

Human recombinant erythropoietin improves motor function in rats with spinal cord compression myelopathy

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Short title: Erythropoietin improves motor function in rats with developing compression myelopathy

Abstract

OBJECTIVE

Erythropoietin (EPO) is a clinically available hematopoietic cytokine. The aim of this study was to evaluate the effect of EPO on a rat model of cervical cord compression myelopathy and to explore the possibility of its use as a pharmacological treatment.

METHODS

To produce the chronic cervical cord compression model, thin polyurethane sheets were implanted under the C5-C6 laminae of rats and gradually expanded due to water absorption. In this model, motor functions significantly declined from 7 weeks after surgery. Based on the result, EPO administration was started 8 weeks after surgery.

Motor function as seen with rotarod performance and grip strength was measured 16 weeks after surgery, and then motor neurons were stained with H-E and NeuN staining, and counted. Apoptotic cell death was assessed with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining. To assess transfer of EPO into spinal cord tissue, the EPO level in spinal cord tissue was measured with an enzyme-linked immunosorbent assay for each group after subcutaneous injection of EPO.

RESULTS

High-dose EPO (5000 IU/kg) administered from 8 weeks after surgery markedly restored and maintained motor function in the Compression groups ($P < 0.01$). EPO significantly prevented loss of motor neurons in the anterior horn ($P < 0.05$) and significantly decreased the number of TUNEL-positive apoptotic cells ($P < 0.05$). The EPO level in spinal cord tissue was significantly higher in the High-dose EPO group than other groups.

CONCLUSIONS

EPO improves motor function in rats with progressive chronic compression myelopathy. EPO protects anterior horn motor neurons and inhibits neuronal cell apoptosis in spinal cord compression. The neuroprotective effects can be produced through transfer of EPO into spinal cord tissue. These findings suggest that EPO has high potential as a treatment for developing compression myelopathy.

Key words: compression myelopathy, erythropoietin, chronic cervical cord compression, medical treatment, motor neuron, apoptosis, rat.

1 **Introduction**

2 As the population ages, degenerative changes in the cervical spine progress. The spinal
3 canal gradually narrows due to cervical spondylosis, disc hernia, and ossification of the
4 posterior longitudinal ligament [1, 2]. This chronic compression of the cervical spinal
5 cord causes cervical myelopathy. The symptoms of chronic compression myelopathy
6 such as motor weakness, sensory disturbances, decreased fine motor coordination, and
7 spastic gait gradually progress over time. The main pathogenesis is presumed to be local
8 compression and spinal cord ischemia at the compressed segment [3]. At this time,
9 surgical decompression is often performed to treat cervical compression myelopathy [4-
10 6]. However, no optimal medical treatment is available to improve the neurological status
11 in patients with worsening compression myelopathy.

12 To elucidate the biological mechanism of chronic compression myelopathy and develop
13 a treatment strategy for it, a co-author, Kim, established a novel experimental model of
14 chronic cervical cord compression [7]. This model is created by inserting a sheet of water-
15 absorbing urethane-compound polymer under the laminae of rats. This model induces
16 delayed motor dysfunction and reproduces the characteristic course of clinical delayed
17 cervical myelopathy. Using this model, we have previously demonstrated that
18 pharmacological agents such as *Limaprost alfadex*, prostaglandin E1 derivative, and

19 *Cilostazol*, a selective type III phosphodiesterase inhibitor, prevent the onset of cervical
20 compression myelopathy [8, 9]. However, functional recovery from developing
21 compression myelopathy has not been elucidated in those studies.

22 We recently confirmed that granulocyte colony–stimulating factor (G-CSF) improves
23 motor function in the progressive phase of compression myelopathy and preserves
24 anterior horn motor neurons in the rat chronic spinal cord compression model [10].
25 However, in healthy people, G-CSF causes marked leukocytosis, which commonly
26 results in fever, arthralgia, and rarely, thromboembolism and splenomegaly [11].

27 Erythropoietin (EPO) is a physiological hematopoietic cytokine like G-CSF. EPO is a
28 30.4-kDa glycoprotein secreted from the kidney that stimulates red blood cell (RBC)
29 production (erythropoiesis) after binding to the EPO receptor in the bone marrow
30 [12]. EPO is commonly used in anemic patients undergoing chronic hemodialysis or
31 suffering from cancer and undergoing chemotherapy [13, 14]. EPO is also used for
32 preoperative autologous blood donation in hematologically healthy individuals [15].
33 Therefore, EPO can often be used safely, even in elderly patients or those with critical
34 disease.

35 In addition, EPO has multifunctional tissue-protective effects, including anti-apoptotic,
36 anti-inflammatory, anti-oxidative, and angiogenic effects [16-18]. During the last two

37 decades, quite a few reports have described its neuroprotective effects in cerebral
38 infarction, brain contusion, and acute spinal cord injury (SCI) in laboratory investigations
39 [19-23]. Those papers reported its effect of neuroprotection, angiogenesis, and anti-
40 apoptosis in the brain and spinal cord [18, 24]. Recently, recombinant human EPO
41 (rhEPO) was preliminarily used in a randomized clinical trial of acute SCI, and results
42 indicated the possibility of treating acute SCI with EPO [25].

43 However, no reports have shown the neuroprotective effect of EPO for compression
44 myelopathy in experimental or clinical studies.

45 Here, we investigated the neuroprotective effects of EPO for chronic cervical
46 compression myelopathy using our established rat model of spinal cord compression
47 [7].

48

49 **Materials and methods**

50 **Animal maintenance**

51 This study was approved by the Institutional Animal Care and Use Committee of
52 Yokohama City University School of Medicine (IRB: F-A-15-022). Male Wistar rats
53 (12 weeks old, weight 250-300 g; Japan SLC Inc., Hamamatsu, Japan) were housed in
54 cages for 3 weeks before surgery for adaptation to the environment. All rats were

55 trained to exercise on the rotarod device and to undergo forepaw grip strength
56 measurement for 2 weeks before surgery. Throughout this experimental period, the rats
57 had free access to water and food. Body weight was recorded every week during this
58 study.

59

60 **Surgical procedure to create the chronic compression model**

61 The detailed surgical procedure to create the chronic cervical compression model has
62 been described [7]. Under general anesthesia with 2% isoflurane, a midline incision was
63 made in the nuchal area, and the C3-Th1 laminae were exposed. A sheet of expandable
64 urethane compound polymer (size $2 \times 6 \times 0.7$ mm; Aquaprene C[®], Sanyo Chemical
65 Industries, Ltd., Tokyo, Japan) was inserted into the sublaminar space of C5-C6 (Fig
66 1A, B). This sheet gradually expands to 230% of the original volume over 48-72 hours
67 by absorbing water in the tissue. In this model, the decline in motor function is delayed,
68 with a latency period after compression introduction, and then gradual progression,
69 whereas no acute damage suggestive of SCI is observed. This model reproduces the
70 characteristic course and features of clinical cervical spondylotic myelopathy [17].

71

72

73 **Fig 1. The Chronic Compression Model**

74 A: Computed tomography (CT) axial view at the C5 level, 0.5 mm above the
75 intervertebral foramen.

76 B: CT sagittal view of the cervical spine. Aquaprene[®] (expandable urethane compound
77 sheet, size $2 \times 6 \times 0.7$ mm) was inserted under the C5-C6 laminae.

78

79

80 **Experimental design**

81 **Preliminary experiment**

82 As a preliminary experiment, we confirmed the course of motor function decline in
83 this model to determine when to administer EPO in the treatment experiment.

84 Briefly, 40 rats were allocated to two groups; Sham operation group (n = 15) and
85 Compression group (n = 25). In the Sham group, rats underwent a sham operation; the
86 polymer sheet was placed under the laminae and removed immediately. In the
87 Compression group, this polymer sheet was left in place and continued to compress the
88 spinal cord chronically (Fig 1A, B). The motor functions were evaluated once a week
89 from 1 week before surgery to 26 weeks after surgery.

90

91 **Treatment experiment (Fig 2)**

92 In the treatment experiment, 48 rats were allocated to four groups; Sham group (sham
93 operation + normal saline [NS]; n = 12), Vehicle group (compression + NS; n = 12),
94 Low-dose EPO group (compression + EPO low dose; n = 12), and High-dose EPO
95 group (compression + EPO high dose; n = 12). From the results of the preliminary
96 experiments, the motor function was significantly decreased 8 weeks after surgery.
97 Therefore, administration of rhEPO or NS was started from 8 weeks after surgery and
98 lasted until 16 weeks; the frequency of administration was twice a week. In the Sham
99 group, rats underwent the sham operation and received administration of NS
100 subcutaneously. In the Vehicle group, rats underwent polymer sheet implantation and
101 received administration of NS subcutaneously. In the Low-dose EPO group, cervical
102 compression model rats received rhEPO 500 IU/kg/day (rhEPO; kindly provided by
103 Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) subcutaneously. In the High-dose EPO
104 group, cervical compression model rats received administration of rhEPO 5000
105 IU/kg/day subcutaneously. The motor functions were also evaluated once a week from 1
106 week before surgery to 16 weeks after surgery. Histological assessment of the anterior
107 horn was evaluated at 16 weeks after surgery (Fig 2A).

108

109

110

111

112 **Fig 2. Treatment Experiment: Experimental Design**

113 Administration of low-dose and high-dose EPO and normal saline was started twice a
114 week at 8 weeks postoperatively.

115 **A:** Forty-eight rats were divided into four groups (Sham, Vehicle, High-dose EPO,
116 Low-dose EPO). The motor functions of rotarod performance and grip strength were
117 evaluated once a week before surgery to 16 weeks after surgery. Every rat was
118 sacrificed, and histological analysis was performed (H-E staining and NeuN staining).

119 **B:** Another 18 rats were divided into three groups (Sham, Vehicle, High-dose EPO).
120 Treatment was done from 8 weeks to 10 weeks after surgery, and all rats were sacrificed
121 at 10 weeks after surgery. Apoptotic cells were evaluated with TUNEL staining at 10
122 weeks after surgery.

123 **C:** Another 12 rats were divided into three groups (Vehicle, High-dose EPO, Low-dose
124 EPO). Each single treatment was done 8 weeks after surgery. All rats were sacrificed 12
125 hours after injection, and the EPO level in the spinal cord was measured using a rhEPO
126 enzyme-linked immunosorbent assay (ELISA).

127

128

129 **Motor function analysis**

130 *Rotarod performance*

131 Rotarod performance was assessed by using the rotarod device (ENV-557, Med
132 Associates Inc., St. Albans, VT). Based on our previous research, a moderate rotation
133 speed of 10 rpm was set [7-10]. All rats could walk on the rotarod for more than 300
134 seconds before surgery. Therefore, 300 seconds was set as the cut-off. Three trials in
135 each session were performed for all rats. We recorded the longest duration time of the
136 three trials.

137

138 *Forelimb grip strength*

139 Forelimb grip strength was assessed by using a digital force meter (MK-380CM/F,
140 Muromachi Kikai, Tokyo, Japan). We assessed grip strength according to the methods
141 of Meyer et al [26]. The animals were evaluated before surgery and once a week after
142 surgery. All rats also performed three trials in each session, and the maximum score (in
143 newtons: N) was used for data analysis.

144

145 **Histological analysis**

146 ***Hematoxylin and eosin (H-E) staining***

147 At 16 weeks after surgery, transcardial perfusion was performed with 4%
148 paraformaldehyde in phosphate-buffered saline (PBS) in all rats. The spinal cord
149 segment at C5-6 was removed en bloc and placed in 4% paraformaldehyde solution for
150 3 days. After this process, these C5-6 segments were embedded in paraffin and
151 sectioned at a slice thickness of 5 μm and a gap interval of 5 μm over 1000 μm length,
152 according to stereological considerations of motor neurons [7-10]. One hundred
153 specimens of all rats were stained with H-E. Motor neurons have large nuclei and well-
154 developed, densely stained Nissl bodies in the cytoplasm. The characteristic large
155 nucleolus has a uniform diameter of approximately 5 μm [27, 28]. In H-E-stained
156 sections, we regarded such cells as motor neurons. Motor neurons on both sides of the
157 anterior horn gray matter were counted.

158

159 ***NeuN staining***

160 NeuN protein appears in neuron-specific nuclei. The nucleus of the motor neuron is
161 more clearly detected with NeuN staining compared with H-E staining.

162 In the treatment experiment, 10 specimens (thickness = 5 μm , gap interval = 50 μm) of
163 five rats in all groups were stained with immunohistochemistry. Both sides of the
164 anterior horn were also evaluated with NeuN staining following application of the
165 chromogen diaminobenzidine (Dako North America, Santa Clara, CA, USA, 1:100)
166 using the labeled streptavidin biotin technique [29]. Rabbit anti-NeuN (EMD Millipore
167 Corporation, Burlington, MA, USA, 1:100) was used as the primary antibody. NeuN-
168 positive cells on both sides of the anterior horn gray matter were counted.

169

170 ***Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin***
171 ***nick end labeling (TUNEL) staining***

172 Apoptotic cell death was investigated 10 weeks after surgery. Another 18 rats (Sham
173 group; n = 6, Vehicle group; n = 6, high-dose EPO group; n = 6) were perfused
174 transcardially with 4% paraformaldehyde in PBS (Fig 2B). The C5-6 segment of the
175 spinal cord was embedded in optimal cutting temperature compound and frozen in
176 liquid nitrogen. Three sections from C5-6 segments (thickness = 20 μm , gap interval =
177 50 μm) were cut in a cryostat and stained with the In Situ Cell Death Detection Kit,
178 POD (Roche, Basel, Switzerland) according to the manufacturer's recommendations.
179 Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:5000 in PBS)

180 (Molecular Probes, Eugene, OR). The TUNEL stain signal was observed under an
181 FV300 confocal microscope (Olympus Optical Company, Ltd., Tokyo, Japan). TUNEL-
182 and DAPI-positive cells were counted, and the ratios of apoptotic cells to total nuclei
183 were evaluated in each group.

184

185 **Hematological assessment**

186 Another 12 rats were divided into three groups. All these rats underwent the operation
187 to place the polymer sheet under the C5-6 laminae and were treated with NS or rhEPO
188 twice a week from 8 weeks after surgery. The Vehicle group, Low-dose EPO group, and
189 High-dose EPO group were examined. All rats were subjected to inhalation anesthesia
190 with 2% isoflurane. Blood samples (0.5 ml/body) were collected by venipuncture from
191 the tail vein at 2, 4, and 6 weeks after the first EPO administration. Blood samples were
192 collected into blood collection tubes with EDTA 2K (BD Microtainer, Japan Becton,
193 Dickinson and Company, Tokyo, Japan) immediately. RBC, hemoglobin (Hb), and
194 hematocrit (Ht) values were assessed with an automated hematology analyzer (XE
195 2100, Sysmex, Hyogo, Japan).

196

197 **The rhEPO level in spinal cord tissue**

198 To assess whether subcutaneously injected EPO was transferred to the spinal cord, we
199 measured EPO levels in the spinal cord with an enzyme-linked immunosorbent assay.
200 Another 12 rats were divided into three groups; Vehicle group, Low-dose EPO group,
201 and High-dose EPO group. All these rats underwent the operation in which the polymer
202 sheet remained under the C5-6 laminae. They received NS or rhEPO 8 weeks after
203 surgery. Twelve hours after subcutaneous injection of NS or rhEPO, all rats were
204 sacrificed under anesthesia, and blood was completely removed by transcardial
205 perfusion with PBS to exclude rhEPO from blood (Fig 2C). The spinal cord segment at
206 the C5-6 level was removed en bloc. These tissues were homogenized in IP buffer with
207 an ultra Turrax homogenizer and centrifuged at 12000 rpm at 4°C for 5 min.
208 Supernatants were removed and analyzed to determine the levels of rhEPO in the spinal
209 cord tissue. The total protein of the spinal cord tissue was determined using bovine
210 serum albumin as a standard. The rhEPO concentration in spinal cord tissue was
211 measured with a rhEPO enzyme-linked immunosorbent assay kit (R&D Systems
212 Europe, Abingdon, UK) according to the manufacturer's instructions. The concentration
213 was described as the rhEPO level per 1 g tissue (mIU/g) and tissue dose % of injected
214 dose (%ID).
215

216 **Statistical Analysis**

217 GraphPad Prism 6 software for Windows (GraphPad Software Inc., La Jolla, CA,
218 USA) was used for statistical analysis. Data are expressed as the means \pm standard error
219 of the mean. The duration of walking on the rotarod, forelimb grip strength, and
220 hematological data were analyzed using two-way repeated-measures analysis of
221 variance. The number of anterior horn motor neurons with H-E, NeuN, and TUNEL
222 staining and the rhEPO level in spinal cord were tested using one-way analysis of
223 variance. P values <0.05 were regarded as significant.

224

225 **Results**

226 **Motor function**

227 **Preliminary experiment**

228 Rotarod performance declined gradually with a latency period of 4 weeks in the
229 Compression group. At 7 weeks after surgery, the walking duration significantly
230 decreased in the Compression group compared to the Sham group ($P < 0.001$). In the
231 Compression group, the duration declined gradually and reached a plateau after 16
232 weeks (Fig 3A).

233 Forelimb grip strength increased until 5 weeks after surgery and started to decrease
234 from 6 weeks in the Compression group. The strength gradually declined and
235 significantly decreased after 7 weeks compared with the Sham group ($P < 0.001$) (Fig
236 3B).

237 Based on these results, we decided to administer EPO beginning 8 weeks after surgery
238 as a treatment experiment.

239

240

241 **Fig 3. Preliminary experiment**

242 A: Time course of rotarod performance measured by walking time on a rotarod (cut-off
243 300 seconds). In the Compression group, the walking time gradually started to decline
244 from 4 weeks, and showed a significant decrease at 7 weeks after surgery ($P < 0.001$).

245 The performance reached a plateau with a low duration of about 50 seconds after 15
246 weeks.

247 B: Time course of grip strength. In the Compression group, grip strength decreased at 1
248 week after surgery, but gradually increased as body weight increased. However, grip
249 strength gradually declined from 6 weeks, and showed a significant difference at 7

250 weeks after surgery ($P < 0.0001$) After that, the strength continued to decrease

251 gradually, reaching approximately 10.5 N at 26 weeks postoperatively.

252

253

254 **Treatment experiment**

255 The rotarod performance of the Compression groups (Vehicle, Low-dose EPO, High-

256 dose EPO groups) declined gradually from 5 weeks after surgery, and a significant

257 decrease was seen from 7 weeks compared with the Sham group ($P < 0.005$) as in the

258 preliminary experiments (Fig 3A).

259 After EPO administration beginning 8 weeks after surgery, rotarod performance

260 started to improve in the treatment groups (Low-dose EPO, High-dose EPO group).

261 Especially in the High-dose EPO group, rotarod performance significantly improved

262 compared with the other Compression groups (Vehicle, Low-dose EPO groups),

263 although the performance gradually declined from 13 weeks. The effects of EPO

264 continued for 5 weeks after EPO administration ($P < 0.01$). Furthermore, the High-dose

265 EPO group improved to the level at which no significant difference in motor function

266 was seen between the Sham and High-dose EPO groups at 9 weeks after surgery. The

267 Low-dose EPO group showed slightly improved rotarod performance, but did not show
268 significant improvement compared with the Vehicle group (Fig 4A).

269 The forelimb grip strength of the Compression groups decreased at 1 week after
270 surgery but started to recover gradually from 2 weeks after surgery. The strength of the
271 Compression group showed an improvement course equal to that of the Sham group
272 from 2 weeks after surgery and then started to decrease gradually from 7 weeks; at this
273 time, the strength was significantly decreased compared with the Sham group ($P <$
274 0.001) (Fig 4B).

275 After EPO administration at 8 weeks after surgery, grip strength started to improve in
276 the treatment groups (Low-dose EPO, High-dose EPO groups).

277 In the High-dose EPO group, the strength significantly improved compared with the
278 other Compression groups (Vehicle, Low-dose EPO groups) ($P < 0.0001$). Its effects
279 continued throughout the period of EPO administration (9 to 16 weeks after surgery),
280 although the strength gradually decreased from 4 weeks after EPO administration.

281 In contrast, the Low-dose EPO group showed a slight improvement in strength, but did
282 not show significant improvement compared with the Vehicle group (Fig 4B).

283

284

285 **Fig 4. Treatment experiment: Motor function**

286 A: Time course of rotarod performance measured by walking time on a rotarod (cut-off
287 300 seconds). In the Compression models (Vehicle, Low-dose EPO, and High-dose
288 EPO groups), rotarod performance gradually declined from 3 weeks after surgery, and
289 showed a significant difference at 7 weeks after surgery. After administration of EPO
290 from 8 weeks, rotarod performance improved in the EPO groups. Especially in the
291 High-dose EPO group, performance markedly improved. This effect was maintained
292 with a significant difference by week 13 after surgery ($P < 0.01$). In the Low-dose EPO
293 group, slight improvement in rotarod performance was observed, but it did not reach
294 statistical significance compared with the Vehicle group.

295 B: Time course of grip strength. In the Compression groups, the strength started to
296 decline from 6 weeks after surgery, and a significant decline was observed at 7 weeks.
297 EPO was administered at 8 weeks, and grip strength improved, especially in the High-
298 dose EPO group. Significant improvement was seen from 9 weeks in the High-dose
299 EPO group ($P < 0.0001$) and continued up to 16 weeks after surgery. In the Low-dose
300 EPO group, grip strength slightly improved, but no significant difference was found
301 compared with the Vehicle group.

302

303

304 **Histopathological analysis**

305 *H-E staining*

306 At 16 weeks after surgery, loss of anterior horn motor neurons and vacuolar
307 degeneration in the spinal cord were observed in H-E-stained sections from the
308 Compression groups (Vehicle, Low-dose EPO, and High-dose EPO group) (Fig 5A).
309 The numbers of motor neurons were 1834.7 ± 115.4 (Sham group), 1421.6 ± 50.1
310 (Vehicle group), 1484.7 ± 74.2 (Low-dose EPO group), and 1640.0 ± 66.9 (High-dose
311 EPO group). The number of motor neurons on both sides of the anterior horn was
312 significantly decreased in every Compression group compared to the non-compression
313 Sham group ($P < 0.0001$). In the High-dose EPO group, however, the motor neurons
314 were significantly preserved compared with the other Compression groups (Vehicle and
315 Low-dose EPO group; $P < 0.0001$, $P < 0.0005$) (Fig 5B).

316

317

318 **Fig 5. Treatment experiment: Anterior motor neurons**

319 A:

320 Top panels: CT axial view in C5. In the Compression groups (Vehicle, Low-dose EPO,
321 and High-dose EPO), the spinal cord was compressed by Aquaprene® (expandable
322 urethane compound sheet, size $2 \times 6 \times 0.7$ mm). The yellow dotted figure shows the
323 outline of the spinal cord.

324 Second panels: The spinal cord at the C5 level was sliced into 5- μ m thick sections at 16
325 weeks after surgery. Hematoxylin and eosin staining of cross sections of spinal cord is
326 shown (original magnification $\times 4$, scale bar = 100 μ m). In the Compression groups, the
327 spinal cord was flattened. Black box shows the region of the anterior horn.

328 Third panels: The black box in the second panel was magnified ($\times 10$, scale bar 20 μ m).
329 Cells with large nuclei and well-developed, densely stained Nissl bodies in the
330 cytoplasm indicate motor neurons. In the Vehicle and Low-dose EPO groups, motor
331 neurons decreased, and vacuolar degeneration was obvious. In the High-dose EPO
332 group, motor neurons were preserved, although vacuolar degeneration was present.

333 Bottom panels: NeuN staining of the anterior horn ($\times 10$, scale bar 20 μ m). The nuclei of
334 motor neurons are clearly detected with NeuN staining compared with H-E staining.
335 Motor neurons decreased in the Vehicle and Low-dose EPO groups, but were preserved
336 in the High-dose EPO group.

337

338 B: Counting of anterior horn cells in H-E-stained tissue. The number of cells with large
339 nuclei in the anterior horn was counted in every group. The number was significantly
340 decreased in the Compression groups compared with the Sham group (*P < 0.0001).

341 However, in the High-dose EPO group, the number was significantly preserved
342 compared with the other two Compression groups (Vehicle and Low-dose EPO group)

343

344 C: Counting of anterior horn cells in NeuN-stained tissue. NeuN-positive cells were
345 significantly decreased in the Compression groups compared with the Sham group (*P

346 < 0.0001). However, in the High-dose EPO group, the number was significantly
347 preserved compared with the other two Compression groups (*P < 0.0001). The

348 tendency in the cell count was similar to that with H-E staining.

349

350

351 *Cell counting of NeuN-positive cells*

352 The number of NeuN-positive cells in 10 slices of each group was 286.8 ± 17.6 (Sham
353 group), 176.0 ± 14.3 (Vehicle group), 178.0 ± 17.1 (Low-dose EPO group), and $220.4 \pm$

354 9.4 (High-dose EPO group). NeuN-positive cells in each Compression group decreased

355 compared with the Sham group, but the number in the High-dose EPO group was

356 significantly preserved compared with the Vehicle and Low-dose EPO groups. This
357 tendency was similar to that of the number of motor neurons in H-E-stained sections
358 (Fig 5B, 5C).

359

360 *TUNEL staining*

361 TUNEL-positive cells were significantly increased in the Vehicle group compared
362 with the other two groups (Sham and High-dose EPO groups) ($P < 0.0001$) (Fig 6A,
363 6B). We found no significant difference between the Sham and High-dose EPO groups.
364 The ratios of TUNEL-positive cells to DAPI-positive cells (%) were $1.72 \pm 0.59\%$
365 (Sham group), $35.01 \pm 9.17\%$ (Vehicle group), and $5.66 \pm 2.27\%$ (High-dose EPO
366 group). The ratio in the Vehicle group was significantly higher than that in the other two
367 groups ($P < 0.0001$), and we found no significant difference between the Sham and
368 High-dose EPO groups (Fig 6A, 6C).

369

370

371 **Fig 6. Treatment experiment: TUNEL staining**

372 A: TUNEL staining was performed to detect apoptotic cells at 10 weeks after surgery.

373 DAPI/TUNEL double staining is shown in each group (DAPI staining, TUNEL

374 staining, DAPI/TUNEL staining, Bar = 100 μ m). The Vehicle group showed the highest
375 number of TUNEL-positive cells.

376 B: The number of TUNEL-positive cells was counted in each group. The number of
377 TUNEL-positive cells in the Vehicle group was significantly higher than in the other
378 two groups (*P < 0.0001), with no significant difference between the Sham group and
379 High-dose EPO group.

380 C: The percentage of TUNEL-positive cells in each group. The percentage in the
381 Vehicle group was significantly higher than in the other two groups (*P < 0.0001).

382

383

384 **Hematological data**

385 After administration of EPO, the RBC, Hb, and Ht values increased immediately in the
386 EPO-administered groups (Low-dose and High-dose EPO groups) (P < 0.0001). The
387 trend in RBC and Hb values showed a similar increasing tendency after EPO
388 administration (Fig 7A, B). Eventually, the RBC and Hb values increased to
389 approximately 1.2 and 1.4 times in the Low-dose and High-dose EPO groups,
390 respectively, compared to the baseline value (Vehicle group). The values were

391 significantly higher in both EPO-administered groups than the Vehicle group until 6
392 weeks after administration ($P < 0.0001$) (Fig 7A, B).

393 The Ht value in the EPO-administered groups was the highest at 4 weeks and
394 increased to approximately 1.3 and 1.4 times in the Low-dose and High-dose EPO
395 groups, respectively, compared to the baseline value ($P < 0.0001$). At 6 weeks after
396 administration of EPO, the Ht value of the EPO-administered groups started to peak.

397 The Ht value of the High-dose EPO group was significantly higher than that of the other
398 two groups ($P = 0.005$) at 6 weeks (Fig 7C).

399

400

401 **Fig 7. Treatment experiment: Hematological data**

402 A: Time course of red blood cells (RBCs). RBCs increased immediately in the Low-
403 dose and High-dose EPO groups ($P < 0.0001$). From 4 weeks after administration, we
404 found a significant difference between the Low-dose and High-dose EPO groups ($P <$
405 0.0001). Eventually, RBCs increased up to approximately 1.2 and 1.4 times in the Low-
406 dose and High-dose EPO groups, respectively, compared with the Vehicle group.

407 B: Time course of hemoglobin (Hb). The Hb value increased immediately in the Low-
408 dose and High-dose EPO groups ($P < 0.0001$). The time course was similar to that of

409 RBCs. Eventually, the Hb value increased up to approximately 1.2 and 1.4 times in the
410 Low-dose and High-dose EPO groups, respectively, compared with the Vehicle group.
411 C: Time course of hematocrit (Ht). The Ht value increased immediately in the Low-dose
412 and High-dose EPO groups ($P < 0.0001$). The Ht value was the highest at 4 weeks, and
413 then peaked. The maximum Ht value was approximately 1.3 and 1.4 times in the Low-
414 dose and High-dose EPO groups, respectively, compared with the Vehicle group.

415

416

417 **rhEPO level in spinal cord tissue**

418 The rhEPO level in the spinal cord 12 hours after subcutaneous injection of rhEPO
419 was less than 0.10 mIU/g in the Vehicle group, 1.07 ± 0.46 mIU/g in the Low-dose EPO
420 group, and 8.67 ± 2.33 mIU/g in the High-dose EPO group. The rhEPO level was
421 remarkably higher in the High-dose EPO group than the other two groups ($P < 0.0001$).
422 In the Low-dose EPO group, the rhEPO level was slightly increased, but that of the
423 Low-dose EPO group did not show a significant difference compared with the Vehicle
424 group (Fig 8A).

425 The tissue % ID was 4.4 ± 1.2 ($10^{-4}\%$) in the High-dose EPO group and 5.4 ± 2.3
426 ($10^{-4}\%$) in the Low-dose EPO group. We found no significant difference between the

427 two groups (Fig 8B). This result shows that the rhEPO level in the spinal cord was dose
428 dependent.

429

430

431 **Fig 8. Treatment experiment: ELISA of recombinant human EPO (rhEPO)**

432 A: The amount of rhEPO per 1 g spinal cord tissue. The rhEPO level was significantly
433 higher in the High-dose EPO group compared to the other two groups (*P < 0.0001).

434 B: The tissue rhEPO level for injected dose (%ID) in the Low-dose and High-dose EPO
435 groups. No significant difference was found in the %ID between the two groups.

436

437

438 **Discussion**

439 The present study demonstrated that EPO improved motor functions and preserved
440 motor neurons, even in developing myelopathy due to spinal cord compression.

441 Furthermore, EPO was transferred into spinal cord tissue following subcutaneous EPO
442 administration.

443 Some studies reported that EPO improves motor function in an experimental acute SCI
444 model [21, 22, 30, 31]. EPO and the EPO receptor (EPO-R) are highly expressed in

445 both the central and peripheral nervous systems [32]. The roles of EPO in these areas
446 are in neuroprotection, angiogenesis, anti-apoptosis, and anti-inflammation [18, 24, 33].
447 Clinically, a preliminary randomized comparative trial was performed in patients with
448 acute SCI. In this trial, the effect of EPO treatment was compared with high-dose
449 methylprednisolone treatment. EPO had higher efficacy and fewer side effects than
450 methylprednisolone, indicating a therapeutic effect for acute SCI patients [25].

451 In contrast, the neuroprotective effect of EPO for compression myelopathy remains
452 unknown. We previously demonstrated that blood flow in the compressed segment is
453 markedly reduced, indicating the presence of local spinal cord ischemia in the chronic
454 compression myelopathy model [32]. Consistent with our previous studies [9, 10], this
455 study also demonstrated that chronic spinal cord compression induces apoptotic cell
456 death. In hypoxic stress conditions, endogenous EPO is produced in response to low
457 oxygen partial pressure and protects neurons [34]. Importantly, cell apoptosis induced
458 by spinal cord compression is inhibited by high-dose EPO administration, indicating
459 anti-apoptosis and anti-inflammatory effects of EPO [18, 24, 33]. Additionally, a rapid
460 increase in RBC, Hb, and Ht values following EPO administration may improve the
461 local oxygen supply and restore motor function (Fig 7). Liem et al. reported that blood
462 transfusion for anemia improves cerebral oxygenation in newborn infants [35].

463 Although we could not directly evaluate local oxygen pressure, we speculate that
464 improvement in cervical myelopathy is due to anti-apoptotic effects of EPO and
465 improvement in local ischemia in the spinal cord with an increased oxygen supply.

466 In the current study, both high-dose and low-dose EPO increased hematopoietic values
467 including RBC, Hb, and Ht. However, functional recovery was observed with high-dose
468 EPO treatment in particular. High-dose EPO may have passed through the blood-spinal
469 cord barrier. EPO is a high-molecular weight glycoprotein (30.4 kDa) [12]. In classic
470 papers, the blood–brain barrier (BBB) was considered to be impermeable to large
471 glycosylated molecules like EPO [36]. However, some recent studies have reported that
472 EPO can pass through the BBB due to a high concentration and after BBB disruption
473 such as that which follows brain and spinal cord contusion [37-39]. EPO can cross the
474 BBB at 450 IU/kg or more in rats [37] and crosses the BBB in a dose-dependent manner
475 in a rat brain contusion model [40]. In the current study, in fact, high-dose EPO was
476 predominantly transferred into the spinal cord tissue 12 hours after EPO subcutaneous
477 administration, probably resulting from passing through the blood-spinal cord barrier.

478 Transfer of EPO into spinal cord tissue was dose dependent (Fig 8A), and the transfer
479 activity was almost the same between the Low-dose and High-dose EPO groups (Fig
480 8B). This finding demonstrates that the higher the dose of EPO that was administered,

481 the more EPO can transfer into spinal cord tissue. This result indicates that EPO directly
482 affected the spinal cord to provide neuronal protection as well as indirectly affected the
483 cord by increasing RBC, Hb, and Ht values.

484 The dosage of EPO (500 IU/kg or 5000 IU/kg) in this study was decided based on
485 previous reports in acute or subacute SCI with no side effects including hematological
486 complications [21, 41-43].

487 EPO has been used in clinical practice for a long time, and knowledge of the
488 hematopoietic effect, clinical safety, and side effects of EPO has accumulated. The
489 possible side effects of EPO in humans include hypertension, coagulation disorders, and
490 polycythemia. [44] However, no adverse effects occurred in brain injury patients treated
491 with 10000 IU/kg for 7 consecutive days [45]. In a recent preliminary randomized
492 comparative trial (EPO versus methylprednisolone) for human acute SCI, EPO (500
493 IU/kg) had a predominant effect and no adverse effects compared with high-dose
494 methylprednisolone. Based on these data, EPO may be a clinically acceptable agent for
495 progressive compression myelopathy as well as a hematopoietic cytokine.

496 Polycythemia vera (erythemia) is defined as a Hb value more than 18.5 in males and
497 16.5 in females by WHO guidelines [46]. In practical clinical use, EPO should be used
498 while monitoring of RBC, Hb, and Ht values, especially in hematologically healthy

499 people. Administration of EPO is indicated for patients with anemia and those waiting
500 for surgery and expecting preoperative hematopoietic effects.

501 The effect of EPO treatment gradually declined at 4 weeks after EPO administration in
502 this rat model of compression myelopathy, although the group given high-dose EPO
503 was finally superior to the group given NS in terms of motor functions. Therefore, the
504 best treatment period may be limited to several weeks after EPO administration, and
505 surgical decompression may be considered during that period.

506 Certainly, continuous administration of EPO to patients with simple cervical
507 spondylosis over a long period seems unrealistic considering the side effects and high
508 costs. Practical clinical use of EPO may occur for a limited period, especially in patients
509 with worsening symptoms of compression myelopathy who have higher systemic risks
510 such as severe anemia, older age, and diabetes mellitus, and those who live far from a
511 hospital that performs spinal surgery. Furthermore, EPO may be effective against
512 surgical complications such as compression myelopathy due to postoperative epidural
513 hematoma and spinal alignment failure.

514 The detailed mechanisms of the neuroprotective effect of EPO for compression
515 myelopathy remain to be elucidated. In addition, in this study, the changes in local
516 blood flow and oxygen partial pressure in the spinal cord were not elucidated. However,

517 this study strongly suggests that EPO has potential for treating patients with developing
518 compression myelopathy, and may be worth reconsidering for clinical use to provide
519 both neuroprotective and hematopoietic effects. Further investigations including larger
520 randomized controlled trials with long-term follow-up surveys are required to establish
521 the clinical efficacy of EPO treatment and elucidate therapy-related adverse events.

522

523 **Conclusions**

524 EPO improved motor function in rats with developing myelopathy due to chronic
525 spinal cord compression. EPO protected anterior horn motor neurons and decreased
526 neuronal cell apoptosis. The neuroprotective effects were produced following transfer of
527 EPO into the spinal cord tissue. These findings suggest that EPO has high potential as a
528 treatment for developing compression myelopathy.

529

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531

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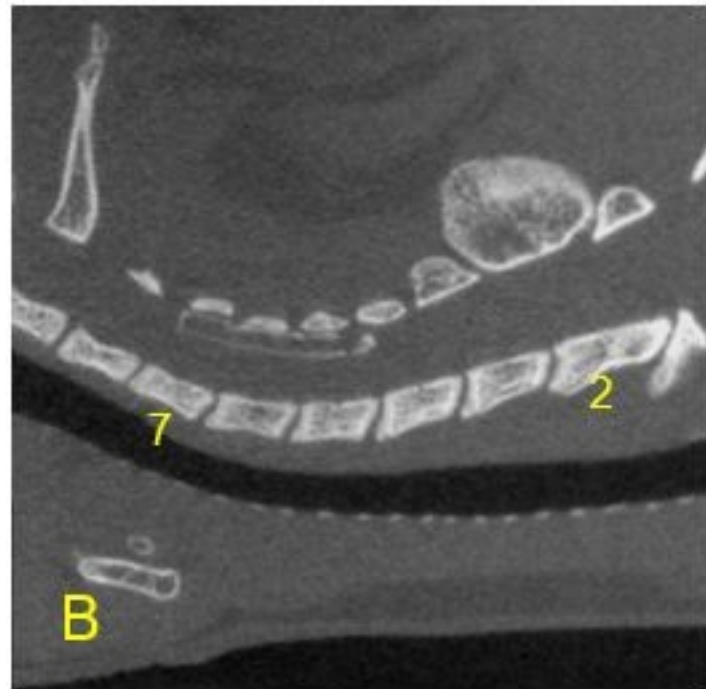
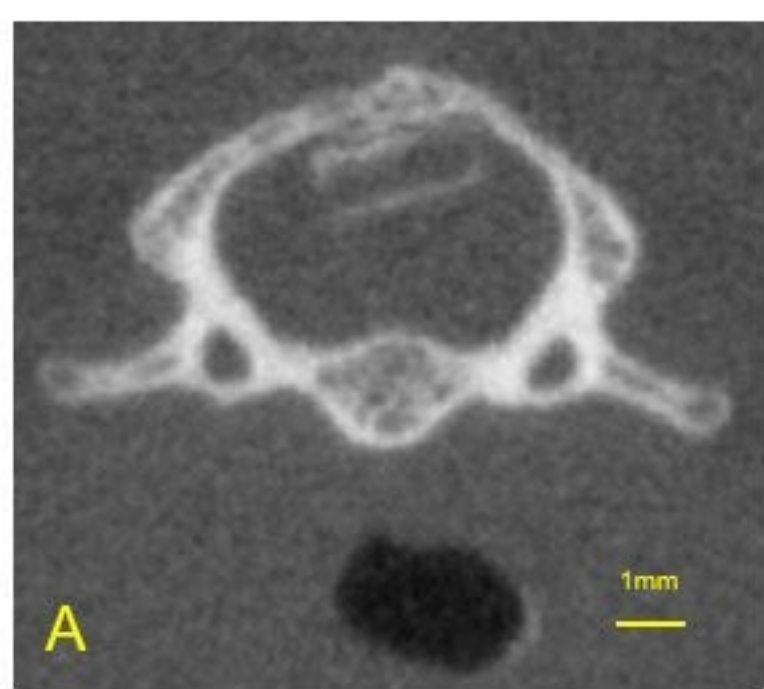
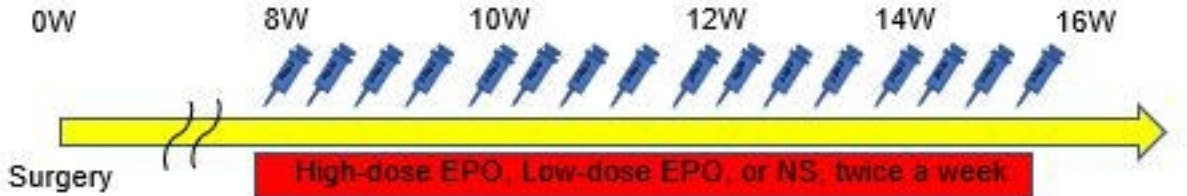
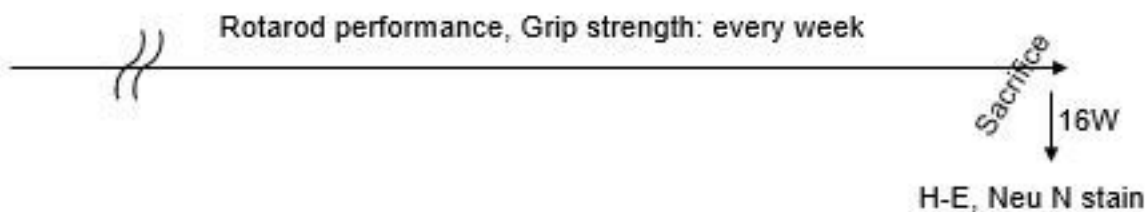


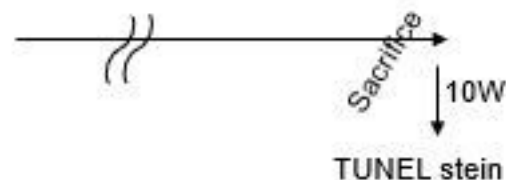
Figure 1



A Motor function, Motor neuron counting (n=12)



B Apoptotic cell counting (n=6)



C EPO level in spinal cord (n=4)

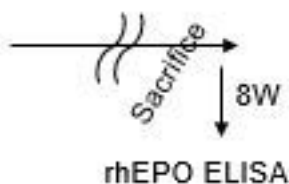
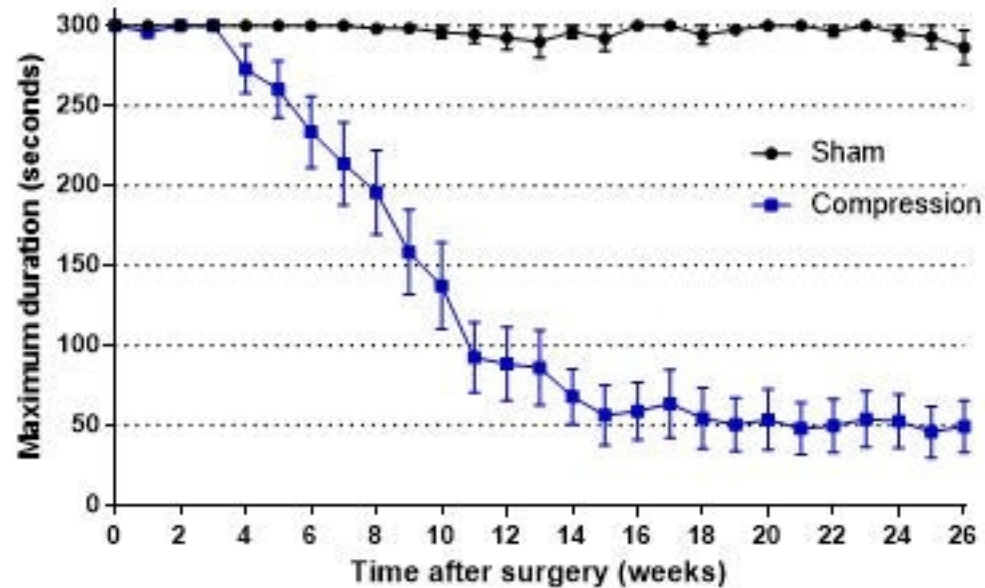


Figure 2

A

Rotarod Performance



B

Forelimb motor function

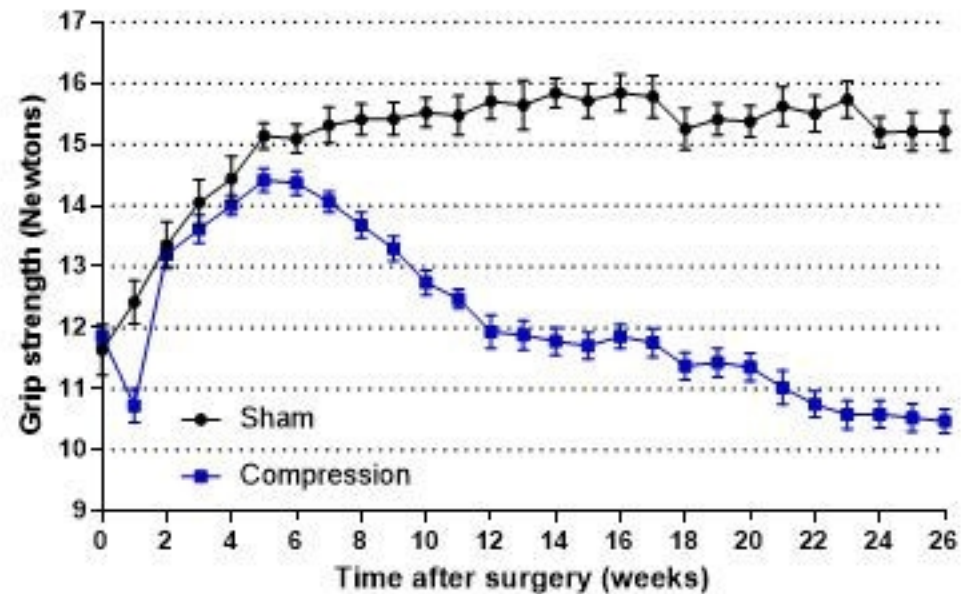


Figure 3

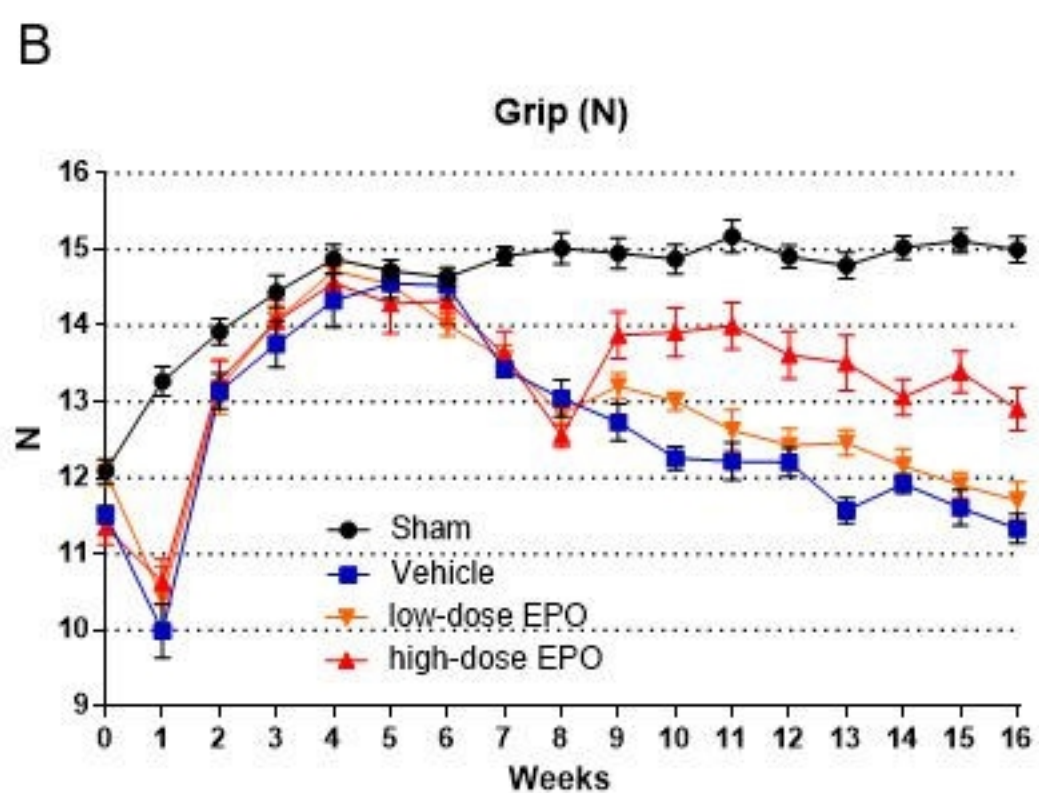
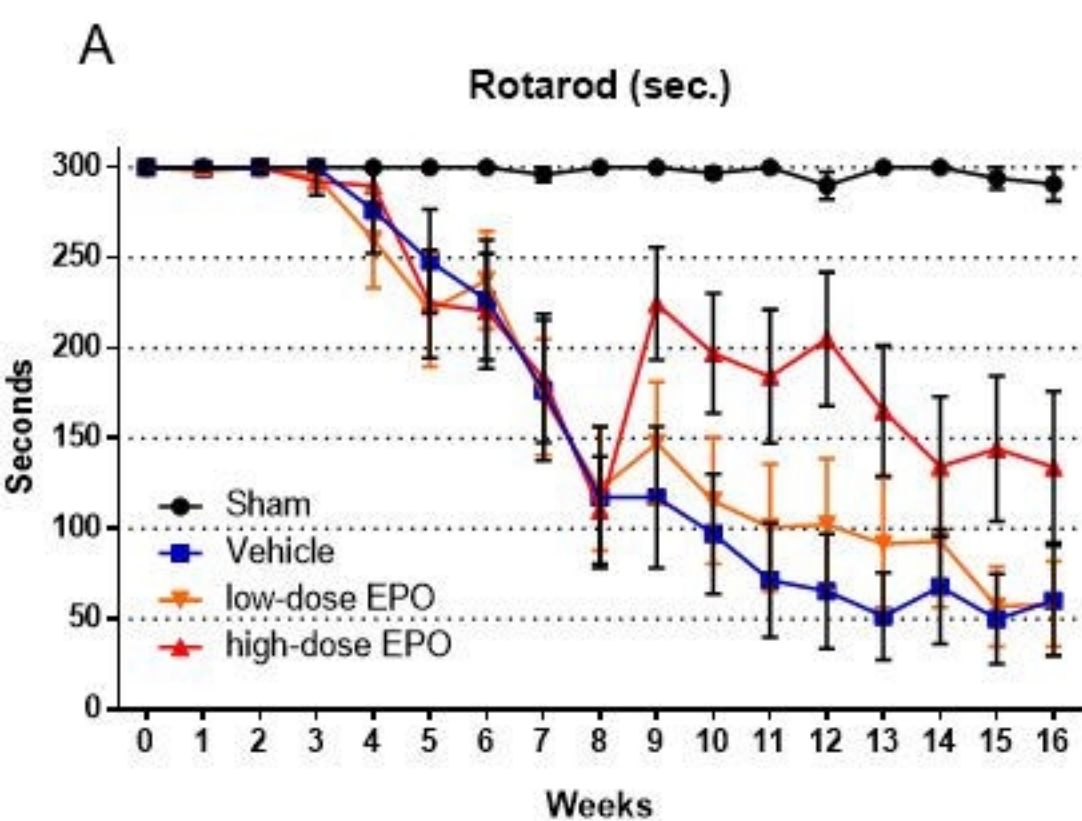
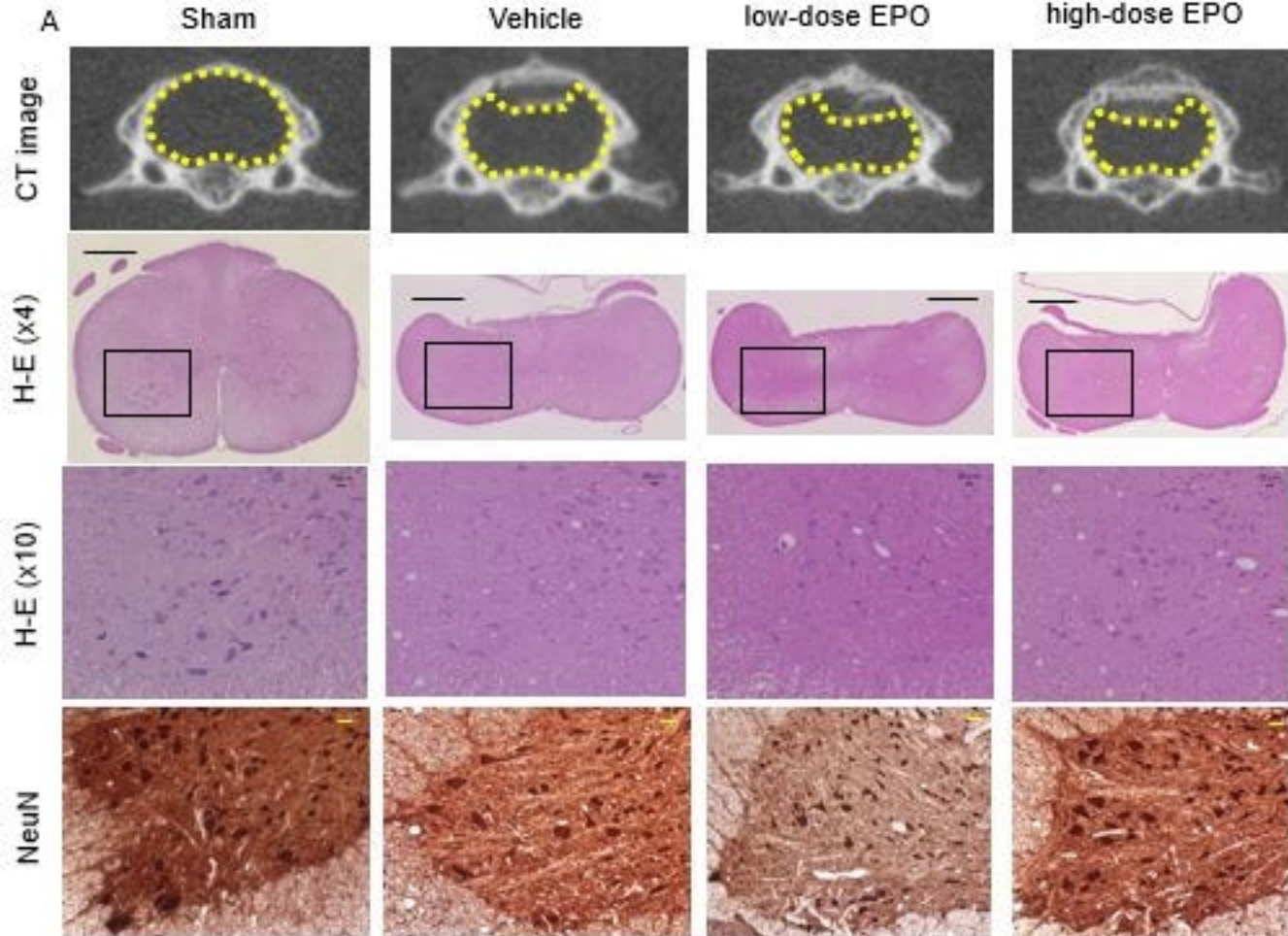
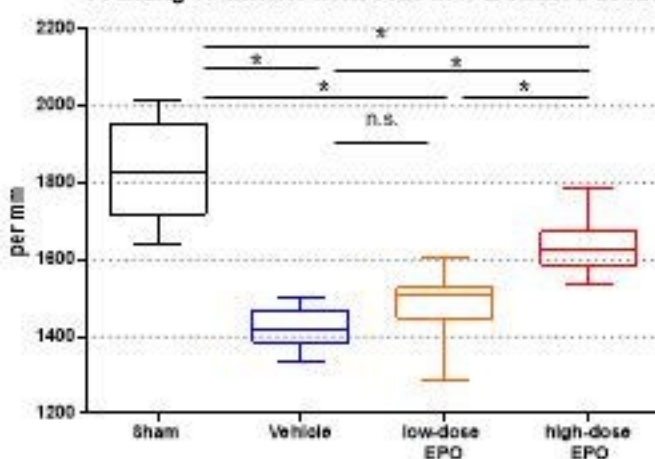


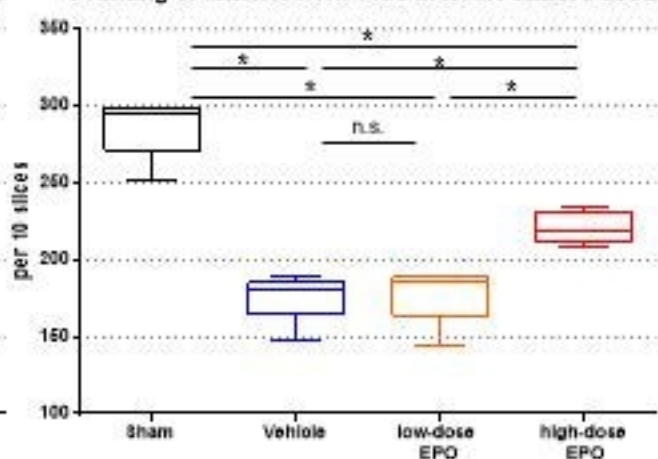
Figure 4



B Counting of anterior horn cells in H-E stained tissue

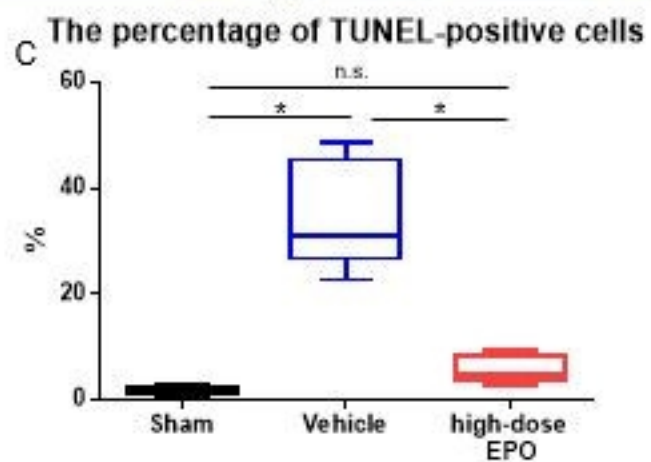
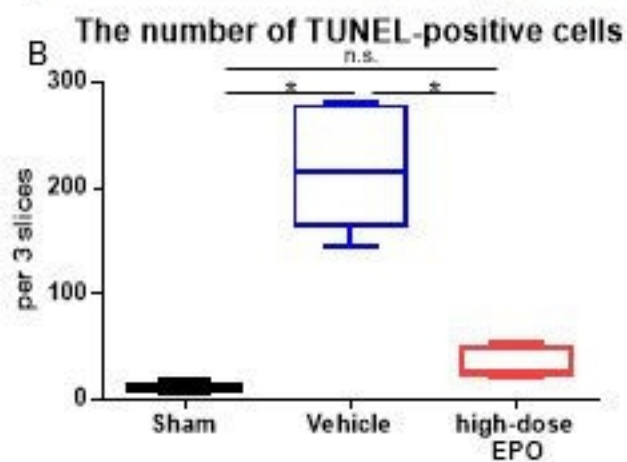
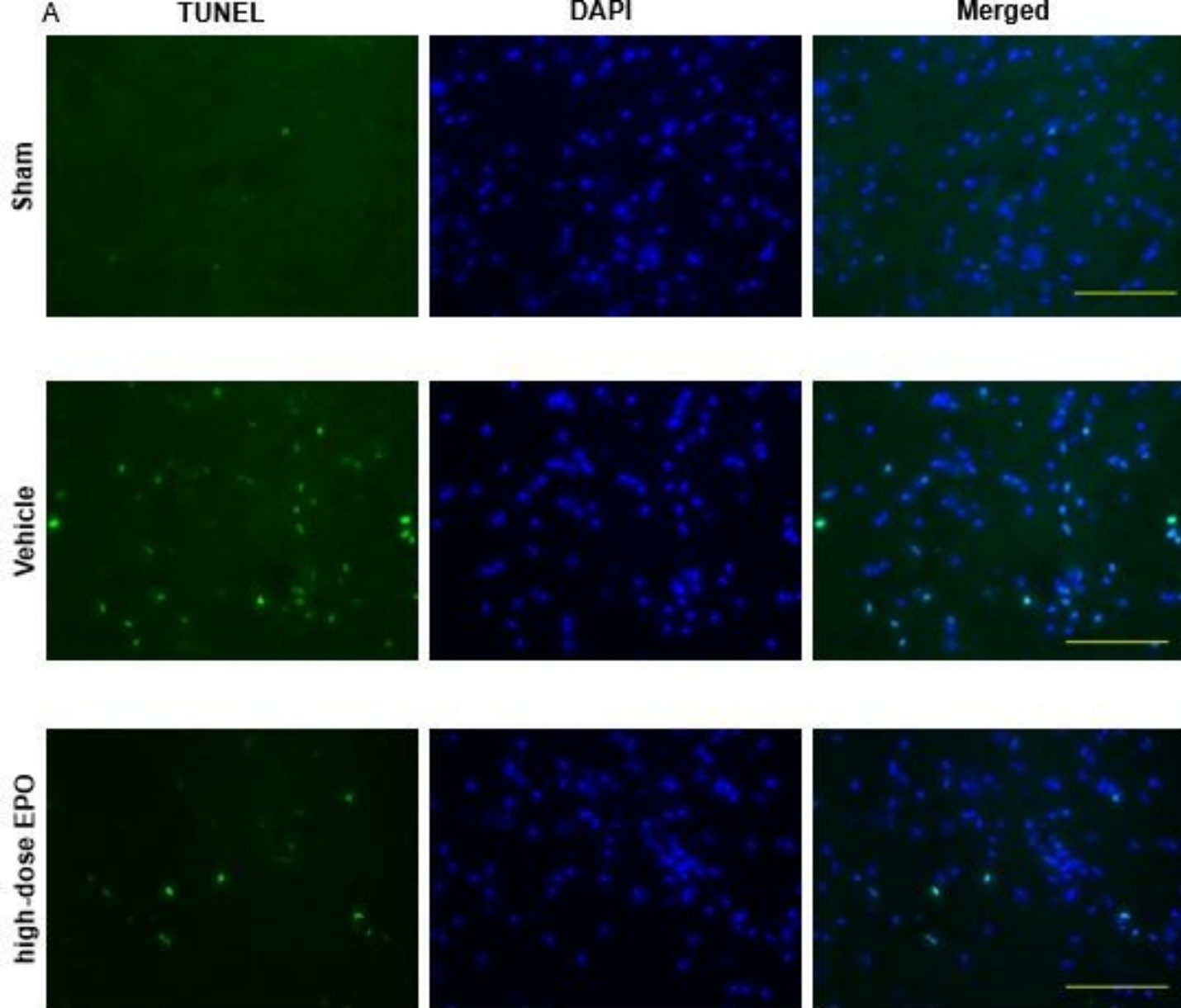


C Counting of anterior horn cells in NeuN-stained tissue



*: $P < 0.0001$, n.s.: not significant (two-way analysis of variance)

Figure 5



*: $P < 0.0001$, n.s.: not significant (one-way analysis of variance)

Figure 6

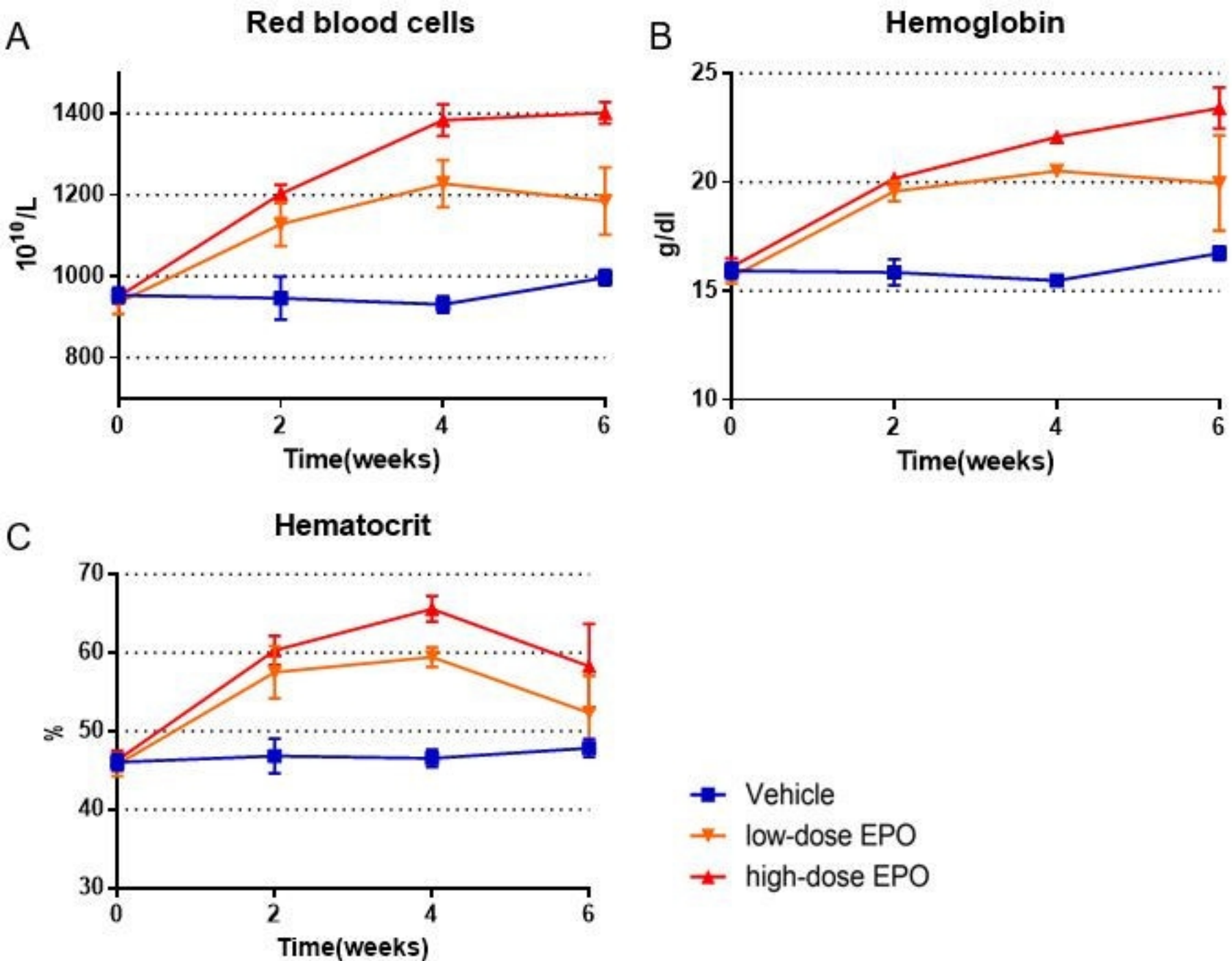
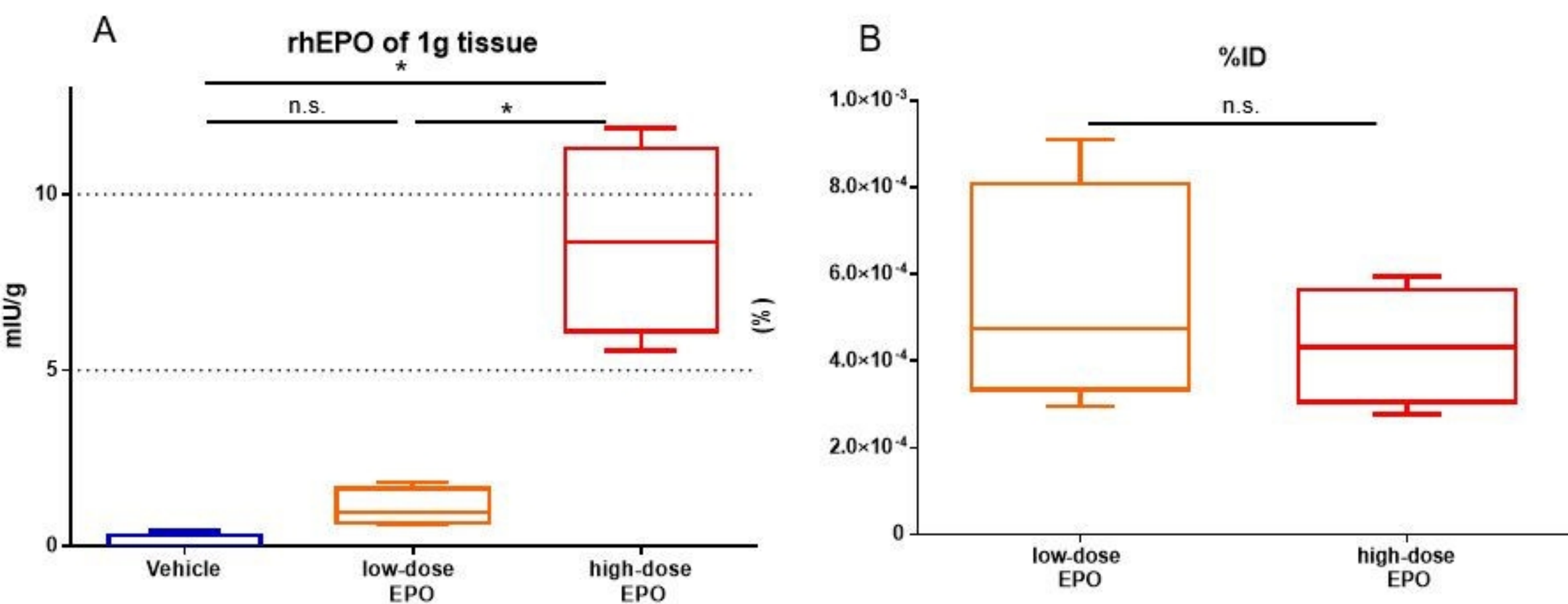


Figure 7



*: $P < 0.0001$, n.s.: not significant

(A: one-way analysis of variance, B: Mann-Whitney U-Test)

Figure 8