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# Human Muscle-Derived Stem Cells. Effectiveness in animal models of faecal incontinence. Research scheduling

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SUMMARY: **Human Muscle-Derived Stem Cells. Effectiveness in animal models of faecal incontinence. Research scheduling.**

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*Researchers believe that human muscle-derived cells are able to restore leak-point pressure to normal levels by differentiating into new muscle fibres that prevent anal sphincter muscle atrophy. Laboratory data are needed to identify exactly how these cells work to regenerate muscle. The objective of this study is to test whether stem cells can be employed to treat internal anal sphincter (IAS) injuries in humans; to this end, this work will use a two-step process to study: first, the effectiveness of the treatment in a sample of animals with artificial injuries to the IAS and then to verify the results in a population of selected humans affected by pathology.*

RIASSUNTO: **Cellule staminali derivate dal muscolo umano. Efficacia in modelli animali di incontinenza fecale. Programma di ricerca.**

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*I ricercatori ipotizzano che sia possibile utilizzare cellule derivate dalla muscolatura umana capaci di differenziarsi in nuove fibre muscolari per riparare lesioni dello sfintere anale. Per identificare esattamente come queste cellule possano rigenerare il muscolo sono necessari dati sperimentali. L'obiettivo di questo studio è quello di verificare se le cellule staminali possano essere utilizzate per trattare le lesioni dello sfintere anale interno nell'uomo. A tal fine, è stato programmato un processo in due tappe: inizialmente si testerà l'efficacia del trattamento in animali con lesione artificiale dello sfintere anale interno e successivamente si procederà su una popolazione umana affetta da tale patologia.*

KEY WORDS: Faecal incontinence - Muscle-Derived Stem Cells - Electromyography - Anal pressure - Sphincterotomy - Pudendal nerve.
Incontinenza fecale - Cellule staminali derivate dal muscolo - Elettromiografia - Pressione anale - Sfinterotomia - Nervo pudendo.

## Introduction

Faecal incontinence is a clinical condition that can have devastating social, psychological, and economic consequences both for children and adults. Between 2% and 15% of the population is affected by this pathology as assessed by many epidemiologic studies (1,2).

Many factors influence faecal continence in adults and children, such as stool consistency and volume, colon transit time, rectal compliance, sphincter injuries, and obstetric and ano-rectal surgery (3-5). Surgical intervention has been advocated for patients with faecal incontinence in appropriate cases (6,7). There have also been continuous improvements in surgical care and development of new surgical options.

Three structures (muscles) are responsible for anal canal closure: the smooth muscle internal anal sphincter (IAS), the striated muscle external anal sphincter, and the striated muscle puborectalis muscle. The IAS is a specialized circular smooth muscle with elevated basal tone compared with the rectum, and it plays a significant role in recto-anal continence.

The IAS is characterized by its ability to contract (maintain tone) and to relax; in this case the passage of stool is allowed. Although the major tone is due to myogenic properties, a number of neuro-humoral and hor-

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monal responses of the muscle cells also contribute to the tone in the IAS.

Stem cells are special cells that have self-renewing properties and are able to differentiate into one or many different specialised cell types. During the past five years, they have received a lot of interest owing to their potential use in cell-based treatments for human neurodegenerative diseases, stroke, and muscular dystrophies (8-12).

Toti-potent or pluri-potent stem cells are generally plentiful in embryonic or fetal tissue, although their use can lead to certain ethical problems.

Adult stem cells can differentiate into a wide variety of cell types: skin stem cells can differentiate into neurons and smooth-muscle cells; fat stem cells can produce osteogenic, myogenic, and chondrogenic cells; bone-marrow mesenchymal stem cells can differentiate into blood, myogenic, vascular, and neurogenic cells.

In the last two years, it has been observed that skeletal-muscle satellite cells, normally quiescent, can be activated in response to muscle trauma; thus it was hypothesized that they could regenerate muscle fibres.

However, recent studies have shown that adult skeletal-muscle tissue contains progenitors or pluripotent stem cells that are able to differentiate into several phenotypes of muscle cells, suggesting that the pool of skeletal-muscle derived stem cells include distinct subpopulations of precursors, with different differentiating capacities and that satellite cells may represent only one of these subsets.

## Objective

The objective of this work is to test whether stem cells can be employed to treat internal anal sphincter (IAS) injuries in humans. To this end, the study will use a two-step process to study first the effectiveness of the treatment in a sample of animals with artificially-induced injuries to the IAS and then to verify the results in a population of selected humans affected by the pathology.

The study will follow a quantitative approach to evaluating the effectiveness of the treatment, and will integrate certain well-known techniques (such as EMG, tissues analysis, manometry, etc.). It will also employ an index of effectiveness.

## Methods

In both the animal study and the human study an appropriate Design Of Experiment (DOE) will be employed. The animal study will be planed in two stages; in the first one the total sample of Wister rats will be divided into three groups: treated with stem cells, with placebo and not treated.

The results obtained in this stage of the first step will be used to establish an estimate of the variability of the effectiveness index that will also be used in order to find the most appropriate size of the sample (and also consider further factors such as sex, age, etc.) of the second stage of the first step.

Regarding the protocol used to isolate and to culture adult human muscle derived stem cells, the authors will refer to small samples of muscle taken from patients and/or Mesenchymal Stem Cells (MSCs) from Human Umbilical Cord Blood (UCB).

*Human muscle derived-stem cell isolation*

The study protocol requires that the human muscle-derived stem cells (hMDCs) be isolated from a primary culture. After the informed consent of the patient, tumour-free skeletal muscle sections (10×5×5 mm) will be taken during abdominal surgery (rectus abdominis).

Muscle samples will be minced, digested at 37°C by 0.2% collagenase, trypsiniz, filtered through 70 m filters, and cultured in cell culture dishes (35 mm diameter) at 37°C in F12 complete medium with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After culturing the cells for 1 hour, the adherent fibroblasts will be discarded and non-adherent cells will be collected and re-plated in fresh collagen coated cell culture dishes. When 30–40% of the cells have adhered to each tissue dish, the serial re-plating of the supernatant will be repeated.

The culture will be enriched with small, round cells after 3–4 serial plantings.

Purification of the stem cells will be performed by CD34 cell selection; cells will be subjected to immune-magnetic separation using the Dynal CD34 progenitor cell selection system, according to the protocol provided by the manufacturer. Briefly, cells will be incubated with CD34 microbeads (microbeads conjugated to monoclonal mouse anti-human CD34 antibody) at room temperature for 30 min and will then be placed in a magnet for 2 min. The supernatant will be discarded and the bead-bound cells will be gently washed four times with buffer 1 (PBS w/0.1% BSA, 2 mM EDTA, pH 7.4, without $Ca^{2+}$and $Mg^{2+}$). Finally, the cells will be re-suspended in complete medium and cultured in T-60 cell culture dishes. Cultures of human muscle-derived stem cells will be used after five to nine in-vitro passages (2–3 months).

*Human Umbilical Cord Blood (UCB): collection and preparation of Mesenchymal Stem Cells (MSCs)*

Human UCB samples will be collected from term or preterm deliveries at the time of birth with the mother's informed consent.

Blood samples will be processed within 24 hours of collection. The mononuclear cells will be separated from

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UCB using Ficoll-PaqueTM PLUS and will be suspended in culture (High-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 g/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, EGF 10ng/ml, -mercaptoetanolo 55 μM).

The cells will then be seeded at a density of $1x10^6$ cells/cm$^2$ in culture flasks. After 5 days of culture, suspended cells will be removed and adherent cells will additionally be cultured. Cultures will be kept at 37ºC in a humidified atmosphere containing 5% carbon dioxide, with a change of culture medium every 5 days. Approximately 50%–60% of confluent cells will be detached with 0.1% trypsin–EDTA and replaced at a density of 6 $x10^3$/cm$^2$ in culture flasks.

*Myogenic differentiation*

Myogenic differentiation will be induced by culturing MSCs in myogenic medium (culture medium supplemented with 5% horse serum, 0.1 M dexamethasone, and 50 M hydrocortisone) for up to 6 weeks, as described by Zuk and colleagues [1–3]. Myogenic differentiation will be analyzed by FACS (Fluorescence Activated Cell Sorting) for MyoD1, myogenin (MYOG) , and myosin heavy chain (MHC). For FACS, mesenchymal stem cells will be detached and stained sequentially with primary antibodies (mouse-anti MyoD and anti-myogenin antibodies; and FITC-conjugated secondary antibodies (FITC-rat anti-mouse IgG1). Stem cells will be fixed with 2% formaldehyde until analysis by FACS. In order to detect the MHC protein, cells will be permeabilized with cold methanol/PBS for 2 minutes at -20ºC before being stained with primary mouse anti- myosin heavy chain (fast, Sigma) and FITC conjugate secondary antibody.

*Animals*

This study will be performed under the supervision of the local ethics committee and all procedures will be conducted in accordance with European legislation and with the guidelines for the care and use of laboratory animals established by the National Institutes of Health.

In the first phase, the authors will use a sample of SCID Wistar rats (weight range, 250–300 g), which will be divided into three groups.

Each rat of the sample will be initially analyzed using rectal manometry and EMG of the IAS muscle; after this the three groups (called A, B and C) will receive an internal sphincter injury.

Group A will not receive any treatment. Group B will receive saline injections in the damaged zone. Group C will receive an injection of differentiated MSC in the same zone.

All the rats will undergo a further EMG and manometry after one week.

All the animals will be housed in single cages with a natural night and day cycle and with free access to water and commercial pellet diet (Harlan, Udine, Italy).

Preoperative and postoperative clinical evaluations will be performed, and the feeding and defecation behavior will be observed daily to verify fecal continence and detect possible complications. Three week after the injury, a further EMG and Manometry will be carried out.

After four weeks (30 days), all the animals will be sacrificed with anesthetic overdose followed by exsanguinations.

A histological evaluation will be carried out on half of the animals in each group, while the remainder will be stored for in vitro functional studies.

*Surgical procedure and MSC injection*

Animals will be anesthetized with an intra-peritoneal injection containing a combination of tiletamine, zolazepam and xilazine.

The perineum will be shaved and the skin washed with povidone-iodine solution. Injury will be carried out under an operating microscope by a left-lateral full-thickness IAS section.

A left circum-anal incision will be performed on the rats and the mucosa of the anal canal will be separated from the sphincters by soft dissection.

Both the external (EAS) and internal anal sphincter (IAS) will be isolated, exposed and then the IAS will be divided using a knife at the 3.00 o'clock position. The skin wound will be closed with absorbable interrupted suture.

MSC will be labelled with Luciferase and GFP/LacZ to permit the identification of grafted cells in the host tissue.

Using a 50- l Hamilton microsyringe, an injection of GFP/LacZ labeled MSC (10 l with approximately $0.75\times10^6$ cells) or normal saline solution (10 l) will be made into the cut end of the IAS (2 injections). In group B, two injections of saline solution (10 l) will be given at the 3.00 o'clock position.

*Tissue processing and immuno-histochemical analysis of grafted MSC*

Four weeks after the MSC transplantation, the tissues will be dissected out, fixed and incubated overnight at 4°C with primary antibodies diluted in PBS containing 1.5% normal goat serum.

The following antibodies will be used; monoclonal anti-skeletal muscle myosin (1:250) and monoclonal anti-smooth muscle actin (1:1000). After washing, sections will be incubated for 1 hour at room temperature with Trich-conjugated secondary antibodies (1:200 dilution). After 2 more washes, the sections will be covered with Fluorsave mounting medium. Staining controls will be produced by omitting the primary antibodies.

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*The evaluation index*

In order to evaluate the effectiveness of the stem cells, an appropriate index will be used that can summarize the result of the tests performed before, during and after the treatment.

The index will be built by considering that the tests are composed of subjective and objectives analysis; it should be noted that more than one expert will be involved when referring to a subjective analysis (such as the histological exam).

## Conclusion

The objective is to show that significant results will be obtained only in the rats treated with MSC. To this end we will consider the T-test and its associated probability for the evaluation index regarding groups A, B and C.

Once encouraging results have been observed, we will start to identify the factors that could be considered significant in order to repeat the experiment on a human sample.

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