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Erythropoietin enhances neurological recovery after experimental spinal cord injury

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Abstract. *Purpose:* Erythropoietin is a hematopoietic growth factor. It is also involved in neurodevelopment and neuroprotection. The purpose of this study was to evaluate the effectiveness of erythropoietin in enhancing the neurological recovery following experimental spinal cord injury.
Methods: Rats were randomly divided into 4 groups. Group 1 received only laminectomy. Group 2, 3, and 4 have undergone a spinal contusion injury of 50 gr/cm. Group 2 received no medication. Group 3 received 30 mg/kg methylprednisolone. Group 4 received 5000 IU/kg recombinant human erythropoietin. Following injury, neurological recovery was evaluated for 14 days, using a swimming test.
Results: At day 1, there was no difference between mean motor scores of group 2, 3, and 4. Following day 1, rats in group 4 exhibited a marked improvement in motor score, and this was maintained throughout the study. When compared to group 2, mean motor score of group 4 was significantly higher at day 4, 7, 10, and 14. When compared to group 3, mean motor score of group 4 was significantly higher at day 7, 10, and 14.
Conclusion: These findings suggest that erythropoietin enhances neurological recovery following spinal cord injury and it seems to be more effective than methylprednisolone at the given doses.

Keywords: Erythropoietin, neuroprotection, spinal cord injury

1. Introduction

The process of spinal cord injury (SCI) has two components: primary injury, resulting from the mechanical insult, and secondary injury, resulting from a cascade of biochemical reactions resulting in tissue destruction, initiated by the primary insult [25]. The rationale for pharmacological intervention in SCI is the possibility of interrupting the biochemical cascade leading to secondary injury, thereby improving the spinal cord tissue survival, and thus preserving the necessary anatomic substrate for functional recovery to take place. Currently, methylprednisolone (MP) regimen is the only accepted protocol in the treatment of acute SCI, but it provides only a minimal benefit at best [15]. Therefore, many agents are still being investigated in experimental SCI models [18].

Erythropoietin (EP) is a hematopoietic growth factor, which stimulates proliferation and differentiation of erythroid cells. It is also found to be involved in neurodevelopment and neuroprotection [6]. Although extensively studied in brain ischemia and injury, studies regarding treatment in SCI are relatively recent and limited [11]. The purpose of this study was to evaluate

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the effectiveness of EP in enhancing the neurological recovery following experimental SCI.

2. Materials and methods

The study protocol was designed to meet the guidelines of European Communities Directive (86/609/EEC) of 24 November 1986, regarding the protection of animals used for experimental and other scientific purposes, and was approved by the ethics committee. Seventy male Wistar rats weighing 180–220 gr were obtained. The rats were randomly divided into 4 groups. Group 1 had 10 rats, and the remaining groups had 20 rats each. All the rats have undergone general anesthesia using 60 mg/kg ketamine hydrochloride and 0.05 mg/kg fentanyl citrate, administered intramuscularly. The rats were placed in prone position and fixed to the operating board. Following a vertical skin incision from T4 to T9, paravertebral muscles were dissected, and T6–T7 laminectomy was performed. The dura was kept intact. Following laminectomy, surgical procedure was ended for the rats in group 1. The skin was closed as a single layer, using 2.0 silk sutures.

In the remaining groups, experimental spinal trauma was performed. A 5 gr steel bullet was dropped through a 10 cm tube onto the dorsal surface of the spinal cord, exerting an impact of 50 gr/cm. After this, the surgical procedure was ended and the skin was closed as a single layer with 2.0 silk sutures.

The rats in group 2 received no medication following spinal trauma. In group 3, 30 mg/kg MP was administered intraperitoneally, within 60 minutes following trauma. In group 4, 5000 IU/kg recombinant human erythropoietin (rhEP) was administered intraperitoneally, within 60 minutes following trauma.

All the rats were followed up for 14 days. Manual bladder evacuation was performed twice daily until spontaneous voiding had resumed. Neurological motor function was evaluated by an independent observer in a blinded fashion, twice daily, starting from day 0.

To evaluate motor function, a swimming test described by Gorio et al. [11] was used. A swimming pool with a diameter of 50 cm and a water depth of 30 cm has been designed. A wire mesh ladder was attached to the side of the pool. The rats were placed into the pool, one at a time, and were observed when swimming in and climbing out of the pool. Motor scoring was performed as follows; “0” if there was no use of hind limbs, “1” if there was partial use of hind limbs, and “2” if there was normal use of both hind limbs.

Statistical analysis was performed using SPSS 12.0 database and independent “t” test. $P < 0.05$ was considered as statistically significant.

3. Results

All the rats in group 2, 3, and 4 were profoundly affected immediately after injury. Mean motor score in all these three groups were 0 at day 0. On the other hand, rats in group 1 have never demonstrated motor weakness, and mean motor score of this group was 2 throughout the study, including day 0.

First traces of motor recovery were noted at day 1 in group 4, at day 2 in group 3, and at day 3 in group 2. At day 1, mean motor score of group 2 and 3 were 0, and mean motor score of group 4 was 0.2. There was no statistically significant difference between mean motor scores of group 2, 3, and 4 at day 1.

Following day 1, rats in group 4 exhibited a marked improvement in motor score, and this was maintained throughout the study. At day 4, mean motor score of group 2 was 0.2, group 3 was 0.3, and group 4 was 0.7. At day 7, mean motor score of group 2 was 0.5, group 3 was 0.6, and group 4 was 1.2. At day 10, mean motor score of group 2 was 0.6, group 3 was 0.8, and group 4 was 1.4. At day 14, mean motor score of group 2 was 0.7, group 3 was 0.9, and group 4 was 1.6.

When compared to group 2, mean motor score of group 4 was significantly higher at day 4, 7, 10, and 14. When compared to group 3, although the mean motor score was slightly higher at day 4, it was not statistically significant. But at day 7, 10, and 14, mean motor score of group 4 was significantly higher than group 3.

Mean motor score of group 3 was slightly higher than group 2, starting from day 2. But the difference could not reach to a statistical significance throughout the study (Table 1).

4. Discussion

The process of SCI has two components: primary injury, resulting from the mechanical insult, and secondary injury, resulting from a cascade of biochemical reactions resulting in tissue destruction, initiated by the primary insult [25]. Primary insult damages the spinal cord as a result of contusive, compressive or stretch injury. But most of the time, it actually do not lead to the physical transection of the cord. Typically, residual

Table 1
Mean motor scores of group 2, 3, and 4 at day 1, 4, 7, 10 and 14

	Mean motor scores			Statistical analysis		
	Group 2	Group 3	Group 4	p 3-2	p 4-2	p 4-3
Day 1	0	0	0.2	*	0.036	0.036
Day 4	0.2	0.3	0.7	0.478	**0.001**	0.011
Day 7	0.5	0.6	1.2	0.537	$<$ **0.001**	$<$ **0.001**
Day 10	0.6	0.8	1.4	0.176	$<$ **0.001**	$<$ **0.001**
Day 14	0.7	0.9	1.6	0.120	$<$ **0.001**	$<$ **0.001**

*P value can not be calculated because both means are zero.

white matter containing portions of the ascending sensory and descending motor tracts remains intact, allowing for the possibility of neurological recovery [18]. However, during the first minutes following primary insult, a cascade of biochemical reactions are initiated, which is proportional to the magnitude of primary injury, leading to a secondary injury. The rationale for pharmacological intervention in SCI is the possibility of interrupting the biochemical cascade leading to secondary injury, thereby improving the spinal cord tissue survival, and thus preserving the necessary anatomic substrate for functional recovery to take place.

The most immediate event following primary injury is the mechanically induced depolarization leading to opening of the voltage dependent ion channels. This leads to release of various neurotransmitters, including glutamate, which further leads to opening of the glutamate receptor operated ion channels. The most important outcome of this cascade is the accumulation of intracellular calcium leading to; mitochondrial dysfunction, activation of nitric oxide synthase, phospholipase A2, and cysteine protease [15]. One of the consequences of above events is the formation of reactive oxygen species including peroxynitrite anion. Although it can trigger cellular damage by a variety of mechanisms, lipid peroxidation (LP) has been conclusively demonstrated to be a key mechanism [13]. LP occurs in neurons, directly impairing neuronal and axonal membrane function and integrity. It also takes place in blood vessels, causing microvascular damage and secondary ischemia which contributes to secondary injury [14].

Although many pharmacological agents have been studied for the treatment of SCI, limited number of drugs has been tested in phase 3 trials: MP, tirilazad, naloxone, monosialoganglioside, thyroid releasing hormone (TRH), gacyclidine and nimodepine [15]. First multi-center, randomized study for MP in the treatment of SCI was called National Acute Spinal Cord Injury Study (NASCIS l) [4]. The results were disappointing. But later, in early 80's, an experimental study in cats have demonstrated that, very high doses of MP can inhibit post-traumatic LP in spinal cord [12]. This led to another clinical trial called NASCIS ll, and 24 hour treatment with MP was found to decrease functional injury, when compared to placebo [3]. Although it was considered as a break-through at the period, articles stating that the clinical effectiveness of MP treatment is negligible or weak at best, have been published [16]. For today, 24 hour MP treatment (30 mg/kg IV bolus followed by 23 hour infusion of 5.4 mg/kg per hour), started within the initial 8 hours of injury, is the only accepted treatment for SCI [15].

Because of the fact that, MP treatment provides a modest benefit at best, many pharmacological agents are still being investigated. EP is a hematopoietic growth factor, which stimulates proliferation and differentiation of erythroid cells. But it is also found to be involved in neurodevelopment and neuroprotection [6]. Among from liver, kidney and uterus, EP is also produced in brain [9]. It is found in the cerebrospinal fluid of the preterm newborn, and the level decreases with gestational age [17]. In addition, EP levels increases in CSF, in cases of brain ischemia, regardless of serum levels [19]. Masuda et al. [20] were first to describe that astrocytes produce EP, which is more potent than rhEP, and it binds to EP receptors (EPR) on neurons.

Due to these facts, EP investigations were initiated. The first consideration was that the EP could not pass blood brain barrier (BBB) due to its size and glycoprotein structure. Therefore, exogenously given EP could not reach to central nervous system (CNS). But it's then shown that, cerebral capillary endothelium possesses EPR, and EP crosses BBB by endocytosis first, and then by translocation into the brain [26]. Also it's shown that, EPR mRNA is up-regulated in the periphery of the cerebrocortical infarct [22]. EP has been studied extensively in brain injury. In ischemic brain injury, it decreases infarct volume, decreases cerebral edema, and increases neurological score [5]. In traumatic brain injury, it decreases the dissociation between blood flow and metabolism [10]. In subarachnoid hemorrhage

(SAH), it decreases mortality and increases functional recovery [7].

Although extensively studied in brain ischemia and injury, studies regarding treatment in SCI are relatively recent and limited. It is expected to prevent or limit secondary injury by decreasing the formation of free radicals and preventing LP [23]. It also decreases glutamate mediated neuronal apoptosis [21]. It may also modulate angiogenesis leading to an increase in tissue oxygenation [1]. It also reduces inflammatory infiltration, which is accused of late damage, especially involving oligodendrocytes [24].

Kaptanoglu et al. [18] have studied EP in the treatment of SCI, and they stated that, EP inhibits LP better than MP, and ultra-structural neuroprotection of EP is similar to MP. Celik et al. [8] have studied EP in ischemic SCI, and they concluded that it has both acute and delayed beneficial effect. Gorio et al. [11] have studied the effectiveness of EP both in ischemic SCI and in blunt trauma. They reported that it provides an early recovery of function in ischemic trauma and reduced cavitation in cord after blunt trauma.

In this study, weight-drop technique was used to create a SCI. It seems to mimic a mechanical SCI in a better fashion. In aneurysm clip models, a compressive-occlusive injury characterized by transient vascular occlusion and pressure-related block of axonal conduction, but often with minimal disruption of myelin is produced. Restoration of axonal conduction is likely to occur sooner for an axon possessing an intact myelin sheath [2]. On the other hand, in the weight-drop model, injury is mechanical in nature, and it differentially injures large myelinated fibers, with the most severe damage occurring at the nodes of Ranvier. Additionally, significant hemorrhage occurs from the severe parenchymal disruption eliciting pronounced inflammatory and degenerative processes as a secondary phase of injury [18]. Also, a laminectomy-only group was included in the study, to eliminate any suspicion of a possible injury resulting from laminectomy itself.

For evaluation of the effectiveness of EP treatment, a functional evaluation was preferred. In histological examinations, white matter may not appear greatly damaged, but it may still be unable to support transmission. Therefore, motor read-outs seem to be more sensitive [11]. As a result, although the motor recovery was no better than the untreated group in the first day, functional outcome was significantly better in the EP treated group, at day 4 and become profoundly better there after. When compared to MP treated group, EP treated group was significantly better at day 7, and it was maintained throughout the study. When MP treated group was compared to the untreated group, the difference in functional recovery could never reached a statistical significance.

MP is the only available treatment for SCI for today. Its beneficial effects are minimal. EP seems to be effective in the treatment of experimental SCI. RhEP, which is known to be a well-tolerated agent, can be the next candidate for the clinical trials.

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