Ischemic spinal cord injury induced by aortic cross-clamping: prevention by riluzole

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Abstract

Objective: Recent studies confirmed the deleterious role of glutamate in the pathophysiology of spinal cord ischemia induced by aortic cross-clamping. We investigated the effect of riluzole, an anti-glutamate drug, in a rat model of spinal cord ischemia.

Materials and methods: Spinal cord ischemia was induced in normothermia for 14 min in Sprague-Dawley rats using direct aortic arch plus left subclavian artery cross-clamping through a limited thoracotomy. Experimental groups were as follows: sham-operation (n = 15), control (n = 15) receiving only vehicle, riluzole (n = 15) receiving riluzole (4 mg/kg) before clamping and at the onset of reperfusion. Separate animals were used for monitoring physiologic parameters in the sham-operation (n = 3), control (n = 5), and riluzole (n = 5) groups. Neurologic status was assessed at 6, 24 h, and then daily up to 96 h. Rats were randomly killed at 24, 48, or 96 h (n = 5 for each time). Spinal cords were harvested for histopathology, immunohistochemistry for microtubule-associated protein 2 (MAP-2), TUNEL staining, and analysis of DNA fragmentation by agarose gel electrophoresis.

Results: All sham-operated rats had a normal neurologic outcome, whereas all control rats suffered severe and definitive paraplegia. Riluzole-treated rats had significantly better neurologic function compared to the control. Histopathology disclosed severe neuronal necrosis in the lumbar gray matter of control rats, whereas riluzole-treated rats suffered usually mild to moderate injury. Riluzole particularly prevented motor neurons injury. MAP-2 immunoreactivity was completely lost in control rats, whereas it was preserved either completely or partly in riluzole-treated rats. TUNEL staining revealed numerous apoptotic neurons scattered within the whole gray matter of control rats. Riluzole prevented or dramatically attenuated apoptotic neuronal death in treated rats. DNA extracted from lumbar spinal cords of sham-operated and riluzole-treated rats exhibited no laddering, whereas spinal cords from control rats showed DNA laddering with fragmentation into <180 multiples of base pairs.

Conclusions: Riluzole may protect the spinal cord in a setting of severe ischemia by preventing neuronal necrosis and apoptosis. This drug may therefore be considered for clinical use during ‘high risk’ surgical procedures on the thoracoabdominal aorta.

Keywords: Riluzole; Spinal cord ischemia; Excitotoxicity; Aorta

1. Introduction

Spinal cord ischemia with resulting paraplegia remains a devastating complication after repair of thoracoabdominal aortic aneurysms or dissection [1,2]. Recent studies demonstrated the deleterious role played by excitotoxic mechanisms in the pathophysiology of spinal cord ischemia [3,4]. Riluzole is an anti-excitotoxic drug that has demonstrated efficacy in several models of cerebral ischemia and spinal cord injury [5]. Moreover, this drug is in clinical use among patients with amyotrophic lateral sclerosis, since 1995 [5,6].

The goal of this study was to observe whether riluzole could consistently prevent spinal cord ischemic injury in a rat model of transient spinal cord ischemia induced by aortic cross-clamping and to analyze whether this drug could prevent neuronal necrosis or apoptosis or both.

2. Materials and methods

2.1. Materials

Sixty-eight Sprague-Dawley male rats weighing 350–
400 g were used in this study. All animals were allowed free access to laboratory chow and tap water in day/night regulated quarters at 25°C. Animal care and experiments complied with the European Convention on Animal Care and was approved by the local Sub-Committee for Research and Animal Care.

2.2. Surgical procedure

Anesthesia was induced in a chamber containing 3% halothane and was maintained by inhalation through a facial mask of 1.5% halothane driven by oxygen 2 l/min. Rectal temperature was continuously monitored with a flexible probe inserted 7 cm into the rectum, and supported by a thermal pad and a heating lamp. Body temperature was maintained at 37.5 ± 0.5°C during the surgical procedure (Table 1). The rats were placed in the supine position and a longitudinal incision was made through the skin in the sternal region. The left jugular vein was used for intravenous injection of drugs or solutions. The chest wall was incised from the apex of the manubrium caudal along the left sternal border, to the third rib. Care was taken to avoid the left superior vena cava and the left internal mammary artery. The thymus was retracted superiorly and the aortic arch was isolated between the left common carotid and the left subclavian arteries, avoiding the left recurrent laryngeal nerve. Heparin (100 U/kg) was administered intravenously 10 min before aortic clamping. Spinal cord ischemia was induced by aortic arch plus left subclavian artery cross-clamping for 14 min, using two Micro Vessel Clips (Catalog No 14-1020, Biomedical Research Instruments, Rockville, MD). Then the clips were removed and the chest was closed in layers. Five milliliters of 0.9% saline were injected intraperitoneally after completion of the surgical procedure. Sham-operated animals underwent an identical surgical procedure except for aortic arch and left subclavian artery clamping. Animals were allowed to recover in a plastic box at 28°C for 3 h, and were placed in their cages. The créder’s maneuver was used twice daily to empty the urinary bladder of paraplegic animals.

2.3. Study groups

Animals were separated in different study groups as follows:

- sham-operation (n = 15): operation was performed using similar conditions except for aortic and subclavian artery clamping;
- control (n = 15): animals received only vehicle;
- riluzole (n = 15): riluzole (4 mg/kg) was injected 30 min before aortic clamping and at the onset of reperfusion.

All rats received a similar volume of solutions: 1 ml for all the procedure. Riluzole (Research Biochemicals International, Natick, MA) was first dissolved in 0.1 N hydrochloric acid then diluted in distilled water.

2.4. Measurement of mean arterial blood pressure (MABP)

Separate groups of animals were anesthetized using similar conditions: sham-operation (n = 3), control (n = 5), riluzole (n = 5). An intravenous catheter (24 G) was inserted in the tail artery and connected via a transducer to a pressure monitor (Hewlett-Packard, Palo Alto, CA) for continuous monitoring of distal MABP. Distal arterial blood pressure was monitored throughout the procedure. Catheter was removed after 30 min of reperfusion, and the tail incision was closed.

2.5. Evaluation of neurobehavioral outcome

Serial assessments of motor and sensory functions in the hindlimbs of all rats were performed at 6, 24 h, and then daily up to 96 h of reperfusion, using the score of Le May et al. [7] with modifications:

(a) walking with lower extremities:
- 0, normal;
- 1, toes flat under body when walking but ataxia is present;
- 2, knuckle walking;
- 3, movements in lower extremities but unable to knuckle walk;
- 4, no movement, drags lower extremities.

(b) Pain sensation:
- 0, normal, withdrawal to toe pinch;
- 1, squeals to toe pinch but does not withdraw;
- 2, no reaction to toe pinch.

A motor sensory deficit index (MSDI) was calculated for each animal at each time point. The final index was the sum of the scores (a) and (b), and the maximum deficit was indicated by a score of 6.

### Table 1

<table>
<thead>
<tr>
<th>Physiologic parameters*</th>
<th>Sham (n = 3)</th>
<th>Control (n = 5)</th>
<th>Riluzole (n = 5)</th>
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<tr>
<td>MABP (mm Hg)</td>
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<td></td>
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<tr>
<td>Preischemia</td>
<td>97 ± 2</td>
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<td>93 ± 2</td>
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<tr>
<td>30-min reperfusion</td>
<td>86 ± 2*</td>
<td>63 ± 4</td>
<td>68 ± 5</td>
</tr>
<tr>
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<tr>
<td>Preischemia</td>
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<td>37.5 ± 0.6</td>
<td>37.6 ± 0.2</td>
</tr>
<tr>
<td>End ischemia</td>
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<td>37.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>30-min reperfusion</td>
<td>37.6 ± 0.6</td>
<td>37.6 ± 0.3</td>
<td>37.2 ± 0.4</td>
</tr>
</tbody>
</table>

* MABP, distal mean arterial blood pressure, measured in the tail artery; preischemia, value measured within 1 min before clamping; 5 min ischemia, value measured after 5 min of aortic clamping; 30 min reperfusion, value measured 30 min after unclamping. Physiologic data were analyzed with one way ANOVA analysis of variance. Statistically significant difference among experimental groups is expressed by *.
2.6. Euthanasia

Animals were randomly assigned to sacrifice at 24, 48, or 96 h of reperfusion (n = 5 for each time, in each group). Animals were anesthetized with 3% halothane and transcardially perfused with 100 ml of 0.9% saline solution at 4°C. Spinal cords were quickly harvested, and placed either in fresh 4% paraformaldehyde at 4°C for 48 h, or frozen in isopentane on dry ice and stored at −80°C until use.

2.7. Histopathology

Spinal cords were removed from 4% paraformaldehyde after 48 h fixation. Specimens were dehydrated in alcohol 95% for 30 min, followed by four changes of 100% alcohol for 1 h each and five changes of toluene for 1 h each under vacuum at 37°C. Spinal cords were infiltrated with paraffin and embedded in paraffin at 57°C under vacuum and pressure. Transverse sections were made on a microtome (Leica). Ten micrometer-sections were obtained through the lumbar spinal cord. Sections were stained with hematoxylin and eosin (H&E) and the luxol fast blue staining method and were examined under the light microscope. All animals had their spinal cord examined. The neuropathologist who performed examination was blinded to the experimental conditions.

The Rexed’s classification was used to describe the locations of damaged neurons in gray matter [8].

2.8. Immunohistochemistry for MAP-2

Sections were immersed in 0.3% H2O2 for 10 min, blocked with 5% goat serum (Vector Labs) and 3% Triton for 1 h at room temperature, followed by a rinse in PBS. Then sections were incubated with the primary antibody overnight. The antiserum used for the study of cytoskeletal protein expression was a monoclonal mouse anti-MAP-2 (Clone HM-2, Sigma, diluted 1:500). After the primary incubation and three rinses in PBS, sections were incubated in biotinylated horse anti-mouse IgG (diluted 1:100, Vector Labs.) for 3 h. MAP-2 expression was visualized by 3,3′-diaminobenzidine and nickel chloride (DAB-Ni) staining using the Vectastain ABC kit (Vector Labs.). All sections were washed a final time in PBS and distilled water, and then mounted with glycerol. Sections were examined under the light microscope.

2.9. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) staining method

Coronal 10 μm sections were used and processed according to the TUNEL method as described by Gavrieli et al. [9]. The in situ cell death detection kit (Hoffmann–La Roche Ltd., Basel, Switzerland) and VectaStain ABC kit (Vector Laboratories, Inc., Burlingame, CA) were used for this purpose. Cells with nuclei frankly stained by the TUNEL method or containing apoptotic bodies were considered to be apoptotic. Neurons which nuclei exhibited very faint TUNEL staining and that did not contain apoptotic bodies were considered to be necrotic.

2.10. Analysis of DNA fragmentation by agarose gel electrophoresis

Six separate animals were operated: sham-operation (n = 2), control (n = 2), and riluzole (n = 2) and sacrificed at 24 h of reperfusion using the technique described previously. Rats were perfused with 100 ml of 4°C cold saline and the lumbar spinal cord harvested quickly and flash frozen in liquid nitrogen before storing at −80°C. The Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) was used to extract DNA from lumbar spinal cords. DNA electrophoresis was performed as described by Du et al. [10].

2.11. Statistical analysis

Statistical analyses of measured physiologic data were performed by one-way ANOVA analysis of variance. All physiologic data are expressed as mean ± SE. Neurologic scores were analyzed with Kruskal–Wallis tests followed by Mann–Whitney U-tests when significant. Differences were considered statistically significant for P < 0.05.

3. Results

3.1. Neurobehavioral outcome

Three rats in the control group (20%) and one (6.6%) in the riluzole group died either during or after operation (left superior vena cava wound 1, right pneumothorax 1, postoperative bowel ischemia 2). Those rats were replaced by other rats to reach the appropriate n in each group. All rats survived the entire randomly assigned observation period. All sham-operated rats had a normal postoperative neurologic outcome, whereas all control rats had a MSDI of 6 at 6 h, and remained severely paraplegic throughout the observation period with no improvement in either sensory or motor function. Control rats had flaccid paraplegia after recovery from anesthesia and either developed spasticity within 6 h or exhibited flaccid paraplegia until sacrifice. They also suffered urinary bladder dilatation. Five of 17 rats (29%) suffered temporary pain-like response to light touch known as allodynia during the first 24 h of reperfusion. Neurologic outcome was significantly better in riluzole-treated rats at any time considered (Table 2). All riluzole-treated rats had delayed recovery from anesthesia (1–3 h) consistent with anesthetic properties of riluzole [5]. All riluzole-treated rats had some form of flaccid paraplegia after recovery from anesthesia, but began to recover motor function between 3 and 6 h postoperatively. At 24 h, 66.6% of rats had a completely normal motor function, whereas...
33.3% suffered mild to middle deficit. Partial or complete sensory deficit was diagnosed in 60% of rats. These results persisted at 48 h, and at 96 h 60% of rats had a normal motor and sensory function, whereas 40% suffered middle motor deficit and severe sensory deficit.

3.2. Histopathology

H&E and luxol fast blue staining were used to analyze neuronal cell death in gray matter. The extent of ischemic damage was grossly proportional to the neurologic score. Ischemic damage was observed almost exclusively in gray matter, which contained typically necrotic neurons with eosinophilic cytoplasm (‘red neurons’) and loss of cytoplasmic structures (‘ghost neurons’), but also neurons demonstrating apoptotic features as shrinkage, chromatin condensation, and apoptotic bodies. Sham-operated rats had normal spinal cords. All control rats had very severe ischemic injury with global necrosis of gray matter. In most severe cases, white matter surrounding gray matter was also damaged in spinal cord sections of rats sacrificed at 48 h, or later. Most riluzole-treated rats (MSDI 0–2) had either no or mild neuronal damage predominantly in laminae 3 to 7 and 10 in gray matter. Riluzole-treated rats with MSDI 3–4 had variable-size confluent necrotic areas predominantly in laminae 3 to 7 and 10. Laminae 1 and 2 in dorsal horns. Lamina 8 and lamina 9 containing motor neurons were variably affected depending on the neurologic status.

![Fig. 1. Histopathology (original magnification × 400). Representative photomicrographs of lumbar spinal cord sections stained with Klüver–Barrera stain from sham-operated (A), control (B), and riluzole-treated (C) rats that underwent 14 min of spinal cord ischemia and 48 h of reperfusion. (A) Normal appearance of motor neurons and surrounding gray matter in the ventral horn. (B) Necrotic motor neurons with vacuolization of gray matter in the ventral horn of a control rat. (C) Motor neurons in the ventral horn appear grossly normal despite 14 min of ischemia and 48 h of reperfusion in a riluzole-treated rat with a MSDI of 2.](https://academic.oup.com/ejcts/article-abstract/18/2/174/425608)
Motor neurons were usually not injured with MSDI of 3, whereas less than 50% were injured with MSDI being 4 (Fig. 1).

3.3. Immunohistochemistry for MAP-2

The cytoskeletal protein MAP-2 is involved in maintaining neuronal structural integrity. It is extremely sensitive to ischemic insult and immunoreactivity for MAP-2 has been demonstrated to be a sensitive, accurate and early marker of central nervous system injury following ischemia [11]. The spatial distribution for loss of MAP-2 immunoreactivity paralleled ischemic injury defined by H&E staining. MAP-2 immunoreactivity was strongly expressed throughout gray matter of sham-operated rats, particularly in neuronal perikaryon and dendrites. Spinal cord from control rats exhibited a complete loss of MAP-2 immunoreactivity throughout gray matter (laminae 1 to 10) as early as 24 h following ischemia. Spinal cord from riluzole-treated rats exhibited either grossly normal MAP-2 immunoreactivity (MSDI 0–2), or focal decrease of MAP-2 immunoreactivity in dorsal horns body (laminae 3 to 6) and intermediate zone (lamina 7) (MSDI 3–4) (Fig. 2).

3.4. TUNEL staining

Spinal cords from sham-operated rats showed no evidence of TUNEL staining. When treated with DNAase, those cords demonstrated TUNEL-stained nuclei (data not shown). Spinal cord samples from control rats showed numerous TUNEL-positive neurons scattered within gray matter. Numbers of TUNEL-positive neurons increased in the ventral to dorsal direction. They were most highly concentrated in laminae 1 and 2, then in laminae 3 to 7 and 10. Laminae 8 and 9 contained usually less TUNEL-positive cells. The number of TUNEL-positive cells increased from 24 h, reached a peak at 48 h and decreased after that. Spinal cords from riluzole-treated rats showed very few to numerous TUNEL-positive neurons scattered within intermediate gray matter and dorsal horns body, depending on the neurologic status of the rat (Fig. 3). There were usually no TUNEL-positive neurons in ventral horns. The total number of apoptotic neurons within gray matter was less in all riluzole-treated rats compared to control rats (Fig. 3).

3.5. DNA fragmentation study

DNA extracted from the whole lumbar spinal cord from sham-operated rats exhibited no laddering, whereas DNA from control rats showed a ladder of multiples of ≈180-bp-sized fragments after electrophoresis in agarose gel. DNA extracted from riluzole-treated animals exhibited no laddering (Fig. 4).

4. Discussion

Paraplegia resulting from spinal cord ischemia remains a dreaded complication of thoracoabdominal aortic surgery despite various surgical adjuncts and pharmacological interventions [1,2,12]. The rat model used in this study has been valid for investigating the effects of ischemia on spinal cord function. The results presented in this study suggest that riluzole, a drug currently used to treat amyotrophic lateral sclerosis, can be effective in reducing ischemic damage to the spinal cord. Further studies are needed to determine the mechanism by which riluzole exerts its protective effects and to explore the potential of riluzole as a therapeutic agent for spinal cord ischemia.
adapted from Le May et al. [7]. It is highly reproducible, because 100% of control rats operated in strict normothermia and clamped for 14 min suffered severe and definitive paraplegia. Operative mortality was low (3%) and severe bowel ischemia occurred in only 10.5% of control rats. Spinal cord arterial vascularization is almost similar in rat and man, with an heterosegmental aorta and few anterior radicular arteries reaching the anterior spinal artery [13]. Combined clamping of both aortic arch and left subclavian artery reproduces the operative situation encountered during repair of the descending thoracic or thoracoabdominal aorta. Blood flow not only to the spinal cord, but also to liver, bowel and kidneys is dramatically reduced during the clamping period [12,13]. Thus, no specific problem such as metabolic acidosis, bacterial translocation, coagulopathy, or renal failure, is occulted in this model. This is of importance because in the rabbit model of spinal cord ischemia, that has been the most widely used for the past 15 years, the aorta is cross-clamped below the renal arteries, occulting by this fact additional problems that may interfere with the level of neuronal damage in the spinal cord [14].

Recent studies demonstrated the deleterious role played by excitotoxic mechanisms in the pathophysiology of spinal cord ischemic injury due to aortic cross-clamping [3,4]. Drugs aimed at blocking excitotoxicity as NMDA and non-NMDA receptor antagonists have demonstrated efficacy in several animal models of cerebral or spinal cord ischemia [3,15–17]. However, none of these drug consistently proved efficacy in clinical trials and the potential for

![Fig. 3. TUNEL staining. TUNEL staining at L4 after sham-operation (A), or after 14 min ischemia + 48 h reperfusion (B and C). After sham-operation (A) there are no TUNEL-labeled neurons. After ischemia (B) there are numerous TUNEL-positive neurons scattered within the gray matter (dorsal horn, intermediate gray, ventral horn). There is no TUNEL-positive motor neuron. Riluzole treatment (C) resulted in a dramatic decrease in TUNEL labeling within the almost whole gray matter, especially intermediate zone and ventral horn. Only few small-sized neurons are TUNEL-positive in dorsal horn. Scale bar 200 μm.](image)

![Fig. 4. DNA fragmentation measured by agarose gel electrophoresis. Gel electrophoresis of DNA extracted from animals that underwent sham-operation (S), 14-min ischemia (C), 14-min ischemia + riluzole treatment (R), and DNA extracted from a normal rat that was sacrificed without any operation (N). DNA extracted from sham-operated rats (S), normal rat (N), and riluzole-treated rats (R) exhibit no laddering, whereas DNA extracted from control (C) rats exhibit laddering consistent with the presence of DNA fragments with lengths being multiples of 180 base pairs (bp). DNA marker positions in base pairs are shown on the left. (+) or (−) indicates the presence or absence of laddering.](image)
serious adverse effects and morphological neuronal changes associated with this class of compounds limit their clinical use [17,18].

Riluzole is a neuroprotective drug that has demonstrated anti-ischemic properties in several models of cerebral ischemia [5]. The exact molecular target of riluzole is unknown yet, but its mechanism of action include an inhibition of presynaptic release of glutamate, an inactivation of both sodium and calcium ion voltage-dependent channels, a direct but noncompetitive inhibition of NMDA receptors, and an activation of a recently discovered class of background potassium ion channels that are highly expressed in brain and spinal cord [5,19,20]. In addition, recent studies indicated that riluzole could improve mitochondrial function and increase sodium ion-dependent glutamate reuptake after spinal cord injury [21]. In vitro, riluzole has been reported particularly efficient for motoneuronal protection [5]. Moreover, as a sodium ion channel blocker, riluzole may also be effective on white matter injuries that have been reported after incomplete spinal cord ischemia [17,22,23]. Although neuronal death carried by excitotoxic mechanisms has been classically attributed to necrosis, recent reports demonstrated that apoptosis, a form of programmed cell death, may also be triggered by excessive excitatory amino-acids release, free radicals formation occurring after spinal cord ischemia/reperfusion, or activation and cleavage of caspases [24–26]. The present study demonstrates that riluzole injected intravenously before aortic clamping and at unclamping may prevent both necrosis and apoptosis of spinal cord neurons. Histopathologic examination of spinal cords harvested on most riluzole-treated animals showed either no or very little evidence of necrotic injury, whereas spinal cords from control rats exhibited extensive necrotic injury throughout gray matter. Those findings were confirmed by MAP-2 immunohistochemistry: riluzole-treated rats had preserved immunoreactivity, whereas control rats had evidence of extensive cytoskeletal protein MAP-2 breakdown, that is known to be an early and sensitive marker of acute ischemic injury. Compared with control rats, there were fewer TUNEL-positive neuronal cells in the gray matter of most riluzole-treated rats, and no laddering could be found in DNA samples obtained from those rats, suggesting that neuronal apoptosis was also efficiently prevented or attenuated by riluzole (Table 3).

Neither perioperative hypothermia nor increase in arterial blood pressure can account for the protective effect of riluzole observed in this study, because rectal temperature and arterial blood pressure were closely monitored and did not differ among experimental groups (Table 1). Riluzole is currently in clinical use among patients with amyotrophic lateral sclerosis since 1995 [5,6]. Except for one case of temporary liver dysfunction, no major side effects have been reported with chronic treatment 100–200 mg/day over a 4-year period [27]. Although higher dose may be required for adequate neuroprotection (4–8 mg/kg), it seems highly improbable that a single 8 mg/kg injection may result in major adverse effects in closely monitored patients undergoing major aortic surgery. We anticipate that riluzole may have important implications in clinical situations as thoracoabdominal aortic surgery or aortic arch surgery, that can trigger excitotoxicity in the spinal cord or brain, respectively [4,28]. In these particular situations a single injection of riluzole before aortic clamping or circulatory arrest may prevent neuronal necrosis and apoptosis by extending ischemic tolerance before adequate brain or spinal cord perfusion could be reestablished. Riluzole may also be of special interest during repair of traumatic ishmic aortic rupture, by extending ischemic tolerance of the spinal cord and making distal perfusion methods and high dose heparin unnecessary.

5. Conclusions

Riluzole provided robust protection against neuronal necrosis and apoptosis in a highly reproducible rat model of spinal cord ischemia. We anticipate that riluzole may have important clinical implications during ‘high risk’

Table 3

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>Sham-operation</th>
<th>Control</th>
<th>Riluzole</th>
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<tr>
<td>Dorsal horn</td>
<td>Normal</td>
<td>Necrosis/apoptosis</td>
<td>Necrosis/apoptosis</td>
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surgical procedures on the descending thoracic or thoracoabdominal aorta.

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References


