Age-Associated Changes on Axonal Regeneration and Functional Outcome after Spinal Cord Injury in Rats

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Abstract- This study was conducted to evaluate the association between aging and regenerative potential of spinal cord injury. Three groups of male Sprague–Dawley rats, including young (40 days), mature (5-6 months) and old (28-29 months) were spinally hemisected at the L1 level. The locomotor performance was assessed weekly for eight weeks after lesion using locomotors' rating scale developed by Basso, Bresnahan and Beattie (BBB). In the tracing study, retrograde labeled neuron was counted in the lateral vestibular nucleus for axonal regeneration. From 4-8 weeks, the functional recovery of the young and mature age rats was significantly increased in comparison to the old age group. At 8 weeks, young and mature animals achieved a plateau score of (mean ± SD), 17 ± 1.47 and 16.8 ± 0.70 respectively, and the old rats reached an average score of 13.8±1.63 (P<0.05). The mean number of labeled neurons in the vestibular nucleus in the young group (mean ± SD): 32.05 ± 1.03 increase significantly compared to the older age group 5.01 ± 1.31 (P<0.05). Current findings suggest that axonal repair and functional improvement decrease in aged animals after partial spinal cord injury. Thus, the aging process may affect the regenerative capacity of the injured central nervous system, and axonal regeneration is age dependent.

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Keywords: Age; Axonal regeneration; Functional recovery; Spinal cord hemisection

Introduction

Worldwide, an estimated 130,000 new cases have been affected by the different severity of spinal cord injury (SCI) each year (1). Traumatic spinal cord of the adult mammal leads to permanent loss of motor and somatosensory functions below the injury site (2). The lack of functional repair is mainly due to the inability of neurons to regenerate their axon through the inhospitable extraneuronal environment and glial scar at the lesion site. During the natural course of aging the central nervous system go through the intrinsic changes. Probably, age at the time of spinal cord injury is an important factor in the outcome of axonal regeneration and functional recovery (3,4).

It has been shown the influence of aging on the axonal regeneration capacity of the motor nucleus in the central nervous system (CNS) after SCI is not clear (5). Thus, identification of the nerve repair with advancing age could be an esteemed area of research that plays an important role in clinical practice (6). Further, the natural course of the axon is affected in aging by a variety of a neurodegenerative condition such as swelling and spheroid (7). These swellings have been reported in human and different animals such as dog, mouse, and rhesus monkey (7-9). Neuroaxonal Dystrophy (NAD) with axonal spheroids, swellings, dysmyelination and axon aberrations were seen in advance age, and it may lead to deficit function in aging (10,11). The vestibulospinal tract is of prime

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importance in regulating the tones of muscle involves in posture, so that the balance is maintained (12,13). Stimulation of the lateral vestibular nucleus causes excitation of motor neurons that supply extensor muscles of the ipsilateral lower limb (13). Although, both injury and aging of the CNS have deep changes in motor function, gait, posture and balance, but more experimental studies are needed to gain conclusive results. Therefore, to determine the association between the age and axonal regeneration and functional recovery, we studied the retrograde tracing in the vestibulospinal tract and assessment of locomotion in different age groups of rats after spinal cord injury.

Materials and Methods

Surgical procedure

All experiments were performed according to the guidelines of Iranian Syndicate for application and Care of Animals and were approved by the Animal Research Ethical Committee of Yasuj University of Medical Sciences. Thirty-six male Sprague -Dawley rats in three groups (n=12), including young (40 days), mature (5-6 months) and old (28-29 months) were used in this study. Before surgery, the animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Under an operating microscope, a dorsal laminectomy of the T12 vertebrate was performed and the superior articular joint on the left side was removed, then the L1 spinal segment was identified and Median sagittal vein was used as an anatomical landmark to demarcate the median plane. As previously described, hemisection on the left side was performed using a pair of iridectomy scissors, effectively disrupting all major unilateral descending pathways (14). After homeostasis, the muscle and skin were sutured in layers, at the end of surgery all groups were kept under the same conditions with free access to food and water. Postoperative treatments which included saline (1.0 cc s.c.) for rehydration and penicillin-G (0.35 ml/kg i.m) as a prophylactic antibiotic. Their bladders were manually expressed twice daily for the first three days.

Open field locomotor test

Hindlimb motor function was assessed based on the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (15) at one day post-surgery and weekly for a total of eight weeks. For BBB assessment, the rats were allowed to move individually for five minutes on a smooth, non-slip floor in an open field (200 x 100 cm). Hindlimb motor function was scored from 0- 21 based on the performance of the ipsilateral hindlimb by an observer who was oblivious to the identity of the groups. The movement of the joints, frequent to the consistent weight supported plantar steps and paw position were scored according to the 21 point BBB locomotion scale (15).

Retrograde tracing of vestibulospinal tract and histological procedures

At the end of behavioral assessment, five rats from each group were randomly chosen for retrograde tracing. Then animals were anesthetized via intraperitoneal injection of a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg) after that under an operating microscope laminectomy was performed to expose the lumbar enlargement at the spinal segment with L3-L4, two or three segments distal to the primary lesion site. For retrograde tracing, using an 11 scalpel blade, a stab incision was made on the left side of the midline at the depth of 1.5 mm (ventral funiculus) at the L4 spinal segment. After haemostasis, one crystal of DiI (1, 1-dioctadecyl-3, 3, 3, 3-tetramethylindocarbocyanin perchlorate, Molecular Probes, Leiden, The Netherlands), with diameter of 1 mm, were inserted into the depth of the spinal cord incision with forceps and then covered with gel foam.

The overlying muscles and adipose tissues were returned to their original positions and the skin was sutured. After two weeks, all animals were perfused transcardially with 10% formalin which was dissolved in sodium phosphate buffer under deep pentobarbital anesthesia (40 mg/kg, i.p.). The whole left brain stem was dissected out and sliced into 50 mm thickness cross sections on a freezing microtome (Leica CM 3000), and examined under a fluorescent microscope (Olympus Ax70). The retrogradely labeled cells with intact nucleus and soma, which were considered to be live neurons, were counted in the lateral vestibular nucleus. As previously mentioned, the number of labeled neurons of any section in each lateral vestibular nucleus were summed together to give the total number of motor neurons for each rat (16). The same procedure was performed on four uninjured rats, and the results in three groups were expressed as the percentage compared to the normal or uninjured rats.

Statistical analysis

All data were expressed as mean±SD. One-way ANOVA was used for data analysis, followed by the Tukey test for post hoc analysis. Differences between groups were considered significant at $P<0.05$.

Results

Recovery of the locomotor function

Figure 1 shows the locomotor performance of the three groups. One day after the operation, hemisected animals displayed loss of ipsilateral hindlimb function with no observable movement, Followed by a phase of rapid spontaneous recovery up to the second week. BBB scores increased considerably at the end of three weeks of post surgery for each of the three groups (mean ± SD): 12.85 ± 1.28, 12.02 ± 0.43 and 11.92 ± 1.61 for young, mature and old groups, respectively. From a 4th week till 8th the young and mature rats showed a significant increase in movements of their hindlimbs compared to the aged group (P<0.05).

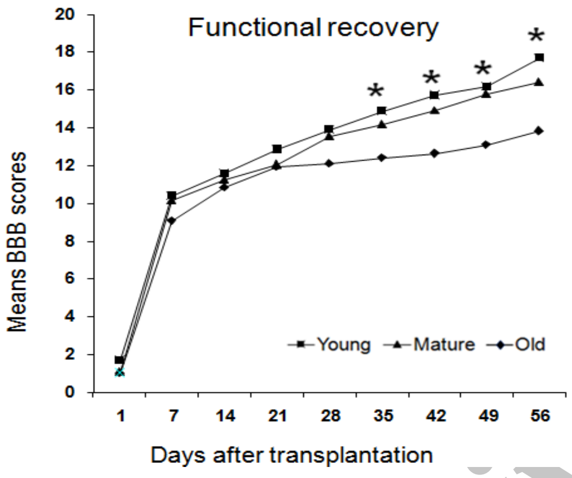

Figure 1. The Mean BBB motor score for ipsilateral hind limbs in various age groups after hemisection surgery. During the three first weeks post injury all groups recovered nearly at similar rates, but from 4-8 weeks the young and mature groups show spontaneous recovery of function, and significant difference is seen (*P<0.05) over the old rats. Values represent means ± SD, n=12

The young and mature groups recovered nearly at similar rates during the end of 5th week post-injury although the young rats showed slightly improved their walking behavior in comparison to the mature group. Furthermore, the young group showed consistent plantar stepping and consistent forelimb–hindlimb (FL-HL) coordination, whereas the old rats demonstrated limited FL–HL coordination. At the end of eight weeks, young and mature animals achieved a plateau score of (mean ± SD), 17 ± 1.47 and 16.8 ± 0.70 respectively, and the old rats reached an average score of 13.8 ± 1.63. One-way ANOVA results showed a significant difference between young and mature groups compared to the aged group during weeks four to eight (P<0.05).

Axonal regeneration in the vestibulospinal tract

After 10 weeks of injury, some regenerated fibers transported the DiI tracer in retrograde directions, and we observed retrograde labeled somata in the lateral vestibular nuclei. Ten weeks after the lesion, in uninjured rats (n=4), the numbers of labeled neurons (mean ± SD) were 102.7 ± 3.8. Although this number was decreased considerably in injured rats, but in young rats compare to the other groups, more neurons of vestibular nucleus regenerated their axons to the lesion site and to the retrograde substance, distal to the lesion site and transported DiI to their nucleus (Figure 2). The labeled of neurons for each of the three groups were (mean ± SD): 32.05 ± 1.03, 17.8 ± 0.36 and 5.01 ± 1.31 for young, mature, and old groups, respectively (Figure 3). The statistical analysis reveals a significant difference only between the young group and the aged group. Although there was a trend toward an increased number of DiI labeled neurons in the young group compared to the adult group, but there are not any significant differences between them. The results of this retrograde tracing study have shown that the different rate of axonal regeneration after spinal cord injury differentially develops with normal aging.

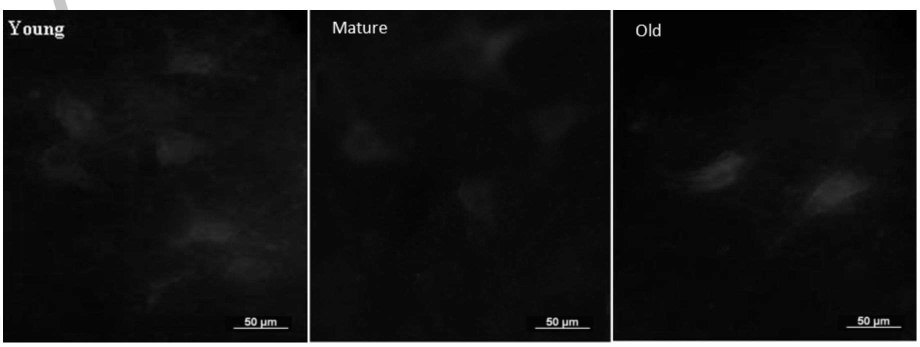

Figure 2. Fluorescence photomicrographs of retrogradely DiI labeled motor neurons in the left vestibular nucleus. The presence of the retrograde tracing in the motor neurons is indicative of regeneration of the some transected axons into the lesion site. The number of DiI labeled motor neurons in young rats was significantly higher than old ones, n=5, P < 0.05, Scale bar: 50 μm

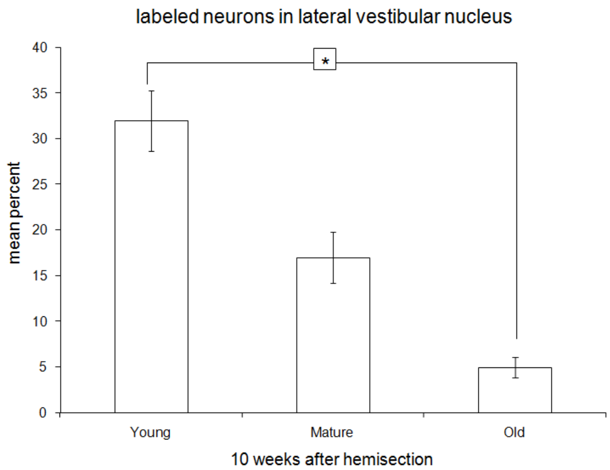

Figure 3. The Mean of the retrograde labeled neuron in left lateral vestibular nuclei (LVN) in different groups after injury. After ten weeks, some neurons of LVN extended their axons beyond of injury site to reach the lumbar enlargement. There were a respectively reduced by 95% of detectable neurons in old rats compared to 84% and 69% cell in mature and young groups. A significant difference ($P<0.05$) was found only in the young group and old one. Values represent means ± SD, n=5

Discussion

The Current behavioral assessment showed that one day after the spinal cord hemisection no hindlimb movements were found in all injured rats. Thus, this data shows that the animals in three groups had completely hemisected spinal cords. Hemisections were considered complete when the dorsal column, Lissauer's tract, lateral and ventral column, and gray matter were sectioned (14). Three weeks after injury all group recovered nearly the plantar placement without coordination of fore-hind limb coordination. As indicated previously, spinal cord hemisection of the thoracolumbar region resulted in pronounced paralysis on the ipsilateral hindlimb (impaired hindlimb), with partial recovery by three weeks (17). Present data showed the number of DiI labeled neuron of the lateral vestibular nucleus in uninjured rats were (mean ± SD) were 102.7 ± 3.8. These findings are consistent with the Asada and Bernstein-Goral results that used the same procedure for retrograde tracing to counting the supraspinal labeled neurons (15,18). The number of DiI labeled neuron in young and aged groups were about 30% and 5% respectively compared to the uninjured rats. Importantly, these findings indicate that the functional recovery is correlated with axon regeneration because the rats in the young and adult group had more axon regeneration and task recovery compared to the aged group. It has been reported that little axon regeneration can considerable impact on the recovery of the function (19). Different alterations such as morphological change, protein synthesis and gene expression have seen in the central nervous system with aging (20-22). The mechanisms might link the age participation to nerve fiber growth and functional recovery after spinal cord injury has not been identified, but it has been shown with advancing age the total number of sympathetic and parasympathetic neuron have decreased (23). Although, some evidence indicated the aging could be result of the environment effect including radiation, free radicals and chemical toxins, etc. (21,24). The experimental study showed after traumatic brain injury the capacity for plasticity and repair reduced with aging (25). Moreover, the excitability of the dorsal horn increased and descending pathways impaired following SCI in advancing age rats (26). Also, accumulations of proteins during the aging lead to swelling of axons in several disease models including Parkinson's disease and giant axonal neuropathy (27). Present results showed that positive correlation presents between aging and its associated functional deficits. In advanced age a few of motor neurons have been undergoing atrophy process and anatomical changes such as reduction of dendritic tree zone, decrease synaptic input and axonal swelling (28,29). These features of the axonal pathology may be indicative of block in retrograde axonal transport. In this regard, the potential of intrinsic regeneration mechanisms, signaling pathway and expression of regeneration associated genes are correlated with the age of the neuron (24). Some studies have shown that neuronal atrophy, axon dystrophy and degeneration have seen in the CNS with advancing age and the increase of GFAP in elderly may be a response to demyelination and degeneration in CNS (24,30).

Furthermore, current finding indicated that the axonal regeneration and functional recovery after spinal cord injury is not only dependent on extracellular matrix molecules, sufficient growth-promoting molecules, or physical and chemical barrier, but also on the innate capacity of a neuron to respond to these factors. Parallel to this result, other experiments report that after spinal transaction axon can regenerate in immature mammals whereas in the adult mammals are abortive (31). Besides, aberrations in axon guidance pathway of cholinergic and monoaminergic fibers in the spinal cord in the aged rats could limited the recovery of locomotor function following SCI (32). However, this experimental study indicates that the age contributed significantly to th axonal regeneration and functional recovery after SCI. But,further investigation is needed to understand the mechanisms of endogenous factors participating in axonal re-growth after spinal cord injury.

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