Restoration of Spermatogenesis by Adenoviral Gene Transfer into Injured Spinal Cords of Rats

Mohammad A. Khalili, Ph.D.1,2, Alexander G. Rabchevsky, Ph.D. 2

1Research and Clinical Center for Infertility, Yazd University of Medical Sciences, Yazd, Iran. 2 Department of Physiology, University of Kentucky College of Medicine, Lexington, KY, USA

Background: Spinal cord injury (SCI) has a significant impact on male reproductive functions which may lead to infertility. A large number of spinal cord injured men suffer from impaired spermatogenesis. Currently, in vivo gene transfer of molecules with potential therapeutic value has been recognized as a viable method for inducing functional recovery after SCI. This study characterized the role of adenovirus-mediated gene transfer into experimentally injured spinal cords of rats on possible restoration of spermatogenic cell lines.

Materials and Methods: Young adult Sprague-Dawley rats (200-250g) were assigned into one of the three different groups of control, SCI, and adenovirus transfer (Ad) (n=3/group). Control rats received no injury, nor any surgery. For SCI rats, SCI was produced by a 10g brass rod with a tip diameter of 2 mm which was dropped from a height of 12.5 mm onto exposed spinal cord at level of T10 with NYU impactor. Animals were perfused transcardially 43 days post SCI. Both spinal cord and testicular tissues were cryo-sectioned and ultra thin-sectioned, respectively. Cellular morphology and morphometry were done for spinal cord tissues. The testicular samples were processed for both light and transmission electron microscopy (TEM). The third group of rats underwent SCI first, followed by microinjection of LacZ adenoviral vectors (5x106 p.f.u./µl) along the T6-T10 dorsal root entry zone bilaterally. The immune system of animals were suppressed before the Ad administration. Each Ad injection was done using a glass micropipet and a Nonoject injector. Rats were killed 43 days after Ad injections, and the tissues were studied as for other groups.

Results: The spinal cord lesion extents for SCI and Ad groups were 8.1±3 and 5.8±2.2 mm, respectively (p<0.05). The testicular tissue of controls revealed a normal arrangement of spermatogenesis cell types. However, impaired spermatogenesis including vacuolization of germ cells along with incomplete spermatogenesis were noted in the tubules of SCI group. Also, nuclei and cell membranes of spermatozoa were damaged. In Ad rats, relatively active spermatogenesis, ranging from reappearance of proliferating spermatogonia to the presence of mature spermatozoa were observed in some seminiferous tubules.

Conclusion: Bilateral adenovirus-mediated gene transfer into experimentally injured spinal cords of rats can restore the ultrastructure of spermatogenesis including mature spermatozoa.

Key Words: Spinal cord injury, Gene therapy, Spermatogenesis, Rat

Introduction

At present, spinal cord injury (SCI) is one of the major public health problem worldwide. In the United States alone, over 10,000 new cases of SCI occur annually. Eighty-two percent of the victims are males, and the majority are in their prime reproductive years. Infertility due to SCI is a common problem which result from a combination of ejaculatory dysfunction and abnormal semen parameters of sperm count, progressive motility, and morphology (Rajasekaran and Monga, 1999). With advancements of the assisted reproductive technology (ART), some spinal cord injured men have become the biological father of their children. Despite these clinical advances in recent years, there are still a large number of victims suffering from prolonged infertility. Therefore, a significant amount of basic research has been directed towards potential strategies for improving axonal regeneration following SCI which subsequently improve the fertility potential of victims (Romero and Smith, 1998). The application of gene therapy for SCI has become a...
relatively recent development. Less than a decade ago, gene therapy was considered only for the treatment of genetic disorders. Today, gene therapy is being considered for both neurological and reproductive disorders that are not due to genetic abnormalities. Gene therapy will become an intrinsic part of spinal cord therapy in the future for the following reasons: First, spinal cord regeneration requires manipulation through manipulation of cellular environment by changing the genetic expression of spinal cord cells. Second, regeneration takes a long time, probably years in humans. It may be more efficient to administer factors through gene therapy rather than through drug administration which may indirectly influence the male reproductive system (Stibley et al., 2002; Romero and Smith, 1998).

In conclusion, gene therapy is another effective tool which can be applied non-invasively. It can be used to augment or alter the expression of many factors in the target cells. Finally, gene therapy will greatly accelerate progress towards an effective "cure" of SCI in human (Stibley et al., 2002; Robbins and Ghivizzani, 1998; Romero and Smith, 1998).

### Materials and Methods

Young adult male Sprague-Dawley rats (200-235g) were assigned to one of the three groups of control, SCI and Ad (n=3/group).

#### Control Group

Rats

- Xylazine (10mg/kg) before a laminectomy was performed at T10. The exposed vertebral column was stabilized by clamping both T9 and T11 vertebral bodies with Adson delicate forceps.

- The testicular and spinal cord specimens were collected for further assessment.

#### SCI Group

- Rats were first anesthetized with ketamine (80mg/kg) and xylazine (10mg/kg) before a laminectomy was performed at T10. The exposed vertebral column was stabilized by clamping both T9 and T11 vertebral bodies with Adson delicate forceps

- Following SCI, Ad microinjection was performed. Ad microinjections were done as described by Romero and Smith, 1998 (Romero and Smith, 1998). Before, the Ad administration, the animals received 100 µg intraperitoneal of combined solution of rat CD-4 (W3/25) and CD-45 (MRC OX-22) anti-sera to suppress the immune response. Each rat then received eight bilateral injections (4 µl; 0.5 mm apart and 0.5 mm deep) of adenoviral vectors of encoding LacZ (5x10^6 p.f.u./µl) along the T6 - T10 dorsal root entry zone (DREZ). Each injection was done with a nano injector 2000 attached to a beveled micropipette. Just before injection, the micropipette was filled with colored mineral oil followed by viral suspension (Romero and Smith, 1998), following injections, post-operative care was done for each animal. Animals were sacrificed 43 days post surgery, and both testicular and spinal cord specimens were collected for further assessment.

#### Tissue Processing for TEM

The testicular samples were cut in small pieces and stored in 3.1 M PBS in 10% sucrose at 4°C. The specimens were washed in 0.1 M PBS, and then post-fixed in 2% aqueous osmium tetroxide in above buffer.
Figure 1. Normal seminiferous tubule with active spermatogenesis from control group (solochrome stain).

Figure 2. Vacuolization of spermatogonial cells (arrowheads) with reduction in number of sperm in SCI sections (solochrome stain).

The specimens were subsequently dehydrated in a graded series of ethanol solutions, and embedded in Araldite. Ultra-thin sections were cut on a Reichert Ultramicrotome (OMU3). The ultra-thin sections were picked up on 200 mesh copper grids and stained with uranyl acetate for 12 min in dark, and lead citrate for 2 min in a CO2 free atmosphere. The micrographs were finally taken using a Philips TEM at an accelerating voltage of 60 kV.

Histology for Spinal Cord

A modified eriochrome cyanine (EC) staining protocol for differentiation of white matter and cell bodies was used to calculate the amount of spared tissue in sections of injured cords. Briefly, air-dried sections were cleared and hydrated before being placed for 10 min into a solution consisting of 2 ml 10% FeCl3 and 40 ml of 0.2% EC (Sigma Co. OM) in 0.5% aqueous H2SO4 brought to a final volume of 50 ml with dH2O. This was followed by washes in water and differentiation for 2 min in 0.5% aqueous NH4OH. The reaction was terminated with rinses in water before sections were dehydrated and cleared for covering with permount (Fisher Scientific Co., OH).

Statistical Analysis

Measurements of the lesion extents were compared using a 2-way ANOVA. The Mann-Whitney test was performed to determine significant differences between the groups. Significance was set at p<0.05.

Results

Spinal Cord Tissue

The morphological evaluation of spinal cord sections from control rats showed a normal appearing cytoarchitecture. However, the injured spinal cord showed an abnormal gray matter area with dramatic reduction in intact tissue. In addition, the group differences in the percentage of spared tissue (white and gray combined) were statistically significant when compared to the controls (P<0.05, Table I).

Testicular Tissue

a) Light Microscopy: Complete Spermatogenesis was observed in testis of control rats (Figure 1). Normal appearing spermatogonia cell adjacent to the basement membrane as well as tremendous number of spermatozoa filling the lumen of the seminiferous tubules were observed in testicular sections of controls. However, in SCI animals, vacuolization of the majority of spermatogonial arranged in an abnormal fashion (Figure 2). Figure 3 represent a cross section of seminiferous tubule from Ad group with complete spermatogenesis. Rare vacuolization of spermatogonia...
expression determines the production of the therapeutic gene product in the cell (Stribley et al., 2002; Robbins and hivizzani, 1998; Romero and Smith, 1998). Currently, delivery stems can be divided into viral and non-viral vectors. Among e most used viruses, adenoviruses are usually applied more in urotrauma diseases such as SCI. Host cells infected with wild-type adenovirus undergo cell sis, resulting in viral load release. Therefore, in present aly adenoviral vectors of encoding LacZ gene was injected ong the DREZ of injured spinal cords (Romero and Smith, 1998). The inflammatory response of the spinal cord to enovirus depends on viral titer (dose) and rat strain. Wood et al. (1996) found that administration of high viral titers (>10^6 p.f.u.) to spinal cord produced severe tissue damage. However, lower viral titers produced lasting expression of the gene that they sought to express, beta-galactosidase, with minimal immune response. They concluded that the immune responses to adenovirus administration are both dose- and strain- dependent (Wood et al., 1996). Therefore, in this study we injected only 5x10^6 p.f.u. Ad vector into DREZ of Sprague-Dawley rats which are the most common species used in the gene therapy studies on SCI. Also, adenoviruses may encode certain proteins that allow them to evade the immune system.

A sympathetic center, located in spinal cord segment T11-L2 and prostatic smooth muscle fibers rise to the peristalsis necessary for ejaculation. Also, a para-sympathetic center Allocated in S2-4 with efferents in nervi perigents supplies the prostate glands leading to formation of seminal fluid. This indicates that the SCI directly influence the reproductive system in men (lisenmeyer and Perkash, 1991). Another reason postulated for poor semen quality after SCI is intrinsic damage of the testicles. Bors and associates found abnormal testicular histologies in 31 of 34 men with SCI. The most common finding was atrophy of the seminiferous tubules. In addition, the biopsies revealed abnormalities that varied from absence of spermatogenic cells to rare spermatids and spermatozoa. Our results indicate that there was a marked reduction in spermatogenesis in spinal cord injured rats at 43 days post injury. 90% of azoospermic sperms are only. Bors and associates found abnormal testicular histologies in 31 of 34 men with SCI. The most common finding was atrophy of the seminiferous tubules. In addition, the biopsies revealed abnormalities that varied from absence of spermatogenic cells to rare spermatids and spermatozoa. Our results indicate that there was a marked reduction in spermatogenesis in spinal cord injured rats at 43 days post injury. A sympathetic center, located in spinal cord segment T11-L2 and prostatic smooth muscle fibers rise to the peristalsis necessary for ejaculation. Also, a para-sympathetic center Allocated in S2-4 with efferents in nervi perigents supplies the prostate glands leading to formation of seminal fluid. This indicates that the SCI directly influence the reproductive system in men (lisenmeyer and Perkash, 1991). Another reason postulated for poor semen quality after SCI is intrinsic damage of the testicles. Bors and associates found abnormal testicular histologies in 31 of 34 men with SCI. The most common finding was atrophy of the seminiferous tubules. In addition, the biopsies revealed abnormalities that varied from absence of spermatogenic cells to rare spermatids and spermatozoa. Our results indicate that there was a marked reduction in spermatogenesis in spinal cord injured rats at 43 days post injury. 90% of azoospermic sperms are only.

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The present study demonstrated derangement of the tubules in experimentally injured rodents. Additionally, our study carried out function with special reference to 529 patients with spinal cord injury. The results from our study is in agreement with the study done by Liu et al (1997) that introducing recombinant adenovirus into injured spinal cord may have transduced cells surrounding the lesion site and induce them to synthesize and release neurotrophins to the nerve fibers and neuronal cells which subsequently could improve the nerve supply to the testis of rats (Liu et al.,1997). Hirsch et al. (1999) suggested that spermatogenic defects may occur soon after SCI (early phase). Altered testicular function following SCI may result from abnormal thermal regulation associated with denervation, resulting in elevated scrotal temperature. Additionally, spermatogenic insult in early phase of SCI may result from endocrine alterations. While, the present study did not consider hormonal parameters, Linsenmeyer et al. (1994) reported lower serum testosterone in injured rats (Lisenmeyer et al.,1994). Moreover, the same group noted a significant alteration in serum gonadotropin and testostrone levels, which resolved in 2 weeks. These findings would not support a hormonal etiology for the histologic abnormalities that persist in our SCI animals. Therefore, while its exact etiology remains unclear, spermatogenic deficit following clinical and experimental SCI is a commonly observed sequela. Hirsch IH, Huang B, Chancellor MB, Rivas DA, Salzman SK, Jost LK, Evenson DP. Spermatogenesis in early and chronic phases of experimental spinal cord injury in the rodent model. J Androl 1999; 20: 63-71. Holstein AE, Saverwein D, Schirren U. Spermatogenesis in patients with traumatic transverse paralysis. Urologe 1985; 24: 208-11. Linsenmeyer TA, Perkash I. Infertility in men with spinal cord injury. Arch Phys Med Rehabil 1991; 72: 747-54. Linsenmeyer TA, Pogach L, Ottenweller JE, Huang HF. Spermatogenesis and the pituitary testicular hormone axis in rats during acute phase of spinal cord injury. J Urol 1994; 152: 1303-7. Therefore, while its exact etiology remains unclear, spermatogenic deficit following clinical and experimental SCI is a commonly observed sequela. While, the present study investigated the potential role of adenovirus in improving the spermatogenesis which can result in testicular hormone axis infertility in spinal cord injury. J Androl 1999; 20: generally alters following SCI. Significant dysfunction occurred in spinal injected rats. Therefore, it is concluded that altered spermatogenesis can be reversed following Ad injection. Romano MI, Smith GM. Adenoviral gene transfer into the in the spinal cord segments and a spermatogenic cycle which takes over forty days in rats.

Acknowledgment

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