Penile reconstruction using mesenchymal stem cells[1]

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ABSTRACT

PURPOSE: To compare the reconstruction of corpus cavernosum segments when seeded with mesenchymal stem cells and when stem cells are infused intravenously.

METHODS: Sixteen New Zealand rabbits were submitted to reconstruction of the corpus cavernosum and distributed in Group A - decellularized matrices, Group B - decellularized matrices seeded with mesenchymal stem cells Group C - decellularized matrices submitted to intravenous infusion of mesenchymal stem cells. The mesenchymal stem cells were obtained by bone marrow aspiration. The venous filling aspect of the distal end of the corpus cavernosum was evaluated and the specimens were submitted to histological analisis and to immunohistochemistry. Cavernosometry was done in one animal of each group

RESULTS: Three animals on B and three animals on C presented full filling of distal end of the corpus cavernosum. No animals in A presented filling of the distal end of corpus cavernosum. At cavernosometry the animal on B attained 50 cmH_2O, on C 110 cmH_2O and on A 20 cmH_2O. Trabeculae forming cavernous sinuses were found in groups B and C.

CONCLUSION: The reconstruction of corpus cavernosum using descellularized matrices and mesenchymal stem cells, either by intravenous injection or directly seeded is possible, with growth of corpus cavernosum-like tissue.

Key words: Tissue Engineering. Stem Cells. Regenerative Medicine. Penis. Rabbits.

Introduction

Penile reconstruction is a complex procedure, however necessary in the treatment of congenital anomalies that may involve from total agenesis of the organ to anomalies such as the exstrophy-epispadias complex, sexual differentiation disorders and proximal hypospadias[1]. Acquired anomalies such as in cases of traumas, infections, malignancies or even in cases of sex reassignment may also require genital reconstruction. The objective is to reconstruct an esthetically acceptable phallus that allows for adequate intercourse.

For full reconstruction of the penis, different tactics have been used, almost all involving the confection of skin flaps and using complex microsurgery techniques, that inevitably lead to large morbidity, both in the donor and receptor areas and, although possibly giving the penis a reasonable appearance, rarely do they allow for functional recovery of the organ[2-4].

Tissue engineering is currently an alternative to the functional reconstruction of the penis. In an experimental study using rabbits, homologous corpora cavernosa were decellularized, seeded sequentially by autologous smooth muscle cells and endothelial cells, enabling replacement of a 0,7cm entire cross-seccional segment. Growth of the smooth musculature and an intense neovascularization network were observed in the morphological evaluation[5].

When corpus cavernosum are absent or no native tissue is available to initiate cellular culture, stem cells may come in as a good alternative. Embryonic and other pluripotent stem cells have the inconvenience of forming tumors when implanted "in vivo", in addition to ethic matters involved in their usage. Among the adult stem cells, mesenchymal cells are the ones mostly studied. They can be easily obtained through bone marrow aspiration[6]. The most important characteristic of these cells is believed, actually, to be their capacity to maintain homeostasis and tissues integrity[7-9]. They are kept in a quiescent condition in specific areas, called niches, where they would be able to make normal cell replacement of that specific tissue and, should a lesion occur, they would have capacity to migrate to the site of the aggression, attracted by a series of chemokines repopulating the damaged organs[10]. The immunomodulatory role of mesenchymal stem cells has already been demonstrated. They can suppress the immune response by inhibiting the proliferation of T lymphocytes, B lymphocytes[11,12], "natural killer" cells and damaging the function of antigen presentation of dendritic cell[13-15], through a paracrine secretion mechanism[9].

As they are infused "in vivo" they migrate to the damaged tissue areas[16] and are not recognized by the host's immune system for failing to express major histocompatibility complex (MHC) class II antigens[17-18]. Systemic infusion of mesenchymal stem cells was used with good results in the graft-versus-host disease following transplantation of hematopoietic stem cells, with no difference among syngeneic or allogeneic stem cell transplantation[19].

In this study we used tissue engineering techniques to construct a corpus cavernosum segment by using decellularized matrices repopulated with allogeneic mesenchymal stem cells either directly seeded on the matrix or intravenously infused.

Methods

The experiment was submitted to the Research Ethics Committee of the Universidade Federal de São Paulo – Escola Paulista de Medicina (UNIFESP-EPM) and approved under protocol number: CEP 0924/08.

Sixteen New Zealand rabbits aged six months, approximately, obtained from CEDEME (Centro de Desenvolvimento de Modelos Experimentais) the animal facility from UNIFESP were submitted to reconstruction of the corpus cavernosum and allotted into three groups: Group A (n=4) using decellularized homologous matrix Group B (n=6) Using decellularized homologous matrix seeded with mesenchymal stem cells Group C (n=6) Using decellularized homologous matrix followed by intravenous infusion of mesenchymal stem cells.

Procedures

The animals were given pre-anesthetic medication with acepromazine maleate (2.4mg/Kg) followed by anesthetic medication 2% xylazine chloride (2.0mL) and 1% ketamine chloride (1mL) by intramuscular route. Penile anesthetic block was performed with 0.25% bupivacaine chloride (2.0mL). The same anesthetic procedures were used for the bone marrow punctures.

Preparation of the collagen matrix

Corpora cavernosa were taken from sacrificed rabbits obtained from commercial slaughterhouses. We harvested the discarded corpora tissue within few minutes after death and were kept in PBS on ice until descellularization process was initiated[5].

They were sectioned into segments of approximately 7mm and washed with distilled water in a magnetic stirrer flask for 24 hours at 4°C and then treated with 1% Triton x100 (Sigma Chemical CO, Saint Louis, MO, USA) and 0.1% ammonium

hydroxide in fresh distilled water for 14 days. They were washed with distilled water for 48 hours and put into phosphate buffered saline (PBS) for 24 hours. Histological tests were performed using hematoxylin-eosin to confirm acellularity, and picrosirius red stain to demonstrate preservation of the collagen fibers. The matrices were kept in 87% glycerol at 4ºC. Before used the matrices were washed with PBS and incubated with DMEM (Dulbecco's modified Eagle's medium– Sigma Chemical Company, St Louis, MO, USA) with 10% fetal bovine serum (FBS) (Fetal Bovine Serum – Gibco, Carlsbad – CA, USA) 10.000 Ul/L of penicillin and 50 mg/L streptomycin for 2 days, for glycerol to be removed.

Culture of mesenchymal stem cells

Bone marrow was aspirated from iliac crest of five New Zealand male rabbits under aseptic conditions. These animals were posteriorly used in the penile reconstruction takin care to not make autologous seeding or infusion. The mononuclear cells were separated by density gradient, by using Histopaque solution (Sigma-Aldrich, St. Louis, MO, USA) and placed in 25 cm^2 flasks containing DMEM with 15% FBS and kept in incubators at 37ºC with 5% CO_2. The cultures were expanded until reaching the number of cells necessary either to seed on the matrices or for intravenous infusion according to the animal group. Cells from the third to the sixth passage were used.

Cell seeding on the collagen matrix

Mesenchymal stem cells were seeded on the decellularized matrix in a $20x10^6$ cells/mL concentration, using a 1mL syringe and a 23G needle. The culture was kept in a flask with DMEM supplemented with 10% FBS, in an incubator, for 7 days until implantation time. The culture medium was changed every 24hs.

Corpora cavernosa implant

A longitudinal incision on the dorsal face of the penis was performed and the central portion of the corpora cavernosa dissected, preserving the neurovascular bundles. The corpora cavernosa was cross-sectioned and the penile urethra was dissected and isolated. The 7mm-long matrix was inserted between the two stumps of the corpus cavernosum and sutured with separate 6-0 polyglycolic acid stitches. A 6-0 polypropylene suture marked both anastomosis sites (Figure 1).

Intravenous infusion of mesenchymal stem cells

The mesenchymal stem cells were intravenously infused in a concentration of $0.5\text{-}1x10^6$ cells/Kg, in 1.5mL PBS by auricular vein puncture. Infusions were performed on day one, eight and twenty postoperatively.

Postoperative evaluations

All animals were assessed postoperatively in week 12.

Macroscopic aspect

Cavernous filling of the distal end of corpora cavernosa was observed and every animal was photographed.

Cavernosometry

One animal from every group underwent cavernosometry and all animals have been evaluated before surgical procedure. For normal measures we used animal before surgical proceeding. The penis was clamped in its proximal region and the corpus cavernosum was punctured in its distal portion with a 25 Gauge scalp. Saline solution was infused at 1mL/min (ST670 volumetric infunsion pump; Samtronic, Sao Paulo, Brazil). Proximal puncture was performed with a 21 Gauge scalp, which was connected to the transducer of the urodynamic device (Dynapack MPX816-Dynamed, Sao Paulo, Brazil) to measure the pressure of the corpora cavernosa.

Histomorphometric evaluation

The animals underwent euthanasia in postoperative week twelve through anesthetic overdose. As Normal group we used animals from slaughterhouse before decellularization. The penis was excised, fixed in 10% formaldehyde, and cut into five-micrometer longitudinal sections stained with hematoxylin-eosin and analyzed by optical microscopy. For immunohistochemistry smooth muscle cells were labeled with monoclonal anti a-actin (Clone 1A4, Cell Marque Rocklin, CA - USA) antibodies and endothelial cells with polyclonal anti Factor VIII (BIOSB Santa Barbara, CA 93117 – USA) antibodies. It has been observed the architecture of venous sinus formation.

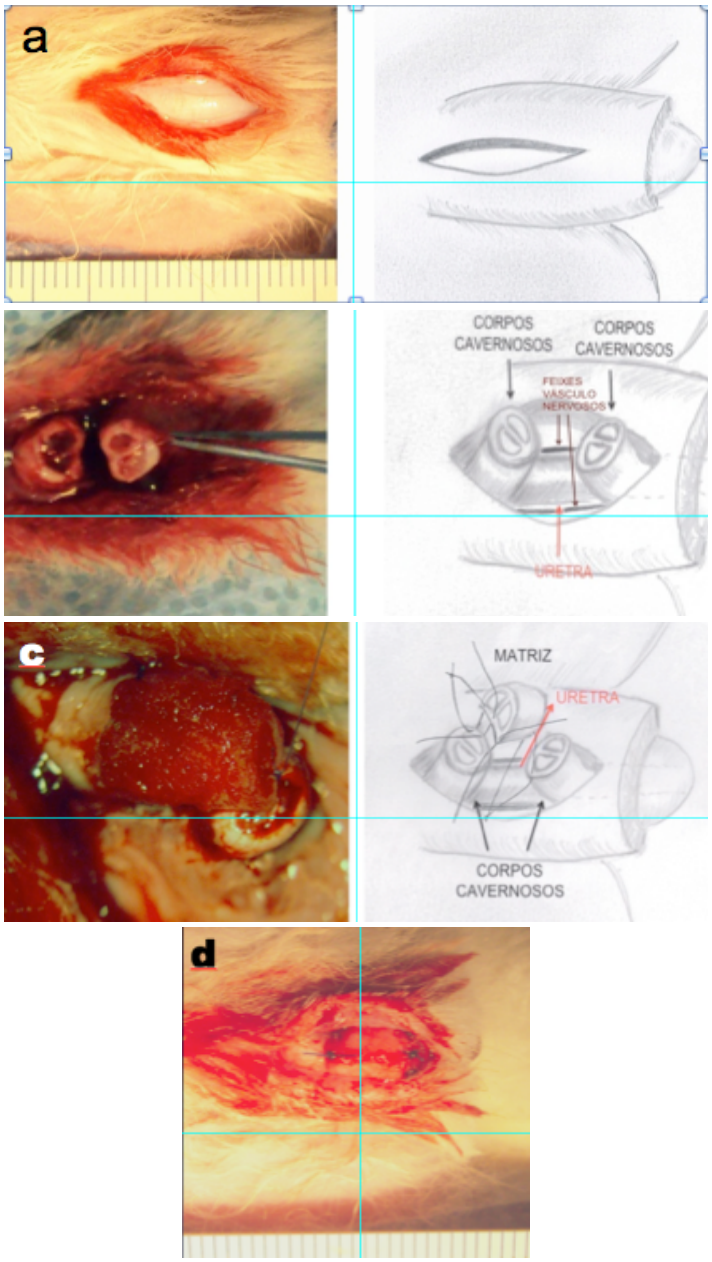

FIGURE 1 – **a**. Incision on the dorsal aspect of the penis. **b**. Cross-section of corpus cavernosum preserving neurovascular bundles and urethra. **c**. Matrix insertion and suture. **d**. Matrix in place.

Results

Sixteen surgical interventions were performed, with one death in group C on postoperative day ten due to wound infection.

Macroscopic evaluation

No animal in group A was found to show filling of the distal end of the corpus cavernosum. Three animals in group B and three in group C was found to show filling of the distal end of corpus cavernosum (Figure 2). All animals had augmented diameter of the corpora cavernosa in the graft area (Figure 3).

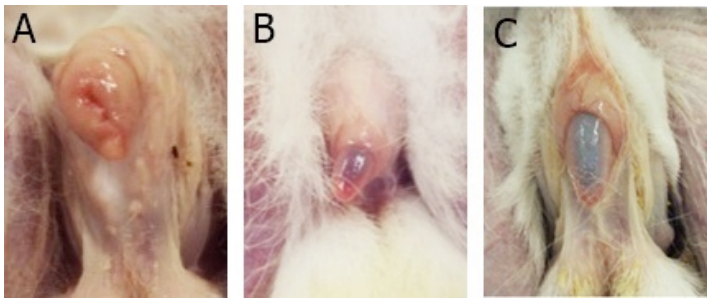

FIGURE 2 - Filling of distal end of corpora cavernosa. **A.** Group A matrix without cells. **B.** Group B matrix with cells seeded. **C.** Group C matrix with intravenous infused cells.

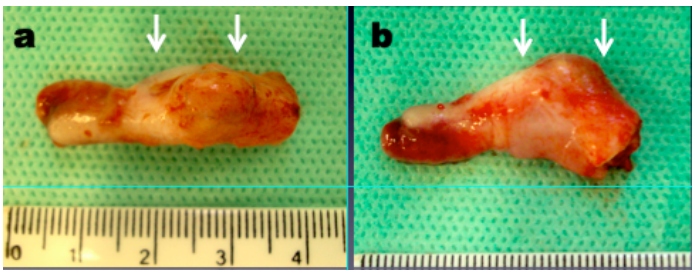

FIGURE 3 – Neocorpora showing augmented diameter of graft intake animal from group C. **a.** Dorsal aspect. **b.** Lateral aspect.

Histological and immunohistochemical evaluation

Histological evaluation showed that the animals in the three groups had healing tissue with fibrosis. Areas similar to trabeculae forming cavernous sinuses with neovascularization, surrounded by scar reaction were found in the animals in groups B and C. In the immunohistochemical evaluation with anti a-actin and anti-Factor VIII antibodies, all animals had formation of smooth muscle tissue with neovascularization; however, we observed areas of organized smooth muscle bundles forming cavernous sinuses in the animals in groups B and C. The animals in group A showed disorganized areas of smooth muscle tissue overlapping fibrous tissue areas, rarely forming cavernous sinuses (Figures 4 and 5).

Cavernosometry

The pressure determined in normal animals was around 305 cmH_2O. The animal from group A had no significant pressure increase, with a peak of 20 cmH_2O. The animal on group B had a pressure peak of 50 cmH_2O and on group C 110 cmH_2O (Figure 6).

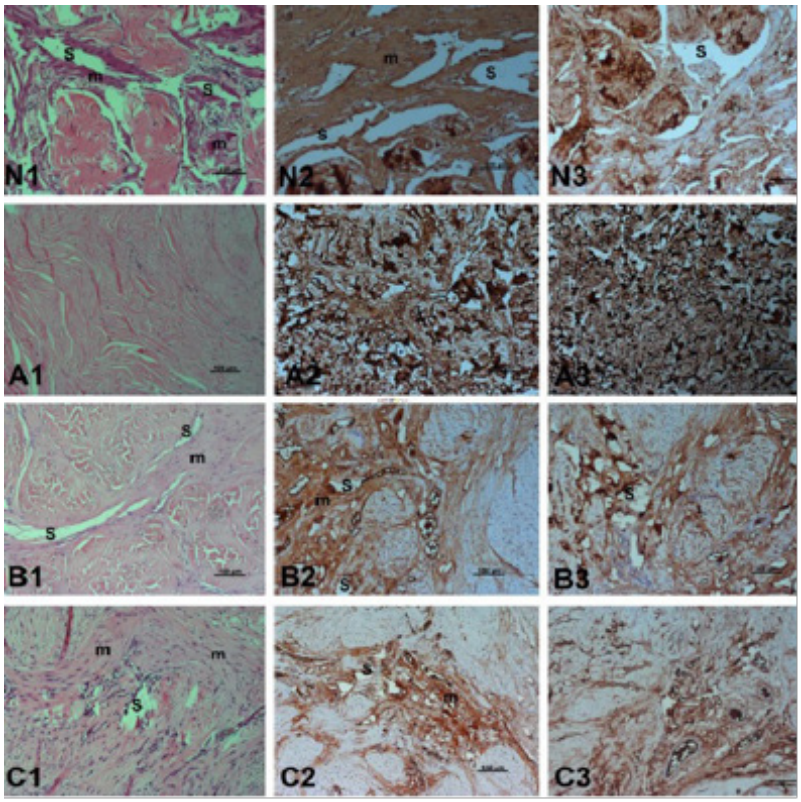

FIGURE 4 - Histological sections **N**= Normal rabbit. **A**= Matrix without cells, **B**= Matrix with cells seeded, **C**= Matrix with intravenous infused cells. **1**= H&E, **2**= antibody anti α-actina, **3**= antibody anti factor VIII. **s**= cavernous sinus, **m**= smooth muscle. Original magnification x100.

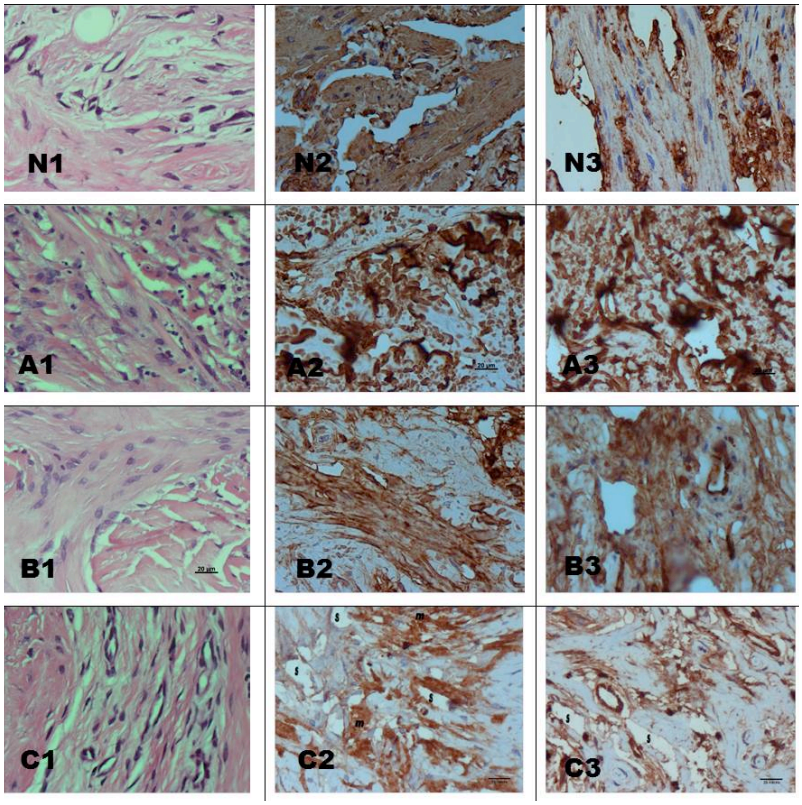

FIGURE 5 - Histological sections **N**= Normal rabbit. **A**= Matrix without cells, **B**= Matrix with cells seeded, **C**= Matrix with intravenous infused cells. **1**= H&E, **2**= antibody anti α-actina, **3**= antibody anti factor VIII. **s**= cavernous sinus, **m**= smooth muscle. Original magnification x400.

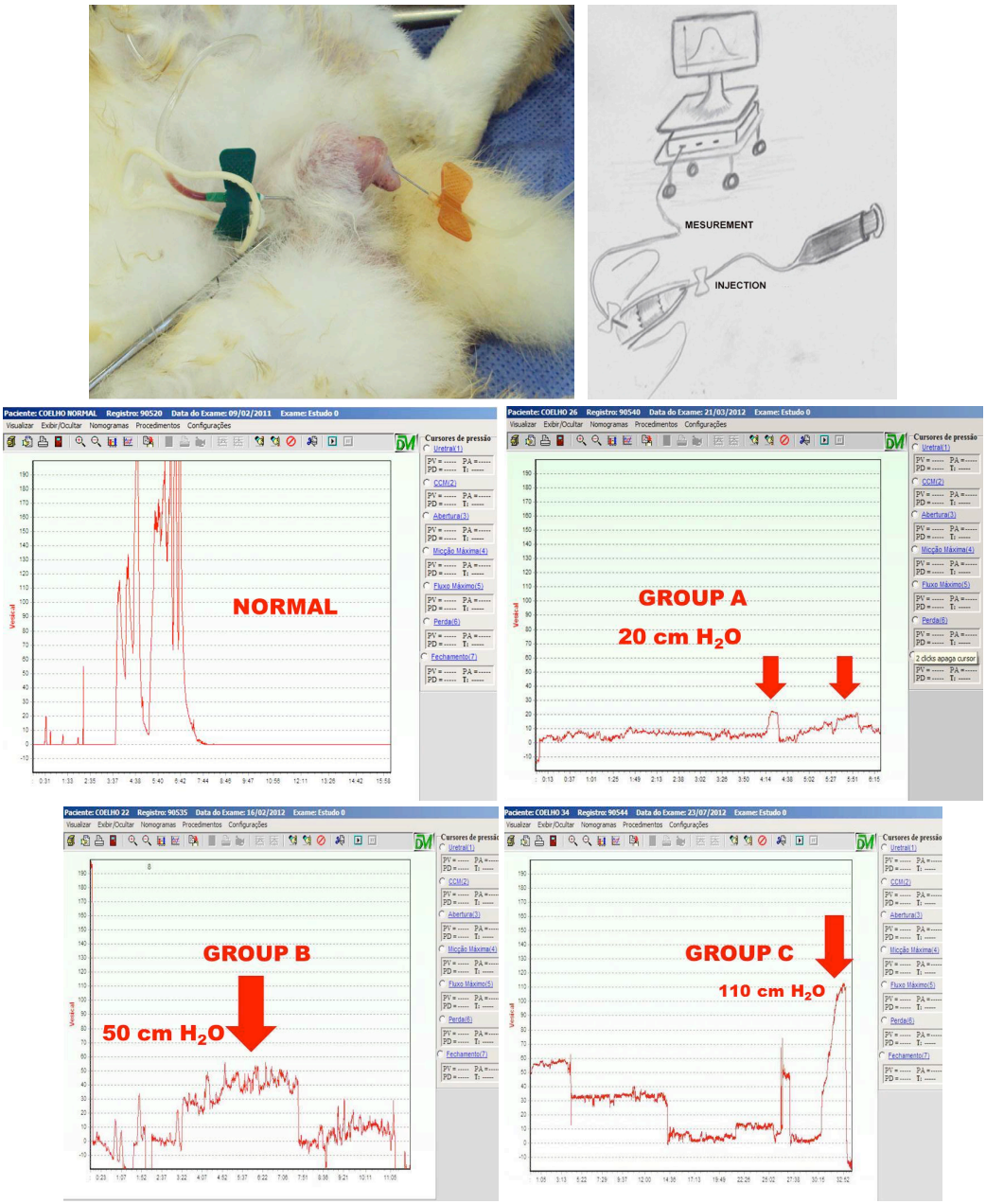

FIGURE 6 – Cavernosometry. Animals from groups A, B and C. Normal – Measures performed before the surgical procedure

Discussion

Scarcity of autologous tissue adequate for corpora cavernosa anatomic and functional reconstruction is the major technical difficulty for adequate penile reconstruction. Regenerative medicine may be a viable alternative and mesenchymal stem cells have been used for their capacity of differentiation into other mesenchymal cells, and of immunomodulation with consequent reduction of the inflammatory process[7,8,12,19]. In this study mesenchymal stem cells either seeded directly on the decellularized matrix or intravenously infused, were able to partially reconstruct cavernous sinuses similar to those of the native corpus cavernosum.

Trials using tissue engineering techniques for penile reconstruction at first used synthetic matrices[20,21]. Although formation of well vascularized smooth muscle tissue occurred, the intricate anatomy of the cavernous tissue could not be reproduced.

The solution found was to use the corpus cavernosum of cadaveric donors that once submitted to the decellularization process, provided the matrices for the receptor's cells to grow. Those structures kept the proper architecture of the penis, and maintained bioactive substances that favor cell migration, adherence, proliferation and differentiation[22-24].

In this study the rabbits' corpus cavernosum decellularized matrices were used to find out whether the topic or systemic use of allogeneic bone marrow mesenchymal stem cells could reconstruct the organ anatomy, therefore avoiding the use of autologous cells.

The dynamic studies of the distribution of infused labeled mesenchymal stem cells showed that 70% of them first accumulate in the pulmonary capillaries that work as a large barrier[25,26]. After forty-eight hours, they could be found in the liver, lungs and kidneys[25]. Cells migration to damaged tissues is favored by chemokines produced by those tissues and their specific receptors facilitate cell adherence and infiltration[8].

In previous research our group investigated the action of mesenchymal stem cells in rats undergoing bladder augmentation with bladder acellular matrices (BAM). In one of the animal groups mesenchymal stem cells were seeded on the BAM before augmentation and in the other group the animals received stem cell infusions on postoperative day one, eight and fifteen. Although in both groups the normal architecture of the bladder was restored, only the groups in which stem cells were infused showed growing of nervous fillets. Notwithstanding such results, labeled stem cells couldn't be demonstrated in the bladder when the organ was examined on postoperative day 28. Those cells are most likely to act rapidly at the site where they migrate to, through immunomodulatory activity[27].

No reports on the use of those cells in penile reconstruction experiments were found, although the use of stem cells taken from striated muscle has been reported by Ji *et al*.[28]. In this case a small fusiform longitudinal graft was used. Evaluation made in the second, fourth and sixth postoperative months showed that smooth muscle fibers were progressively organized around the collagen in the stem cell grafts, clearly forming cavernous sinuses, whereas the animals in which no stem cells were implanted (control group), although presenting muscle growth, did so in a disorganized fashion, overlapping the new collagen fibers[28]. In a recent study transfected cells were added to this experimental design to produce VEGF. A larger number of capillaries in the graft was found in the second month postoperative evaluation[29].

In this study a 7mm long segment of the corpus cavernosum was reconstructed, subjecting the graft to different levels of ischemia depending on its distance from the proximal anastomosis. Blood passage through the graft with the filling of the distal portion of the corpus cavernosum (Figure 1) could be seen in three animals in group B and in three animals in group C. Smooth muscle fibers grew in all three groups, and the groups in which stem cells were used, either seeded directly on the matrix or systemically infused, showed a better organized muscle tissue, with clear formation of smooth muscle trabeculae forming cavernous sinuses, heterogeneously intermixed by fibrosis areas (Figure 2). In group A muscle fibers were formed, arranged in a disorganized fashion, overlapping the fibrous tissue, with no clear formation of cavernous sinuses. It may be speculated that stem cells, through an immunomodulatory paracrine action, were important to keep neoformed tissue organized.

Just one animal from every group underwent cavernosometry, so as to avoid any possible alteration later in the histomorphetic study of the further rabbits. The animal from group C had the highest pressure, followed by the animal from group B. The animal from group A (Figure 3) had no significant pressure increase.

The studies to follow should use animal models that allow for vascular anastomosis for a more adequate vascularization of the new penis, while the use of VEGF, FGF and of other growth factors may enable a more exuberant vascular network to be developed in the graft, or even a more adequate neural network to be established.

Conclusion

Reconstruction of the corpus cavernosum by using decellularized matrices with intravenously infused or directly seeded mesenchymal stem cells was possible, with grown tissue similar to that of the native corpus cavernosum. No significant differences were found, either when stem cells were directly seeded on the matrix or intravenously infused.

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