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Chronic administration of [Pyr^1] apelin-13 attenuates neuropathic pain after compression spinal cord injury in rats

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ABSTRACT

Apelin is an endogenous ligand for apelin receptor (APJ) with analgesic effect on visceral, analgesic and proanalgesic influences on acute pains in animal models. The purpose of this study was to determine the possible analgesic effects of [Pyr^1] apelin-13 on chronic pain after spinal cord injury (SCI) in rats. Animals were randomly divided into three major groups as intact, sham and SCI. The SCI group randomly allocated to four subgroups as no treatment, vehicle-treatment (normal saline: 10 μl, intrathecally) and two subgroups with intrathecal injection (i.t) of 1 μg and 5 μg of [Pyr^1] apelin-13. After laminectomy at T6-T8 level, spinal cord compression injury was induced using an aneurysm clip. Vehicle or [Pyr^1] apelin-13 injected from day1 post SCI and continued for a week on a daily basis. Pain behaviors and locomotor activity were monitored up to 8 weeks. At the end of the experiments, intracardial paraformaldehyde perfusion was made under deep anesthesia in some animals for histological and immunohistochemistry evaluations. Western blot technique was also done to detect caspase-3 in fresh spinal cord tissues. SCI decreased nociceptive thresholds and locomotor scores. Administration of [Pyr^1] apelin-13 (1 μg and 5 μg) improved locomotor activity and reduced pain symptoms, cavity size and caspase-3 levels. Results showed long-term beneficial effects of [Pyr^1] apelin-13 on neuropathic pain and locomotion. Therefore, we may suggest [Pyr^1] apelin-13 as a new option for further neuropathic pain research and a suitable candidate for ensuing clinical trials in spinal cord injury arena.

1. Introduction

Neuropathic pain following spinal cord injury (SCI) is a significant clinical problem worldwide (Clark et al., 2010; Hu and Zhao, 2014; Yu et al., 2013). Traumatic SCI leads to long-lasting neurological deficits below the lesion level and is associated with paraplegia and neuropathic pain symptoms such as hyperalgesia and allodynia (Clark et al., 2010; Santos-Nogueira et al., 2012). These symptoms are serious complications post SCI that persist for long and provoke physical dependence, psychological and social problems with poor quality of life (Clark et al., 2010; Impellizzeri et al., 2012; Liu et al., 2015b; Talac et al., 2004). The exact mechanism underlying neuropathic pain is still obscure (Bruce et al., 2002; Chew et al., 2014; Sun et al., 2012). Spinal cord apoptosis and increased caspase-3 activity in the region may be one leading cause (Liu et al., 2015a; Sakurai et al., 2003; Vaquero et al., 2006). Today, optimal neuropathic pain management is a challenging issue (Clark et al., 2010; Talac et al., 2004). Although numerous analgesic drugs are available, appropriate treatment is still difficult to reach (Hu and Zhao, 2014; Jergova et al., 2012; Sun et al., 2012). Nowadays, SCI neuropathic pain researchers have focused on neuroprotective agents as a new therapeutic window targeting secondary injury events to protect the cells against apoptosis (Aziz et al., 2014; Bai et al., 2012; Kakinohana et al., 2011). Apelin, an endogenous ligand for APJ receptor, initially isolated from bovine stomach tissue (Chen et al., 2015; Zeng et al., 2010). This peptide is usually derived from a 77 amino acid precursor protein by angiotensin-converting enzyme II and cleaved into 13, 17 and 36 active amino acid forms (Chen et al., 2015; Zeng et al., 2012) with high binding affinity to APJ receptor (Hatzelmann et al., 2013). Among different apelin isoforms, apelin-13 and [Pyr^1] apelin-13 are more potent than others (Pope et al., 2012; Zeng et al., 2010). Efficient distribution of the apelinergic system in CNS pain-related sites may be a clue for apelin pain modulator role (Lv et al., 2012; Xu et al., 2009). In addition, in some studies apelin is shown to have analgesic effects in visceral (Lv et al., 2012) and some acute pain models (Turtay et al., 2015; Xu et al., 2009) in mice and rats. However, to the best of our

Abbreviations: SCI, spinal cord injury; i.t, intrathecal; SNL, spinal nerve ligation; MW, molecular weight; BBB, Basso Beattie, and Bresnahan; PyrAP13, pyroglutamyl apelin-13; WK, week; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, poly vinylidene fluoride; i.p, intraperitoneal; i.c.v, intracerebroventricular; CCI, chronic compression of the dorsal root ganglia; BSCB, blood spinal cord barrier; BMSCs, bone marrow-derived mesenchymal stem cells.

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knowledge, literature is completely empty, of papers on apelin and neuropathic pain post SCI in spite of bunch of articles showing apelin anti-apoptotic and neuroprotective effects (Chen et al., 2015; Zeng et al., 2012; Zeng et al., 2010; Zhao et al., 2011). For example, Zhao et al., have recently showed low apelin expression post SCI led to apoptosis induced neuronal death. They also depicted reduced sciatic nerve ligation (SNL) induced motor neuron apoptosis in the spinal anterior horn post apelin injection at proximal nerve stump (Zhao et al., 2011).

Based on our literature review, apelin with neuroprotective and anti-apoptotic properties may have potential to provide a robust therapeutic strategy for alleviation of neuropathic pain. There's no published paper on the effect of apelin in chronic pain situation post SCI. Therefore, in this study we investigated the analgesic and anti-apoptotic role of [Pyr^1] apelin-13 in a central neuropathic pain model.

2. Materials and methods

2.1. Experimental animals

Adult male Wistar rats (140–160 g) were kept at a room temperature of 22 ± 1 °C with free access to fresh food and water in a 12 h light/dark cycle. All experimental protocols performed in accordance with guidelines and policies of the International Association for the Study of Pain in conscious animals (Zimmermann, 1983) and approved by the ethics committee of animal study in Iran University of Medical Sciences (Approved number: 23621). Rats were randomly divided into three major groups as intact, sham and SCI groups. In SCI group, the animals were allocated into four subgroups as SCI, that underwent spinal cord compression injury, SCI + vehicle which received intrathecal injection of vehicle (0.9% saline solution) for one week and apelin treated groups, which received [Pyr^1] apelin-13 intrathecal injections in 1 μg and 5 μg doses for one week (n = 8).

2.2. Intrathecal catheter implantation

Intrathecal catheter implanted using a modified Yaksh and Rudy method (Yaksh and Rudy, 1976). Briefly, a 7 cm polyethylene (PE-10) catheter first immersed in 70% ethanol and then flushed with 0.9% sterile saline. Anesthesia was inducted with a mixture of ketamine/xylazine (80/10 mg/kg, i.p). Upon anesthesia completion, animals were placed in a stereotaxic instrument. A midline 1.5–2 cm rostral to caudal incision was made on the skull skin. After exposing atlanto-occipital membrane, dura punctured with the tip of insulin syringe. The catheter was then slowly conducted 2.5–3 cm into the subarachnoid space where its tip placed close to the injury site. The catheter was firmly fixed to the surrounding tissue. Muscles and skin were separately sutured with 3–0 chromic and silk surgical threads respectively. The catheter was then flushed with 10 μl of 0.9% saline solution and closed with heat at the external end. Animals were then returned to their cages for one week recovery period before spinal cord injury induction.

2.3. SCI induction procedure

For spinal cord injury induction, deep anesthesia was done first. Surgical area was clean shaved and disinfected with 70% ethanol and povidone iodine solution. A midline longitudinal incision was made at T6-T8 dorsal surfaces. Laminectomy was carried out at T6-T8 segments and the spinal cord then exposed. SCI was induced according to the method explained by Hama and Sagan (Hama and Sagen, 2012). Briefly, the spinal cord was extradurally compressed at T7–T8 level for 1 min using an aneurysm clip (Harvard Apparatus, MA) with 20 g closing force. Sham animals were only subjected to laminectomy without spinal cord compression. Urinary excretion was manually carried out twice daily until natural voiding reflex recovery.

2.4. Drug

[Pyr^1] apelin-13 was purchased from Biorbyt company (MW: 533.85). Stock solutions were prepared with 0.9% sterile saline and then stored at −20 °C until use. Stock solution thawed and diluted before injection on a daily basis (Xu et al., 2009).

2.5. Drug administration

Apelin and vehicle were intrathecally administered in a volume of 10 μl within 60 s using a Hamilton syringe (Ray et al., 2011) once a day from day 1 post SCI and continued for a week.

2.6. Behavioral tests

Behavioral tests were performed 30 min post acclimation period on a weekly basis by a person blinded to the experiment starting from the first week post SCI for 8 weeks.

2.6.1. Heat hyperalgesia

Noxious heat sensitivity assessed using a test apparatus consisting a portable heat source and a timer (Ugo Basile, Italy) according to Hargreaves et al. method (Hargreaves et al., 1988). In brief, rats were individually placed in chambers on a smooth glass surface. The heat source under the glass floor was positioned directly under the mid-plantar surface of hind paw. A 25 s cut-off time was used to prevent tissue damage. Withdrawal latencies were measured for both hind paws with at least three minutes interval between each. Three trials were carried out for each paw and the mean was calculated and used for the statistical analysis.

2.6.2. Mechanical allodynia

Mechanical allodynia was measured with a series of Von Frey filaments (Stoelting Co., USA). Rats were placed in a specific chamber on a metal mesh. The filaments were perpendicularly applied to the plantar area of the hind paw to buckle at a specific force. Fifty percent paw withdrawal threshold was determined with a set of eight specific filaments of 3.61, 3.84, 4.08, 4.31, 4.56, 4.74, 4.93 and 5.18 equivalent to 0.4, 0.6, 1, 2, 4, 6, 8, and 15 g respectively using up–down method (Chaplan et al., 1994). Abrupt withdrawal like lifting, licking and shaking considered as a positive response.

2.6.3. Cold allodynia

To assess cold allodynia, animals were placed on a wire mesh floor cage in specific chambers. Acetone (0.1 ml) was then sprayed to hind paw mid-plantar surface. Paw lift, lick or shake were considered as positive response. The test was performed five times with 5 min interval between each for each hind paw. Withdrawal responses were measured and reported as positive response percentage (Hosseini et al., 2014).

2.6.4. Mechanical hyperalgesia

Mechanical hyperalgesia was measured using Randall-Selitto analgesimeter (Ugo Basile, Italy) (Santos-Nogueira et al., 2012). Increasing mechanical pressure was used to determine the hind paw pressure threshold. Briefly, rats were immobilized with a towel and the stimuli applied with a dome shaped tip to the plantar surface of the hind paws. Test was done two times for each paw with 5 min interval. The force in gram at which the animal screams or withdraws the paw was measured. Threshold scores for two trials on each paw were averaged and used for statistical analysis.

2.7. Locomotor function testing

The Basso, Beattie, and Bresnahan (BBB) scoring method was used to assess locomotor activity (Basso et al., 1995). This test is a 21-point scale

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starting from 0 that means total paralysis to 21 with normal locomotion, permanent coordination, normal gait and weight-support.

To perform this test, rats were placed in a circular box (90 cm × 24 cm) with an open top. Hind-limb movement, gait, weight-support, trunk stability, paw placement/position, stepping, coordination, toe clearance and tail position were all evaluated for 4 min and scored accordingly.

2.8. Histological study

Eight weeks post SCI, half of the animals were deeply anesthetized with a mixture of ketamine/xylazine (100/20 mg/kg, i.p). Immediately after intraventricular injection of 0.1 ml of heparin (5000 IU/ml), transcardial perfusion of 300 ml of 0.9% saline solution (37 °C) was started. Perfusion was followed by 300 ml of cold 4% paraformaldehyde (4 °C) in 0.1 M phosphate buffer (pH 7.4). Following laminectomy, 1.5 cm of spinal tissue on injury site removed and stored in fixative solution at 4 °C overnight. Five micrometer thick paraffin embedded sections were made, stained with cresyl violet and visualized under the light microscope to evaluate cavity existence in the spinal cord tissues (Aziz et al., 2014; Poon et al., 2007).

2.9. Quantitative image analysis procedure

Images of the spinal cord were captured using a digital camera (Olympus) connected to the light microscope (Olympus). Cavity size and lesion area were measured and reported as spinal cord injury index using image J software (Fujimoto et al., 2000) and following formula:

$$\text{Cavity size}\ (\%) = \text{Lesion area}/\text{total cord area} \times 100$$

2.10. Western blot analysis

Western blot analysis was done to detect caspase-3 expression in the spinal tissues. Eight weeks post SCI, half of the animals in each experimental group (n = 4 rats/group) were sacrificed under deep anesthesia with a mixture of ketamine/xylazine (100/20 mg/kg, i.p). Vertebral laminectomy was done at the level of T6–T8 to dissect 1 cm long spinal tissue centered to the injury site. Samples were homogenized on ice in a lysis buffer and centrifuged at 12,000 rpm for 20 min at 4 °C. Tissue supernatants were extracted and stored at −80 °C until use. Dissected tissue protein concentration was determined with Bradford method (Bio-Rad Laboratories, Germany). To do so, an equal volume of loading buffer added into the samples and boiled for 5 min. Sample proteins separated by 4% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF (poly vinylidene fluoride) membranes (Millipore). Membranes were then blocked in 5% non-fat powdered milk and Tris-buffered saline containing 0.1% Tween-20 (blocking buffer, TBS-T, PH 7.5) for 2 h. Being incubated with primary polyclonal antibody anti-caspase-3 (1:100; biorbyt) and anti-β-actin (1:100; Sigma), samples were kept overnight at 4 °C. The day after, samples washed with buffer (0.1% Tween-20 in TBS) solution to incubate blots with horseradish peroxidase-conjugated secondary antibody (1:100; Abcam) at room temperature for1h. Finally, immunoblotting complexes were visualized with chemiluminescence immunoassay using Amersham ECL plus Western blotting detection kit (GE Healthcare Bio-Sciences, USA) according to the manufacturer's instructions. EC3 imaging system (UVP Inc. Upland, USA) was used to catch up caspase-3 and β-actin protein bands. The optical density of the bands was also measured using gel analysis software.

2.11. Statistical analysis

In behavioral studies, statistical comparisons were carried out between groups using repeated measures analysis of variance (ANOVA) followed by Tukey's post-hoc test. For histological and Western blot studies, multiple comparisons were made using one-way ANOVA with Scheffe's post-hoc test. Data were presented as mean ± SEM. p-Values <0.05 considered statistically significant. All data analysis was done using SPSS statistical software (version 21).

3. Results

3.1. Effect of [Pyr1] apelin-13 on mechanical allodynia

Spinal cord injury provoked significant mechanical allodynia (SCI 4.47 ± 0.67 vs intact 13.01 ± 0.77 g, $p < 0.001$) one week post SCI (Fig. 1). Although, slight increase was made in allodynia threshold over time (4.47 ± 0.67 g one week post injury to 5.40 ± 0.67 g six weeks post injury in SCI group), significant mechanical hypersensitivity persisted up to 8 weeks (4.96 ± 0.56 g, $p < 0.001$). Laminectomy alone had no effect on mechanical allodynia in sham animals compared to intact ones. Vehicle administration had no effect on mechanical allodynia compared to SCI group at any time point, while [Pyr^1] apelin-13 (1 μg and 5 μg) significantly alleviated mechanical allodynia. Notably, *anti*-allodynia effect of [Pyr^1] apelin-13 was maintained throughout the experiment.

3.2. Effect of [Pyr^1] apelin-13 on cold allodynia

One week post SCI, rats developed a marked cold hypersensitivity compared to intact animals (85.56 ± 5.80 vs 11.67 ± 4.01, $p < 0.001$) as indicated by increased paw withdrawal percentage (Fig. 2). In sham group, laminectomy had no effect on cold allodynia compared to intact animals. Vehicle administration had no effect on cold hypersensitivity compared to SCI group (86.67 ± 5.58 vs 85.56 ± 5.80 $p > 0.05$). Cold allodynia persisted for 8 weeks post SCI in vehicle and SCI groups. Administration of 1 μg and 5 μg of [Pyr^1] apelin-13 provoked a slight but non-significant decrease in paw withdrawal response at any given weeks compared to SCI or vehicle group ($p > 0.05$).

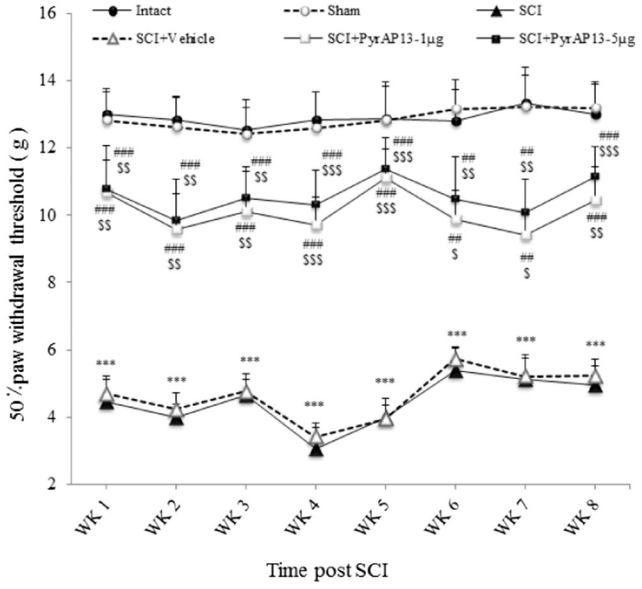

Fig. 1. Effect of i.t administration of [Pyr^1] apelin-13 on paw withdrawal threshold evaluated by Von Frey filaments. PyrAP13; [Pyr^1] apelin-13, WK; week. Data presented as means ± SEM (n = 8 rats/group). $^{***}p < 0.001$ for SCI and vehicle vs intact. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ for apelin (1 μg and 5 μg) vs SCI. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ and $^{\$\$\$}p < 0.001$ for apelin (1 μg and 5 μg) vs vehicle.

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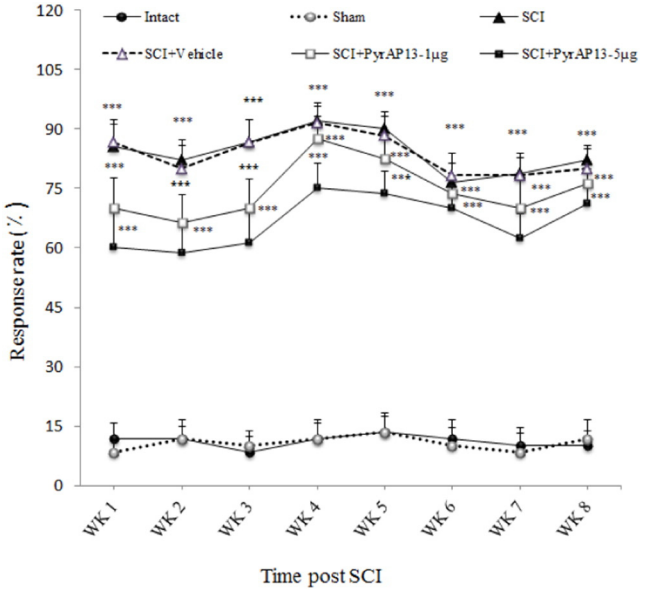

Fig. 2. Effect of i.t administration of [Pyr^1] apelin-13 on paw withdrawal response percentage to acetone droplet in experimental groups. PyrAP13; [Pyr^1] apelin-13, WK; week. Data presented as means ± SEM (n = 8 rats/group). ***p < 0.001 for SCI, vehicle, apelin (1 µg and 5 µg) vs intact.

3.3. Effect of [Pyr^1] apelin-13 on thermal hyperalgesia

Marked thermal hypersensitivity was developed in SCI group as noted in hind paw withdrawal latency compared to intact animals (10.47 ± 0.15 vs 18.83 ± 0.33, $p < 0.001$) (Fig. 3). In sham animals, laminectomy had no effect on thermal hyperalgesia in comparison to intact ones. Vehicle administration had no effect on thermal hypersensitivity compared to SCI group ($p > 0.05$). In SCI and vehicle-treated animals, thermal hypersensitivity persisted throughout the experiment. Administration of 1 µg and 5 µg of [Pyr^1] apelin-13 significantly increased thermal paw withdrawal latency. This response lasted up to the end of the experiment. Significant differences were seen between [Pyr^1] apelin-13 treated groups (1 µg and 5 µg) at 2nd,3rd , 5th and 6th weeks post SCI ($p < 0.01$ and $p < 0.05$).

3.4. Effect of [Pyr^1] apelin-13 on mechanical hyperalgesia

Marked mechanical hyperalgesia was developed in SCI animals compared to intact group (6.71 ± 0.19 vs 19.50 ± 0.67, p < 0.001) (Fig. 4). Vehicle administration had no effect on mechanical hypersensitivity compared to SCI group (p > 0.05). [Pyr^1] apelin-13 injection (1 µg) provoked a non-significant reduction in paw withdrawal threshold at first and second weeks post SCI. However, this response became statistically significant from the third week compared to SCI and vehicle groups. [Pyr^1] apelin-13 in 5 µg dose produced significant attenuation in paw withdrawal threshold compared to SCI animals from the second week post SCI. This response started at the third week post SCI in comparison to vehicle group.

3.5. Effect of [Pyr^1] apelin-13 on locomotor function

One week post SCI, rats demonstrated considerable loss in hind limbs locomotor function compared to intact group (6.28 ± 0.25 vs 21 ± 0.00, p < 0.001) (Fig. 5). Vehicle injection did not change locomotor function compared to the SCI animals (p > 0.05). During the first week post SCI, mean BBB score was markedly reduced in SCI group (6.28 ± 0.25) but spontaneous functional improvement in SCI group was noted over time. At 8th week post SCI, average BBB score in SCI group was 12.44 ± 0.24. In [Pyr^1] apelin-13 (5 µg, i.t) treated animals locomotor function drastically improved compared with SCI and vehicle treated groups (p < 0.01 and p < 0.001). Intact and sham animals showed a mean BBB score of 21 during 8 week study. Significant difference between [Pyr^1] apelin-13-treated (5 µg) and SCI and vehicle animals was noted (p < 0.01 and p < 0.001). However, there was no statistically difference between animals treated with 1 µg of [Pyr1] apelin-13 and SCI (p > 0.05) or vehicle group (p > 0.05) during 8 week experiment.

3.6. Effect of [Pyr^1] apelin-13 on cavity size

Histological study with Nissl staining on injured spinal tissues showed large cavity with progressive necrosis at the site of injury. The

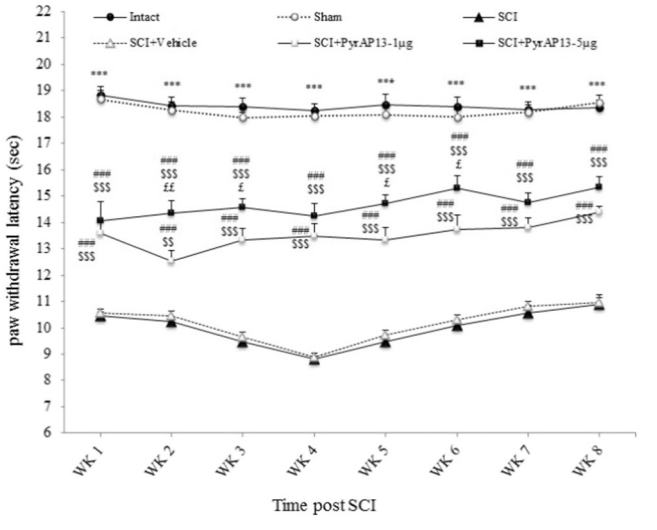

Fig. 3. Effect of i.t administration of [Pyr^1] apelin-13 on paw withdrawal latency in radiant heat test. PyrAP13; [Pyr^1] apelin-13, WK; week. Data presented as means ± SEM (n = 8 rats/group). ***p < 0.001 for SCI, vehicle, apelin (1 µg and 5 µg) vs intact. $^{###}$p < 0.001 for apelin (1 µg and 5 µg) vs SCI. $^{\$\$}$p < 0.01 and $^{\$\$\$}$p < 0.001 for apelin (1 µg and 5 µg) vs vehicle. $^{£}$p < 0.05 and $^{££}$p < 0.001 for apeline 5 µg vs apeline 1 µg.

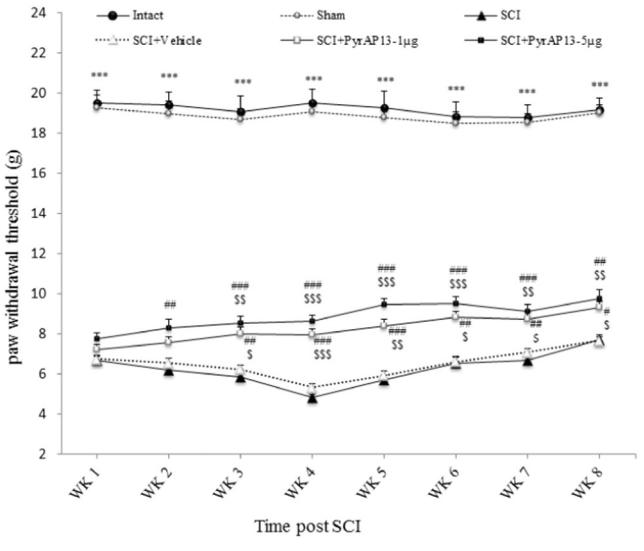

Fig. 4. Effect of i.t administration of [Pyr^1] apelin-13 on paw withdrawal threshold with Randall-Selitto test. PyrAP13; [Pyr^1] apelin-13,WK; week. Data presented as means ± SEM (n = 8 rats/group). ***p < 0.001 for SCI, vehicle, apelin (1 µg and 5 µg) groups vs intact. $^{\#}$p < 0.05 & $^{\#\#}$p < 0.01 and $^{\#\#\#}$p < 0.001 for apelin (1 µg and 5 µg) vs SCI. $^{\$}$p < 0.05 & $^{\$\$}$p < 0.01 and $^{\$\$\$}$p < 0.001 for apelin (1 µg and 5 µg) vs vehicle.

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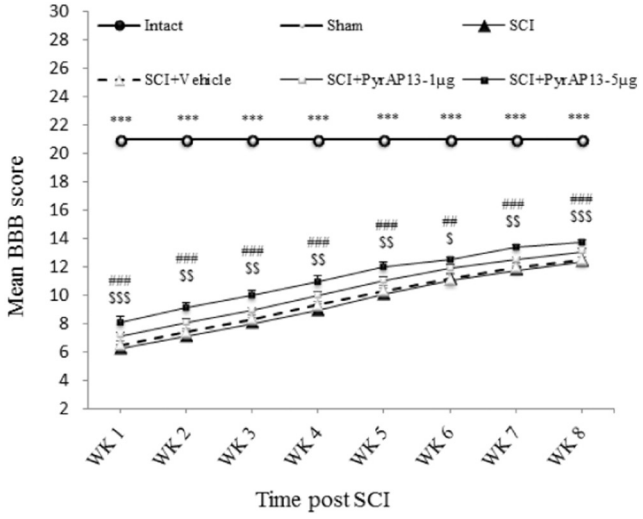

Fig. 5. Effect of i.t administration of [Pyr1] apelin-13 on locomotor function with BBB test in experimental groups. PyrAP13; [Pyr^1] apelin-13, WK; week. Data presented as means ± SEM (n = 8 rats/group).***p < 0.001 for SCI, vehicle, [Pyr^1] apelin-13 (1 μg and 5 μg) vs intact. $^{\#\#}$p < 0.01 and $^{\#\#\#}$p < 0.001 for [Pyr^1] apelin-13 (5 μg) vs SCI. $^{\$}$p < 0.05 & $^{\$\$}$p < 0.01 and $^{\$\$\$}$p < 0.001 for [Pyr^1] apelin-13 (5 μg) vs vehicle.

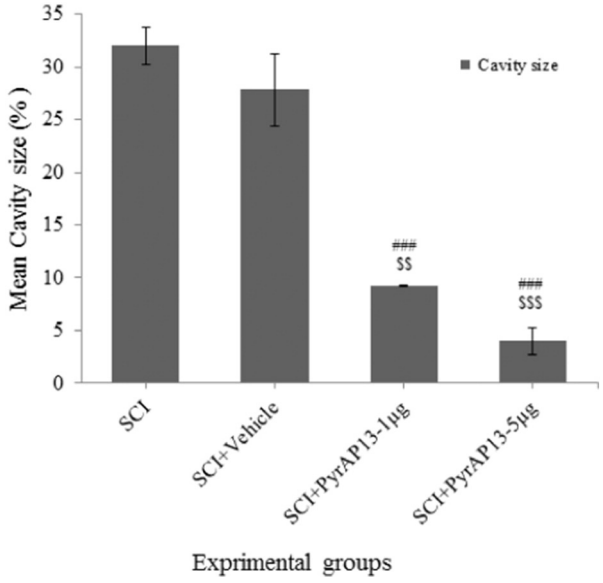

Fig. 7. Effect of [Pyr^1] apelin-13 on cavity size in experimental groups 8 weeks post SCI (n = 4 rats/group). PyrAP13; [Pyr^1] apelin-13, WK; week. Graph shows cavity size mean percentage (n = 4), $^{\#\#\#}$p < 0.001 [Pyr^1] apelin-13 (1 μg and 5 μg) vs SCI). $^{\$\$}$p < 0.01 and $^{\$\$\$}$p < 0.001 for [Pyr^1] apelin-13 (1 μg and 5 μg) vs vehicle.

cavity was located in dorsal column and extended to the grey matter and white matter in SCI and vehicle groups (Fig. 6C, D). Cavity formation was probably due to fiber degeneration and neuronal death. In [Pyr^1] apelin-13 treated animals, cavity size became smaller and restricted to the dorsal column only (Fig. 6E, F). In intact and sham animals, there were no cavity and the spinal tissues were normal (Fig. 6A, B). Quantitative image analysis showed no difference in cavity size between vehicle and SCI group ($p > 0.05$) (Fig. 7). [Pyr^1] apelin-13 (1 μg and 5 μg) injection significantly decreased cavity size compared to both vehicle and SCI groups ($p < 0.01$, $p < 0.001$ respectively))Fig. 7).

3.7. Effect of [Pyr^1] apelin-13 on apoptotic cell death post SCI

To investigate protective mechanism of [Pyr^1] apelin-13 on SCI, we carried out Western blot analysis on spinal tissues to detect the activity of the key apoptotic enzyme, caspase-3, at week 8 post SCI. Statistical and quantitative analysis showed significant caspase-3 up regulation in SCI group compared to intact animals ($p < 0.001$) (Fig. 8A, B). Administration of vehicle had no effect on caspase-3 expression when compared with SCI group ($p > 0.05$). [Pyr^1] apelin-13 (1 μg and 5 μg) significantly attenuated caspase-3 expression in comparison with SCI or vehicle group ($p < 0.001$). There was a significant difference in caspase-3 expression between 1 μg and 5 μg of [Pyr^1] apelin-13 animals ($p < 0.05$). No significant differences in caspase-3 expression were seen between intact and sham animals ($p > 0.05$).

4. Discussion

To the best of our knowledge, current study furnishes for the first time pile of evidence indicating potential attenuating properties of intrathecal administration of [Pyr^1] apelin-13 on nociceptive responses,

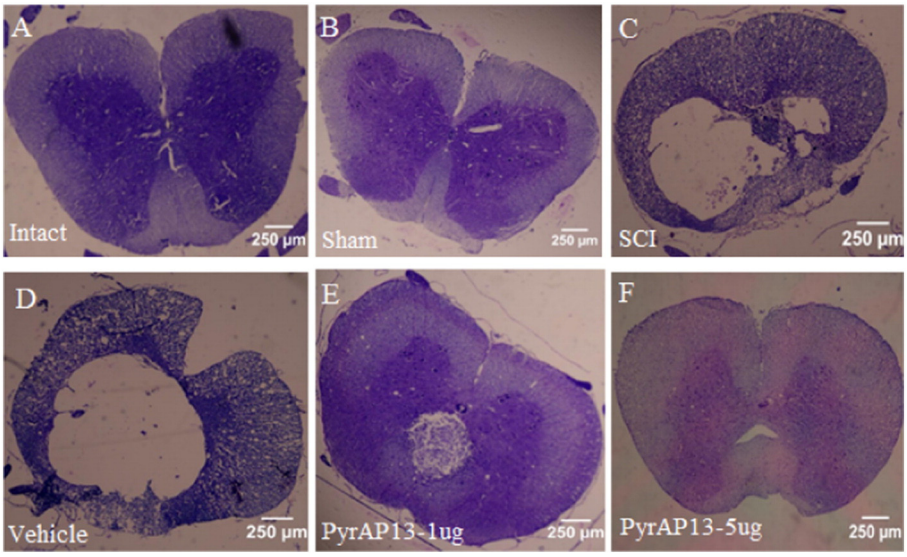

Fig. 6. Spinal cross sections stained with cresyl violet showing epicenteral cavities at the site of lesion 8 weeks post SCI. Spinal tissues in sham and intact groups have normal appearance. Vacuolar changes and cell loss indicates large cavity in SCI and vehicle treated groups. Note the smaller cavity size in [Pyr^1] apelin-13 treated groups compared with vehicle and SCI groups. PyrAP13; [Pyr^1] apelin-13, Scale bar = 250 μm.

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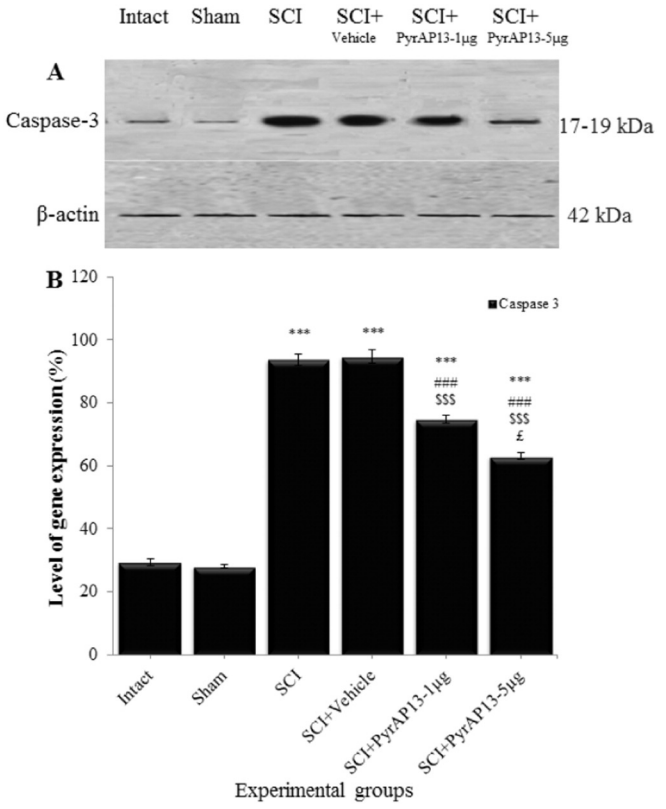

Fig. 8. Western blot analysis for caspase-3 expression in spinal tissues of experimental groups 8 weeks post SCI (A). Caspase-3 detected at 17 and 19 kDa. β-actin (42 kDa) is the loading control (A). Graph shows caspase-3 to β-actin expression ratio (B). PyrAP13; [Pyr^1] apelin-13, WK; week. Data presents as mean ± SEM (n = 4 rats/group). $^{***}p < 0.001$ for SCI, vehicle, [Pyr^1] apelin-13 (1 μg and 5 μg) vs intact. $^{###}p < 0.001$ for [Pyr^1] apelin-13 (1 μg and 5 μg) vs SCI. $^{\$\$\$}p < 0.001$ for [Pyr^1] apelin-13 (1 μg and 5 μg) vs vehicle. $^{£}p < 0.05$ for [Pyr^1] apelin-13 (5 μg) vs [Pyr^1] apelin-13 (1 μg).

locomotor dysfunction, and histological damages in rats with neuropathic pain post SCI. In this study, SCI was induced using clip-compression model. The model thus mimics ischemia-reperfusion injury resulting from transient vascular occlusion (Saadoun et al., 2008) and provides reliable results for rat acute and chronic SCI evaluation (Kang et al., 2011; Poon et al., 2007). In our study, SCI animals showed robust withdrawal responses to noxious stimuli applied to their hind paws one week post injury which is a sign for hyperalgesia presence. The SCI rats also exhibited allodynia in response to harmless stimuli in the same way (Bruce et al., 2002; Nasirinezhad et al., 2015). Hyperalgesia and allodynia are the most important chronic reliable pain-related behaviors post SCI (Bruce et al., 2002; Clark et al., 2010; Hama and Sagen, 2007; Raghavendra et al., 2003). These symptoms lasted 8 weeks post injury almost the same way in our previous experiment (Nasirinezhad et al., 2015). Our results also showed that i.t administration of 1 μg and 5 μg of [Pyr^1] apelin-13 produced marked attenuation of neuropathic pain symptoms. Interestingly, one week administration of apelin could induce a pronounced analgesic effect that lasted 8 weeks post injury. The effect was dose-dependent on mechanical allodynia as well as mechanical and thermal hyperalgesia but not cold allodynia. We used i.t injection since i.t administration of [Pyr^1] apelin-13 provided better drug localization and higher concentration to the site of injury (Hamann et al., 2003; Khoshdel et al., 2016). The [Pyr^1] apelin-13 doses and time course were chosen based on our pilot and other previous study (Baumann et al., 2010; Jaszberenyi et al., 2004). Although [Pyr^1] apelin-13 is a water-soluble peptide, it can cross the blood-spinal cord barrier (BSCB) due to its small molecular size (Kleinz and Davenport, 2005; Malavolta and Cabral, 2011). Thus, [Pyr^1] apelin-13 i.t administration may lead to drug leakage into the blood stream. Usually direct drug delivery close to the lesion site causes rapid tissue penetration through receptor mediated cellular uptake and limits CSF drug distribution (Calias et al., 2014). Although we did not measure plasma [Pyr^1] apelin-13 level, it is unlikely to be drastically changed due to its short half-life (<8 min) (Aydin et al., 2014; Serpooshan et al., 2015),low injection volume (10 μl), high spinal APJ density (Kleinz and Davenport, 2005) and high binding affinity of [Pyr^1] apelin-13 to the APJ receptor (Lv et al., 2012; Pope et al., 2012; Xu et al., 2009).

Apelin expression on neuronal and glial cells in the pain related areas of the CNS is a clue for its role on pain modulation (Cheng et al., 2012). There were several reports on antinociceptive effects of intracerebroventricular (i.c.v) and i.t apeline-13 injections (Lv et al., 2012; Xu et al., 2009). Turtay reported that rat intraperitoneal administration of apelin-13 decreased thermal hyperalgesia in hot-plate and tail-flick tests (Turtay et al., 2015). However, in Canpolat et al. study, acute intraperitoneal injection of apeling-13 in mice decreased acute pain threshold (Canpolat et al., 2016). This controversy could be due to different pain models, animal species, different routes of administration and apelin complicated regulatory mechanisms. Similar controversial results have been reported on antinociceptive effect of spironolactone. Systemic injection of spironolactone decreased acute nociceptive pain threshold in tail flick, hop plate and tail electric stimulation tests. (Abdel-Salam et al., 2010). Whereas it attenuated inflammatory visceral pain (Abdel-Salam et al., 2010) and neuropathic pain in chronic compression of the dorsal root ganglia (CCD) model (Sun et al., 2012).

In our study, locomotor dysfunction was recovered to some extent over time among SCI animals. This finding is similar to several other studies with clip-compression injury model (Hama and Sagen, 2007; Kang et al., 2011; Poon et al., 2007). In this study, apelin provoked a better recovery in comparison with SCI and vehicle treated animals. This finding is in congruent with Xin et al. study in which apelin-13 could decrease rat neurological dysfunction following cerebral I/R damage (Xin et al., 2015). There is a good correlation between [Pyr^1] apelin-13 antiapoptotic activity and long-term locomotor recovery. This finding is also confirmed by our histological and molecular data. Following SCI, spinal tissue underwent a sequence of pathological changes due to apoptotic cell death and cavity formation (Aziz et al., 2014). We found that cavity size in apelin-treated groups remained smaller compared with SCI or vehicle treated group. This finding is paralleled with decreased spinal cord caspase-3 level and is in agreement with other similar studies in which apelin-13 administration significantly decreased rat infarct size post cerebral I/R damage (Khaksari et al., 2012; Yan et al., 2015).

Other in vivo (Chen et al., 2015; Khaksari et al., 2012) and in vitro (Zeng et al., 2012; Zeng et al., 2010) studies on apelin and caspase-3 showed the same track of changes. Western blot study revealed caspase-3 up regulation in SCI rats 8 weeks post injury. Treatment with [Pyr^1] apelin-13 had an inverse effect on caspase-3 level and suppressed secondary injury process via caspase-3 dependent pathway. It has been reported that increased caspase-3 activity, generation of ROS, Ca^{2+} and glutamate accumulation and apoptosis are all involved in secondary injury mechanisms following SCI induction (Dumont et al., 2001; Hall and Springer, 2004; Zhang et al., 2012). Apoptosis in oligodendroglia causes axonal demyelination and long-term neurologic deficits. Neuronal apoptosis in turn produces cell loss with negative effects on SCI outcomes (Dumont et al., 2001; Hall and Springer, 2004). Neuroprotective agents are routinely used to treat SCI via secondary damage inhibition (Bai et al., 2012; Hall and Springer, 2004). Zhao et al. showed antiapoptotic effect of apelin on rat primary SCI model (Zhao et al., 2011). Zeng also reported protective effect of apelin-13 on bone marrow-derived mesenchymal stem cells (BMSCs) through antiapoptotic and MAPK/ERK1/2 and PI3K/Akt signaling pathways inhibition (Zeng et al., 2012). Therefore, we may conclude that motor-sensory improvement and histological changes observed in our study are partially related to [Pyr^1] apelin-13 anti-apoptotic property and caspase-3 down regulating activity.

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Taken together, profound improvement in behavioral and histological outcomes in rats treated with [Pyr^1] apelin-13 post spinal cord compression injury may be interpreted as its fair neuroprotective effects. This may make it a candidate for further serious studies to clarify the exact mechanisms and pave the way for neuropathic pain clinical trials.

5. Conclusion

Our findings suggest that [Pyr^1] apelin-13 can be a possible therapeutic approach to alleviate long-lasting neuropathic pain, locomotor deficit and histological damages post SCI. However, complementary research is needed to elaborate on apelin precise mode of action and involved signaling pathways.

Conflict of interest

The authors have no conflict of interest to declare.

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