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research paper

Effect of Genistein After Spinal Cord Injury in Male Rodents

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Abstract

The post-injury administration of estrogen has been shown to provide protection and promote functional recovery in a rat model of spinal cord injury (SCI) but has potentially problematic feminizing effects. The administration of estrogen post-SCI reduced neuronal cell loss, reduced inflammation, reduced apoptosis, increased white matter sparing, and improved the functional recovery due to the activation of estrogen receptors α and β. Because of structural similarities to estrogen, genistein, a phytoestrogen derived from kudzu, activates the non-feminizing estrogen receptor β, which is present throughout the adult spinal cord. It is hypothesized that the post-SCI administration of genistein will reduce secondary injury and increase tissue sparing in male rats. To test this hypothesis, a SCI was generated in adult male Sprague-Dawley rats by contusion at the cervical vertebral level 5 (C5 level). Beginning at 30 minutes post-SCI and once daily for 14 days, genistein was injected at a dose of 5mg/kg to injured rodents. Skilled and unskilled locomotion and skilled and unskilled forepaw use for all spinal cord injured male rats were evaluated once pre-SCI and then once weekly for 4 weeks. Tissue samples were also evaluated to determine the amount of inflammation, apoptosis, lesion spread, and white matter sparing. The post-SCI administration of genistein caused an increase in the ipsilateral dominant paw use and tissue sparing, which suggests an increase in functional and physical recovery. Therefore, genistein may act as a protective and therapeutic treatment in male subjects post-SCI.

Introduction

In all spinal cord injuries (SCI), a primary injury or initial lesion later results in a secondary injury or continuation of the lesion along the spinal cord over time. This secondary injury drastically decreases a person's functionality and independence. Currently, no pharmacological treatment effectively prevents the inevitable secondary or sub-acute injury. This debilitating condition currently affects 280,000 to 300,000 people in the United States alone, with an estimated 12,000 new cases each year (National Spinal Cord Injury Statistical Center, 2008). After receiving a SCI, most people experience deficits in skilled and unskilled limb function and locomotion along with permanent tissue damage to the spinal cord. A recent study showed that female rats exhibit less tissue damage and improved function than male rats after suffering from a SCI (Farooque et al., 2006; Hauben et al., 2002). This result led to the hypothesis that estrogen reduces secondary injury and increases tissue sparing in humans after a SCI. However, estrogen treatment causes feminizing and other problematic hormonal effects on male rats (Chaovich et al., 2006). Estrogen reduces neuronal cell loss, reduces inflammation, reduces apoptosis, increases white matter sparing, and improves the functional recovery after receiving a SCI through the activation of estrogen receptors α and β. Genistein activates the non-feminizing estrogen receptor β, which is present throughout the spinal cord. Administration of genistein may be considered a possible treatment in providing a protective and functional recovery in males with SCI.

Hypothesis

The hypothesis of this experiment is that post-SCI treatment with genistein will improve functional recovery, reduce neuronal cell loss, reduce injury inflammation, reduce apoptosis, and increase white matter sparing. The null hypothesis is that administration of genistein will have no positive or preventive effect on the overall recovery post-SCI male rat.

Materials and Methods

Four behavioral tasks including unskilled and skilled forepaw and locomotion tasks were performed on 12 male Sprague-Dawley rats prior to receiving a cervical vertebral level 5 (C5) SCI. Three trials were administered for each behavior task for each animal. The dominant paw was assigned to each rat based on its individual results from the unskilled forepaw or paw preference behavioral task.

The rodents received a C5 hemicontusion SCI on the dominant side. Beginning at 30 minutes post-SCI and once daily for 14 days, genestein was injected at a dose of 5mg/kg body weight. Once weekly for 4 weeks, the animals were observed performing the 4 behavioral tasks including the skilled forepaw task (vermicelli handling task), skilled locomotor function task (horizontal ladder task), unskilled locomotor function task (catwalk gait and analysis task), and unskilled forepaw test task (paw preference task).

For the paw preference task, the rodents were placed in a clear Plexiglas cylinder for 5 minutes. Ipsilateral and contralateral paw placements were defined as when one of the corresponding paws supported the body against the wall of the cylinder without contact of the other paw or when the other paw contacted the wall more than 0.5 seconds after the initial paw contact. It was also noted if both paws contacted the wall within 0.5 seconds of the initial paw placement.

For the skilled forelimb function (vermicelli task), the rats were placed in individual cages and were given 3 pieces of 7 cm long

uncooked vermicelli. The animals were given a maximum of 10 minutes to consume the 3 pieces, and each trial was recorded with a Sony Handycam DCR-DRV280, which allowed for detailed analysis of the amount of paw adjustments per piece of pasta. The unskilled locomotor function task was performed using the CatWalk® gait analysis system. As each animal crossed a glass walkway in a dark room, light from a fluorescent bulb was internally reflected within the glass walkway and scattered when the surface of the paw contacted the glass floor. The resulting paw prints were recorded with a high-speed CCD camera. This task was performed by each animal three times. All animals were required to cross the walkways without stopping or changing directions.

For the skilled locomotor function, each rat was positioned so as it crossed the ladder, the ipsilateral side was recorded with a Casio Exilim EX-F1 camcorder to determine the dominant forepaw and hindpaw placements. The ladder was 129 cm long and 16.5 cm wide with removable rungs placed 2.5, 3.2, or 5.7 cm apart. Each week the 15 rungs were repositioned to prevent the animals from learning the spacing pattern. At least 3 runs in which the animal did not change direction or stop were recorded for each animal. The paw placements were later analyzed and labeled as: correct placement, touch, slip, or miss. A correct placement was defined as weight-supported paw placement on the rung and a subsequent removal from the rung. A touch was defined as initial contact and immediate paw removal without any weight support. A miss was defined as a paw movement below the level of the rung without any contact. A slip was defined as initial contact with a rung immediately followed by wrist or ankle movement below the rung level.

The 4 behavioral tasks determined the extent of functional recovery in each rodent. After 28 days of behavioral analysis, the 12 rats were euthanized and spinal cord tissue was harvested for histological analysis. Eighty sections were made from each rodent's tissue sample using a cryostat and were randomly assigned for either cresyl violet staining or luxol fast blue staining to analyze gray or white matter sparing, respectively.

Data Collection and Analysis

For the paw preference task, the average dominant paw use for 3 trials was calculated using the formula:

$$\begin{matrix} average \\ dominant \\ paw\ use \end{matrix} = \frac{\#\ of\ dominant\ paw\ uses}{\#\ dominant\ paw\ use + \#\ of\ nondominant\ paw\ use + \text{total}\ paw\ use}$$

An increase in the percentage of dominant paw use post-SCI signifies functional recovery. A simple t-test was used to compare the control and genistein treated groups.

A pre-SCI assessment was conducted to obtain the baseline average values for skilled forelimb function (vermicelli task) in each animal. Data was recorded as the number of dominant paw adjustments per vermicelli piece averaged over the 3 trials. A paw adjustment was defined as any grasp and re-grasp motion or movement of the digits. The number of grasps were quantified and averaged for the control and genistein groups. After the catwalk gait task, the paw prints were then analyzed and labeled "right front paw," "left front paw", "right hind paw", or "left hind paw" depending on the specific paw print using the CatWalk® analysis software (version 7.1.6) and the area of each dominant paw was also recorded. The average paw area was compared between the control and genistein group over the 4 weeks using a simple t-test. Post-SCI the forepaw had a clubbing effect, and a greater increase in paw print size , signifying an increase in functional recovery.

For the skilled locomotion task, the amount of rung usage and percent correct were analyzed using the following formulas.

$$\%\ correct = \left(\frac{\#\ of\ correct\ placements}{\#\ of\ correct\ placements + \#\ of\ misses)}\right) x\ 100$$

$$\begin{matrix} rung \\ usage \end{matrix} = \frac{\#\ of\ correct\ placements + \#\ of\ touches + \#\ of\ slips + \#\ of\ misses}{\#\ of\ rungs}\ x\ 100$$

An increase in percent correct signifies improvement in functional recovery.

After the cresyl violet stain, the number of surviving neuronal cell bodies in the ventral horn was calculated. The luxol fast blue stain, which stains the myelin basic protein, allowed for a comparison in the percentage of intact white matter. The amount of surviving cells and white matter in the spinal cord tissue were compared between the control and genistein treated groups.

Statistical Analysis

A t-test with 95% confidence level was used to compare the average functionality improvement overtime between the control and genistein groups in all behavioral tasks and tissue samples. The paw preference task was used to compare the average percentage of unskilled dominant paw use while touching the side of a Plexiglas cylinder. The vermicelli task (skilled forepaw use task) was used to compare the average number of dominant paw re-grasps while each rodent consumed three pieces of vermicelli. The catwalk task (unskilled locomotion) compared the average area of the dominant paw while crossing a glass catwalk. The horizontal ladder task (skilled locomotion) compared the average percentage of correct placements on each rung while traversing a ladder. The amount of intact white matter and of surviving neurons in gray matter were compared across groups.

Results

For both dominant paw forelimb tasks, the genistein treated group showed a statistically significant difference in functional

improvement. In the paw preference task, both groups showed an initial percentage of dominant paw use at 80%. Beginning at 2 weeks post-SCI, the genistein group showed a significant increase in dominant paw use from 0% to almost 20%. For the next 2 weeks, the genistein group continued to show improvement while the control group showed no improvement in dominant paw use (shown in Figure 1).

The skilled forelimb task showed that the genistein group had a statistically significant difference in the number of paw adjustments per piece of pasta compared to the control group. Pre-SCI assessment showed that each rodent made on average 12 paw adjustments per piece, and post-SCI this number decreased to 5 and 2 for the genistein and control group, respectively. For the remaining 2 weeks, the genistein treated group continued to show an increase in number of dominant paw adjustments (shown in Figure 2).

After comparing the dominant paw area between the groups, there was no statistically significant difference, but there was a trend toward improvement for the genistein treated group (shown in Figure 3).

The t-test result for the horizontal ladder task and percentage of correct dominant paw placements showed no significant difference or trend between the genistein and control groups (shown in Figure 4).

After the comparison of gray matter and number of surviving neuronal cell bodies in the ventral horn (Figure 5), the genistein treated group showed a significant increase in surviving neurons (Figure 6). The genistein group also demonstrated statistically significant differences in percentage of white matter remaining post-SCI compared to the control group (Figure 7 and 8).

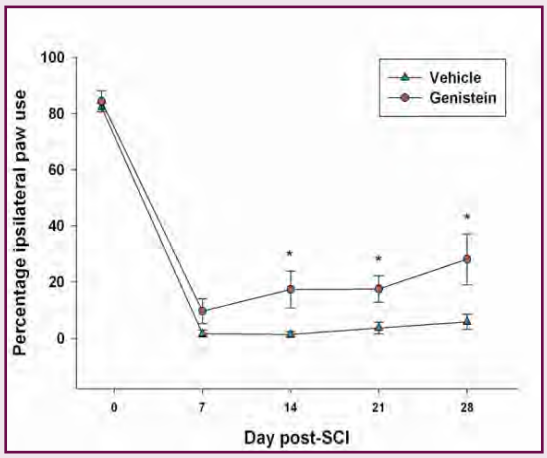


Figure 1. Quantification of Paw Preference Task. From Days 14 - 28 there was a significant difference in the percentage of ipsilateral paw use, suggesting that the administration of genistein improves unskilled forepaw use.

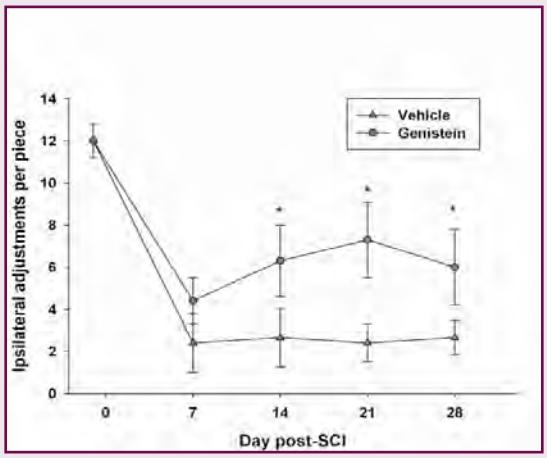


Figure 2. Quantification of Vermicelli Task. From Days 14 - 28 there was a significant difference in the percentage of ipsilateral paw use, suggesting that the administration of genistein improves skilled forepaw use.

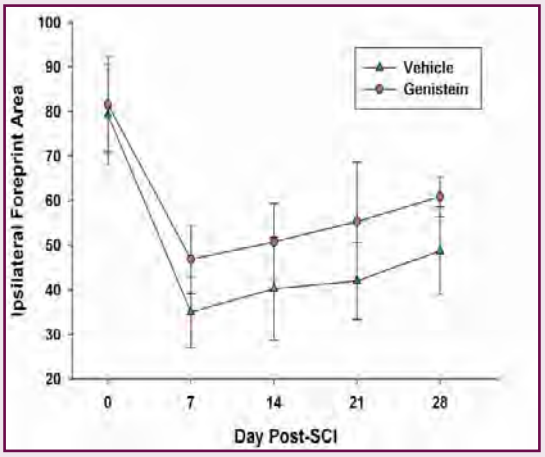


Figure 3. Quantification of CatWalk® gait task. There is no significant difference between the post-SCI administration of genistein and the vehicle groups, but there is a trend toward improvement in the genestein group.

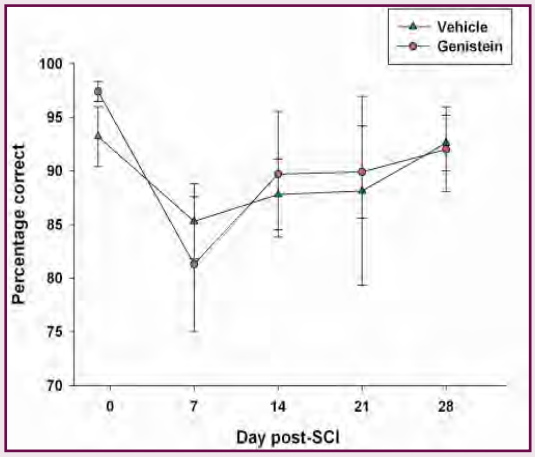


Figure 4. Quantification of Horizontal Ladder Task. There is no significant difference in the percentage of correct placements between the post-SCI administration of genistein and the vehicle. Therefore genistein did not improve skilled locomotor function.

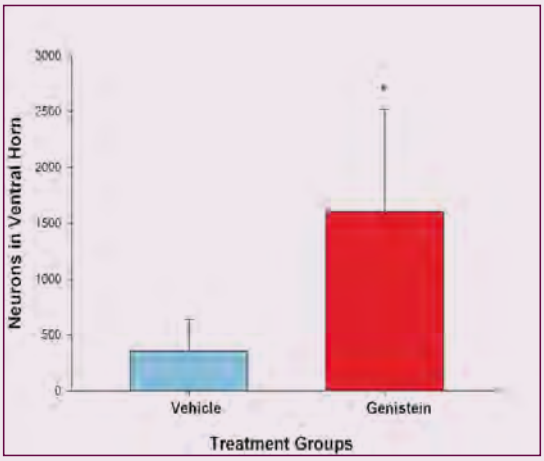


Figure 5. Quantification of neurons in ventral horn. There was a statistically significant difference in the number of neurons present between the vehicle and genistein groups. Genistein treated groups demonstrated a larger neuronal cell survival post-SCI.

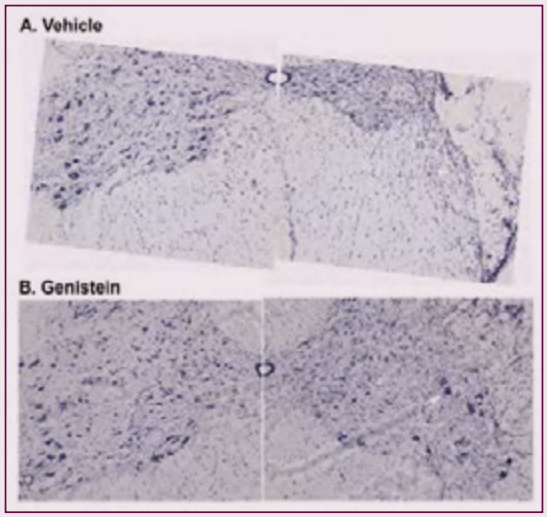


Figure 6. Comparison of the ventral horn between the two groups. The arrow in (B) points to an intact neuron.

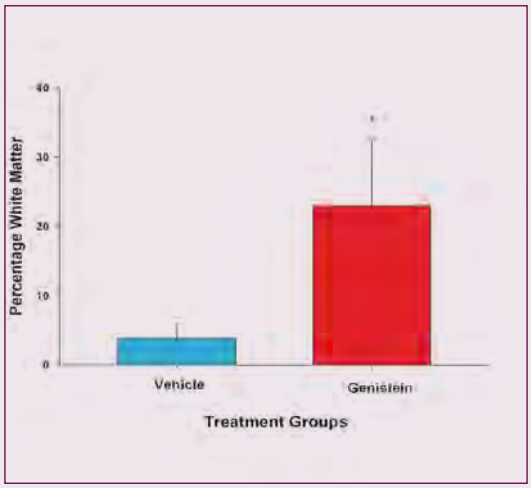
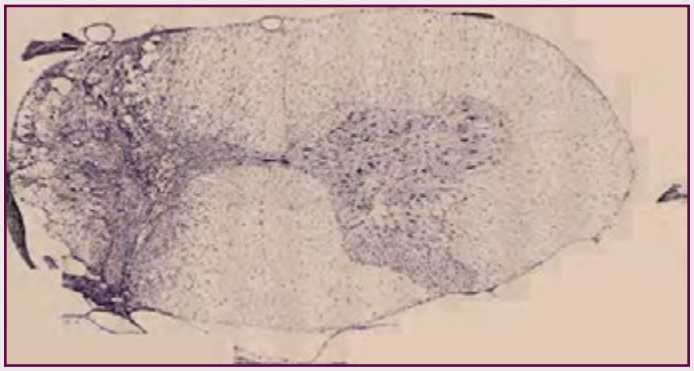
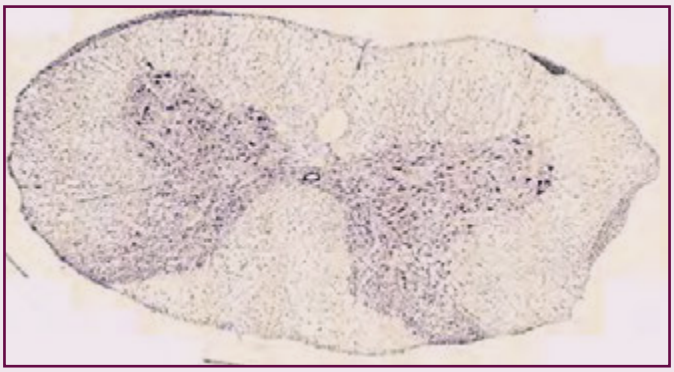


Figure 7. Percentage of white matter. There was a statistically significant difference in the amount of white matter remaining at the hemicontusion site between the vehicle and genistein groups. Genistein treated groups demonstrated a larger percentage of white matter mass post-SCI.



A. Vehicle



B. Genistein

Figure 8. Comparison of the amount of white matter between the two groups post-SCI.

Discussion

The results were consistent with the original hypothesis that the post-SCI administration of genistein will increase functional recovery and tissue sparing in male rodents following a clinically-relevant cervical SCI. The group of male rats that were administered genistein show an increase in paralyzed ipsilateral dominant functionality, and the histological results confirmed a reduction of secondary injury through the improvement in gray and white matter sparing post-SCI. The four behavioral tasks illustrated the physical recovery, while the histological analysis illustrated less nervous tissue damage and more recovery. Therefore, this experiment indicated that genistein is an effective pharmacological treatment for rat spinal cord injuries.

Conclusion

The genistein treated groups showed a statistically significant difference in an increase in the dominant paw forelimb usage and a positive trend in unskilled locomotion. The experimental group also showed decreased apoptosis and prevented the secondary injury as shown by the gray and white matter sparing. Therefore, genistein improved functionality and provided protection to the spinal cord post-SCI.

Because the post-SCI administration of genistein has been shown to be effective in male rat models, it should be considered as a viable treatment for human males as well. The genistein administration timing in the rat model also has real world applications. The initial administration at 30 minutes post-injury is paralleled with an emergency medical technician reaching the scene of an accident and immediately administering genistein if he or she suspects a SCI. Also, the behavioral tasks were continually performed for two weeks after the genistein was no longer being administered as treatment. This allowed for the analysis of relatively long term effects of the genistein treatment. If genistein is able to reach human male trials, the dosage and administration times would require more testing to ensure the maximal functional recovery.

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