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Research report

Electroacupuncture improves gait locomotion, H-reflex and ventral root potentials of spinal compression injured rats

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ABSTRACT

This study explored the effect of electroacupuncture stimulation (EA) on alterations in the Hoffman reflex (H-reflex) response and gait locomotion provoked by spinal cord injury (SCI) in the rat. A compression lesion of the spinal cord was evoked by insufflating a Fogarty balloon located in the epidural space at the T8–9 spinal level of adult Wistar male rats (200–250 gr; n = 60). In different groups of SCI rats, EA (frequencies: 2, 50 and 100 Hz) was applied simultaneously to Huantiao (GB30), Yinmen (BL37), Jizhong (GV6) and Zhiyang (GV9) acupoints from the third post-injury day until the experimental session. At 1, 2, 3 and 4 post-injury weeks, the BBB scores of the SCI group of rats treated with EA at 50 Hz showed a gradual but greater enhancement of locomotor activity than the other groups of rats. Unrestrained gait kinematic analysis of SCI rats treated with EA–50 Hz stimulation showed a significant improvement in stride duration, length and speed ($p < 0.05$), whereas a discrete recovery of gait locomotion was observed in the other groups of animals. After four post-injury weeks, the H-reflex amplitude and H-reflex/M wave amplitude ratio obtained in SCI rats had a noticeable enhancement (217%) compared to sham rats (n = 10). Meanwhile, SCI rats treated with EA at 50 Hz manifested a decreased facilitation of the H-reflex amplitude and H/M amplitude ratio (154%) and a reduced frequency-dependent amplitude depression of the H-reflex (66%). In addition, 50 Hz-EA treatment induced a recovery of the presynaptic depression of the Gs-VRP evoked by PBSt conditioning stimulation in the SCI rat (63.2 ± 8.1%; n = 9). In concordance with the latter, it could be suggested that 50 Hz-EA stimulation reduced the hyper-excitability of motoneurons and provokes a partial improvement of the locomotive performance and H reflex responses by a possible recovery of presynaptic mechanisms in the spinal cord of experimentally injured rats.

1. Introduction

Spinal cord injury (SCI) is one of the most common impairments of the central nervous system that results in complete or partial loss of both sensory and motor functions due to mechanical spinal damage, which often leads to permanent paralysis (Silverman et al., 2012). Traumatic and non-traumatic lesions represent the two presentations of SCI. The first results from contusion, compression or stretching of the spinal cord, and the second is associated with vertebral spondylosis, tumor compression, vascular ischemia, and inflammatory spinal cord disorders (New et al., 2002). In both humans and animals, SCI induces physiological changes in the motor system which included hyperreflexia and abnormalities of the locomotor behavior (Silverman et al., 2012; Yablon and Stokic, 2004). Several researchers use the Hoffman reflex (H-reflex), to analyze the hyperreflexia following SCI (Milanov, 1994; Yablon and Stokic, 2004). The H-reflex is an electromyographic (EMG) response that results from activation of a synaptic pathway conformed by

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the afferent-motoneuron-muscle circuit (Reyes et al., 2007). In SCI, changes associated with hyperreflexia and spasticity included: alpha motor neuron hyperexcitability (Lin et al., 2007; Milanov, 1994), changes in the intrinsic properties of motoneurons (Bennett et al., 2001) and loss of presynaptic inhibition (Hultborn, 2006). It has been shown that the measurement of H-reflex rate-sensitive depression acquires a particular importance in the assessment of hyperreflexia following SCI (Thompson et al., 1992; Chen et al., 2001).

In general, treatment for functional recovery of SCI included surgery (Bregman et al., 2002), physical therapy (Silverman et al., 2012), drugs (Attal et al., 2009), hormonal treatment (Calderón et al., 2015; Osuna et al., 2016), behavioral therapy (Norrbrink et al., 2006), and supportive treatment (Huston et al., 2011). Because there is still a lack of effective treatment for spinal cord injuries, there has been an increased interest in alternative medical treatments.

Acupuncture is a therapeutic modality that emerged from Traditional Chinese Medicine. The World Health Organization recommends the use of acupuncture for the treatment of a wide variety of diseases (Zhang et al., 2014; Barnes et al., 2008). A relatively novel form of acupuncture is the electrical stimulation of acupuncture points (APs), also known as electroacupuncture (EA), which has been widely used in both clinical and experimental reports (Vickers et al., 2012; Zhao, 2008). In previous studies, it has been shown that application of EA (*Dazhui* (DU14), *Mingmen* (DU4), *Sanyinjiao* (SP6), *Huantiao* (GB30), *Zusanli* (ST36) and *Kunlun* (BL60) as a treatment for SCI contributes to the recovery of several neurologic and functional alterations (Min et al., 2015). It has been found that EA produces an improvement in the locomotor pattern (Peng et al., 2007), which is accompanied by reductions in the process of glial scarring (Yang et al., 2005), oxidative stress (Politis and Korchinski, 1990), laminin expression (Zhu, 2002) and aquaporin transport (AQP-4; Xie et al., 2006). It is thought that EA evokes its effects through the activation of peripheral sensory afferents that in turn synaptically interact with sets of dorsal horn sensory neurons in the spinal cord (Quiroz et al., 2014a,b). However, EA effects on experimental animal models of SCI, particularly at the motoneuron level, are scarcely studied. In this study, we analyzed the effect of EA on locomotor behavior (evaluated with the Open Field Test and gait kinematics analysis), H reflex facilitation and H-reflex frequency-dependent depression evoked in adult rats after a spinal cord compression injury. In addition, to disclose possible presynaptic mechanisms in the effect of EA stimulation, we also analyzed the changes produced by conditioning stimulation of the posterior biceps and semitendinosus (PBSt) on the amplitude of ventral root potentials (VRP) evoked by gastrocnemius nerve (Gs) stimulation, as a test for presynaptic inhibition.

2. Materials and methods

2.1. Animals

Male Wistar rats (n = 60) weighing 200–250 g (8–10 weeks old) obtained from our institution were used. All animals had free access to food and water and were housed under identical environmental conditions of light and dark cycles (12:12 h) and temperature (22–24 °C). All experiments were performed in accordance with the guidelines contained in NIH publications No. 80-23 (revised 1996) and the Mexican Official Norm (NOM-062-ZOO-1999) on the Principles of Laboratory Animal Care. The study protocol was approved by the institutional bioethics committee for the Care and Handling of Laboratory Animals (Protocol 0267-05, CINVESTAV).

2.2. Surgical procedures and animals groups

Initially the rats were randomly assigned into five groups by using a random number table: (1) sham control group (n = 10), (2) compression injury without EA treatment group (SCI-UT n = 12), (3) compression injury with EA treatment at 2 Hz (SCI-EA 2 Hz group; n = 11), (4) 50 Hz (SCI-EA 50 Hz group; n = 14) and (5) 100 Hz (SCI-EA 100 Hz group; n = 13). The method used for producing a compression injury was similar to that described previously (Lonjon et al., 2010). Animals were anesthetized with an intraperitoneal injection of a mixed solution of ketamine (100 mg/kg) and xylazine (2 mg/kg). To provoke the compression injury in the rat spinal cord, a Fogarty catheter (2 French x 60 cm, size: 0.67 mm, Ethimed) was inserted into the epidural space through a hole drilled in the posterior arch of the T-10 vertebra, and a groove was drilled on T-11 to guide the insertion of the catheter. The balloon was positioned at the T8–9 level, inflated with sterilized water (10 μl) using a Hamilton syringe, and left in place for 5 min. The sham group of rats (n = 10) underwent the same protocol, except for balloon inflation.

After surgery, the back skin and muscles were sutured with a sterile thread and stainless wound clips, and the animals were allowed to recover from surgery and anesthesia in a clean, heated cage. All rats received an intramuscular injection of penicillin and procaine (5,000,000 U/ml/day), and Furazolidone was spread onto the lesion to prevent infection. The animals were housed in individual cages and individually received intensive care, which included manual expression of urine and excrement until the day of the experiment. Because nine rats died after SCI, and six died during the electrophysiological recording session, the final number of animals included in each group was the following: SCI-UT (n = 10); SCI-EA 2 Hz group (n = 8), SCI-EA 50 Hz group (n = 9); and SCI-EA 100 Hz group (n = 8).

2.3. Electroacupuncture treatment

EA was applied simultaneously at two pairs of acupoints. One pair was situated at the Jizhong (GV6, anode) and Zhiyang (GV9 cathode) acupoints and the other pair were Huantiao (GB30 anode) and Yinmen (BL37 cathode), bilaterally (Fig. 1). These acupoints were selected because they are needling points in acupuncture treatments following SCI in humans (Heo et al., 2013a,b) and contributed to neuroplasticity in the spinal cord after injury (Min et al., 2015; Ding et al., 2011). The *Huantiao* (GB30) acupoint was located at the posterior upper border of the hip joint of the hindlimbs. The *Yinmen* (BL37) acupoint was situated where the long head of the biceps femoris muscle and the semitendinosus muscle converged (upper to the middle part of the popliteal crease), whereas the *Jizhong* (GV6) acupoint was located posterior to the midline, below the spinous process of the eleventh thoracic vertebra (maintaining the animal in the prone position). The *Zhiyang* (GV9) acupoint was situated on the posterior midline and in the depression below the spinous process of the seventh thoracic vertebra in the prone position. EA was applied daily (resting on weekends) by means of an electroacupuncture stimulator (AWQ-104L, China), and it consisted of a sequence of biphasic pulse trains of 2.5 s in duration followed by periods of 2.5 s with no stimulation (Fig. 2). The biphasic pulse stimuli was chosen in this study to avoid the polarization of each needle because of electrolysis and break off in tissue (Pomeranz, 1995). Another advantage of biphasic stimulus is that each pair of needles receives symmetrical current pulses which appears to be very useful to neuromodulate the physiological state of the spinal cord in humans (Gerasimenko et al., 2015). The intensity of the EA current pulses was adjusted to induce slight twitches in hind limbs (1 mA).

Because it has been showed that EA stimulation induced neuroprotective, remyelination, antioxidative and anti-inflammatory

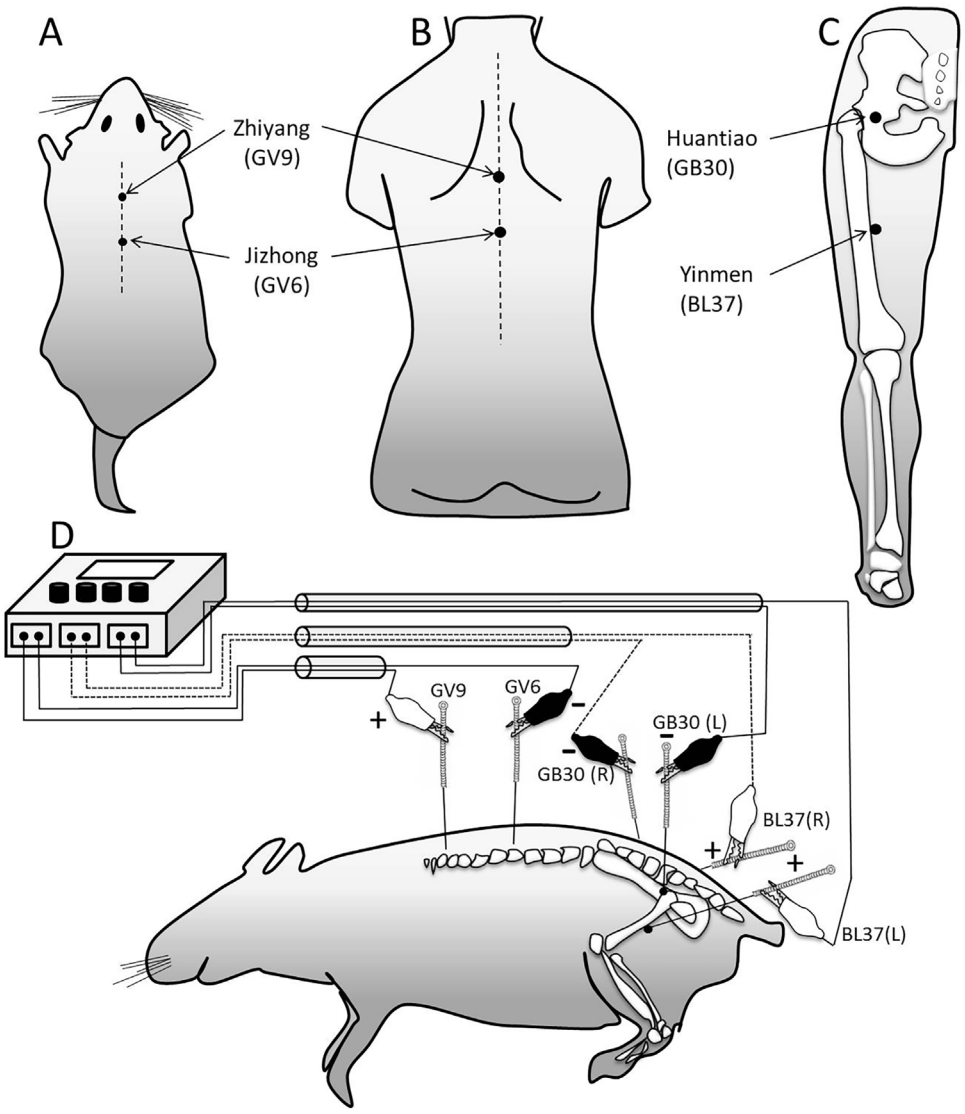

FIGURE1

Fig. 1. Schematic diagram indicating the relative position of the selected acupoints Zhiyang (GV9), Jizhong (GV6) in the rat (A), and their corresponding acupoints in humans: Huantiao (GB30) (B), and Yinmen (BL37) (C);Diagram illustrating the operation process of electrode needles at the selected pairs of acupoints with their respective cathode (+) and anode (−) polarity. Right (R), Left (L).

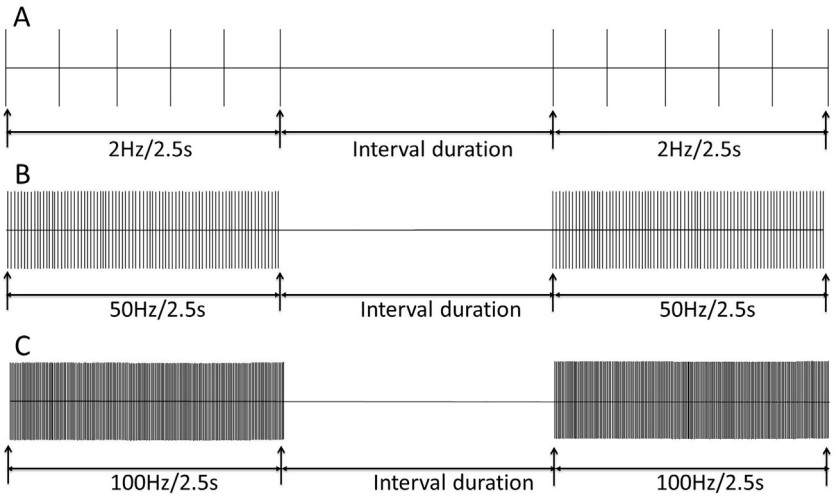

FIGURE2

Fig. 2. Parameters of EA stimulation treatment applied to the three groups of SCI rats. The mode of stimulation was a discontinued biphasic pulse (0.37 ms) applied at 2 (A), 50 (B) and 100 (C) Hz with a duration interval of 2.5 s per pulse over 30 min.

effects during the acute phase of SCI, starting from the third post-injury day (Mo et al., 2016; Huang et al., 2015; Liu et al., 2013) we decided to analyze the effect of EA treatment on H-reflex and ventral root potentials during a period of 4 weeks after the spinal cord injury. In this way, EA was administered at 2 Hz, 50 Hz and 100 Hz frequencies as pointed out previously every other day (session lasted 40 min) for 30 days (four weeks), starting from the second post-injury day until the day prior to the experiment.

2.4. Functional assessments

The locomotive activity was evaluated with the Basso–Beattie–Bresnahan (BBB) open field test and with a Kinematic analysis of gait locomotion. All motor evaluations were made between 9:30–10:30 a.m. and no details of the operative procedure or treatment protocol of animals were offered to the two independent examiners who made the scoring of animals ("double-blinded analysis"). Subsequently, the scores provided by the two independent researchers were averaged

2.4.1. Locomotor performance evaluation (BBB test)

Animals were placed on one open-field arena 90 cm above ground where voluntary movements of the trunk, tail and hind limbs were recorded by a single commercial digital video camera and subsequently evaluated by the Basso, Beattie and Bresnahan (BBB) scale (Basso et al., 1995). BBB scale values varied from 0 (complete hindlimb paralysis) to 21 points (normal hindlimb locomotion). The experimental sessions were 3 days and 1, 2, 3 and 4 weeks post-injury and lasted 5 min each.

2.4.2. Kinematic analysis of gait locomotion

Unrestrained gait locomotion of the different groups of rats was evaluated at 28 days post-injury (4 weeks) by a kinematic method previously used in other studies (Calderón et al., 2015; Osuna et al., 2016). Briefly, gait locomotion of both the sham-operated and experimental animals was recorded with a single commercial digital video camera located on one side of a transparent acrylic passageway. After shaving, the limbs, iliac crest, hip (e.g., greater trochanter), knee, ankle (e.g., lateral malleolus), and fifth metatarsal phalangeal joints were marked with ink (Fig. 3A). The videotapes were digitized (30 frames/s), and their frames were individually captured as digital photographs using a commercial video editing program (Pinnacle Studio V.7, Pinnacle systems Inc.). Limb movements consisting of 4–6 step cycles were analyzed frame by frame using the Image J program (Scion Corporation, NIH), and the X and Y coordinates of each articulation joint were calculated and introduced in a computer program (developed in our laboratory in LabView environment) that calculated the angle and spatial location of each joint articulation. Line drawings constructed between articulation joints illustrated the spatio-temporal sequences of hind limb movements during gait locomotion. Swing and stance phases were identified from each stride cycle, and the duration, length and speed of strides determined. The data obtained from sham-operated and experimental groups of animals were pooled separately, and their corresponding average and standard error values were calculated.

2.5. H reflex recording

At the recording day, the rats were anesthetized with an initial dose of ketamine (100 mg/kg of weight, i.p.) supplemented with additional doses of 75 mg/kg i.p. as required to maintain an adequate level of anesthesia, which was assessed routinely by verifying that the withdrawal reflex was absent after a strong pinch of the right hindlimb. The animal's body temperature was monitored using a thermal probe located in the back muscles and connected to an automatic feedback control unit and heating blanket to maintain the animal's body temperature at 37 °C. All left hindlimb nerves were dissected and sectioned with the exception of the distal branch of the tibial nerve (first series of 10 experiments) or the lateral plantar nerve (second series of 6 experiments in which the medial plantar nerve was also sectioned) which were left intact with their insertion to the plantar muscles ("closed loop condition"). Single voltage pulses (0.05 ms duration) were delivered from a pulse generator every 8 s to avoid rate depression of the H reflex (Gozariu et al., 1998) and applied to the sciatic or proximal lateral plantar nerve through a pair of fine silver hook electrodes over one hour ($n = 100$ trials) and averaged. The stimulus strength was gradually adjusted from the minimal voltage strength needed to evoke a barely discernible electroneurogram response (one times threshold, 1xT) in the distal lateral plantar nerve ($1xT = 0.43 \pm 0.19$ V) to several multiples of the threshold ($1–3 \times T$).

To reduce the possible mechanical artifacts derived from muscle contraction, electroneurogram (ENG) and electromyogram (EMG) recordings were performed with fine "floating" chloride silver electrodes located in the distal lateral plantar nerve and lateral plantar muscles of the foot (between the 4th and 5th digit) (Reyes et al., 2007). The recording electrodes were connected to separate low noise, high gain pre-amplifiers (band pass filters set at 0.3 Hz in the low range and 10 KHz in the high range), which were subsequently connected to a digital oscilloscope and to a digital computer through a data acquisition system (ATMIO 16E2, National Instruments; 12 bits resolution). EMG responses were digitized and stored by means of a specially designed computer program (at 100 bins of resolution and a digitization rate of 3000 samples per second). Typically, EMG responses consisted of two components, the M wave and the H-reflex (Reyes et al., 2007). Subsequently, the Hmax/Mmax amplitude ratio was calculated to estimate the relative proportion of motor units recruited through the monosynaptic reflex loop (Côté et al., 2014; Mazzocchio et al., 2001).

2.6. Rate-dependent depression of H-reflex

Low frequency-dependent depression of the H-reflex is defined as the gradual decrease in the H-reflex amplitude that occurs when a series of reflexes are elicited between 1 and 10 Hz. It has been shown that measures of H-reflex frequency-sensitive depression is of particular importance in the assessment of hyper-reflexia following SCI (Thompson et al., 1992; Chen et al., 2001). To induce the frequency-dependent depression of the H-reflex, sequences of stimulus pulses were applied to the exposed plantar nerve at different frequencies (0.2, 1, 5, and 10 Hz), and alterations in the reflex excitability level of the plantar spinal motoneuron pool were inferred from changes in the Hmax/Mmax amplitude ratio (Côté et al., 2014).

2.7. Stimulation and ventral root recordings

The central ends of the posterior biceps and semitendinosus (PBSt) and gastrocnemius nerves (GS) were mounted on pairs of silver hook electrodes connected to isolated-current-pulse generators (Isoflex D 4030) and square-current pulses (0.05 ms duration; 1 Hz) of graded intensity were applied to the nerves. The pulses were monitored by recording the voltage drop across a resistor (1000 Ω) that was placed in the current return path to ground. The electrical threshold of the evoked spinal potentials (one times threshold, $1 \times T$) was established as the minimum current intensity necessary to evoke a barely discernible response in the ventral roots.

To record the VRP, a previously dissected L6 ventral rootled was placed on two fine silver Ag-AgCl wire electrodes, one located very close to, but not touching, the spinal cord and the other, distally on the severed end of the rootlet (Fig. 1A). Next, the record-

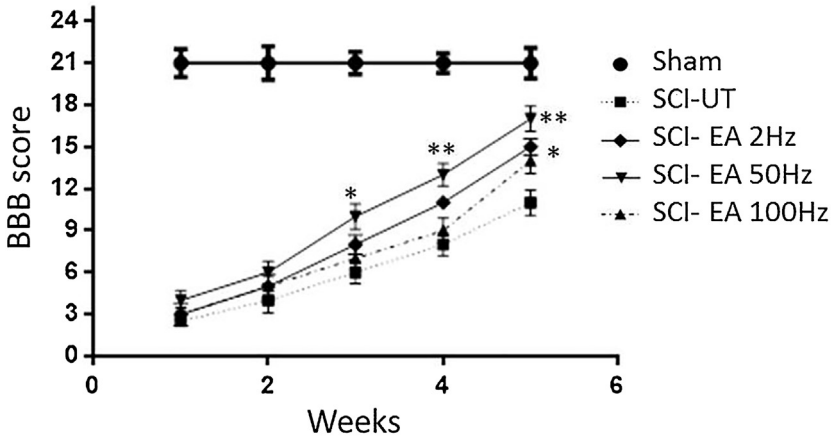

FIGURE3

Fig. 3. BBB performance of the different groups of rats: sham-operated (black circles; n = 10), untreated injured spinal cord (compression of the 8–9th segment of the thoracic spinal cord) (squares SCI-UT; n = 10), and treated with electroacupuncture (EA) at frequencies of 2 Hz (diamonds; n = 8), 50 Hz (inverted triangles; n = 9) and 100 Hz (triangles; n = 8). The baseline BBB score of sham-operated rats (control) was 21, * $p < 0.05$ (two-way repeated measures ANOVA with Bonferroni's post-test).

ing electrodes were connected to low-noise, high-gain differential amplifiers (Grass, model P511; band-pass filters set at 0.3–10 kHz), which in turn, were attached to a digital oscilloscope and personal computer.

To determine the time course of the effect produced by the conditioning stimulation on test responses, four conditioning stimuli (at 300 Hz) were delivered to the PBSt nerve at different interstimulus time intervals (between 10 and 200 ms) before the test stimulus applied to the GS nerve. The conditioning stimulation started 10 ms prior to the test stimulus to reduce the occlusive effect that was provoked by the preceding stimuli on test potentials when short time intervals were used (Enríquez-Denton et al., 2004; Rudomin and Schmidt., 1999).

2.8. Histology

At the end of the recording session, animals were perfused intracardially with a mixed solution of Krebs saline and paraformaldehyde (4%). Subsequently, the compressed spinal T8-T9 segments were dissected and placed in a solution of PBS + 30% sucrose. The segments were placed on an agar block and sliced (50 μm thick) with a vibratome (Leica SM 2000R). Tissue slices were stained with the crystal violet method for Nissl substance, and spinal cord sections showing the traumatic injury were photographed with a digital camera to characterize the damaged spinal areas.

2.9. Data analysis

All the data are expressed as the mean ± standard deviation, and the motor performance (BBB test and kinematic gait analysis) and frequency-dependent depression of the H-reflex values were analyzed using a 2-way ANOVA followed by Bonferroni's multiple comparison test. The significance level was established at $p < 0.05$. All statistical analyses were performed using Graph-Pad Prism (version 5 San Diego Ca.) software.

2.10. Ethics statements

All experimental procedures were performed in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010, National Institutes of Health, Bethesda, MD, USA; Animal Welfare Assurance # A5036-01) and the Mexican Official Norm (NOM-062-ZOO-1999) and approved by the Institutional Bioethical Committee for Care and Handling of Laboratory Animals (Protocol 0267-05, CINVESTAV).

3. Results

3.1. Locomotor performance (BBB test)

At the third day after the spinal cord injury, all rats with spinal lesions showed noticeable locomotor impairment in both the left and right hind limbs, as determined using the BBB test (Fig. 3). At 1, 2, 3 and 4 post-injury weeks, a gradual and progressive motor recovery was observed in all groups of animals. SCI-UT rats had a very poor improvement in locomotor activity (Fig. 3), whereas SCI rats treated with EA stimulation at 2 and 50 Hz showed continuous and significant enhancement in their motor performance ($p < 0.05$, Fig. 3), which was characterized by the occurrence of movements in the knee and ankle joints combined with slight movements of the hip joint during locomotor activity compared to SCI-UT rats ($p < 0.05$, n = 10). Moreover, SCI-EA 100 Hz rats exhibited a noticeable but not significant improvement in locomotor activity at the fourth week post-injury (Fig. 3).

3.2. Kinematic gait analysis

At the first week post-injury, all animals showed no gait locomotion (Fig. 4B). After 4 weeks, the SCI-UT group of animals showed a slight but noticeable recovery in stride parameters during unrestrained gait (stride duration: 28 ± 4.2%, length: 32 ± 4.8% and stride speed: 42 ± 2.1%. Fig. 4B–F), whereas SCI animals treated with EA at 2 and 100 Hz display significant stride improvement (stride duration: 44 ± 5.1% and 47 ± 5.8%; stride length: 45 ± 4.1% and 47 ± 4.9% and stride speed: 58 ± 6.1% and 55 ± 5.2%, respectively. $p < 0.05$. Fig. 4B–F). However, SCI rats treated with EA at 50 Hz showed a relatively better stride performance (stride duration: 73 ± 5.2%; stride length: 78 ± 4.6% and stride speed: 81 ± 5.5%) than the other groups of animals (Fig. 4B–F).

3.3. H-reflex responses

As illustrated in Fig. 5A, EMG responses evoked by single electrical stimulus pulses (1 x T = 0.43 ± 0.19 V) applied at increasing intensities (from 1 to 3 × T) to the plantar nerve and recorded on plantar muscles are usually composed of two components. The first one of short latency (2–4 ms) is called the M wave (Fig. 5A), which corresponds to the activation of muscle fibers by the direct stimu-

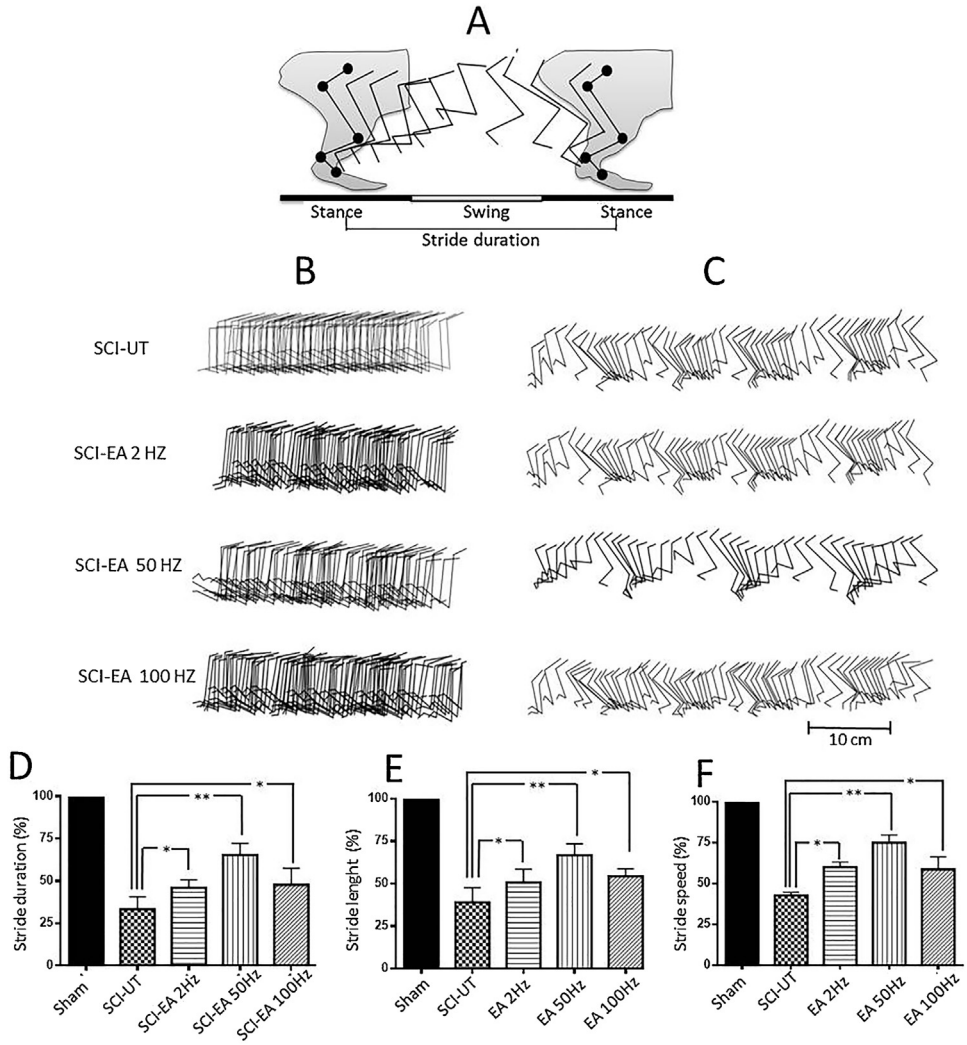

FIGURE4

Fig. 4. Kinematic analysis of hindlimb gait locomotion in sham-operated and experimental animals with SCI. (A) Articulation joints are illustrated (circle label), from which spatio-temporal sequences of hind limb movements during gait were constructed. The stance and swing phases from each step cycle are also shown. (B) Spatio-temporal sequences of hind limb movements during gait at the third day after SCI and (C) 4 weeks after SCI. (D) Graphic representation of the averaged stride duration, (E) stride length, and (F) stride velocity during the unrestrained gait locomotion from untreated injured spinal cord (SCI-UT, n = 10) rats and rats treated with electroacupuncture (EA) at frequencies of 2 Hz (n = 8), 50 Hz (n = 9) and 100 Hz (n = 8) at 4 weeks post-injury weeks. *p < 0.05, ** p < 0.01.

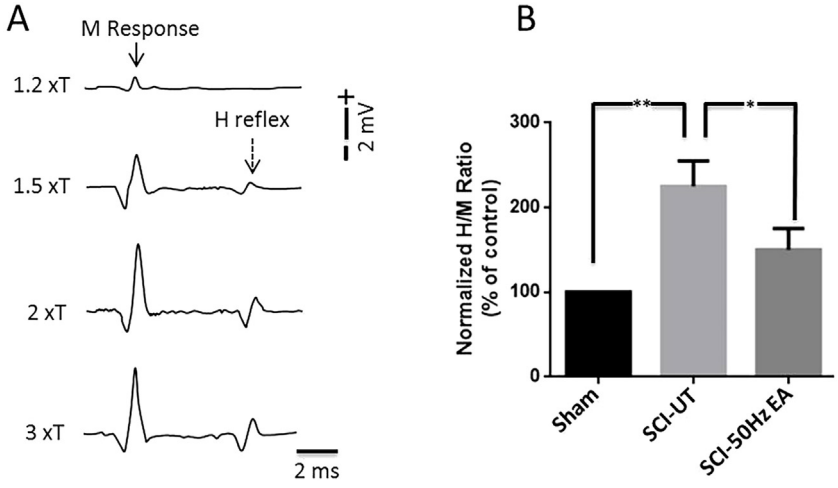

FIGURE5

Fig. 5. Electromyographic responses elicited by a stimulus applied to the lateral plantar nerve and recorded in the plantar muscles. (A) M response and H reflex recording induced by gradual stimulus at 1.2–3 threshold (xT). (B) Normalized H/M ratio (percent of the sham groups, n = 10) 4 weeks after the SCI with no EA treatment (SCI-UT, n = 10) or treated with 50 Hz of EA stimulation (n = 9). *p < 0.05.

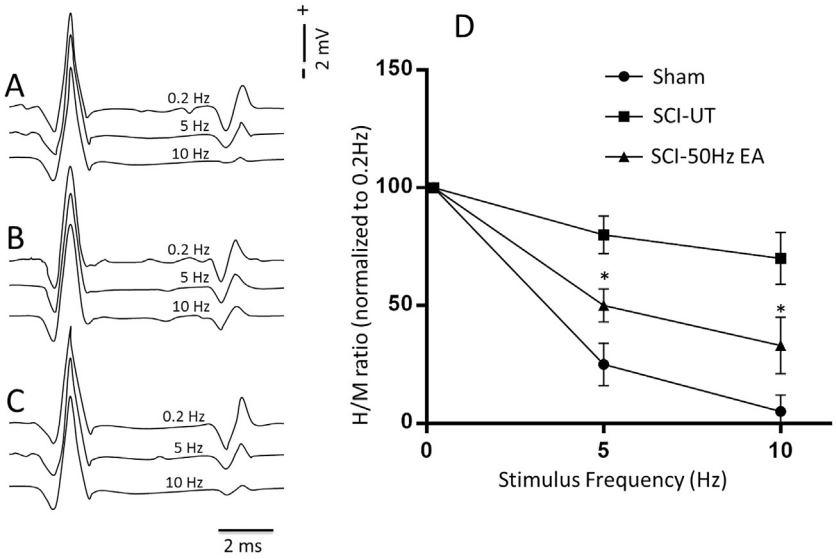

FIGURE6

Fig. 6. Rate-dependent depression of the H-reflex. (A) Examples of recordings of the depression of the H-reflex at 0.2, 5, and 10 Hz of lateral plantar nerve stimulation in sham, (B) untreated spinal cord injury, or (C) treated with 50 Hz of EA stimulation (n = 9). (D) Graphic representation of the average H/M ratio depression in sham rats (filled circles, n = 10), after 4 weeks of spinal cord injury without EA treatment (filled square SCI-UT, n = 10) or with EA treatment at 50 Hz (triangles SCI–50 Hz EA). The amplitude is expressed as a percent of the amplitude at 0.2 Hz designated 100 percent; * indicates $p < 0.05$.

lation of efferent motor axons. The second component, called the H-reflex, had a longer latency (8–12 ms) and results from the activation of muscle fibers by reflex excitation of spinal motor neurons (Mazzocchio et al., 2001; Fig. 5A). After five weeks post-injury, SCI-UT rats showed a significant facilitation of the Hmax/Mmax amplitude ratio ($p < 0.05$), whereas SCI-EA 50 Hz rats exhibited a smaller facilitation with regard to that of sham rats (217% and 154%, respectively. Fig. 5B).

3.4. Frequency-dependent depression of the H-reflex

Fig. 6 illustrates a series of H-reflex responses evoked by increased frequencies of stimuli at 0.2, 5, and 10 Hz applied to the plantar nerve of sham (Fig. 6A), SCI-UT (Fig. 6B) and SCI-EA 50 Hz treated rats (Fig. 6C). At five post-operation weeks, sham operated animals showed a considerable depression of the H-reflex amplitude (Fig. 6A), concurrent with a reduced Hmax/Mmax amplitude ratio (Fig. 6D), as the frequency of plantar nerve stimulation was increased (0.2 Hz: 100%, 5 Hz: 24 ± 4.6%, 10 Hz: 2 ± 1.8%). In contrast, SCI-UT rats exhibited a rather small depression in the H-reflex amplitude and relatively small percentage values of the Hmax/Mmax amplitude ratio (50 Hz: 28 ± 4.6%, 45 ± 3.2%, respectively. Fig. 6B–D), whereas SCI rats treated with EA at 50 Hz showed an enhanced H-reflex amplitude (Fig. 6C–D) and increased frequency-dependent H-reflex depression (5 Hz: 43 ± 2.5% and 10 Hz: 64 ± 4.4%. Fig. 6D).

3.5. Inhibition of Gs evoked ventral root potentials by conditioning stimulus applied to the PBSt nerve

It is well known that activation of GABAergic interneurons making axo-axonic contacts with intraspinal ramifications of sensory fibers produced presynaptic inhibition on terminals of such afferents (Rudomin and Schmidt, 1999). Several authors suggest that decreased presynaptic inhibition is linked to a decreased frequency-depression of the H-reflex (Chen et al., 2001; Pierrot, 1990; Thompson et al., 1992). As a test for presynaptic inhibition, in this study we examined the effect of PBSt conditioning stimulation on the amplitude of the ventral root potentials provoked by Gs nerve stimulation (Enríquez-Denton et al., 2004) in animals under spinal cord injury and treated with EA. Fig. 7 shows the recording and graphical representation of the average ventral root potentials produced by stimulation of the Gs and PBSt nerves. In sham animals, conditioning stimulation of the PBSt nerve (4.5 × T) produced a maximal depression of the GS evoked VRP (43.4 ± 8.6%; n = 6) between 20 and 40 ms interval and such depression lasted up to 200 ms (Fig. 7B, E). Such effect was minor in animals with SCI (78.6 ± 11.2%; n = 5) without significant differences in duration (Fig. 7C, E). The depression of the VRP was partially recovered after four weeks of 50 Hz-EA treatment (63.2 ± 8.1%; n = 9) and lasted 200 ms (Fig. 7D, E).

3.6. Histology

The extent of the SCI provoked by compression of the spinal 10th thoracic segment was similar in all untreated and EA treated rats (Fig. 8A–D), and it includes regions of the dorsal columns (fasciculus gracilis and fasciculus cuneatus), the dorsal corticospinal tract and the left and right dorsal horn gray matter (Fig. 8A–D). In some cases, it extended to the rubrospinal tract and partially to spino-cerebellar tracts in both sides of the thoracic spinal cord (Fig. 8A–D).

4. Discussion

To evaluate the possible use of EA as an experimental procedure for the treatment of spinal cord injuries, we analyzed the effect of electroacupuncture on the locomotor, H reflex and Gs-evoked VRP alterations produced by a compressive spinal lesion at the 10th spinal thoracic segment of rats. SCI in experimental animals was evoked by an insufflated Fogarty catheter, which has been considered suitable to produce a highly reproducible and reliable compression injury in the spinal cord of the rat (Metz et al., 2000; Lonjon et al., 2010). Such reliability was confirmed in the present study because of the similarity in the extent of the lesions evoked in most of the animals.

It is well known that partial spinal cord injuries provoke severe alterations in the locomotor performance of humans and animals (Hiersemenzel et al., 2000). The mechanisms underlying such recovery are not fully understood but include recuperation from the spinal shock (Holaday and Faden, 1983), remyelination and axonal

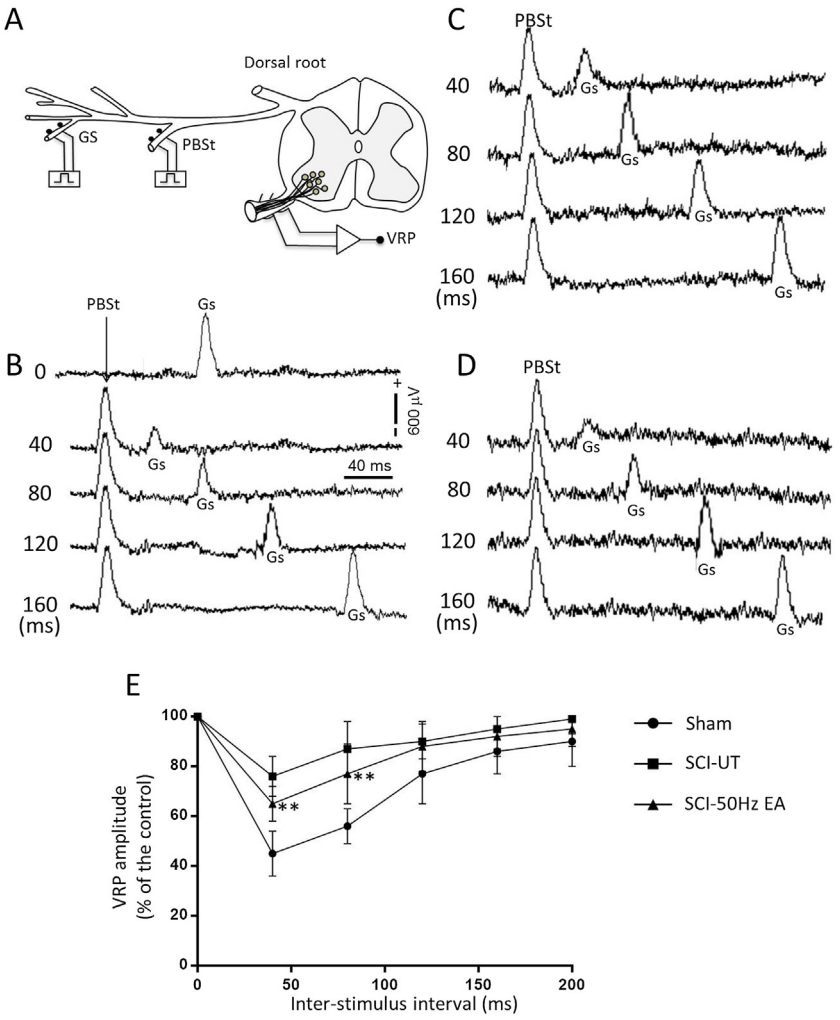

FIGURE7

Fig. 7. Amplitude depression of ventral root potentials (VRP) evoked by stimulus pulses applied to the gastrocnemius nerve (GS, 4.5 × T) by conditioning stimulation of the posterior biceps and semitendinosus nerve (PBSt, 4.5 × T). (A) Diagram of the experimental arrangement; (B) PBSt and Gs VRPs recordings evoked at different conditioning-test time intervals (20–180 ms) in sham animals; (C) Same recordings in rats with five weeks post-SCI without treatment; (D) VRP recordings obtained in rats with five weeks post-SCI with 50 Hz of EA treatment. (E) Graph showing the average percent depression in amplitude of conditioned Gs-VRP, which was measured at different time intervals in sham rats (filled circles, n = 10), SCI rats without EA treatment (filled square SCI-UT, n = 10) or with EA treatment at 50 Hz (filled triangles SCI–50 Hz EA n = 9). The amplitude of the unconditioned VRP responses was considered as 100% (0 ms, upper trace in B). ** indicates $p < 0.01$.

sprouting (Schwab and Batholdi, 1996). Several experimental and clinical therapies or combinations of them have been developed with the objective to alleviate the multiple complications associated with SCI, but most of the therapies are not completely successful (Varma et al., 2013). In this study, we found that EA stimulation at a frequency of 50 Hz that was applied simultaneously at *Huantiao* (GB30), Yinmen (BL37), Jizhong (GV6) and Zhiyang (GV9) acupoints during 5 weeks post-injury provided significant improvement in locomotor activity (according to the BBB test and kinematic analysis of gait locomotion) and H-reflex responses when compared with 2 Hz or 100 Hz EA stimulation applied to SCI rats.

The BBB test is a very useful tool for analyzing the free locomotive behavior of animals with spinal cord injury. One advantage of this test is that it does not need preoperative training for its application in animals (Metz et al., 2000). The kinematic gait analysis without restrictions is recommended to analyze the temporal and spatial sequence of hind limb movements that occur during individual strides of animals in terms of displacement, time, speed and acceleration (Metz et al., 2000). In our study, we found that SCI provokes alterations in length, duration and speed of strides executed during the unrestrained gait locomotion of sham and experimental animals and that such kinematic changes are substantially reverted by EA stimulation at 50 Hz. Taken together, the data obtained from the BBB test and unrestrained gait kinematics suggest that EA stimulation at 50 Hz provokes a substantial improvement in the locomotor activity of SCI animals. However, the probable cellular or physiological mechanisms responsible for such motor recovery associated with EA stimulation remains to be explored.

On the other hand, several studies have shown that after a spinal cord lesion occurs, there is a substantial facilitation of the spinal monosynaptic reflex and H reflex (Lee et al., 2005; Thompson et al., 1992). The H reflex results from the activation of a di-synaptic pathway composed of afferent fibers, motoneurons, and muscle fibers (Sanes and Lichtman, 1999; Chen et al., 1998; Reyes et al., 2007). The Hmax/Mmax amplitude ratio estimates the fraction of motoneurons recruited via the H-reflex with respect to the activation of the entire motor pool (inferred from the maximal M wave response). The ratio is facilitated after an SCI (Côté et al., 2014) and provokes the occurrence of spasticity in animals (Charlotte et al., 2007). In our study, it was found that the H reflex amplitude and the Hmax/Mmax ratio are substantially increased in SCI rats, and the reflex facilitation was significantly reduced by EA stimulation

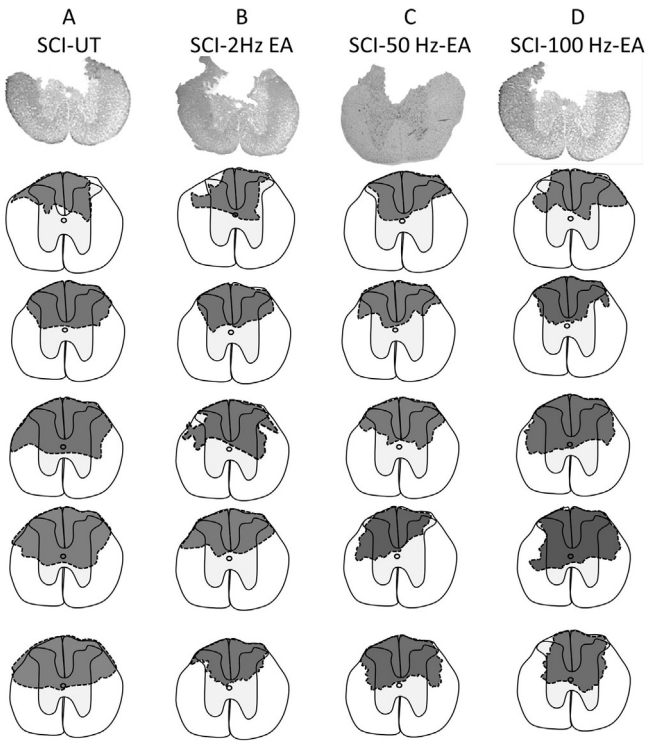

FIGURE8

Fig. 8. Spinal lesioned areas produced by the experimental insufflation of a Fogarty catheter at the T8-T9 spinal cord segments. Upper row: spinal cord slices showing the traumatic compression injury area. Lower rows: Camera lucida diagrams illustrating the injuried areas in spinal cord sections of (A) rats without treatment (SCI-UT); (B) rats with 4 weeks of 2 Hz-EA treatment (SCI–2 Hz EA); (C) with 50 Hz-EA treatment (SCI–50 Hz EA) and (D) with 100 Hz EA treatment (SCI–100 Hz EA). Damaged spinal regions are indicated by shadow areas and dotted lines.

at 50 Hz after 4 weeks post-injury. Our results suggest that EA stimulation decreases the facilitation of the H reflex and that this effect reduced spasticity and improved the motor performance of SCI rats.

Frequency-dependent depression is described as the gradual decrease of H reflex amplitude when a series of repetitive reflexes occurs (between 1–10 Hz of frequency). It has been shown that measures of H-reflex frequency-sensitive depression is of particular importance in the assessment of hyper-reflexia following SCI (Reese et al., 2006; Thompson et al., 1992; Chen et al., 2001). Several studies have shown that a significant decrease in frequency-dependent depression of the H-reflex is observed after 14 and 30 days post-injury in a complete spinal cord transection rats (Charlotte et al., 2007) and after 28 and 60 days in spinal cord contused rats (Thompson et al., 1992). Our data is also indicative that the frequency-dependent depression of the H-reflex in sham, SCI and EA treated animals occurred between 10 and 28 days post-injury. These observations could suggest that the transition to hyperreflexia occurs between 6 and 28 days post-injury (see below).

In both humans and animals, motor alterations caused by SCI include hyperreflexia caudal to the level of the lesion (Lin et al., 2007; Milanov, 1994). It has also been proposed that several processes are associated with hyperreflexia, such as the following: (1) enhanced stretch reflex excitability (Nakazawa et al., 2006); (2) changes in the intrinsic properties of alpha motoneurons (Hultborn, 2003); (3) reduced post-activation depression of synaptic transmission from fibers (Li et al., 2004); (4) alterations in the morphology of alpha motoneurons (Kitzman, 2005); (5) changes at the neuronal gap junction protein level (Yates et al., 2008); and (6) decreased presynaptic inhibition of terminals after SCI (Nielsen et al., 1995).

Several authors suggest that decreased presynaptic inhibition is linked with the decreased frequency-depression of the H-reflex (Chen et al., 2001; Pierrot, 1990; Thompson et al., 1992). In this way, the loss of descending inputs due to SCI could lead to a reorganization of spinal circuitries, leading to hyperreflexia. In a previous study, we proposed that low frequency EA stimulation depresses non-painful sensory pathways through activation of specific inhibitory pathways receiving modulatory actions from other sensory and muscle afferent inputs in the rat spinal cord (Quiroz et al., 2014a,b). The duration and magnitude of the observed depressive effect was quite similar to the time course of primary afferent depolarization and presynaptic inhibition. We also found that Picrotoxin, an antagonist of $GABA_A$ receptors, reduced the depressive actions of EA.

It is well established that the VRP produced by Gs nerve stimulation is depressed by the conditioning stimulation of PBSt nerve, and such effect is mainly attributed to primary afferent depolarization (PAD) and presynaptic inhibition (Enríquez-Denton et al., 2004; Rudomin and Schmidt 1999). Such inhibition exert its highest effect at 10–30 ms and last 200 ms (Rudomin and Schmidt, 1999). We found in this study that 50 Hz-EA treatment induced a recovery of the presynaptic depression of the Gs-VRP evoked by PBSt conditioning stimulation in the SCI rat. In concordance with the latter, it could be suggested that 50 Hz-EA stimulation reduced the hyper-excitability of motoneurons and provokes a partial improvement of the locomotive performance and H reflex responses by a possible recovery of presynaptic mechanisms in the spinal cord of experimentally injured rats. However, other mechanisms could not be excluded. Charlotte et al. (2008) indicates that hyperreflexia, a component of spasticity, does not arise immediately after SCI but emerges over time. These authors proposed that the delay in the generation of hyperreflexia is a consequence of the onset of other mechanisms. They found a relationship between changes in the neuronal gap junction protein levels (Cx-36) and the delayed onset of hyperreflexia (Yates et al., 2008) and proposed that increasing the electrical coupling between motoneurons helps to prevent hyperreflexia. In this regard, it has

been reported that EA stimulation modified the protein expression of connexin 43 and improved the synaptic reorganization of the marginal zone of focal cerebral ischemia in rats (Luo et al., 2011). In addition, Lee et al. (2007) observed an increase in the H-reflex amplitude after 4 weeks post-injury which was positively correlated with an increased expression of 5-HT_2R in the contused spinal cords of rats, and EA stimulation was able to modify the serotoninergic system in the spinal cord (Zhang et al., 2014). In this way, it could be proposed that EA stimulation induces its effects on motoneuron hyper-excitability by modulating multiple pre- and postsynaptic mechanisms at the spinal cord levels.

The significant effect of 50 Hz of EA on the motor function recovery of SCI animals could offer several clinical paradigms for neurological recovery of SCI human patients. However, it is important to clarify that we cannot assume that acupoints, electrical parameters of EA such as intensity, amplitude of the stimulation pulse (the resistance for current electricity is different in the human than in the rat), and that the model of SCI compression in rats are totally equivalent to those corresponding to humans. In this way our results needs to be corroborated in further studies in humans, alone or in combination with other pharmacological or physical therapeutic procedures.

In addition, there are several limitations in our study that needs to be considered. One limitation is the lack of a group that received EA on non acupoints. It could be possible that the effects ascribed to a simple peripheral electrical stimulation or specific acupuncture stimulation on the acupoints located on the back and hindlimb, are related to a combination of acupoint and non-acupoint actions. However, in a previous study we have showed that EA on non-acupoint sites does not evoke significant changes in the SU nerve evoked cord dorsum potentials, suggesting a specific acupoint effect of EA (Quiroz et al., 2014a,b). In such study we proposed that EA reduces the activation of dorsal horn neurons provoked by low-threshold cutaneous afferent fibers by the activation of specific sensory pathways in the spinal dorsal horn of the rat (Quiroz et al., 2014a,b). In this study we only analyzed the effect of different frequencies of EA at the same model of SCI and with acupoints which previously has been reported to have neuroprotective effect on SCI (Heo et al., 2013a,b; Min et al., 2015; Ding et al., 2011). However, further studies are needed to analyze the acupoint specificity of 50 Hz of EA stimulation for spinal cord injury.

A second limitation is that our study do not contemplate the effect of muscle activation by EA stimulation on the improvement of locomotive performance of SCI rats. It could be proposed that EA stimulation provokes the electrical activation of muscles near to acupoints and such action induce an improvement in the locomotive performance of SCI-rats. Previous research support that EA stimulation has the capacity to produce movement in denervated, paralyzed, or spastic muscles, but it is inherently less efficient than human movement (Doucet et al., 2012). In this way our study did not contemplate the analysis of muscle properties in SCI rats to exclude the possible involvement of muscle activation in the improved motor performance of SCI rats with EA treatment.

Another issue that has not been contemplated at our study was the evaluation of a combined treatment of EA with physical therapy or pharmacological interventions that could maximize the therapeutic effects of EA on SCI. Although biphasic pulse is the most common mode of EA stimulation on basic and clinic practice other parameters as pulse width, electrode placement, variable frequency pulse, amplitude patterns and program duration needs to be characterized. The proper characterization of an adequate treatment or combinations of treatments, in turn, will define how quickly these advances can become commonplace in the clinic

5. Conclusions

The results obtained in this study demonstrated that EA stimulation applied at 50 Hz improves the motor performance and H-reflex amplitude by a possible recovery of presynaptic mechanisms in the spinal cord of experimentally injured rats. However, other mechanisms could not be excluded. The significant effect of 50 Hz of EA on the motor function recovery of SCI animals could offer several clinical paradigms for neurological recovery of SCI human patients.

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