LONG-TERM STABILITY, FUNCTIONAL COMPETENCE AND SAFETY OF MICROENCAPSULATED SPECIFIC PATHOGEN-FREE (SPF) NEONATAL PORCINE SERTOLI CELLS: A POTENTIAL PRODUCT FOR CELL TRANSPLANT THERAPY.

Running Title: Microencapsulation of SPF Sertoli cells

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Abstract

Background: porcine Sertoli cells **(**pSCs) have been employed for cell therapy in pre-clinical studies for several chronic/immune diseases because deliver molecules with trophic and antiinflammatory effects.

In order to be employable for human long-term xenografts, pSCs products have to grant safety and stability. To fulfill such requirements, we employed a microencapsulation technology to increase pre-transplant storage stability of specific pathogen free pSCs (SPF-pSCs) and evaluated the in vivo long-term viability and safety of grafts.

Methods: SPF-certified neonatal pigs underwent testis excision under sterility. pSCs swere isolated, characterized by immunofluorescence (IF) and cytofluorimetric analysis (CA), and examined in terms of viability and function [in terms of production of anti-mullerian hormone (AMH), inhibin B, transforming growth factor beta-1 (TFGbeta-1)].

After microencapsulation in barium-alginate microcapsules (Ba-MC), long-term SPF-pSCs (Ba-MCpSCs) viability and barium concentration were evaluated over 0, 24 and 40 hours to determine pre-transplant storage conditions.

Results: The purity of isolated pSCs was about 95% with negligible contaminating cells. Cultured pSCs monolayers, both prior and after microencapsulation, maintained high function and viability up to 24 hours of storage. At 40 hours post encapsulation, pSCs viability decreased to 80%. Barium concentration in Ba-MCpSCs lagged below the normal maximum daily allowance and were stable for 4 months in mice with no evident side effects.

Conclusions: Such results suggest that this protocol for the isolation and microencapsulation of pSCs is compatible with long-haul transportation and that Ba-MCpSCs could be potentially employable for xenotransplantion.

Key words: Xenotrasplantation; Microcapsules; Sertoli cells; Specific Pathogen Free.

Abbreviations:

SPF: Specific pathogen free

pSC: porcine Sertoli cells

SCs: Sertoli cells

SPF-pSCs: specific pathogen free porcine Sertoli cells

Ba-MCpSCs: barium alginate-based microspheres

IF: immunofluorescence

CA: cytofluorimetric analysis

PERVs: porcine endogenous retroviruses

AMH: Müllerian inhibiting substance

INSL3: insulin-like 3

ASMI: alpha-smooth muscle actin

1. **Introduction**

Successful xenotransplantation of cells, tissues or vascularized organs from nonhuman species into humans could provide an unlimited supply of organs for permanent replacement protocols or tissues and cells for other'carry-over' life-saving strategies. **(Vorrebbe far scrivere DOES NOT SUPPLY).** However, the widespread diffusion of xenotransplantation is limited by many hurdles such as immunologically mediated graft rejection of xenogeneic tissues and the possible metabolic or molecular incompatibilities between donor organs/tissues and humans[1]. Nevertheless, progress accomplished in understanding these events might render the xenotransplantation a well established technique. Unfortunately, the positive outcomes of xenograft protocols are currently limited by the need for life-long multidrug systemic immunosuppression therapy regimens to prevent the rejection by the recipient immune system [2]. In this respect, a safe or even drug-free tolerance induction for xenografts would be the most effective way to overcome these problems, and in particular, to prevent the hazardous side effects of chronic immunosuppression, the risk of chronic rejection and the dangerous acquisition of debilitating chronic infections. A new approach to induce tissue/cell xenograft acquired tolerance could be the transplantation of "ghost" cells, cells not detected by immune system. One such promising cell type is the testis-derived SCs. In fact, over the past 30 years there has been a growing interest in SCs biology upgrading these cells from simple mechanical support/nursing elements of the testis to potent immunosuppressive, immunomodulatory and trophic factors delivering cells [3-6]. These cells have been used in treatment of several chronic and degenerative diseases, such as secretory infertility [7], Parkinson disease [8], amyotrophic lateral sclerosis [9], Huntington disease [10], type 1 [11] and type 2 [12] diabetes mellitus, Laron syndrome [13] and skin allograft transplantation [14], in animal models. A different and better regulated protocol for transplanting viable SCs, as compared to the transplantation of naked SCs, is the use of microencapsulated SCs [11-14].

In fact, the use of alginate-encapsulated SCs, assuring the retrievability of the graft, may be a desirable feature for the product, allowing removal of the cells if there are complications with them.

Since the employment of SCs from human pre-pubertal testes encounter substantial ethical constraints, a major concern is which animal source would best be suited to derive SCs to be transplanted into humans. In fact. zoonotic agents, that are benign in the donor, could become opportunistic and aggressive in a human host [1]. The U.S. Food and Drug Administration precluded use of nonhuman primates as a source of cells because of the risk of transmission of viruses known to be infectious for humans (plus high costs and breeding limitations) [15]. On the other hand, pigs, while immunologically dissimilar, are easier to breed and may be grown under very clean and controlled conditions, with high standards of animal husbandry, to exclude extracellular organisms and bacteria from the animal herds, thus overall providing a well-characterized source of cells for transplantation purposes [1]. However, attention has been drawn on a family of PERVs, capable of infecting human cells *in vitro* [16], data, however, not confirmed *in vivo*[17-20]. Monitoring of patients upon extracorporeal connection to pig kidneys [20] or following injection of pig islets into either the portal vein or under the kidney capsule [21] did not demonstrate PERVs presence.

In our work, because transplanted "naked" SCs would be impossible to retrieve, thereby disregarding an important regulatory issue, we have (a) isolated pure, viable and functional neonatal pSCs obtained from a SPF pig herd in order to prevent any transmission of infectious adventitious agents to the host cells, (b) enveloped these pSCs in highly biocompatible (endotoxin levels ≤0.5 EU/mL) Ba-MC [22],(c) determined the in vivo long term viability of Ba-MCpSCs, and storage at room temperature in sterile tubes, to determine the feasibility of eventual long-haul transportation of these cells and (c) evaluated the barium content of microcapsules to test their safety for graft purposes.

2. Materials and Methods

2.1 Experimental animals

BKS.Cg-Dock7^{m}+/+Lepr^{db}/J mice, purchased from Charles River Laboratories (Lecco, Italy), were used as recipients. All animals were housed individually and studies *in vivo* were conducted in agreement with national (Italian Approved Animal Welfare Assurance A-3143-01) and the University of Perugia Animal Care and Use Committee guidelines.

2.2 Sertoli cell isolation

SPF-certified (Table 1) Large White neonatal pigs (20-25 days old,) grown in a unique SPF herd in Italy, the Experimental Zooprophylactic Institute of Lombardia and Emilia Romagna, were hauled in sterile boxes and transported to the surgical suite of the Veterinary Medical College, University of Perugia, where after general anesthesia with 40 mg/kg KETAMINE (ketavet 100, Intervet, Milan, Italy) and 40 μg/kg DEXMEDETOMIDINE (dexdomitor, Orion Corporation, Finland), they underwent bilateral orchidectomy. Subsequently, pSCs were isolated, according to previously established methods, modified in our laboratory [22,23]. Briefly, the testes were finely minced, after removal of the fibrous capsule, to obtain a homogeneous dense tissue. Thereafter, the tissue underwent a step-wise enzymatic digestion with a Hanks' balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO) containing trypsin and DNase I. After washing, the tissue was incubated with 2 mg/mL Collagenase P (Roche Diagnostics S.p.A., Monza, Italy) in HBSS solution. The pellet was passed through a 500-μm stainless steel mesh and resuspended in glycine buffer to eliminate any residual Leydig as well as peritubular cells [24]. The resulting isolation was collected into culture ware and maintained in HAM'sF-12 (Euroclone, MI, Italy), supplemented with 0.166 nM retinoic acid, (Sigma-Aldrich, St. Louis, MO) and 5 mL/500 mL insulin-transferrin selenium (ITS; Becton Dickinson cat. no. 354352) in 95% air/5% CO2 at 37°C. At 3 days of culture, SCs

were incubated with 10mM trishydroxylmethylaminomethane hydrochloride (TRIS, Sigma-Aldrich, St. Louis, MO) buffer, as previously described, to eliminate any residual germ cells [25].

2.3 Sertoli cell characterization

To detect the presence of AMH (a unique pre-pubertal SCs marker), INSL3 (Leydig cells marker) and ASMI (peritubular cells marker), immunostainings were performed according to previously reported methods with minor changes [26-29]. Briefly, pSCs monolayers were grown on glass chamber slides (LabTek II, Nunc, Thermo Fisher, Rochester, NY, USA) and fixed in 4% paraformaldehyde–phosphate-buffered saline (PFA-PBS) for 30 min. Ten fixed cells then were subjected to permeabilization (PBS, 0.2% Triton X-100) for 10 min at room temperature and blocked with 0.5% bovine serum albumin (BSA; Sigma-Aldrich, MO, USA) in PBS for 1 hour prior to exposure to the AMH primary antibody (sc-6886, Santa Cruz Biotechnology, CA, goat anti-rat, polyclonal, 1:100), to INSL-3 (NBP1-18706, Novus Biologicals, CO, rabbit anti-human, polyclonal, 1:200), to ASMI (ab 5694, ABCAM, UK, rabbit anti-pig, polyclonal, 1:200) over night at +4°C. The cells were then washed in PBS three times for 5 min and then exposed to the secondary Alexa 488-conjugated donkey anti-goat antibody (Molecular Probes, NY, USA, 1:500) and Alexa 488-conjugated donkey anti-rabbit antibody (Molecular Probes, NY, USA, 1:500). Thereafter, the cells were treated with RNAse (10 mg/mL, Sigma-Aldrich, St. Louis, MO) and counterstained for 1 min with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO). Negative controls bypassed the primary antibody treatment. Cells were mounted on slides with ProLong® Gold antifade reagent (Molecular Probes, NY, USA). To evaluate the percentage of AMH, INSL-3 and ASMI positive cells, chamber slides were analyzed using a BX-41 microscope (Olympus, Tokyo, Japan) equipped with a fluorescence photocamera (F-viewer, Olympus, Tokyo, Japan); images were processed with Cell F imaging software (Olympus, Tokyo, Japan) and 10 different sections, containing at least 500 cells in total, were counted.

For FACS analysis, pSCs monolayers were harvested, centrifuged (400 x *g* for 5 min) to form a cell pellet of approximately 1 x 10^6 cells, and the supernatant was removed. The cells were fixed in 4% PFA-PBS for 30 minutes and after washing in FACS buffer (PBS with 3% BSA), were treated with 0.1% Triton X-100 in FACS buffer for 10 minutes. After centrifugation (400 x *g* for 5 min), the supernatant was removed, and the cells were blocked with 5 % BSA in FACS buffer at RT for 1 h before incubation with primary antibody (AMH, INSL-3 and ASMI, 1 µl antibody per 0.5 – 1.0 million cells, or buffer alone) at RT for 1 h. The cells were washed twice with 2 ml of FACS buffer per tube, pelleted by centrifugation (400 x *g* for 5 min), with the supernatant being removed. Lastly, the cells were exposed to the secondary Alexa 488-conjugated donkey anti-goat antibody (1:500) and Alexa 488-conjugated donkey anti-rabbit antibody (1:500) and suspended in 0.5 ml FACS buffer. Then, the cells were centrifuged (400 x g, 5 min), the supernatant was removed and pellet resuspended in 0.5 ml FACS buffer with 1% PFA for analysis. Data acquisition was performed on 10,000 events per tube based on a total (ungated) count of forward and side light scatter at approximately 200-300 events per sec on a BD FACS ort flow cytometer (BD Biosciences) and analyzed using FACS Diva software (BD Biosciences).

2.4 Preparation of alginate-based microcapsules

Immediately after isolation, at day 3 of culture and before microencapsulation, pSCs viability was assessed by staining the preparations with ethidium bromide (Sigma-Aldrich) and fluorescein diacetate (Sigma-Aldrich, St. Louis, MO) as previously described [30]. The cells were visualized under fluorescence microscopy (Olympus BX-41, Tokyo, Japan) using the filter block for fluorescein in conjunction with F-View camera and Cell-F software (both from Olympus). Dead cells appeared in red while viable cells appeared in green. pSCs were encapsulated in alginate microcapsules according to our previously described method [22]. Briefly, confluent monolayers of pSCs were collected with 0.05% trypsin–EDTA (Gibco, Gaithersburg, MD) (2 min), washed, counted by hemocytometer and assayed for viability as described above. pSCs were suspended in

1.6% aqueous solution of highly purified sodium alginate (AG, endotoxin levels ≤0.5 EU/mL) (Stern Italia, Milano, Italy). The AG–pSCs suspension was continuously aspirated by a peristaltic pump at a flow rate of 12–14 mL/min and then extruded through a microdroplet generator (air flow rate: 5 L/min) under sterile conditions. The cell suspension was continuously mixed by magnetic stirrer to prevent cell aggregation resulting, therefore, in a homogenous distribution of pSCs within the alginate medium. The microdroplets were collected on a $BaCl_2$ (1.2%, w/v) gelling bath which immediately turned them into gel microbeads. The obtained Ba-MCpSCs were washed twice in saline, cultured in HAM'sF-12 (Euroclone, MI, Italy), supplemented with 0.166 nM retinoic acid, (Sigma-Aldrich, St. Louis, MO) and 5 mL/500 mL insulin-transferrin selenium (ITS; Becton Dickinson cat. no. 354352) in 95% air/5% CO_2 at 37°C for 1 hour, washed twice again the aliquots of pSCs-MCs, corresponding to $50x10^6$pSCs/2.5 ml sodium alginate were stored at room temperature in 50 ml tubes filled with HAM'sF-12 (Euroclone, MI, Italy), supplemented with 0.166 nM retinoic acid, (Sigma-Aldrich, St. Louis, MO) and 5 mL/500 mL insulin-transferrin selenium (ITS; Becton Dickinson cat. no. 354352), up to 40 hours, mimicking transport conditions and time necessary to allow their safe long distance transportation. Viability with EB/FD was evaluated at time 0 and upon long term storage (24 and 40 hours at room temperature) in 50 ml tubes to simulate transport conditions and time which usually is necessary for long-haul transportation.

Additionally, functional competence of naked SCs monolayers and Ba-MCpSCs, in terms of AMH, inhibin B, TGF-β1and testosterone secretion (the latter to determine contamination of Leydig cells), was evaluated on 3 X 10^6 naked or encapsulated SCs. Inhibin B, AMH and TGF-β1 secretion were determined by ELISA (kit inhibin B Gen II ELISA, Beckman Coulter, Webster, TX, U.S.A., intra assay CV=2,81%; inter assay CV=4,33%), (kit AMH Gen II ELISA, Beckman Coulter, Webster, TX, U.S.A., intra assay CV=3,89%; inter assay CV=5,77%), (kit TGF-β1, MB 100B, R&D, Minneapolis, MN, intra-assay CV=2,76%; inter-assay CV=7,46%), testosterone secretion was

determined by RIA (kit testosterone, IM 1119, Beckman Coulter, Webster, TX, U.S.A., intra assay CV≤ 8,6%; inter assay CV≤11,9%).

2.5 In vivo studies

Ba-MCpSCs and control empty alginate microcapsules (Ba-MCE), immediately after preparation, were injected into the subcutaneous adipose tissue of BKS.Cg-Dock7^{m}+/+Lepr^{db}/J recipient mice (20 X10^{6} SCs/20 grams of mouse's body weight) through a 16-gauge catheter. The animals were divided into two experimental groups (10 animals each), one transplanted with microencapsulated Ba-MCpSCs and one grafted with Ba-MCE. All the animals were sacrificed after 4 months to retrieve Ba-MCE and Ba-MCpSCs for studies of biocompatibility, morphology, viability and function.

2.6 Ba-MCpSCs barium ions content

Microcapsules were prepared, as reported above, by crosslinking of 2.5 mL 1.6% w/v alginate solution, containing 50x10^{6} cells, in 0.6, 1.2 and 1.8% w/v $BaCl_2$ solution. The obtained microcapsules were placed in 50 mL of HAMF-12 and maintained at room temperature. At 0, 24, and 40 hours, samples were centrifuged to recover the cell pellets in order to evaluate Ba^{2+} content by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) using a Varian 700-Es series spectrometer (Agilent, Milan, Italy). The pellets were centrifuged and submitted to several washing cycles with PBS. Afterwards, the pellets were freeze-dried for 24 hours and then accurately weighed. The cell pellets were dissolved by overnight rotation after addition of 10 mL 1M sodium EDTA in 1M NaOH solution (Sigma-Aldrich, Milan, Italy). The solubilized microcapsules were 10x diluted in the EDTA solution prior to being submitted to ICP-OES analysis in triplicate. Calibration was performed diluting Ba standard stock solution (1 mg/mL Ba^{++} from $BaCO_3$ in 1M HNO_3) (Sigma-Aldrich, Milan, Italy) in the range 1-15 µg/mL. The Ba content was expressed per unit weight of pellet and % of the total amount added and the error expressed as S.D. Moreover, the

obtained values were extrapolated to a theoretical administration of 100 mL microcapsule suspension for a 6 months treatment. The Ba ions content was compared to the reported human reference Ba levels.

2.7 Statistical analysis

Data were analyzed for statistical significance by Student's t test at 95% and 99% significance level from at least three replicates. Data are expressed as Mean ± S.D.

3. Results

3.1 Sertoli cell isolation and characterization

The isolation and purification steps of pSCs were examined by light and fluorescence microscopy, to evaluate the tissue viability and monitor each enzymatic digestion process. The isolates, still surrounded by fragments of interstitial tissue (Leydig and peritubular cells), after enzymatic digestion with trypsin, and DNase I(Fig. 1 panel A), but appeared to be devoid of cell contaminants after the enzymatic digestion with collagenase P (Fig. 1 panel B). Seminiferous tubules viability before plating was very high (Fig. 1 panel C). The percentage of AMH positive cells in pSCs monolayers was about 97% (Fig. 2 panel A), as determined by IF, with negligible contamination with Leydig (Fig. 2 Panel B) and peritubular cells (Fig. 2 Panel C). These data were confirmed by FACS analysis (Fig. 3 Panel A-C) setting the final values of AMH positive cells at 95%, Leydig cells2% and peritubular cells at 3% as the mean value between IF and FACS results (Table 2).In the absence of sure specific markers for germ cells, pSCs monolayers were treated with TRIS buffer, as previously described [25] to eliminate any residual germ cells. Because germ cells are easily identifiable under the light microscope, we established that the percentage of these contaminant

cells was very low, 0,5%. This value was obtained by the average counts of two different operators on 10 different sections, containing at least 500 cells in total.

3.2 Preparation of alginate-based microcapsules

pSCs monolayers, prior to encapsulation, demonstrated high viability (95%) (Fig. 4 Panel A). The obtained microcapsules, elastic and translucent, were fully satisfactory in terms of size, morphology, sphericity and coalescence (Fig. 4 Panel B). In addition, pSCs viability was maintained unaltered after the microencapsulation process both at time 0 (Fig. 4 Panel C) and at 24 hours (Fig. 4 Panel D). There was a slight decreased to 80% at 40 hours (Fig. 4 Panel E).

3.3 In vitro and in vivo studies

The effects of microencapsulation on pSCs function were evaluated by AMH, inhibin B, and TGF-β1 secretion *in vitro*. Ba-MCpSCs TGF-β1 secretion (Fig. 5 Panel C) was not statistically significant at 40 hours respect to the corresponding time 0, but it was slightly higher compared to those found in naked pSCs at each time point (not statistically significant), whereas a reduced secretion of AMH and inhibin B in Ba-MCpSCs was observed in comparison with naked pSCs at each time point ($p<0.05$) (Fig. 5 Panel A-B). Up to 40 hours of culture maintenance, inhibin B and AMH concentrations increased both for naked and encapsulated pSCs, as compared to the corresponding controls at time 0 ($p< 0.001$).

Four months post-implantation, recipient mice were sacrificed, the skin was carefully cut (Fig. 6 Panel A) and the underlying subcutaneous adipose tissue was removed to recover the microcapsules (Fig. 6 Panels B and C). Gross inspection revealed that microcapsules were perfectly preserved (Fig. 6Panel D), appearing morphologically intact (88%±10) and were mostly free of fibrotic tissue overgrowth (Fig. 6 Panels E and F). Inside the microcapsules, pSCs were still viable as demonstrated by inhibin B and TGF-β1 secretion that remained unaltered compared to values at

time 0 (Fig. 6 Panel G),whereas AMH was not detected. Furthermore, to verify the purity of pSCs monolayers, testosterone (the specific Leydig cells hormone) was never detected both in naked and encapsulated SCs or retrieved Ba-MCpSCs.

3.4 Ba-MCpSCs barium ions content

Ba-MCpSCs underwent careful washing cycles before implantation in order to minimize the exposure to toxic barium ions levels. Following washing, the barium content measured in the microcapsules prepared at 0.6, 1.2 and 1.8% w/v $BaCl_2$ concentration was not significantly different ($p=0.729$) among the preparations (Table 3). The mean barium values ranged between 12 and 17 ppm/mg of pellet for all preparations. Moreover, barium content in microcapsules remained steady for 40 hours of culture, thereby suggesting that no release had occurred over time. Hence these microcapsules could be stored safely for up to two days before their use. A release was observed only for Ba-MCpSCs prepared using 1.8% w/v $BaCl_2$, as outlined by the significant drop in the barium content from the initial 26 to 14.6 ppm at 24 hours ($p<0.002$). This observation was explained considering that the much higher amount of barium added and therefore a higher amount of barium adsorbed on the polymer matrix and the inside the capsule, would require more time to be eliminated.

The estimated exposure to barium over a six months treatment of a normal body weight patient suggests that Ba-MCpSCs could be administered safely without expected barium related toxic effects (Table 4). In fact, even considering the maximum administration of 100 ml of Ba-MCpSCs batches intraperitoneally, either the total dose or the daily dose normalized to the average body weight, would be less than half of the daily Ba intake by diet and 1/10 of the tolerable daily intake, respectively, regardless of the initial $BaCl_2$ concentration used for capsule preparation (Table 4). Such an observation is fundamental to confirm safety of the treatment and to ensure maximum efficacy in compliance with regulatory guidelines [31-33].

4.Discussion

In our study, using SPF-certified pigs born and weaned in the unique SPF herd in Italy, we have obtained pure, viable and functional neonatal pSCs monolayers composed of 95% AMH positive cells (AMH is a glycoprotein uniquely produced by prepubertal SCs) [26], a 2% Insl-3 positive cells, specifically expressed by Leydig cells [34], a 3% ASMI positive cells specifically expressed by peritubular myoid cells [28,29] and a 0.5% contamination of germ cells.

In the current study, we have employed our unique microencapsulation technology, based on highly purified sodium alginate [21] and have demonstrated that the deleterious effects of microencapsulation on pSCs viability and function, as evaluated by EB/FDA and *in vitro* inhibin B, AMH, TGF-β1secretion are minimal even after long-term storage (up to 40 hours) at room temperature in 50 ml tubes.

Results from this study have demonstrated that Ba-MCpSCs TGF-β1 secretion was not statistically significant at 40 hours respect to the corresponding time 0, but it was slightly higher compared to those found in naked pSCs at each time point (not statistically significant), whereas a reduced Ba-MCpSCs secretion of inhibin B and AMH was evident at each time point compared to naked pSCs although, up to 40 hours of culture maintenance, AMH and inhibin B concentrations of these function SCs products increased, when compared to the corresponding controls at time 0 for naked and encapsulated SCs.

These observations can be explained by the fact that the diffusion of AMH and Inhibin B is limited by the microcapsules membrane's permeability [35] that is the sum of different factors such as size (AMH: molecular weight of 140 kDa; Inhibin B: 47 kDa at prepubertal period [36]), shape obstruction (quaternary conformation) and ionic interactions with our sodium alginate having the pH adjusted to 7.4 - 7.6 (pI AMH: 7.04, pI Inhibin B: 6.9-7.3). TGF-β1, on the other hand, is a growth factor that may easily pass through the microcapsule barrier due to its smaller dimension (TGF-β1: molecular weight of 25 kDa and pI of 8.83). The results of these different secretory

behaviors are beneficial in that they avoid, the detrimental effects of AMH and Inhibin B on the hypothalamus/hypophysis/gonads axis of the potential human recipient but at the same time, they enable the passage of useful molecules, such as TGF-β1, which have beneficial trophic and or immunomodulatory properties. As previously described [13,14], this latter observation was assessed *in vitro* in experiments showing that Ba-MCpSCs had comparable secretory values with respect to naked SCs and in experiments *in vivo* showing secretion was detectable up to six months in the serum of Laron Ba-MCpSCs transplanted mice (data not shown).

In the present study, at 4 months post-transplant, the recovered microcapsules were perfectly preserved, morphologically intact with the majority of the microcapsules (88%±10) appearing free of fibrotic tissue overgrowth with pSCs still maintaining functional viability as demonstrated by inhibin B and TGF-β1 secretion that remained unaltered compared to values at time 0, while AMH was not detected in our rodents, and in agreement with Tran et al. [37].

In recent years Ba^{2+} has been frequently used to crosslink ions in preparing alginate hydrogels intended for cell encapsulation purposes.[38]. However, barium is known to be toxic at high doses, and chronic toxicity may induce cardiovascular disease, hypertension and renal toxicity [32]. The WHO [33] determined that the dietary daily intake of barium in humans should range between 0.3 to 1.7 mg of barium/day. In the current, we demonstrated that most of the unbound divalent ions were removed during the washing steps. Crosslinking our alginate solution in 0.6, 1.2 and 1.8% w/v $BaCl_2$ solution resulted in the barium content in microcapsules remaining constant in long-term culture (up to 40 hours). Additionally, adhering to the maximum administration of 100 ml of Ba-MCpSCs in suspension residing in the peritoneal cavity of a potential human recipient of normal body weight, the released barium amount would fall below the daily dietary intake recommended by WHO [33]. Furthermore, even in the case of unexpected breakage and disruption of the capsules with total and sudden diffusion of barium (up to 1.8% w/v $BaCl_2$ solution), the total amount released into the body (22.5-24.0 mg) would amount to 11-12% of the acute toxicity dose (200 mg/day).

4. Conclusions

In conclusion, we have uniquely obtained, from SPF herds, highly purified, viable and functional pSCs. Microencapsulation of these cells did not alter viability at 24 hours, with a slight reduction at 40 hours. Barium concentration in these capsules and its eventual release complied with required daily allowance (RDA) within range. This protocol for the isolation and microencapsulation of pSCs is compatible with long-haul transportation of SPF-derived pSCs. Such data together with the observed pre-grafting and post-transplant stability candidate our Ba-MCpSCs as potential cell therapy product for human use (i.e. neurodegenerative diseases, T1D and T2D).

Acknowledgments

This work was supported by a grant from Mr. Gary Harlem (Altucell Inc. 3 Astor Court, Dix Hills, New York, NY) with no involvement in any part of our study design.

We acknowledge for technical assistance Dr. G. Lombardi: Experimental Zooprophylactic Institute of Lombardia and Emilia Romagna, Brescia, Italy.

Authors'role

G. L., F. M. and M. C. performed SC isolation, histologic staining, analyzed data and wrote the manuscript. I. A., G. F. and G.B. performed microencapsulaton of pSC and transplanted SC-MCpSCs. A. B. and V.De M. performed anesthesia and porcine orchiectomy. E. T. supervised breeding, nursing and microbiological assays of SPF-certified pig. C. N., C. L., C. B. and T. B. analyzed and interpreted the data. F.F. performed flow cytometric assays and interpreted data. M.C. A. performed ELISA kits and interpreted the data. S. G. performed ICP-OES and interpreted data. M. B. and R. C. supervised the research and edited the manuscript. All authors discussed the data and the manuscript.

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Figure captions

Fig. 1. Fluorescence and bright field (insets) photomicrographs of pSCs showing cell isolation and purification steps following enzymatic digestion with trypsin and DNase I (A), collagenase P treatment (B) and before plating (C). Fluorescence microphotographs were taken after double staining for viability assessment with ethidium bromide and fluorescein diacetate. PanBar corresponds to 200 or 50 μm in main pictures and insets, respectively.
The images are representative of 4 separate experiments.

Fig. 2, Morphological characterization of cultured pSCs in vitro by fluorescence microscopy. A: pSCs after immunostaining with anti-müllerian inhibiting substance (AMH) antibody and visualized by anti-goat Alexa Fluor 488 (green). B: pSCs after immunostaining with anti- insulin-like 3 (INSL3) antibody and visualized by anti-rabbit Alexa Fluor 488 (green). C: pSCs, after immunostaining with anti-rabbit alpha-smooth muscle actin (ASMI) antibody and visualized by anti-rabbit Alexa Fluor 488 (green). Nuclei are counterstained with 4',6-diamidino-2-phenylindole (DAPI, blue). Bar corresponds to 25 or 10 μm in main pictures and insets, respectively.
The images are representative of 4 separate experiments.

Fig. 3, FACS analysis of cultured pSC in vitro for anti-müllerian inhibiting substance (AMH) (A), anti- insulin-like 3 (B) ant anti-rabbit alpha-smooth muscle actin (ASMI) (C).
The images are representative of 4 separate experiments.

Fig. 4. Fluorescence (A, C) and bright field (B) photomicrographs of pSCs before (A) and after encapsulation in barium alginate-based microencapsules at time 0 (B, C) at 24 (D) and 40 h (E).

Fluorescence microphotographs were taken after double staining for viability assessment with ethidium bromide and fluorescein diacetate. Bar corresponds to 200, 350 or 50 μm in panels A, B-E and inset, respectively.

The images are representative of 4 separate experiments.

Fig. 5. Functional competence of free pSCs (cultured as monolayer) and pSCs encapsulated in barium alginate-based microencapsules (Ba-MCpSC). Cultured cell were evaluated for AMH (A), inhibin B (B) and TGF-β (C) secretion after 1 h (open bars), 24 h (grey bars) and 40 h (black bars) from encapsulation. Data represent the mean of three experiments ± SD.

Fig. 6. Recovery of transplanted Ba-MCpSCs after four months in mice recipients. Skin was carefully cut (Panel A) and the underlying subcutaneous adipose tissue was removed to recovery microcapsules . (Panels B and C). The preliminary visual examination revealed that microcapsules were perfectly preserved in the subcutaneous adipose tissue (Panel D), appearing morphologically intact (88%±10) (Panels E) and free of fibrotic tissue overgrowth (Panel F). Levels of inhibin B and TGF-β1, assessed in the supernatants of the retrieved Ba-MCpSCs, were comparable to the values in vitro at time 0 (Panel G).

The number of animals employed in each experiment (n=4) was 10.

The images from panel A to panel G are representative of 4 separate experiments.