DISEASES OF THE COLON & RECTUM

ORIGINAL CONTRIBUTION

Treatment of Experimental Injury of Anal Sphincters with Primary Surgical Repair and Injection of Bone Marrow-Derived Mesenchymal Stem Cells

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PURPOSE: Sphincter injury is a common cause of anal incontinence. Surgical repair remains the operation of choice; however, the outcome often is poor. We investigated the ability of injected bone marrow-derived mesenchymal stem cells to enhance sphincter healing after injury and primary repair in a preclinical model.

METHODS: Twenty-four inbred Wistar Furth rats were divided into three groups. As a control, Group A underwent sham operation. Group B had sphincterotomy and repair of both anal sphincters plus saline injections. The study group (Group C) underwent sphincterotomy and repair followed by intrasphincteric injections of syngenic bone marrow-derived mesenchymal stem cells. A further group (Group D) of outbred Wistar rats treated with mesenchymal stem cells and immunosuppressive therapy also was evaluated. At 30 days, histologic and morphometric analysis and *in vitro* contractility testing was performed.

RESULTS: A significant decrease of muscle tissue was observed at the site of repair after sphincter injury. However, in Groups C and D, histologic examination demonstrated new muscle fibers and morphometric analysis revealed a significantly greater muscle area fraction than in Group B ($P<0.05$). Moreover, mesenchymal stem cells injection improved contractility of sphincters strips compared with Group B ($P<0.05$). No significant differences were found between Groups C and D.

CONCLUSIONS: In our experimental model, bone marrow-derived mesenchymal stem cells injection improved muscle regeneration and increased contractile function of anal sphincters after injury and repair. Therefore, mesenchymal stem cells may represent an attractive tool for treating anal sphincter lesions in humans. Investigations into the biologic basis of this phenomenon should increase our knowledge on underlying mechanisms involved in sphincter repair.

KEY WORDS: Anal incontinence; Anal sphincter injury; Anal sphincter repair; Bone marrow-derived mesenchymal stem cells.

Anal incontinence is a disabling condition that affects a large number of patients and may have devastating effects on quality of life.[1,2] The mechanism of continence is complex and involves many structures and pathways. Disruption of the anal sphincters is one of the most common causes of anal incontinence and may occur as the result of obstetric injury, anorectal surgery, or trauma.[3–8] Sphincter repair is the operation of choice in patients with damaged anal sphincters, and clinical series have reported a relatively good success rate after treatment. However, the initial symptomatic improvement is not always maintained with time and long-term outcome remains unsatisfactory.[9–14]

Many reports have demonstrated that mesenchymal stem cells derived from bone marrow (MSC) can differentiate into mature cells of many tissues.[15,16] In particular, injected MSC engraft and form multinucleated myotubes in skeletal muscle under appropriate stimuli, *i.e.*, injury, contributing to muscle regeneration.[17–20] Thus, MSC injection may represent a new attractive treatment option for anal sphincter lesions. Moreover, experimental injury of detrusor muscle and urethral sphincter has been successfully treated with stem cells injection as a potential therapy for urinary incontinence.[21–26] To our knowledge, no previous report on the use of stem cells for

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the treatment of anal incontinence is present in the surgical literature.

We developed an experimental rat model of anal sphincters injury treated with primary surgical repair and investigated the contribution of injected MSC on the regeneration and the healing of damaged sphincters using histologic and functional studies.

MATERIALS AND METHODS

This study was approved by the local ethics committee and all procedures were conducted in accordance with the European legislation and with the guidelines for the care and use of laboratory animals established by the National Institutes of Health.

Twenty-four male inbred Wistar Furth rats (weight range, 250–300 g) from Charles River Laboratories (Lecco, Italy) were used and divided into three groups of eight animals each. As a control, Group A received no sphincters injury but had a sham operation plus intrasphincteric saline solution injections. A second group untreated with MSC (Group B), underwent sphincterotomy and direct primary surgical repair of both anal sphincters plus saline injections. The study group (Group C) underwent sphincterotomy and surgical repair of anal sphincters followed by intrasphincteric injections of syngenic MSC. To evaluate the effects of immunosuppressive therapy on the outcome of sphincter repair in animals treated with MSC, a further group (Group D) of eight male outbred Wistar rats (weight range, 250–300 g; Charles River Laboratories) underwent sphincters injury and repair plus injections of MSC obtained from the same strain, and received a daily intramuscular injection of 5 mg/kg of cyclosporine (CsA) (Sandimmun®, Novartis Farma, Varese, Italy) during the experimental period.

All animals were housed in single cages with a natural night and day cycle and with free access to water and commercial pellet diet (Harlan, Udine, Italy) *ad libitum*. Preoperative and postoperative clinical evaluation was performed, and the feeding and defecation behavior was daily observed to verify fecal continence and detect possible complications.

The rats were killed by using an anesthetic overdose followed by exsanguination at 30 days after treatment. Half of the animals of each group were used for histologic evaluation and half for *in vitro* functional studies.

Mesenchymal Stem Cells

Isolation and Culture Expansion. Rat bone marrow cells were collected from both inbred and outbred donor animals following the Dobson procedure [27]: once the femurs and tibiae were extracted, their proximal ends were removed, and the bones were placed in microcentrifuge tubes supported by plastic inserts cut from 1-ml hypodermic needle casings and briefly centrifuged at 700 × *g* for two minutes. The marrow pellet was resuspended in 10 ml of Hank's Balanced Salts Solution (HBSS, w/o calcium and magnesium; Euroclone, Milan, Italy) +1 percent fetal bovine serum (FBS; HyClone, South Logan, UT) and washed (300 × *g* for 7 minutes). After the cells were passed through a 22-gauge needle, they were resuspended in culture medium (Dulbecco's modified Eagles's medium-Low Glucose (DMEM-LG), with L-glutamine, HEPES 25 mM, and pyruvate; GIBCO®-Invitrogen, Milan, Italy, supplemented with 10 percent FBS), counted using a hemocytometer and seeded at 24×10^6/75 cm^2 flask. Cells were incubated at 37°C in a humidified atmosphere containing 95 percent air and 5 percent CO_2. Half of the complete medium was changed after one week and the whole medium every three to four days. When approximately 80 percent of the flask surface was covered, the adherent cells were incubated with 0.05 percent trypsin–0.02 percent EDTA (Eurobio, Courtaboeuf, France) for five to ten minutes at 37°C, harvested, washed with HBSS and 10 percent FBS, and resuspended in complete medium (primary culture, P0). Cells were then reseeded at 10^4 cells/cm^2 in 100-mm dishes (P1): expansion of the cells was obtained with successive cycles of trypsinization and reseeding.

CFU-F Frequency. The number of colony-forming units—fibroblastoid (CFU-F) was used as a surrogate marker for MSC progenitor frequency: two 100-mm dishes were seeded with 1×10^6 total nucleated cells (TNC). After incubation for 14 days at 37°C in 5 percent CO_2 humidified atmosphere, the dishes were rinsed with HBSS, fixed with methanol, and stained with Giemsa; visible colonies formed by 50 or more cells were counted and reported as number of CFU-F/10^6-seeded TNC.

Osteogenic and Adipogenic Differentiation. MSC (10^4 cells/cm^2) were grown near confluence in 25-cm^2 flasks and then incubated in osteogenic medium (DMEM-LG with 10 percent FBS, 10 nM of dexamethasone, 100 μg/ml of ascorbic acid, and 10 mM of β-glycerophosphate; Sigma, St. Louis, MO) or in adipogenic medium (DMEM-LG with 10 percent FBS, 0.5 mM of isobutyl methylxanthine, 10 μM of dexamethasone, 10 μg/ml of insulin, and 70 μM of indomethacin; Sigma). The medium was replaced every 3 to 4 days, and the deposition of mineral nodules or the accumulation of lipid-containing vacuoles was revealed after 21 days with Alizarin Red-S or with Oil Red O staining, respectively.

Immunophenotyping. At the fourth or fifth passage, the morphologically homogeneous population of MSC was analyzed for the expression of particular cell surface molecules by using flow cytometry procedures: MSC

recovered from flasks by trypsin-EDTA treatment and washed in HBSS and FBS 10 percent, were resuspended in flow cytometry buffer consisting of Cell WASH (0.1 percent sodium azide in phosphate-buffered saline (PBS); Becton Dickinson, San Jose, CA) with 2 percent FBS. Aliquots (1.5×10^5 cells/100 μl) were incubated with the following conjugated monoclonal antibodies: CD 45-CyChrome, CD 11b-FITC (to quantify hemopoietic–monocytic contamination), CD 90-PE, CD 106-PE, CD 73-PE, CD 54-FITC, and CD 44-FITC (BD Pharmingen, San Diego, CA). Nonspecific fluorescence and morphologic parameters of the cells were determined by incubation of the same cell aliquot with isotype-matched mouse monoclonal antibodies (Becton Dickinson, San Diego, CA). All incubations were performed for 20 minutes and, after incubation, cells were washed and resuspended in 100 μl of Cell WASH; 7-AAD was added to exclude dead cells from the analysis. Flow cytometric acquisition was performed by collecting 10^4 events on a FACSort instrument (488 nm argon laser equipped; Becton Dickinson), and data were analyzed on DOT-PLOT biparametric diagrams by using CELL QUEST software (Becton Dickinson) on Macintosh PC.

Surgical Procedure

Animals were anesthetized with intraperitoneal injection of a combination of tiletamine and zolazepam (Zoletil®, Virbac, Milan, Italy) and xilazine (Xilor®, Bio 98, Bologna, Italy). The perineum was shaved and the skin washed with povidone-iodine solution (Poviderm®, Nuova Farmec, Verona, Italy). Injury was performed under an operating microscope (OMPI CS XY Carl Zeiss, Milan, Italy) by a left-lateral full-thickness internal and external sphincterotomy. A left circumanal incision was performed, and the mucosa of the anal canal was separated from the sphincters by soft dissection. Both external (EAS) and internal anal sphincter (IAS) were isolated, exposed and then divided one by one using a knife. The continuity of the muscles was restored with an end-to-end suture performed with absorbable stitches (7/0 Vicryl®, Ethicon, Rome, Italy). Using a 50-μl Hamilton microsyringe, an injection of MSC (10 μl with approximately 0.75×10^6 cells) or normal saline solution (10 μl) was made into each cut end of both sphincters (2 injections for each sphincter). On sham-operated animals, two injections of saline solution (10 μl) were performed at the 3-o'clock position of each isolated sphincter. The skin wound was closed with absorbable interrupted suture.

Histologic Evaluation

A ring specimen, including the anal canal and the terminal rectum, was removed *en bloc* and rapidly fixed by immersion in Karnovsky reagent (4 percent paraformaldehyde, 2.5 percent glutaraldehyde in 0.1 M of cacodylate buffer, pH 7.35) for 24 hours at 4°C, dehydrated in ethanol, and embedded in methacrylate (Technovit 7100, Heraeus Kulzer GmbH, Werheim/TS, Germany). Cross-sections at 3-μm to 5-μm thickness were cut with a Reichert Jung microtome (Leica Microsystems, Milan, Italy) along a horizontal plane, stained with 0.1 percent toluidine blue, and then observed under a Nikon Eclipse E600 microscope (Nikon Instruments, Florence, Italy).

To evaluate the regeneration of the sphincters at the site of surgery, a quantitative morphometric study using a computer-assisted image analysis was performed in stained samples. A mean of 12 microscopic fields for each anal sphincter was manually searched at the site of repair at 4x magnification and, subsequently, the muscle area fraction (MAF) was automatically measured with the Nikon Nis-Elements AR 2.10 software package (Nikon Instruments, Florence, Italy). This system creates a binary image through color intensity thresholds and calculates the ratio between the area of muscle elements (identified as smooth or striated at histologic examination) and the measured area of interest. The analysis was performed by two independent operators in a blinded fashion and repeated measurements ensured constant and reproducible results.

In Vitro Functional Studies

The anal canal and the rectum were removed *en bloc* and cleaned of excess connective tissue. Specimen was opened at the 9-o'clock position (opposite to the site of the injury) and pinned out in a Petri dish. Under microscope guide, the mucosa was carefully removed and the IAS and EAS were identified. Strips were dissected following the direction of the muscle bundles and included in the middle of the injured area. One smooth and one striated muscle strip was obtained from each rat. IAS strips measured approximately 5-mm × 1-mm × 1-mm (weight range, 5–10 mg); EAS strips were prepared by using the entire sphincter circumference and measured approximately 10-mm x 1-mm x 1-mm (weight range, 15–25 mg). Strips were tied at each end with fine silk ligatures, mounted in small organ baths (0.2 ml) between two platinum ring electrodes 1-cm apart, and continuously superfused with oxygenated (95 percent O_2, 5 percent CO_2) Krebs' solution (pH 7.2; 37°C) at a flow rate of approximately 1 ml per minute.[28] Krebs' solution consisted of (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, $MgCl_2$ 1.5, NaH_2PO_4 1, $CaCl_2$ 2.5, glucose 11.5. The apparatus allowed four strips to be studied simultaneously. Each strip was placed under an initial tension of 1-g weight and allowed to equilibrate for at least one hour.

Isometric contractions were measured by using mechano-electrical transducers (Ugo Basile, Varese, Italy) and recorded on Chart v3.6 data acquisition system (AD Instruments, Chalgrove, UK). Muscle strips were stimulated by electrical field stimulation (EFS), delivered via a Grass stimulator (Grass Instruments, West Warwick, RI).

Preliminary experiments were performed to establish optimal EFS parameters. Five-second train pulses, 0.01-ms

pulse width, 50 V voltage, with frequency varying from 1 to 20 Hz were selected to stimulate the IAS; 5-second train pulses of different pulse widths (0.01–50 ms), 50 V voltage, and 1 and 20 Hz frequency were selected to stimulate the EAS. The effects of tetrodotoxin (TTX) (3 μM; Sigma) and d-tubocurarine (100 μM; Sigma), on the EFS response, also were assessed. TTX was used to block the IAS nerve mediated muscle contraction; d-tubocurarine, to block the neuromuscular junction at the EAS. For both IAS and EAS strips, a minimum of five minutes recovery between stimulations ensured no diminution of the response.

To investigate the intrinsic contractile ability of the IAS, strips were stimulated with carbachol (10^{-5}M; Sigma). The responses were evaluated by an examiner blinded to the experimental groups.

Statistical Analysis

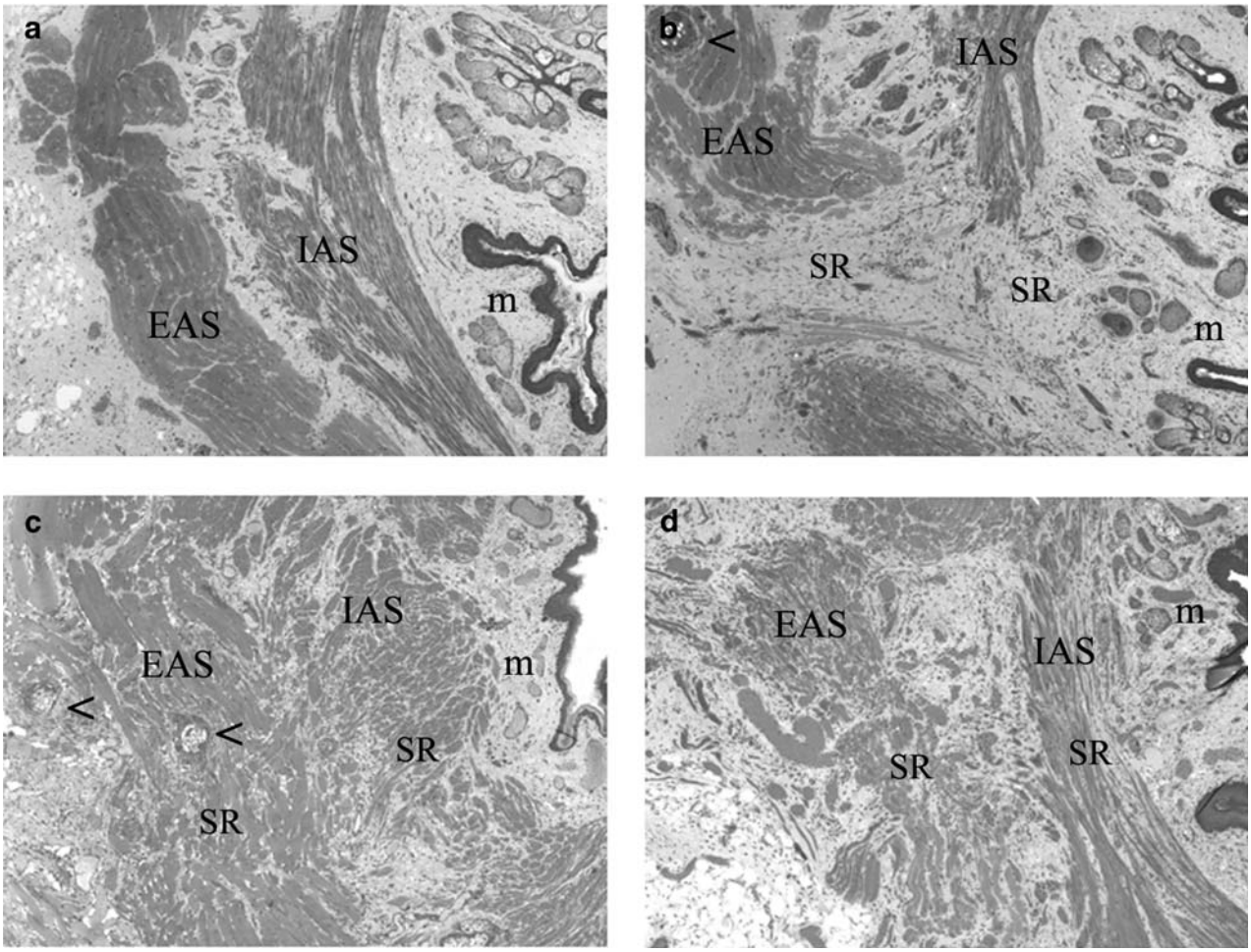
The results of morphometric analysis and the responses of sphincters strips to EFS and chemical agents are expressed as mean ± standard error of the mean (SEM) of four animals. Statistical analysis of the data was performed by Student's *t*-test for unpaired samples, or by one-way ANOVA followed by Dunnett's test for multiple comparisons. $P<0.05$ was considered significant.

RESULTS

MSC Characterization

Rat bone marrow-derived MSC were culture-expanded successfully. Cells were particularly heterogeneous until the fourth-fifth passage in culture and also included numerous lipid vacuoles. Hematopoietic cells were lost during the medium changes. Primary culture cells (14 days) were trypsinized and plated, reaching a cellular expansion up to a factor of 10^9 in three months. After the fifth passage, the cells grew exponentially, requiring weekly passages. The CFU-F assay was used as a surrogate for mesenchymal progenitors. In bone marrow, the total nu-

FIGURE 1. Histologic examination after four weeks. The external anal sphincter (EAS) appeared more compact than the internal anal sphincter (IAS) (Group A, **a**). In Group B, a gap filled with fibrous connective tissue and dilated blood vessels was observed within each muscle layer at the site of sphincter repair (SR) (**b**). Injected MSC led to increased sphincter mass at the site of SR in Group C (**c**) and Group D (**d**). *m* = mucosa; *arrows* = residual suture material (original magnification; 4x) (Group A: sham; Group B: SR + saline; Group C: SR + MSC; Group D: SR + MSC + CsA). SR = sphincter repair; MSC = bone marrow-derived mesenchymal stem cells; CsA = cyclosporine.



cleated cell population (the estimated CFU-F efficiency) was 56 (mean)/10^6 TNC. MSC treated with osteogenic medium formed small deposits of hydroxyapatite stained intensely red with Alizarin S. MSC treated with adipogenic medium were successfully differentiated toward adipogenic lineage: lipid vacuoles started to accumulate in the cytoplasm of the cells after just 2 to 3 days of stimulation and they were orange-red stained after 21 days. FACS analysis was used to assess the purity of MSC and the existence of a homogeneous population of adherent cells (after 4–5 passages). After the exclusion of dead cells (R1 on 7-AAD negative elements), the cell population was uniformly positive for CD 90, CD 44, CD 54, CD 73, CD 106. There was no significant contamination of hematopoietic cells, as flow cytometry was negative for markers of hematopoietic lineage, including CD 11b and CD 45.

Clinical Evaluation

Treatment was well tolerated by all animals. They had a rapid recovery and quickly reestablished a normal defecation behavior with emission of feces of normal consistency, size, and shape. During the postoperative period, no animal had proctologic complications or symptoms of fecal incontinence or obstructed defecation. The feeding behavior and the body weight growth were similar in all rats. Neither death nor side effects were observed during the course of the experiment.

Histologic Analysis

At histologic examination, the EAS appeared more compact compared with the IAS, which contained multiple connective septa between the muscle bundles (Group A; Fig. 1a). The area of injury was easily detected because of the presence of residual suture material surrounded by a flogistic reaction. In Group B, which was damaged and repaired but not treated with MSC, a gap within the muscle layers of both IAS and EAS was always observed at the site of surgery. Fibrous connective tissue with dilated blood vessels filled the gap and pale muscle cells in course of degeneration were seen at the borders (Fig. 1b). In contrast, in Groups C and D, which had been injected with stem cells, there was no evidence of a gap at the site of injury and the area appeared rich in irregularly disposed muscle cells of different sizes (Fig. 1c and d). Moreover, fusiform-shaped cells with a single central nucleus were observed at the site of IAS repair, whereas polynucleated cells with transversal striation, because of sarcomeres, and myotubes were found at the site of EAS repair and indicated the presence of selective regeneration of each sphincter (Fig. 2a and b).

The results of the morphometric analysis are shown in Table 1. In Group B, the MAF of EAS and IAS at the site of sphincter repair was significantly lower than in control rats ($P<0.001$ and $P<0.01$, respectively).

The injection of MSC resulted in increased muscle tissue in the repaired area. In Group C (treated with syngenic MSC), the MAF of EAS and IAS was lower than in control group ($P<0.01$ and $P<0.05$, respectively) but significantly higher compared with Group B ($P<0.01$ and $P<0.05$, respectively). In Group D (treated with MSC plus CsA), statistical analysis showed similar values to Group C. The MAF of EAS was lower than in sham-operated animals ($P<0.05$) but significantly higher than in Group B ($P<0.05$). Moreover, a significant difference was observed between the MAF of IAS in Group D *vs.* Group B ($P<0.05$) but not in Group D *vs.*control rats.

FIGURE 2. Area of repair of the external anal sphincter of an animal of Group D. A number of cells of small and medium size with pale voluminous nuclei aligned in the central position of the myofibers were observed at the site of sphincter repair. They, as well as adjacent round cells with central nuclei (*arrows*), represent immature muscle cells or myotubes (*my*) and indicate the presence of muscular regeneration of the sphincter (**a**, **b**) (original magnification; 40x). Group D: sphincter repair + injection of bone-marrow mesenchymal stem cells + cyclosporine.

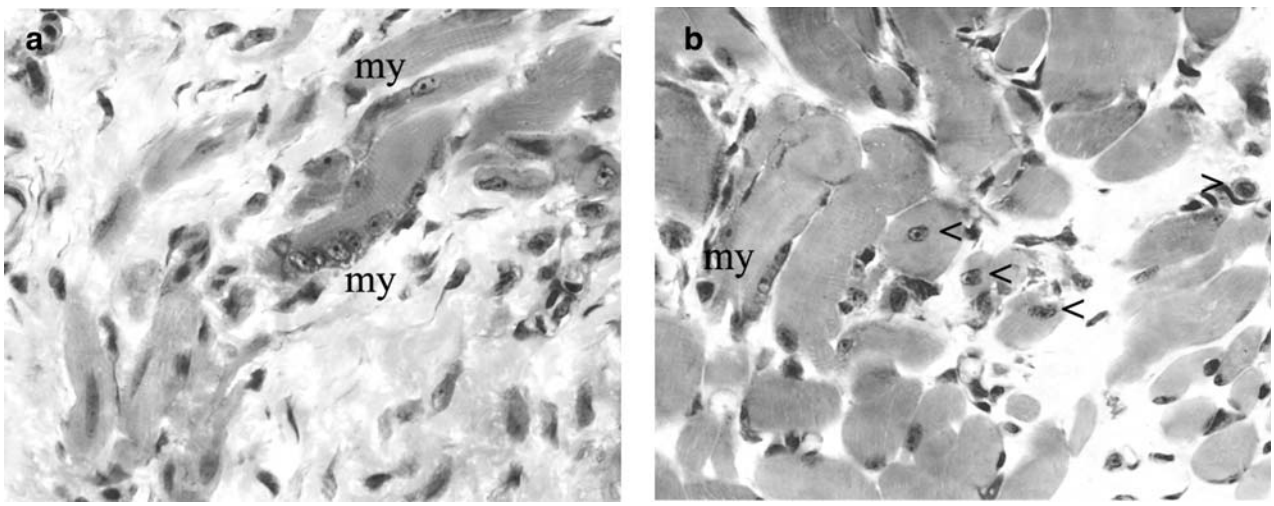


Table 1. Muscle area fraction of internal and external anal sphincter at the site of sphincter repair one month after surgery

Group	Muscle area fraction	
	Internal anal sphincter	*External anal sphincter*
A (sham)	0.7904±0.035	0.8904±0.025
B (SR + saline)	0.449±0.042[*]	0.3799±0.035[‡]
C (SR + MSC)	0.5973±0.004[†,§]	0.636±0.023[*,‖]
D (SR + MSC + CsA)	0.7247±0.058[§]	0.6801±0.062[†,§]

SR = sphincter repair; MSC = bone marrow-derived mesenchymal stem cells; CsA = cyclosporine. • Data are means ± standard errors of the means (4 animals). • [†]$P<0.05$, [*]$P<0.01$, [‡]$P<0.001$ *vs.* Group A; [§]$P<0.05$, [‖]$P<0.01$ *vs.* Group B; Group C *vs.* Group D not significant.

Responses of Sphincters Strips to Electrical and Chemical Stimulations

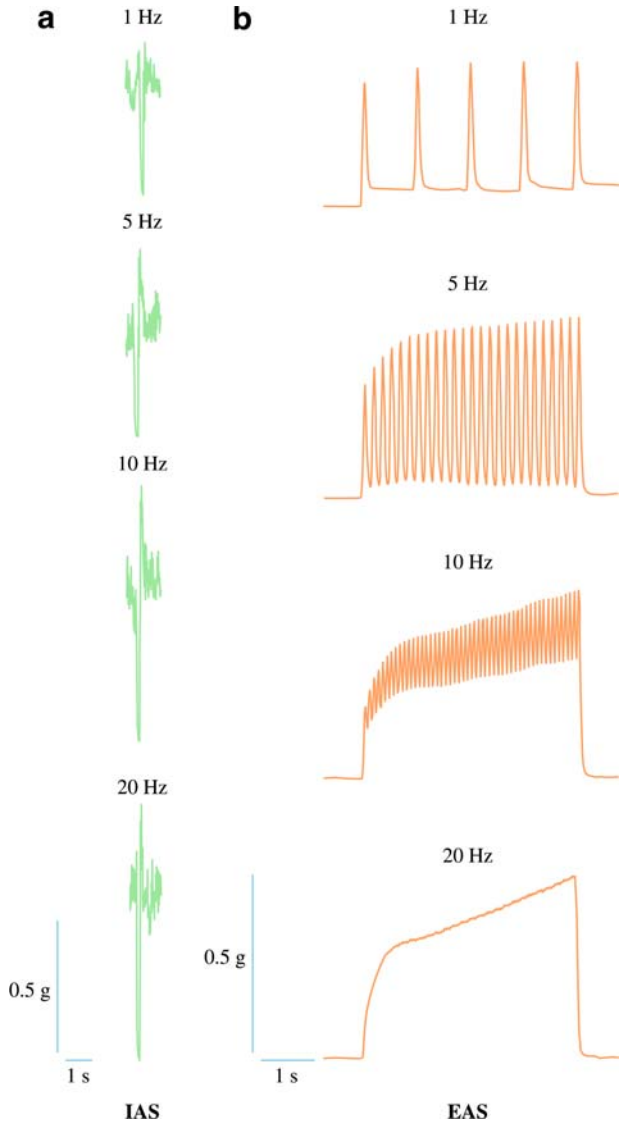
Preliminary studies were performed to decide the length of the anal sphincters strips and the EFS parameters to be used to obtain an adequate response. EAS strips had to be prepared using the whole circumference of the sphincter to preserve activity in the long striated muscle fibers and thus enable a twitch response of 14.6±5.8 mg/mg tissue at 1 Hz stimulation and at low width of impulse (0.01 ms); in contrast, IAS strips of approximately 5 mm, including the injured part in the middle, developed good EFS responses (39.3±17.5 mg/mg tissue at 1 Hz stimulation). The contractile response of the EFS parameters used to stimulate IAS strips (5-second train pulses; 0.01 ms duration; 50 V voltage; frequency, 1–20 Hz) was an initial relaxation followed by a contraction. The relaxation response predominated at low stimulation frequencies, whereas the contractile element became more evident at higher frequencies (Fig. 3a). Both relaxation and contraction was blocked by 3 μM TTX, thus confirming that the response was nerve-mediated.

EAS strips were stimulated by keeping constant the width of the impulse (0.01 ms), while varying the frequency of stimulation (1–20 Hz), or *vice versa* (impulse width increased from 0.01–50 ms; frequency kept to 1–20 Hz). At pulse widths less than 1 ms, simple twitch responses were seen to stimulation frequencies up to 5 Hz, with summation of contraction at higher frequencies leading to partially fused tetanus at 20 Hz stimulation (Fig. 3b). At high frequencies, there was an initial peak followed by a small drop in pressure, which was maintained throughout the stimulation. The peak pressure increased with an increase in frequency. These fast-twitch responses of the striated muscle to EFS were blocked by TTX or d-tubocurarine. With increasing pulse width, some direct activation of the muscle seemed to occur, and these drugs became less effective. Contractions appeared again in the presence of d-tubocurarine with a pulse width of 10 ms (20 Hz), although they were significantly lower than the contractions recorded in its absence.

After 40 minutes equilibration, IAS control strips, but not EAS control strips, had developed a spontaneous tone, which was 22.9±7.5 percent higher than the tension applied. IAS strips of the damaged groups (B, C, and D) had developed a frequent and irregular contractile activity.

To investigate whether the contractile ability of IAS strips differed among the four groups, the strips were exposed to exogenous carbachol. The response to carba-

FIGURE 3. Representative traces showing responses of rat internal (IAS) and external anal sphincter (EAS) to different electrical field stimulation (EFS) parameters (5-second train pulses, 0.01 ms duration, 50 V voltage, 1–20 Hz frequency). An initial relaxation followed by a contraction was observed in IAS strips; the relaxation response predominated at low stimulation frequencies, whereas the contractile element became more evident at higher frequencies (**a**). EAS strips developed simple twitch responses to stimulation frequencies up to 5 Hz, with summation of contraction at higher frequencies leading to partially fused tetanus at 20 Hz stimulation (**b**). Scale bars: horizontal, 1 second (time); vertical, 0.5 g (tension) (Group A: sham).



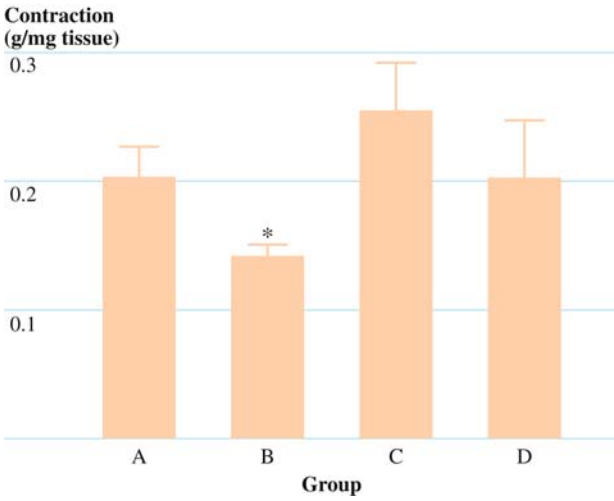


FIGURE 4. Carbachol (10^{-5} M) induced contractile responses of rat internal anal sphincter (IAS). Results are expressed as tension developed g/mg of wet tissue (mean ± SEM for 4 animals). * $P<0.05$ *vs.*all groups (Group A: sham; Group B: SR + saline; Group C: SR + MSC; Group D: SR + MSC + CsA). SR = sphincter repair; MSC = bone marrow-derived mesenchymal stem cells; CsA = cyclosporine.

chol of Group B strips (damage and repair, no MSC injection) was significantly lower than that of Groups A, C, and D (Fig. 4). Groups C and D strips responses were not significantly different from each other, and results from these groups have been pooled.

In Figure 5a, the relaxation developed by IAS strips in response to EFS stimulation is summarized. Relaxation responses were significantly different among the groups. In particular, Group B strips showed the lowest values of relaxation in response to EFS at all frequencies of stimulation. However, the contractile responses to EFS stimulations were not significantly different among the groups at all frequencies of stimulation applied (Fig. 5b).

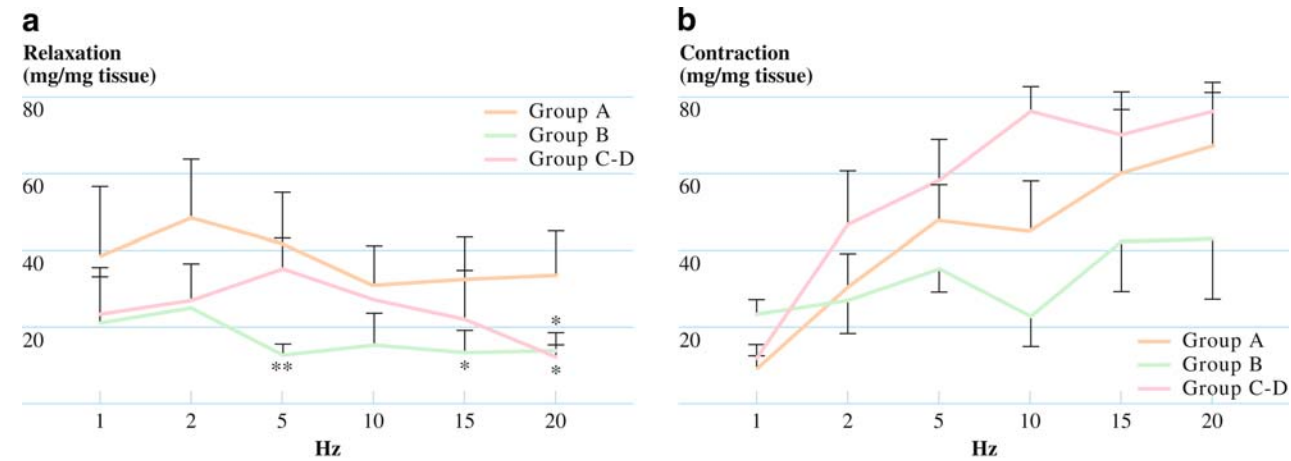
As to the function of EAS strips (Fig. 6), the contraction evoked in the control Group A by EFS was significantly greater than that of strips from Group B. The EAS strips of Groups C and D showed an improvement in their EFS response compared with Group B, which was more evident at mid to high frequency of stimulation and at low width of the stimulus. In contrast, at high impulse width combined with high frequency of stimulation, this improvement was not evident.

DISCUSSION

In recent years, several studies have investigated the ability of MSC to differentiate into mature cells of many tissues, both *in vitro* and *in vivo*, and specific improvement of tissue repair has been described in different organs after injury and stem cells injection.[15,16] In muscular tissue, in particular, injected MSC have the capacity to engraft and form multinucleated myotubes participating effectively in regeneration after injury.[17–20] The injection of stem cells as a potential treatment of urinary incontinence has been already successfully reported in animal models. Injected stem cells have been shown to enhance regeneration and improve contractility of injured detrusor muscle and urethral sphincter.[21–26] Therefore, MSC injection may represent a new treatment option for anal incontinence caused by sphincteric lesions, and we have investigated the contribution of such cells on the regeneration and the healing of anal sphincters after injury and surgical repair in an experimental model.

Several factors have been suggested to influence the outcome of sphincter repair, and heterogeneous findings

FIGURE 5. Electrical field stimulation (EFS) (5-second train pulses, 0.01 ms duration, 50 V voltage, and 1–20 Hz frequency) induced relaxation (**a**) and contraction (**b**) of rat internal anal sphincter (IAS). Results are expressed as mg/mg of wet tissue (mean ± SEM for 4 animals). * $P<0.05$, ** $P<0.001$ *vs.* Group A (Group A: sham; Group B: SR + saline; Group C: SR + MSC; Group D: SR + MSC + CsA). SR = sphincter repair; MSC = bone marrow-derived mesenchymal stem cells; CsA = cyclosporine.



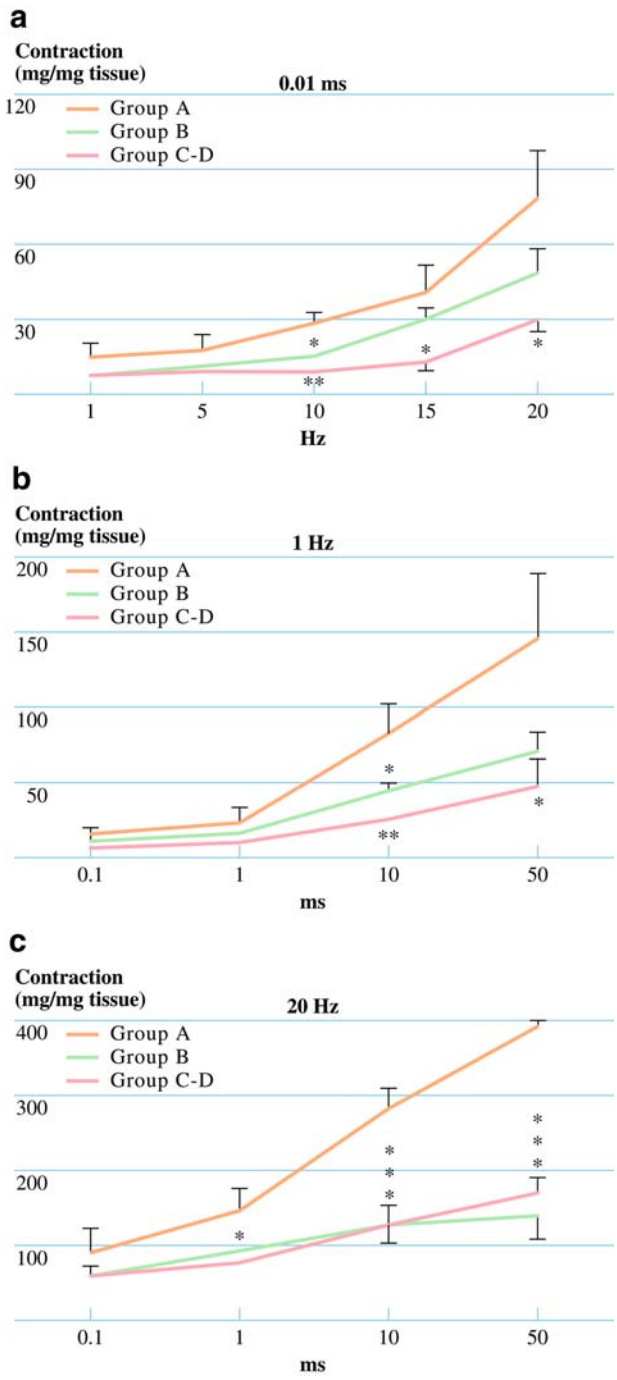


FIGURE 6. Twitch responses of rat external anal sphincter (EAS) induced by different parameters of electrical field stimulation (EFS). **a** 5-second train pulses, 0.01 ms duration, 50 V voltage, and 1–20 Hz frequency. **b** 5-second train pulses, 0.1–50 ms duration, 50 V voltage, and 1 Hz frequency. **c** 5-second train pulses, 0.1–50 ms duration, 50 V voltage, and 20 Hz frequency. Results are expressed as mg/mg of wet tissue (mean ± SEM for 4 animals). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs.* Group A (Group A: sham; Group B: SR + saline; Group C: SR + MSC; Group D: SR + MSC + CsA). SR = sphincter repair; MSC = bone marrow-derived mesenchymal stem cells; CsA = cyclosporine.

have emerged from different series. So far, the patient's age,[11,12,29] the technical failure of the repair,[14] the presence of pudendal neuropathy,[3,30] and the atrophy and fibrosis of the sphincter muscle with associated impaired contractility[10,11,14] have been identified as important negative prognostic factors. Moreover, the type of injury (obstetric, traumatic, or subsequent to anal surgery),[11] the number of the sphincters involved (isolated or combined EAS and IAS injury),[9,14,31] the extent of sphincteric injury (partial or total damage of each sphincter),[31] the surgical technique adopted (ovelapping or end-to-end),[32–36] the time of the repair (immediate or delayed),[34,35] and the addition of a levatorplasty[35] or a stoma[29] have all been considered as potential factors affecting the outcome of surgery. Furthermore, surgical repair of damaged IAS has not been extensively studied, and the few series available reported disappointing results.[37,38]

Relatively little is known about the quality of tissue repair, although this element certainly has an important role on clinical outcome. In our experiment, sphincter muscles were intact, the lesion was linear and the repair immediate, so that all the detrimental effects related to age, pathologies, or delayed repair were absent. Nevertheless, even in such an ideal situation, histologic examination revealed a gap between muscular bundles with fibrous connective tissue and poor regeneration at the site of repair. Moreover, our contractility tests showed complex damage with reduced responses to EFS, presumably resulting from changes to the intrinsic nerves and the neuromuscular junctions at the site of injury.

In our study, injected MSC led to the formation of new myotubes and myofibers and improved the contractility of anal sphincters after injury and repair. After MSC injection, we observed a significant anatomofunctional improvement of tissue repair resulting in increased MAF and higher responses of sphincters strips to electrical and chemical stimulations. The regenerative effect of stem cells may consequently have clinical relevance in the treatment of sphincteric injuries and lead to a better outcome after repair, because the physiologic reparative process normally fails to restore original tissue.

Interestingly, we found that injected MSC had an enhancing effect on the responses of the strips to carbachol rather than to EFS. It is likely that MSC mainly differentiate into the muscle lineage and the repair affects the muscle cells more than the intrinsic nerves, which are less able to regenerate. Neuronal damage seems to be more serious and resistant than muscle damage, although regeneration of nerve endings and neuromuscular junctions has been recently hypothesized after injection of muscle precursor cells into a rat model of urethral sphincter injury[24] and unilateral pelvic nerve transection.[26] The authors showed restoration of functional motor units and autonomic peripheral nerves by stem cells autograft; however, these preliminary results need to be further investigated.

Various mechanisms have been suggested to be involved in the reparative effects of MSC, not only in muscle but also in other organs and tissues. Direct engraftment of injected MSC, which can differentiate and form myotubes or fuse with damaged fibers, has been postulated as well as activation of local quiescent muscle precursor cells.[17–20] Moreover, many mediators and growth factors seem to be involved and promote the regenerative process.[39] A trophic action of injected cells without differentiation and engraftment has been demonstrated in a model of kidney injury.[40] However, the biologic basis of this phenomenon is still not well known. Our main purpose has been to determine whether MSC injection could improve anal sphincter repair after injury and further studies are needed to elucidate the underlying mechanisms involved. Moreover, at four weeks after injury and surgical repair, we observed advanced healing of the lesion; however, the long-term evolution of the regenerative process may have clinical relevance and needs to be assessed at different intervals of time in further experiments.

It also is noteworthy that the number of injected cells may be extremely critical to achieve satisfying results. We chose a number of cells comparable to the amount that other investigators have previously used in rat models of urinary incontinence,[21,22,25] and their effectiveness also could be related to the high degree of purity of injected MSC. Nevertheless, the optimal number of cells remains to be explored.

In the present work, sphincteric injury was directly followed by repair and MSC injection; however, delayed surgery often is required.[13,29,30] Therefore, the contribution of injected MSC to anal sphincter regeneration on a preexisting scar and after delayed repair, with fibrosis and weakness of the sphincter, needs to be assessed in further experiments. Moreover, each sphincter was divided and repaired one by one, and, at histologic examination, we found that MSC improved smooth or striated muscular regeneration if injected into the IAS or the EAS, respectively. Nevertheless, a mixed repair of the sphincter complex may occur after a crushed injury, and the contribution of stem cells to such a repair needs to be further investigated.

Another interesting finding is that there were no significant differences between results of the two groups treated with MSC. Autologous cells undoubtedly present major advantages; however, allogenic cells may be useful when a rapid employement is required, even if a period of immunosuppressive therapy has to be administered.[41] In our experiment, immunosuppression with CsA had no negative effects on the outcome of sphincter repair associated with MSC injection. Furthermore, it has been shown that various stem cell types, including muscle-derived cells, can differentiate into muscle lineage.[42,43] Thus, additional studies will be necessary in the future to examine and compare different stem cells proposed to anal sphincter repair after injury.

CONCLUSIONS

This study provides an experimental basis for potential future clinical application of stem cells therapy for anal sphincter injuries. Stem cells could be injected at the moment of surgical repair or percutaneously by ultrasonographic guide in selected injured areas and improve the regeneration and the healing of damaged anal sphincters. Nevertheless, further studies are required to confirm and expand our preliminary results before attempting this in the clinical setting.

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