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ORIGINAL RESEARCH

Spinal cord hemisection delays the rodent estrous cycle

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The reproductive hormones estrogen and progesterone are suspected to have neuroprotective effects in recovery from spinal cord injury (SCI). As a step toward understanding how SCI effects the regular cycling of these reproductive hormones, which impacts their potential therapeutic use after SCI, this study investigated the effects of lateral spinal cord hemisection on the rodent estrous cycle. Vaginal smears were obtained from a cohort of 8 female rats before and after hemisection injury. Smears were analyzed under a microscope in order to classify the stage of estrous cycle (diestrus, proestrus, estrous, or metestrus) based on the presence of leukocytes, epithelial cells, cornified cells or a mix of leukocytes, epithelial and cornified cells, respectively. The combined cycle data were used to determine whether the estrous cycle was normal or aberrant for 24 days after injury, with each cycle parameter defined by a set number of days. Although the time point differed between animals, spinal cord hemisection delayed the estrous cycle in all animals by a mean of 12.4 days. Concurrent with these experiments, rats were trained in various stepping or stimulation paradigms after hemisection to facilitate locomotor recovery and to assess the effectiveness of quadrapedal versus bipedal training in recovery from SCI. Though our data demonstrate no correlation between the type of post-injury training paradigm and delay of estrous cycle, this work provides foundational information regarding the effect of hemisection on the timing of the rodent estrous cycle. These data are pertinent to future studies investigating the role of reproductive hormones in recovery from spinal cord injuries as well as the relationship between estrous cycle delay and post-injury locomotive recovery.

Introduction

Reproductive hormones, such as estrogen and progesterone, are purported to critically influence recovery from spinal cord injury (SCI), with effects that may be conserved from animal models to humans[1-2]. However, the specific roles these reproductive hormones play in SCI recovery are still unclear. Some studies have demonstrated a neuroprotective effect for estradiol, a form of the hormone estrogen, in rodent SCI, while others have found no influence of the reproductive hormone[3-4]. Recent studies have focused on the estrous cycles of rodents with various types of SCI, such as spinal cord transection and contusion, in order to better elucidate the effects of hormonal variation on the recovery process after SCI[2-5]. Further, these studies have sought to address the potential impact of experimental interventions on the reproductive cycles of rodents. Several groups have demonstrated a delay in the rodent estrous cycle after SCI, and that this cycle delay correlates positively with damage to the spinal cord white matter[2-5]. However, there have been no published reports regarding the effect of lateral hemisection, an incomplete SCI model, on the estrous cycle. Given previous studies concerning estrous cycle delay in other models of SCI, we propose that the rodent estrous cycle will be disrupted or delayed following a lateral hemisection injury that involves transecting only half of the spinal cord. If hormones associated with the reproductive cycle are essential modulators of SCI recovery, an altered or disturbed estrous cycle may provide insufficient levels for maximal recovery[1]. Therefore, the primary purpose of this study is to investigate the effect of spinal cord hemisection on the rodent estrous cycle. While estrous cycle disturbance has a known negative impact on reproductive function[1], an aberrant cycle may also hinder recovery from SCI.

The rodent estrous cycle has been shown to have four fixed stages - diestrus, proestrus, estrous, and metestrus[6]. These stages are defined by the relative proportions of three cell types observed in the vaginal smear. A predominance of leukocytes characterize diestrus, a predominance of epithelial cells characterizes proestrus, a predominance of cornified cells characterizes estrous, and a mix of leukocytes, epithelial and cornified cells chracterizes metestrus[7]. Importantly, the precursors of these cells characterize the presence of a specific estrous cycle phase and precursor proliferation varies in response to changes in endocrine hormonal levels (Fig. 1).

Further, Goldman and colleagues have put forward that 3-4 days (d) of estrous or 4-5d of diestrus indicate an extended cycle, whereas an estrous cycle demonstrating greater than 4d of estrous or greater than 6d of diestrus is irregular. This study employs these established guidelines in examining the presence or absence of the regular estrous

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cycle after hemisection injury in the rat, following animals for a time period of 24d after injury. Animals with spinal cord hemisection were also trained with different step training or stimulation paradigms to facilitate recovery in walking ability after hemisection injury, with subsequent examination of the effect of these training paradigms on the rodent estrous cycle.

Overall, the results of this work demonstrate that the rodent estrous cycle is temporarily disrupted following spinal cord hemisection injury. Rodent estrous cycles were delayed for a mean of 12.4d and returned to normal by 19d after injury. Moreover, we did not observe any correlation between the type of step or stimulation training paradigm used to facilitate motor recovery and the transition from estrous cycle delay to normal cycle after hemisection.

Methods

Animals. Female Sprague-Dawley rats (n = 8, mean weight 250 grams, mean age 16 weeks of age) were used in this study. Animals were housed in separate cages in an environmentally controlled facility, maintained on a 12 hour on/off light cycle, and allowed ad libitum access to food and water. Experiments were conducted according to research protocols approved by the Chancellor's Animal Research Committee of the Office for Protection of Research Subjects at the University of California Los Angeles.

Spinal Cord Hemisection. Animals were acclimated to human contact and estrous cycle control data were collected for 4-5d prior to surgery. Thereafter, animals were subjected to hemisection of the spinal cord at the level of the 10th thoracic vertebra (T10) under aseptic conditions. Briefly, an incision was made on the back of the anesthetized animal and the right half of the spinal cord at the T10 level was transversely sectioned with fine scissors and forceps to produce the hemisection injury. The surgical site was cleaned and the wound was closed. This procedure routinely produced paralysis of the animal's hind limbs. Animals were allowed to recover, and were treated with antibiotic (Baytril), analgesic (Buprenex), and Ringer's lactate solution twice per day for 2-3d following surgery. It is important to note that this study is limited by the absence of sham controls, in which animals undergo all aspects of surgery, except for spinal cord hemisection, to control for the effects of surgery alone on the rodent estrous cycle. However, the results presented herein were obtained from a pilot study that laid the foundation for a more comprehensive and ongoing study to examine the effects of different spinal cord injury models on the rodent estrous cycle. This limitation will be addressed by the inclusion of sham controls in this comprehensive dataset.

Vaginal Smear. To collect estrous cycle data, each animal was handled daily for one week prior to obtaining vaginal smears. These periods of introductory handling served to acclimate rats to human touch and minimize the effects of stress on estrous cycle. Each animal was also handled for one minute before each smear was obtained. The normal estrous cycle of each animal was determined by following the daily cycle for 7d prior to spinal cord hemisection. After hemisection, vaginal smears were obtained daily for a period of 24d. Vaginal smears were obtained from each rat at the same time every day, and placed on slides for analysis under a microscope. Since the estrous cycle is phasic, it is important to take vaginal smears at the same time each day in a sterile environment. In order

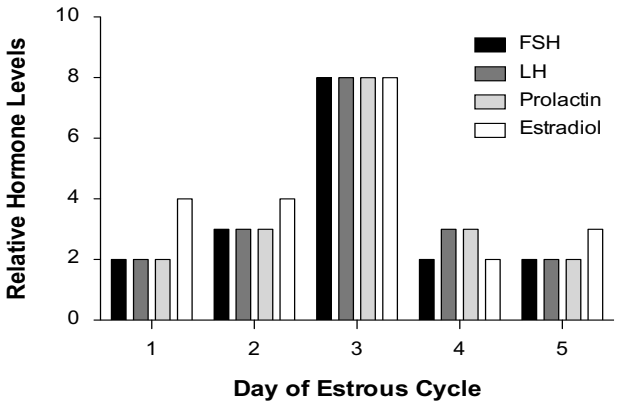

Figure 1 | Hormone levels in rodent estrous. Fluctuations in the relative hormone levels as the normal estrous cycle progresses through the diestrus (days 1, 2), proestrus (day 3), and estrus (days 4, 5) phases. In each case, the respective hormone peaks during the proestrus phase (day 3).

to obtain the smears, glass pipettes were filled with 8-10 µl of warm saline and inserted into the vagina of the rat. Rats were restrained7 and pipettes were inserted no more than 5 mm into the vagina, as deeper insertion may cause injury to the animal or result in enough cervix stimulation to initiate a pseudopregnancy[8]. Unstained slides were examined under a light microscope at magnifications of 4x (to distinguish between leukocytes and epithelial/cuboidal cells) to 20x (to distinguish between epithelial and cuboidal cells). Each day, images were taken of the vaginal smear slides prepared for each animal. Two independent reviewers used these captured images to verify the estrous phase of each rat. In order to decrease risk of infection and contamination, great care was taken to use a sterile method to obtain the lavage from each animal daily.

Step Training. Following surgery all animals were trained for 20m daily with different stepping or stimulation paradigms for 21d. Animals 2 and 6 were trained to walk on their hindlimbs only on a moving treadmill (bipedal stepping paradigm). Animals 4 and 8 were trained to walk on all fours (quadrupedal stepping paradigm). Animal 1 underwent an epidural stimulation procedure, in which the animal's limbs did not contact the treadmill but the animal's spinal cord was stimulated epidurally at the S1 level. Animals 3 and 7 were not trained with any stepping or epidural stimulation paradigm and served as spinal cord hemisection controls. Animal 5 was unable to recover from surgery and died due to trauma-related bleeding and infection.

Results

Estrous Cycle Determination

The phase of the estrous cycle is determined by the dominant cell type present in the vaginal smear. The presence of tiny, round leukocytes characterizes the diestrus phase; round, nucleated epithelial cells characterize the proestrus phase, and cube shaped, anucleated cuboidal cells or needlelike keratinized cells indicate the estrous phase (Fig. 2a-c). Moreover, leukocytes can be present along with epithelial cells, in which case the smear may indicate a transition from diestrus to proestrus. A smear of leukocytes and cuboidal cells may indicate a transition from estrous to diestrus. Similarly, a smear that exhibits a mix of epithelial and cuboidal cells may indicate a

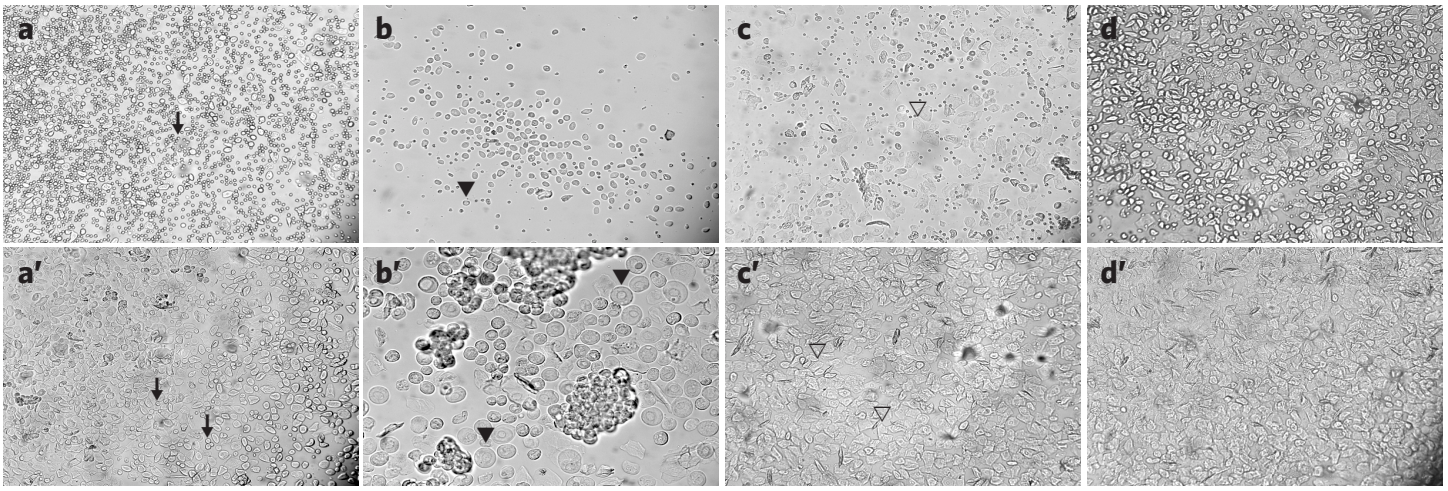

Figure 2 | Characteristic cell types distinguish rodent estrous phases. Representative vaginal smears demonstrate predominant cell types in diestrus (a, a′), proestrus (b, b′), estrus (c, c′), and metestrus (d, d′) phases of the rodent estrous cycle. Arrows indicate small, circular leukocytes that characterize diestrus (a, higher magnification in a′). Filled arrowheads indicate nucleated epithelial cells that characterize proestrus (b, higher magnification in b′). Open arrowheads indicate anucleated cuboidal cells that characterize estrous (c, higher magnification in c′). The metestrus phase (d, higher magnification in d′) is characterized by the presence of leukocytes, nucleated epithelial, and anucleated cuboidal cells in equal proportions.

transition from proestrus to estrous. A smear that exhibits all three cell types in roughly same proportions may be indicative of a fourth phase called the metestrus phase, which is typically seen in between the estrous and the diestrus cycles (Fig. 2d). In this study both the prolonged estrous and diestrus phases (>2d and >3d, respectively) and the combination of mixed cells that do not coincide with occurrence of the metestrus phase, were used to assess a disrupted estrous cycle.

Effects of Spinal Cord Hemisection

Lateral spinal cord hemisection disrupted the estrous cycle of all seven animals in this study. The delayed cycle was determined by comparison with the normal estrous cycle of each animal, which was established during the week prior to injury. Animals 1, 2, 6, and 7 showed a 5-day normal cycle before injury. On the other hand, animals 4 and 8 had 4-day cycles, and animal 3 had an abnormal 7-day diestrus cycle. After hemisection, all seven animals experienced a delay in their normal cycle at some point, after which all animals returned to a normal estrous cycle.

The total time spent in a delayed and/or normal estrous cycle by all animals as a group is represented in Figure 3. Averaged across the cohort of seven rats, the mean delay in estrous cycle was 12.6d, with the delay being most significant one week into the cycle (Fig. 3). The delay of the normal cycle did not begin immediately after surgery for 5 out of the 7 animals. For animals 1, 2, 4, 8 the cycle delay began at days 5, 5, 6, and 7, respectively (Figs. 4a-b). For animal 2, the aberrant cycle is most evident in the prolonged 5-day diestrus phase from day 14 to 19, whereas a similar aberrant cycle in animal 8 is evident in the transition from the diestrus phase to the proestrus phase from days 15 to 16 (Figs. 4a-b). The cycles of animals 6 and 7, however, were delayed the first day after surgery. Animal 3 did not follow either of these patterns; this animal had an abnormal cycle during the week before surgery, while showing a relatively regular cycle after surgery (Fig. 4c). However, this animal experienced a delay in estrous cycle cycle at 13d after hemisection with a return to normal cycle by 19d after injury. There are several environmental factors that can disrupt the rat estrous cycle, including diurnal cycles, stress due to handling, and room temperature. However, these factors were controlled for all animals in this study. Consequently, the original aberrant cycle in animal 3 may have been a result of inherent stress. During the study, the sutures of animal 3 were sprayed several times with antibiotics for fear of chewing, as the animal was known to be stressed. The subsequent return to a normal cycle after hemisection may be attributed to a decrease in stress in this particular animal. The delay in the estrous cycle of animal 3 is most obvious from 14-16d after hemisection, in which the vaginal smear demonstrates mixed cells (Fig. 4c). Although not all of the animals demonstrated a delay in the cycle at the same time, all animals in this study ultimately exhibited some type of delay as a result of lateral spinal cord hemisection.

All seven animals returned to their normal estrous cycle between 16-19d after hemisection (Figs. 3-4). This includes animals 6 and 7, whose normal cycle was disrupted earlier than the other animals in the cohort. Consequently, these two animals had much longer delay periods. Animal 3 returned to a normal 5-day cycle

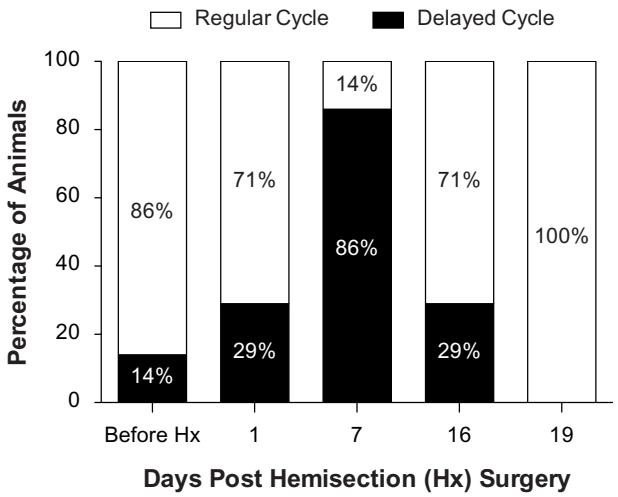

Figure 3 | Percentage of days spent in regular vs. delayed estrous cycle for rats (n=7) before and after lateral spinal cord hemisection surgery.

around 19d after injury and consequently had a much shorter delay period in comparison to the mean delay time. Moreover, all seven animals exhibited 5-day normal cycles when their delayed cycle transitioned into a normal cycle.

Our data found no correlation between type of step training or stimulation paradigm and length of estrous cycle delay.

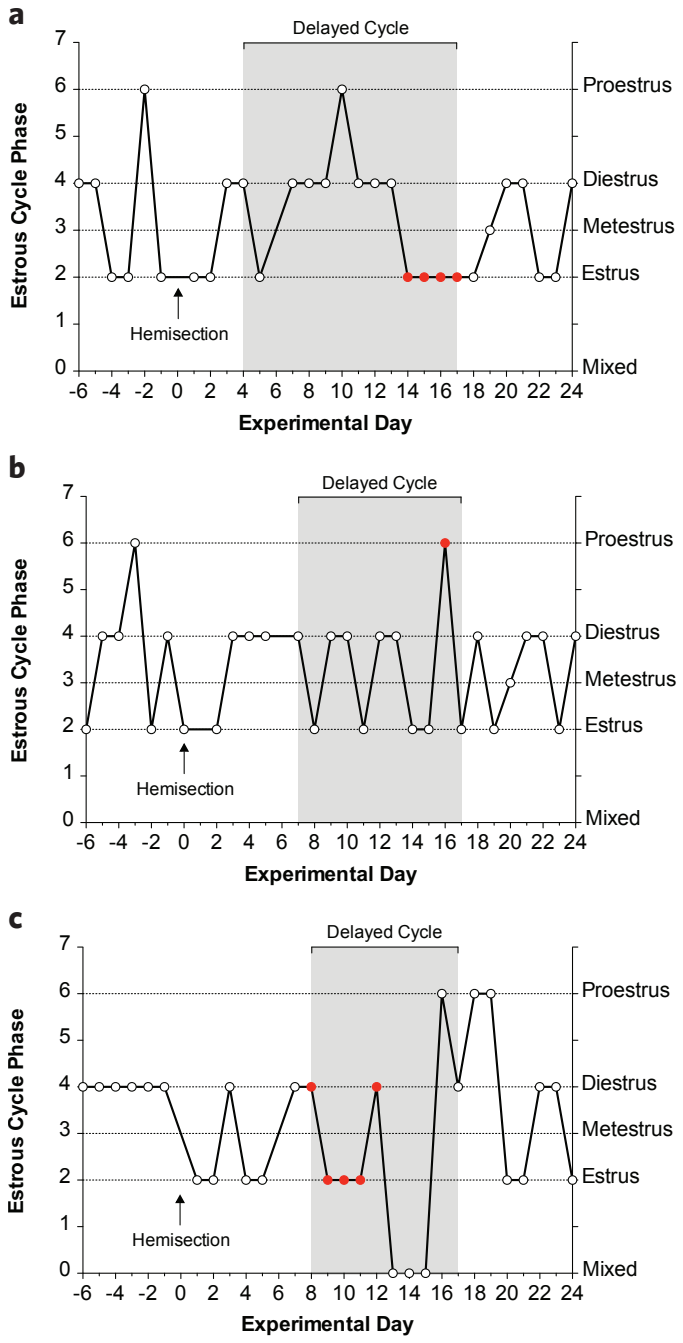

Figure 4 | Daily records of estrous cycles for 3 representative animals. a-c, Estrous cycles were recorded over a period of 30 days, from 6 days prior (d-6) to 24 days after (d24) spinal cord hemisection. Each animal's normal cycle is delayed after surgery for the variable period highlighted in grey. a, Animal 2 demonstrated the longest estrous cycle delay, highlighted by a five-day estrus phase (red data points). b, The aberrant cycle of animal 8 was characterized by an abnormal transition from the proestrus to estrus phases (red data point). c, Animal 3 demonstrated a shorter period of delayed estrous cycle, highlighted by three consecutive days of mixed cells following the proestrus phase (red data points).

Although both step training and epidural stimulation did improve limb mobility for all animals, this improvement did not correlate with changes in timing of the estrous cycle (data not shown).

Discussion

The present work demonstrates that the estrous cycle of adult rats after lateral spinal cord hemisection is delayed by a mean of 12.4d. Although the beginning of the delay differed somewhat among the animals in this study, all animals resumed a normal 5-day cycle between 16-19d after injury. The normal cycle of the animals was established one week prior to surgery. According to Goldman and colleagues, the normal rat estrous cycle is established as either a 4-day cycle or 5-day cycle[6]. In the 4-day cycle, the estrous phase lasts one day (25% of the total cycle time) but in the 5-day cycle the estrous phase lasts 2 days (40% of the total cycle). The diestrus phase lasts either 2 or 3d, respectively, while the proestrus phase lasts about 1d. Generally, the regular cycle of the animals in this study coincided with these established guidelines. Prior to injury, six out of seven animals exhibited a normal estrous cycle length, such that animals 1, 2, 6 and 7 had 5-day normal cycles. Animals 4 and 8 had 4-day cycles, whereas animal 3 had an abnormal 7-day cycle characterized by a prolonged diestrus phase (Fig. 4). Thereafter, however, animal 3 exhibited an estrous cycle that was perhaps the most normal of the cohort.

Our data found no correlation between length of delay of the estrous cycle and the type of step training or stimulation paradigm utilized during the recovery period after lateral spinal cord hemisection. The purpose of employing step training or epidural stimulation paradigms was to facilitate improvement in hindlimb function after hemisection; although training improved animal mobility, it did not correlate with changes in the individual estrous cycle. Animals were trained in two distinct step training paradigms in order to determine whether quadrapedal training promoted locomotive recovery to a greater extent than bipedal training. The purpose of the non-training controls (animals 3 and 7) was to determine whether step training or epidural stimulation paradigms influenced recovery of limb use and locomotion efficiency after spinal cord hemisection. Across the three different types of training (quadrapedal training, bipedal training, and epidural stimulation only) all seven animals resumed a normal estrous cycle at the same time, between 16-19d after hemisection.

Although this study produced reputable data, there were several limitations to the results presented herein. First, technical issues prevented the complete verification of the estrous cycle phases for each animal prior to surgery. Second, this study failed to collect data on both the day of and day after spinal cord injury (0-1d post-hemisection). The reason that for this is that immediately after hemisection, procurement of vaginal smears may have been detrimental to the health and survival of the animals. Finally, the determination of the rat estrous cycle is subjective to a certain extent, as the observation depends on the individual analyzing the smear. However, for the data presented in this paper, all results obtained through analysis by one individual were verified by a second trained individual in the lab to mitigate observational bias.

This works demonstrates that there is a temporary delay of the normal estrous cycle in rats following lateral spinal cord hemisection, an effect similar to the estrous cycle delay demonstrated in complete spinal cord transections and spinal cord contusions.

Previous studies have established that hormones associated with reproductive function fluctuate with different stages of the estrous cycle, and it is suspected that these hormones have a neuroprotective role in recovery from spinal cord injury[7]. Therefore, disruption of reproductive hormone levels via estrous cycle delay after hemisection is likely to have adverse effects on recovery from spinal cord injury. Although this study was able to establish that spinal cord hemisection does result in a delay of the estrous cycle, it did not address how this delay may explicitly impact locomotor recovery in animals with spinal cord hemisection. Future studies will address whether training interventions have an impact on estrous cycle by more closely examining the relationship between locomotive recovery and estrous cycle delay.

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