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

Stem cells are special cells that have self-renewing pro-
erties and are able to differentiate into one or many dif-
ferent 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 neurodegenera-
tive diseases, stroke, and muscular dystrophies (8-12).

Totipotent or pluripotent stem cells are generally
plentiful in embryonic or fetal tissue, although their use
may lead to certain ethical problems.

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

In the last two years, it has been observed that ske-
letal-muscle satellite cells, normally quiescent, can be ac-
tivated in response to muscle trauma; thus it was hy-
pothesized that they could regenerate muscle fibres.

However, recent studies have shown that adult ske-
letal-muscle satellite cells contain progenitors or pluripotent
stem cells that are able to differentiate into several phe-
notypes of muscle cells, suggesting that the pool of ske-
letal-muscle derived stem cells include distinct subpo-
ulations of precursors, with different differentiating ca-
pacities and that satellite cells may represent only one of
these subsets.

Objective

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

The study will follow a quantitative approach to eva-
uating the effectiveness of the treatment, and will in-
egrate certain well-known techniques (such as EMG, tis-
sue analysis, manometry, etc.). It will also employ an in-
dex of effectiveness.

Methods

In both the animal study and the human study an ap-
propriate Design Of Experiment (DOE) will be em-
ployed. The animal study will be planned in two stages;
in the first one the total sample of Wister rats will be di-
vided into three groups: treated with stem cells, with pla-
cebo 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 con-
sider 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, tu-
mour-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, trypsinize, filtered through 70 μm
ilters, and cultured in cell culture dishes (35 mm diame-
ter) at 37°C in F12 complete medium with 15% fetal
bovine serum (FBS) and 1% penicillin/streptomycin. Af-
ter culturing the cells for 1 hour, the adherent fibroblas-
tes will be discarded and non-adherent cells will be col-
lected and re-plated in fresh collagen coated cell cultu-
re 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 cel-
ls 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 tempera-
ture 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 buf-
fer 1 (PBS w/0.1% BSA, 2 mM EDTA, pH 7.4,
without Ca²⁺and Mg²⁺). Finally, the cells will be re-su-
supended 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 pre-
paration 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
Human Muscle-Derived Stem Cells. Effectiveness in animal models of faecal incontinence: clinical trials. Research scheduling

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/cm² 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 x 10^5/cm² 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 -20°C before being stained with primary mouse anti-myosin heavy chain (fast, Sigma) and FITC-conjugated 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 eviscerations.

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 (10^6 with approximately 0.75×10^6 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.
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 objective 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.

References