for lower limb motor functions one hour after the surgical procedure and just before the sacrification at the 24th hour after surgery. Motor evaluation was performed according to the scale defined by Tarlov et al. **(Table 1)** [5].

Sacrificing of Animals and Sample Preparation

The rats were sacrificed by an overdose of pentobarbital after 24 hours. Total laminectomy of T8-T12 was performed on all rats under operation microscope (Zeiss, Germany), and spinal cord segments between T8 and T12 levels were excised and immediately stored in a -76 freezer for further biochemical evaluation of malondialdehyde levels (MDA).

Grade	Motor Function
0	Total paraplegia of the hind limb
1	No spontaneous movement, but response to a hind limb pinch
2	Spontaneous movement, but inability to stand
3	Able to support weight, but unable to walk on a broad, flat surface
4	Normal

Table 1: Tarlov scale

Measurement of Malondialdehyde (MDA) levels

In order to evaluate lipid peroxidation in the spinal cord tissue samples, MDA concentrations were measured using the method of Mihara and Uchiyama [6]. Tissues were homogenized in 10 volumes of tris-KCl (50 mM, pH=7.4) at +4°C. Half a milliliter (0.5 ml) of homogenate was mixed with 3 ml of 1% H_3PO_4 and 1 ml 0.6% thiobarbituric acid. For incubation, the mixture was then heated in boiling water for 45 minutes. The samples were cooled after incubation, and 2 ml of n-butanol was added. The mixture was then centrifuged at 4000 rpm for 10 minutes at room temperature. The absorbance of the organic color layer was then recorded at 532 nm. Using tetramethoxypropane as the standard, tissue lipid peroxide levels were calculated as nanomoles per gram of wet tissue.

Statistical Analysis

Comparisons among motor examination scores of the groups were made using the Kruskal-Wallis analysis of variance and the Mann-Whitney U test. Results were expressed as mean ± SD, median and range. Significance was approved when P values were less than 0.05. Comparisons among MDA levels of the groups were done with Kruskal-Wallis test (as post hoc with Mann-Whitney U test with Bonferroni correction). In corrected as post hoc with Mann-Whitney U test, if 0.05 divided to comparison number 6, significance was approved when the result was less than 0.0083.

Results

Neurobehavioural findings

Figure 1 and Table 2 demonstrate the motor examination scores of the rats at the 1st and 24th hours according to the Tarlov scale. The difference between the groups at the 1st and 24th hours were significant (P=0.005 and P=0.009, respectively). The comparison of the sham group with the other groups also revealed significant differences at the 1st and 24th hours (P=0.008 for both comparison). The difference between the sham group and the treatment group was not significant (P=0.151). At 24 h, difference between trauma and treatment or p-treatment group was statistically significant (P<0.05).

Tissue MDA levels findings

The sham group had the lowest MDA levels (30.2 ± 4.45 nm/g), whereas the trauma group had the highest MDA levels (65.4 ± 14.6 nm/g) **(Figure 2 and Table 3)**. When the MDA levels of the groups were compared with Kruskal–Wallis variance analysis, all of the results were statistically significant (P=0.001). When the groups' MDA levels were compared using the post hoc Mann-Whitney U test, the trauma

group had significantly higher levels than those in the treatment (P= 0.008), p-treatment (P=0.008), and sham (P= 0.008). But the comparison of MDA levels of trauma group and vehicle group had statistically insignificant (P=0.548).

Discussion

Injuries to the spinal cord alter the neurological function through direct and indirect mechanisms. Secondary mechanisms are thought to be preventable. Major biochemical changes (such as glutamate toxicity, accumulation of neurotransmitters, increased arachidonic acid, production of free radicals and lipid peroxidation), as well as electrolyte shifts between the compartments (increase in intracellular calcium and sodium, and extracellular potassium) lead to secondary injury (Tator CH 1996 39/id). Prevention of pathological electrolyte shifts may have positive effects on secondary injury.

There are various animal models of spinal cord ischemia-reperfusion injury. One of the most accepted of these models was described by Zivin [1] and modified in 1982 [1]. According to this model, which has also been used in the present study, occlusion of the abdominal aorta is applied right after the orifice of the left renal artery.

The results of our study revealed that there was a statistically significant difference between the sham group and the other groups regarding motor scores at the 1st hour (P=0.005). This is the result of the ischemia-reperfusion injury. On the other hand, evaluation of the 24th hour motor scores revealed a significant difference between the sham group and the vehicle group (P=0.008); however, there were no difference between the treatment/p-treatment group and the sham

group (P=0.151). At 24 h, difference between trauma and treatment or p-treatment group was statistically significant (P<0.05). From this we can interpret that rifampicin has a positive effect on motor scales.
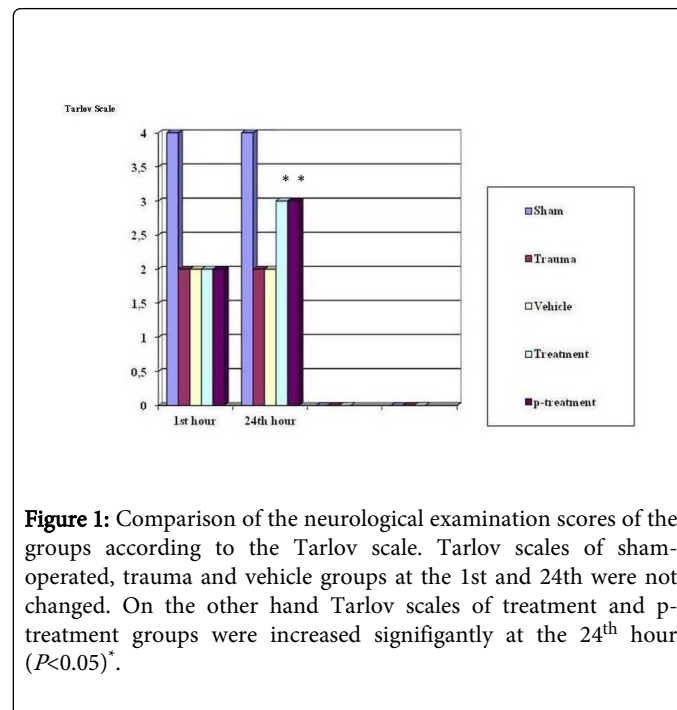
Figure 1: Comparison of the neurological examination scores of the groups according to the Tarlov scale. Tarlov scales of sham-operated, trauma and vehicle groups at the 1st and 24th were not changed. On the other hand Tarlov scales of treatment and p-treatment groups were increased signifigantly at the 24th hour (*P*<0.05)*.

Group	1st hour		24th hour	
	Median	**Min-Max**	**Median**	**Min-Max**
Sham	4	4-4	4	4-4
Trauma	2	1-2	2	2-3
Vehicle	2	1-2	2	2-3
Treatment	2	1-2	3	2-4
p-treatment	2	1-2	3	2-4
KW x2	12.920		11.599	
Df	3		3	
P	0.005		0.009	

Table 2: Median and min-max Tarlov scales of the groups at the 1st and 24th hour

Group	Mean	Standard Deviation	Min-Max
Sham	30.2	4.45	25.5-36.5
Trauma	65.4	14.6	55.0-90.0
Vehicle	64.2	6.34	58.0-72.0
Treatment	46.0	7.20	36.0-53.0
p-treatment	46.2	7.21	36.0-54.2

KW^x 2	15.936			
Df	3			
P	0.001			

Table 3: Distribution of tissue MDA levels (mean ± SD) according to groups
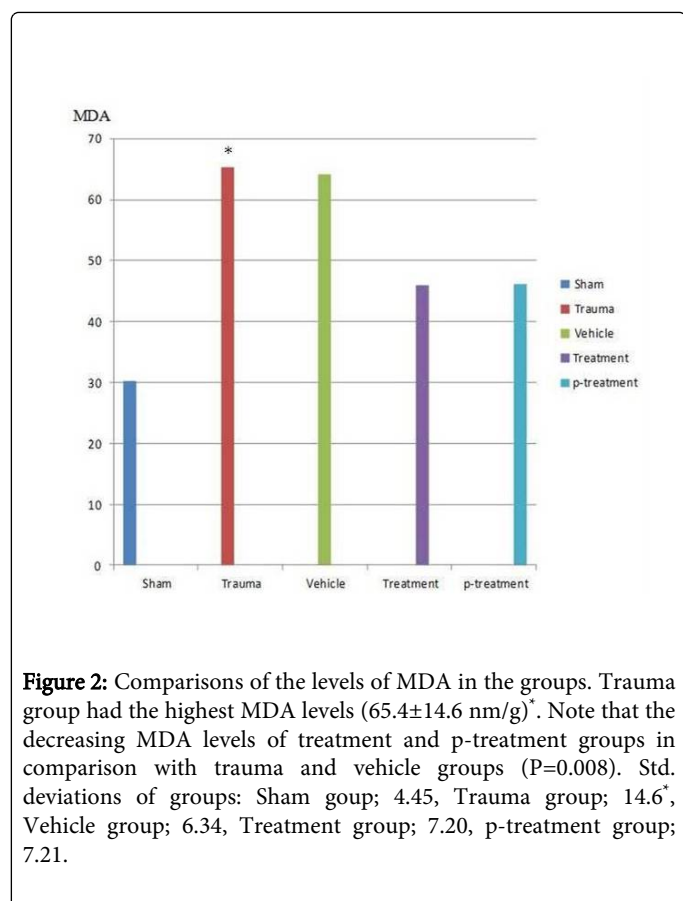
Figure 2: Comparisons of the levels of MDA in the groups. Trauma group had the highest MDA levels (65.4±14.6 nm/g)*. Note that the decreasing MDA levels of treatment and p-treatment groups in comparison with trauma and vehicle groups (P=0.008). Std. deviations of groups: Sham goup; 4.45, Trauma group; 14.6*, Vehicle group; 6.34, Treatment group; 7.20, p-treatment group; 7.21.

Free oxygen radicals arising after the ischemia, depolarize the axonal membrane and increase the intracellular influx of the sodium. This energy difference across the membrane affects the Na+/Ca++ flow across the membrane. The increased calcium leads to irreversible axonal degeneration [7-9]. Additionally, increased Na+ in the intracellular compartment has edematous effects for the cell. This also leads to further cell damage. Another negative effect of the increased intracellular Na+ is alterations in the Na+– glutamate co-transportation. This leads to glutamine secretion and/or decreased re-uptake, which further results in toxicity on NMDA and non-NMDA receptors [10]. Vasoconstriction develops after trauma and increases ischemia and lipid peroxidation.

Rifampicin may function as a hydroxyl radical scavenger with its naphthohydroquinone ring. This provides a neuroprotective effect on spinal cord ischemia. The neuroprotective effects of free-radical scavengers in neurodegenerative disorders and cerebral ischemia are well known. Rifampicin is shown to inhibit Ah1-40 aggregation and neurotoxicity in vitro. By the naphthohydroqunone ring in its molecular structure, rifampicin can inhibit Ah aggregation and neurotoxicity functioning as a hydroxyl radical scavenger [2,3]. It is also shown that rifampicin may have similiar neuroprotective effects with corticosteroids binding to and activating glucocorticoid receptors resulting in the induction of gene transcription [4]. The anti-inflammatory and myocardial protective effects of acute steroid therapy mediated by nontranscriptional activation of eNOS (endothelial nitricoxide synthase) through the PI3K/Akt pathway were previously described [10]. Transcriptional down regulation of eNOS expression and increased systemic blood pressure cause increased risk of stroke after long-term corticosteroid therapy [11]. Neuroprotection is mediated by a rapid nonnuclear effect of glucocorticoid receptor (GR). High-dose corticosteroids applied for acute serebral ischemia results in increased eNOS activity and cerebral blood flow, and reduced cerebral infarct size by 32% [12]. The GR antagonist RU486 and inhibition of phosphatidylinositol 3-kinase (PI3K) act to remove these neuroprotective effects of corticosteroids [12]. Rifampicin-mediated inhibition of apoptosis and activation of caspase-3 and capase-8 via GR activation has been reported [13]. RU486 blocks the rifampicin-mediated inhibition of apoptosis and caspase activation [3]. In addition, while the expression of pro-apoptotic Bax are inhibited by rifampicin, the expression of anti-apoptotic Bcl-2, Bcl-XL, and of anti-apoptotic gene products such as XIAP, cIAP2, FLIPs, playing important roles in the blockage of ischemia-mediated cell death are stimulated by rifampicin. Because RU486 had no effect on the regulation of these gene, the suggestion about induction of changes in the expression of these molecules via a signaling pathway that is independent of GR has been advised by some authors [3,13,14].

There are various studies in the literature evaluating the relation between the MDA levels and the ischemia-reperfusion injury [7,15]. In the present study, the difference between the sham group and the treatment group regarding tissue MDA levels was significant (P=0.016). Furthermore, comparison of the trauma and vehicle groups with the treatment group regarding MDA levels demonstrated significant differences (P=0.008). In our opinion, this is the result of hydroxyl radical scavenger effect of rifampicin after the ischemia-reperfusion injury. We think that rifampicin prevents secondary injury and decreases the MDA levels.

Neurological improvement in the treatment group after administration of 20 mg/kg rifampicin is the result of its neuroprotective and antioxidant effects through its free radical scavenger and activator of glucocorticoid receptor effects.

Conclusions

Rifampicin is known as a potent free radical scavenger and activator of glucocorticoid receptors. This antioxidant and neuroprotective effects of rifampicin were assessed in a rat spinal I/R model. The level of MDA were measured as an indicator of ischemia severity. Further studies need to be conducted, using larger animals and other spinal cord injury models.

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