Improvements in human sperm quality by long-term in vitro co-culture with isolated porcine Sertoli cells

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BACKGROUND: Spermatogenesis is a complex process where spermatogonial germ cells become spermatozoa with the indispensable support of Sertoli cells (SCs), which provide ‘ad hoc’ structural and nutritional support. Unfortunately, for most sperm dysfunctions, no therapies are yet available except assisted reproductive technologies (ART) that are based on the use of different culture media to preserve sperm in vitro. However, sperm culture is only possible for short periods of time, since long-term culture would invariably and irreversibly damage the cells with negative impact on their fertilization potential.

METHODS: Fresh sperm cells (5 ml of 20 × 10^6/ml) were co-cultured with SCs layers, derived from prepubertal pig testes or incubated in cell free SC medium or BWW (Biggers, Whitten and Whittingham) medium for 2, 4 or 7 days. Sperm viability, motility, mitochondrial status, DNA fragmentation, chromatin integrity, intracellular calcium and acrosome status were assessed after every co-culture or incubation time, but capacitation and induction of acrosome reaction (AR) with progesterone was only evaluated after 7 days.

RESULTS: SCs layers derived from prepubertal pig testes (co-culture of sperm and SC feeder, CCSCF) were able to preserve normal sperm viability, motility and normal mitochondrial function, after 7 days of culture; CCSCF did not induce AR or hyperactivation of spermatozoa, keeping the sperm in a quiescent state for 7 days of culture. Nevertheless, the sperm were readily able to initiate AR after stimulation with progesterone.

CONCLUSIONS: CCSCF maintained good sperm viability and motility for 7 days. This approach could improve retention of sperm viability and motility during ART procedures and maintain sperm viability, during transfer between two distant Centres, avoiding the need for cryopreservation.

Key words: ART / culture / sperm / Sertoli cell

Introduction

Spermatogenesis is a fascinating and complex process occurring in the seminiferous tubular compartment of the testis and involving multiple cellular changes that ultimately lead to the formation of motile cells with a haploid number of chromosomes. In particular, over a period of several weeks, undifferentiated spermatogonial germ cells become spermatozoa (Hermo et al., 2010). Sertoli cells (SCs), one of the pivotal somatic cell constituents of the testis, play an essential role in spermatogenesis. They extend from the base to the apex of the seminiferous epithelium, and are in direct physical contact with all the types of germ cells. During adulthood they are entirely committed to supporting spermatogenesis (Clermont, 1967; Russel, 1977). Adult SCs provide germ cells with mechanical and nutritional support, promote their motility, produce seminiferous fluid and induce spermiation (Griswold, 1998).
Sperm cells significantly differ from all other known cells in several respects; they lack physiologically active gene transcription or translation, exhibit rapid motility and tightly condensed DNA, and are produced en masse.

Unfortunately, no treatment for most sperm dysfunctions and male infertility is available. The only realistic therapeutic option for many infertile patients is assisted reproductive technology (ART), principally IVF or ICSI (Barratt et al., 2009). Moreover, at this juncture no treatments are known to improve in vivo motility or to facilitate full maturation of the sperm.

The majority of laboratory protocols for IVF involves culturing them in vitro with different media such as BWW (Biggers, Whitten and Whittingham), Earle’s, Ham’s F-10 or HTF (human tubal fluid), as reported by the World Health Organization (WHO) laboratory manual (Cooper et al., 2010: World Health Organization, 2010) to keep the sperm cells viable in the laboratory. These procedures maintain the sperm alive only for short periods of time and prolonged in vitro culture invariably induces irreversible cell damage, with consequential drastic reduction of their fertilization potential (Schuffner et al., 2002).

In order to maintain sperm viability in long-term in vitro culture, we tested the ability of monolayered SCs, extracted from prepubertal pig testes, to preserve sperm for extended periods of time up to 7 days.

Materials and Methods

Isolation of SCs

Upon approval by Ethical Committee (29 October 2010) Prepubertal ‘large white’ pigs (aged 7–15 days) were used for SCs donors. SCs were isolated according to previously established methods substantially, modified in our laboratory (Korbitt et al., 1997; Luca et al., 2005, 2007). Briefly, the procedure consisted of excising the testes from generally anesthetized piglets. On removal of their fibrous cap, the testes were finely chopped to obtain a homogeneous dense, mud-like tissue that underwent sequential enzymatic digestion with a 2 mg/ml Collagenase P (Roche Diagnostics S.p.A., Monza, Italy) solution in Hanks’ balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO, USA). The digestion continued until physical separation of the seminiferous tubules was achieved. After washing, the tissue suspension was incubated with HBSS solution containing trypsin and DNase I for 15 min (Sigma-Aldrich). On completion of the second digestion, the tissue pellet was washed twice in HBSS and centrifuged at 118 g for 3 min. The pellet was passed through a 500-μm stainless steel mesh and resuspended in 2 mM glycine, 2 mM EDTA buffer, pH 7.2 to eliminate any residual Leydig and peritubular cells (Mather and Philips, 1999). The residual tubules, with no peritubular cells, were collected and culture maintained on 25 cm² flask in HAMF12 (Euroclone), supplemented with 0.166 nM retinoic acid (Sigma-Aldrich) and 5/500 ml of insulin-transforming selenium (Becton Dickinson cat. no. 354352), subsequently referred to as SCs medium, in 95% air/CO₂ (Euroclone), supplemented with 0.166 nM retinoic acid (Sigma-Aldrich) and then cultured in 25 cm² flasks in HAMF12 (Euroclone), Earle’s, Ham’s F-10 or HTF (human tubal fluid), as reported by the World Health Organization (WHO) laboratory manual (Cooper et al., 2010: World Health Organization, 2010) to keep the sperm cells viable in the laboratory. These procedures maintain the sperm alive only for short periods of time and prolonged in vitro culture invariably induces irreversible cell damage, with consequential drastic reduction of their fertilization potential (Schuffner et al., 2002).

In vitro assessment of isolated SCs

After isolation and purification, the in vitro cultured SCs were examined in terms of purity, viability and function [α-aromatase activity and insulin-like growth factor 1 (IGF-1) secretion].

Morphologic characterization and purity

Anti-mullerian inhibiting substance (MIS) immunostaining was performed according to previously established methods with minor changes (Luca et al., 2007). Briefly, SCs were fixed for 30 min in 4% paraformaldehyde in phosphate-buffered saline (PBS). Before immunostaining, the cells underwent permeabilization (PBS, 0.2% Triton X) for 5 min at room temperature, blocked with 0.5% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 10 min and exposed to the MIS goat anti-rat primary antibody (sc-6886, Santa Cruz Biotechnology), 1:100 in PBS for 30 min at room temperature. We included an additional negative control by incubating primary MIS antibody with an appropriate blocking peptide (sc-6886 P, Santa Cruz Biotechnology), 1:1, overnight before use. The cells were then washed with PBS three times for 5 min and thereafter exposed to the secondary fluorescein isothiocyanate (FITC)-conjugated horse anti-goat antibody (1:300 in PBS). The cells were counterstained with 1 mg/ml propidium iodide (PI) for 10 min, after RNase treatment (10 mg/ml for 10 min). Negative immunostaining controls by-passed the primary antibody treatment. Cells were mounted on slides with 0.6% 1,4-diazabicyclo (2.2.2)octane (Sigma-Aldrich) in 90% glycerol in PBS. Immunofluorescent slides were examined via a confocal laser microscope (Zeiss, aoxiplan AX70© Oberkochen, Germany). Assessment of vimentin in SCs was performed as following; SCs were fixed for 20 min in 4% paraformaldehyde in PBS. Before immunostaining, the cells underwent permeabilization (PBS, 0.1% Triton X) for 15 min at room temperature and then were exposed to anti-vimentin Ab (Sigma-Aldrich). We included an additional negative control by incubating the cells with an appropriate mouse isotype control antibody. Negative immunostaining controls by-passed the primary antibody treatment. The cells were then washed with PBS three times for 5 min and exposed to a TRITC (tetrahydroxidamine isothiocyanate)-conjugated (Sigma-Aldrich) (1:50 dilution in PBS) goat anti-mouse IgG. Endogenous alkaline phosphatase activity was assessed, according to Chapin’s procedure, to detect peritubular cells (Chapin et al., 1997). The tissue was fixed in 4% buffered formaldehyde, rinsed and thereafter incubated for 15–30 min in alkaline phosphatase staining. The latter was prepared by dissolving 10 mg of the substrate (naphthol AS-biphosphoric acid) with 40 ml of dimethyl sulphoxide, with the solution being diluted with 5 ml of distilled water. The solution pH was adjusted by adding 5 ml of AMP buffer (25 mM 2-amino-2-methyl-1-propanol containing 1.25 mM MgCl₂, pH 8.9). Immediately before the incubation, 10 mg of the dye (Fast-Red B, tetrafluoroborate salt) was added to the substrate solution; the mixture was carefully vortex-mixed, and the bottom was passed through a 0.2-mm nylon filter. After visualization of the reaction product, the cells were rinsed with AMP buffer and mounted on a glass slide with Aquamount (Gur, Bath, England). Cells that were positive for alkaline phosphatase stained in red. Morphological identification of the Leydig cells was obtained by showing activity of 3β-hydroxysteroid dehydrogenase by a modification of the originally described procedure (Steinberger et al., 1966). The cell pellet, collected from each 1 ml fraction, was incubated for 30 min at 37°C with 0.3 ml of the staining solution (0.07 M phosphate buffer, pH 7.2, containing 1 mg/ml of nicotinamide, 6 mg/ml of b-NAD, 1.5 mg/ml of nitroblue tetrazolium and 100 g/ml of dehydroepiandrosterone). The number of stained versus total cells was counted, allowing quantification on non-Sertoli (peritubular and Leydig) cells.

No evidence of bacteria or porcine endogenous retrovirus (PERV) was found on medium and SCs culture.

Viability and functional competence (α-aromatase activity and IGF-1 secretion)

Twenty-four hours after Tris treatment (before co-culture with sperm), SCs viability was assessed by staining with ethidium bromide (EB; Sigma-Aldrich) and fluorescein diacetate (Sigma-Aldrich) under
Microbiological assessment and PERV transmission

Briefly, as for potential bacterial contamination, medium samples were collected into aerobic and anaerobic blood culture bottles. We used both a negative control (sterile saline) and a positive control (aerobic and anaerobic bottles for Bacillus subtilis and Bacteroides vulgaris, respectively). Bottles were inspected daily for any signs of bacterial growth, turbidity or discoloration.

Fungi were identified via their macroscopic and microscopic characteristics. Criteria for a valid test requires the absence of growth in the negative control bottles and the presence of growth in the positive control bottles.

We have also tested SCs for the presence of PERV, using a standard infectivity method (Patience et al., 1997; Garkavenko et al., 2008) to check the transmission of PERV from primary porcine SCs to human control cells.

Briefly, human embryonic kidney cell line 293 (highly susceptible for PERV infection) was used after induction of neomycin-resistant.

Reverse transcriptase (RT) activity to determine PERV production in the cell culture supernatant was determined using a commercial assay (Cavidi Tech, Uppsala, Sweden), according to the manufacturer’s protocol.

After 6 days, 10^2 porcine SCs primary cells were lethally irradiated, mixed with 10^3 HEK 293 target cells, and plated with appropriate medium. Co-cultures were maintained for up to 20 weeks and aliquots of cell suspension were centrifuged (7 min, 260xg) before RT according to the manufacturer’s instructions. Total RNA was isolated from co-cultured pig SCs and HEK293 cells using TRI Reagent LS (Molecular Research Center, Inc., Cincinnati, OH, USA), following the manufacturer’s instructions. Total RNA was reverse-transcribed by M-MLV RT (Invitrogen) with Random Primers (Invitrogen) at 42°C for 1 h.

Specific primers have been used for COII and PERV pol region and env region amplification. To check the integrity of isolated nucleic acids from HEK293 co-cultures, the following primers for human endogenous retrovirus HERV-K (Y10392) were used: forward 5′-ATGGGGCGCTCTCC AACCCGG-3′; reverse 5′-CGTTTCTGCAGCACATAAAATATCA-3′ for the first round and forward 5′-GCCCTCTCCGGCATGATCC-3′; reverse 5′-TCAATATAGAATAATACAGTCTG-3′ for the second round.

First-round polymerase chain reaction (PCR) was performed for each primer pair in 25-μl reactions containing 2.5 μl of 10× HotMaster Taq Buffer with 25 mmol/l Mg^{2+} (Eppendorf), 0.2 mmol/l dNTPs, 0.1 μmol/l of each primer pair, 1 U Eppendorf HotMaster Taq, and 1 μl cDNA or ~100 ng genomic DNA. A Biometra Thermocycler (Bio- metra, Goettingen Germany) was used for all PCRs with thermocycling conditions as follows: 1 cycle of 94°C for 2 min; 45 cycles of 94°C for 20 s, 55°C for 10 s, 72°C for 30 s and 10 min at 72°C.

Second-round (nested) PCR was conducted under the same conditions as described with specific primers, with 2 μl of PCR product from the first-round amplification. Products were visualized by electrophoresis in 1.5% EB-stained agarose gel (Probiogen, Murrarrie, QLD, Australia).

Semen sample collection, density gradient centrifugation and co-culture of sperm and SCs

Semen was donated by 10 normozoospermic men, who were proven fathers who had been referred to our Centre for male fertility evaluation before initiating an anti-cancer therapy cycle. None of the patients had history of previous genital tract infections, cryptorchidism, testicular torsion and varicocele. All provided informed consent.

Semen samples were obtained by masturbation into sterile containers after 2–5 days of sexual abstinence. Samples were allowed to liquefy for 30 min and were examined according to the WHO criteria for sperm count, motility, morphology and viability (Cooper et al., 2010; World Health Organization, 2010). All samples had normal viscosity and were associated with a leukocyte count <10^6/ml (Cooper et al., 2010; World Health Organization, 2010). Semen culture was negative and anti-sperm antibodies were absent in all subjects.

Density gradient centrifugation

Semen samples were washed twice by centrifugation (1000g, 10 min) in Sperm Washing Medium (SWM; Irvine Scientific, California) to eliminate seminal plasma. Motile spermatozoa were subsequently isolated by centrifugation against discontinuous Ficoll-Paque PLUS (Amersham Biosciences Europe, Freiburg, Germany) density gradients, from top to bottom (50, 75 and 90%). In brief, the sperm pellet was suspended in prewarmed 0.3 ml of SWM and placed on the top of the medium with lower density (50%) and then centrifuged at 1000g for 15–30 min. A sterile Pasteur pipette was used to collect the sperm fraction distributed in the 90% layer that was washed three times with SWM (1000g, 10 min). The specimen was kept at 37°C until use.

Co-culture of sperm and SCs

After Tris treatment, SCs culture medium was refreshed and after 48 h in culture SCs were retrieved, spun at 1600g for 7 min (in order to remove all single cells), and plated on 25 cm^2 flasks with SC medium, in order to obtain a cell monolayer working as feeder. On the top of the feeder, we placed 5 ml of 20 × 10^6/ml sperm resuspended in BWW and incubated the co-culture for 2, 4 or 7 days for subsequent evaluation. As controls we incubated sperm (5 ml of 20 × 10^6/ml) without SCs in SCs medium or in BWW medium (Biggers—Whitten—Whittingham; 95 mM NaCl, 4.8 mM KCl, 1.7 mM CaCl_2, 1.2 mM KH_2PO_4, 1.2 mM MgSO_4, glucose 5.6 mM, sodium pyruvate 20 mM, HEPES-free acid, 25 mM NaHCO_3, 20 mM lactic acid; pH adjusted to 7.5 with NaOH) for the same times. At each time point we aspirated 1.5 ml of sperm suspension in order to evaluate sperm motility, viability, apoptosis, DNA fragmentation, intracellular Ca^{2+} concentration and acrosome reaction (AR) as described later. Induction of the AR by progesterone was only examined after 7 days. For each of the 10 patients, each experimental condition of sperm and SCs feeder (CCSCF) co-culture, cell free SCs medium...
Sperm viability
The test was performed using 0.5% eosin Y following the procedure described in the WHO laboratory manual. Live spermatozoa showed white or light pink heads while the heads were red or dark pink for dead spermatozoa.

We evaluated 200 spermatozoa per condition (CCSCF, CFSCM and BWW), in order to achieve an acceptably low sampling error. Results were expressed as an average percentage of vital cells from the replicate preparations.

Sperm motility analysis
The WHO motility parameters (PR+NP: total motility; PR: progressive motility) were assessed by a Sperm Class Analyser (SCA, Microptic S.L., Barcelona, Spain). Integrated hardware components included a phase-contrast microscope with stroboscopic illumination, a camera, a warm stage, an image digitizer and a computer to analyze and save data. Four microlitre semen aliquots from each condition (CCSCF, CFSCM and BWW) were placed in a Leja 4 analysis chamber (Leja Products B.V., Nieuw-Vennep, The Netherlands), 19.7 mm thick. Six randomly selected microscopic fields were scanned. After every scan, the playback showed the video sequences to determine whether all spermatozoa were identified and to reconstruct their trajectory by SCA system.

Sperm mitochondrial status analysis
Sperm mitochondrial activity was evaluated by staining with JC-1 (5,5'-6,6'-tetrachloro-1,1'-3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Molecular Probes, Invitrogen, Eugene, OR, USA). JC-1 reversibly changes its fluorescence from green (monomeric status) to orange (multimeric status) when the mitochondrial membrane’s potential is high. Ten microlitres of 200 µM JC-1 in DMSO were added to 300-µl semen aliquots (from all the three conditions—CCSCF, CFSCM and BWW), centrifuged, resuspended in PBS and incubated for 20 min at 37°C. One microlitre of 50 mM CCCP (carbonyl cyanide 3-chlorophenylhydrazone, Sigma-Aldrich, Milan, Italy) was added to control tube, and the cells were incubated at 37°C, 5% CO2, for 20 min. After centrifugation and resuspension of the pellet in 500 µl of PBS, the samples were analysed on a flow cytometer Facs Scan (Becton Dickinson, Milano, Italy) with 488 nm excitation using emission filters appropriate for Alexa Fluor 488 dye and R-phycoerythrin. Sperm mitochondrial status was expressed as the percentage of sperm with high mitochondrial membrane potential.

Evaluation of DNA fragmentation
The presence of apoptosis-related DNA strand breaks in spermatozoa was evaluated by terminal deoxynucleotyl transferase-mediated d’UTP nick-end labelling (TUNEL), by use of ‘in situ cell detection kit’ (Roche Diagnostics GmbH, Germany) with FITC-labelled d’UTP. Semen aliquots 10-µl from all the three conditions (CCSCF, CFSCM and BWW) were smeared on microscope glass slides, thereafter fixed with 4% paraformaldehyde in PBS at room temperature for 60 min and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. The cell specimens were washed twice in PBS, incubated in a humidified atmosphere at 37°C for 60 min with TUNEL incubation buffer containing nucleotide mix and terminal transferase enzyme. At the end, the cell samples were rinsed twice with PBS and counterstained with DAPI. The slides were examined under fluorescence microscopy by a 100X oil immersed objective.

For each slide, 200 spermatozoa were assessed. Negative and positive controls were performed, respectively, by omitting the terminal transferase step, and by preincubating fixed and permeabilized sperm cell samples with DNase I (1 mg/ml) for 20 min at room temperature.

Evaluation of chromatin integrity
The acridine orange test was used to assess the integrity of sperm DNA. Briefly, 10-µl semen aliquots from all the three conditions (CCSCF, CFSCM and BWW) were mixed with an equal volume of acridine orange solution (1% AO in distilled water added to a mixture of 40 ml of 0.1 M citric acid and 2.5 ml of 0.3 M Na2HPO40.7H2O and pH adjusted to 2.5) on the surface of a glass slide and covered with a glass coverslip. The samples were then evaluated by a fluorescence microscope with a 490 nm excitation light and 530 nm barrier filter. Nuclei from 100 spermatozoa were examined and scored as green- or red-fluorescent. Spermatozoa may show green fluorescence in the head (bicatenary DNA, normal) or red-fluorescence (monocatenary DNA, denatured).

Sperm intracellular calcium
Sperm intracellular calcium was evaluated by staining 100-µl sperm aliquots (from all the three conditions—CCSCF, CFSCM and BWW), with Calcium Orange (Molecular Probes, Invitrogen, Eugene, OR, USA) at a final concentration of 10 µM. The spermatozoa were washed twice in PBS and after centrifugation for 10 min at 1125 g, were resuspended in 500 µl. The sperm was then incubated with Calcium Orange (Molecular Probes, Invitrogen, Eugene, OR, USA) for 30 min at room temperature. The cells were washed in PBS for 10 min at 1125 g. Finally, the spermatozoa were seeded on glass slides and examined with a epifluorescence microscope at 1000x magnifications (Nikon Eclipse50i, Nikon Instruments, Firenze, Italy). Sperm intracellular calcium was expressed as percentage of cells that resulted positive for calcium orange.

AR assessment
Sperm AR was evaluated by FACS, using FITC-CD46 on 300-µl semen aliquots from all the three conditions (CCSCF, CFSCM and BWW). Spermatozoa were first incubated for 30 min at room temperature with anti-mouse IgG (0.22 mg protein/ml) in order to block non specific sites, followed by washing the blocker off with PBS at 300g for 10 min. The cell pellet was then resuspended in PBS and divided into two aliquots. I:50 FITC-CD46 (1 mg/ml) was added, to the first aliquot, while 1:50 FITC-conjugated mouse anti-human IgG (Vector Laboratories, Burlingame, CA, USA) (1 mg/ml) was added to the other aliquot and served for negative control. After 30 min of incubation at room temperature in the dark, the spermatozoa were washed with PBS by centrifugation at 300g for 10 min, and resuspended in 1 ml PBS containing 1% formaldehyde. Immediately prior to FACS analysis, the supra-vital probe PI (2.5 mg/ml final concentration) was added. Samples of 10 000 cells (considered as 100%) were analysed by flow cytometry Facs Scan (Becton Dickinson, Milano, Italy) at 488 nm excitation using emission filters appropriate for Alexa Fluor 488 dye and R-phycoerythrin. Sperm acrosomal status was expressed as the percentage of total live sperm with AR (PI negative or positive/CD46 positive cells).

Capacitation and induction of AR
Samples of sperm (500-µl) obtained after 7-days SCs co-culture (CCSCF) were incubated in BWW supplemented with HEPES (10 mM, pH 7.4) and 3 mg/ml BSA at 5% CO2, 37°C for 3 h for capacitation, as the previously described (Jaiswal et al., 1998). For AR induction, progesterone (5 mM in DMSO) was added to the capacitated sperm suspensions to a final concentration of 10 µM. DMSO was added to sperm suspensions in control incubations. The tubes were loosely capped and incubated at 37°C and 5% CO2 for 1 h. Subsequently, the spermatozoa were
washed with PBS by centrifugation at 300 g for 10 min. The supernatant was aspirated off and the pellet resuspended in PBS for evaluation by FACS analysis using FITC-CD46 (as described above).

**Statistical analysis**

Sperm from each of 10 patients were studied in all three experimental conditions (CCSCF, CFSCM and BWW) in triplicate at each time period (2, 4, 7 days). Statistical analysis was based on the means of the triplicate observations for each condition and time and \( n = 10 \). Absolute data are expressed as mean ± SD. Comparisons between groups of results were performed by the Student’s t-test for independent variables. \( P \)-values of < 0.05 were considered significant. Statistical analysis was performed by Statistica 7.1 software (Stat Soft Inc., 2004, Tulsa, OK, USA).

**Results**

**In vitro assessment of SC purity, viability and function**

Our method yielded a very high percentage of pure SCs, close to 95%, as shown by specific (anti-vimentin) and unique (anti-MIS) SCs markers (Fig. 1A and B, respectively). Within the non-SC populations, Leydig and peritubular cells were detected at negligible concentrations (about 5%) as shown by Fig. 1C and D, respectively. These residual peritubular and Leydig cell populations could help in the preservation of paracrine interactions that occur in the intact testicle between different cell types. No germinal cells were present in the culture. We also demonstrated that SC viability was very high (>95%) and consistent throughout 14 days (Fig. 2).

Furthermore, SCs functional competence was clearly demonstrated at 3, 7 and 14 days of in vitro culture, by both SCs-derived IGF-I production (79 ng/ml/20 × 10^6 cells) and by significant estradiol production, either basal or after FSH stimulation (150 pg/ml/20 × 10^6 cells versus 298 pg/ml/20 × 10^6 cells, respectively; Fig. 3).

**Microbiological assessment and PERV transmission**

No signs of bacterial or fungal growth were observed under microscopic examination of our cultures throughout 10 days. In addition no PERV transmission from our SCs to target human cells was found.

**Sperm viability, motility and mitochondrial status analysis**

Sperm viability declined over time and had decreased by about 40% after 7 days in cell free SC medium (CFSCM) or BWW. It declined significantly (\( P < 0.001 \), \( n = 10 \), t-test) more slowly and by only 10% after 7 days in SC feeder (CCSCF) conditions (Table 1).

Both total and progressive motility declined rapidly in CFSCM or BWW reaching near zero after 4 days but decreased significantly less (\( P < 0.001 \), \( n = 10 \), t-test) under CCSCF conditions (Table 1). Additionally the sperm mitochondrial status was significantly (\( P < 0.001 \), \( n = 10 \), t-test) better maintained under CCSCF conditions than in CFSCM or BWW (Table 1).

**Evaluation of DNA fragmentation and chromatin integrity**

The percentage of sperm with DNA damage did not differ between the three conditions at any time (Table 1).

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**Figure 1** Morphological characterization of SC preparations under fluorescence (A and B) and light (C and D), microscopy. (A) SC after immunostaining with specific anti-vimentin Ab. (B) SC after immunostaining with anti-MIS Ab (green) and counterstaining with PI. Within the non-SC populations, eliminated during the purification of SC, Leydig cells (C) and peri-tubular cells (D) were detected at negligible concentrations. Bar = 20, 10, 10 and 10 μm in A, B, C and D, respectively.
Sperm intracellular calcium and AR analysis

BWW (a known capacitation inducing medium) increased the percentage of sperm with an intracellular calcium concentration high enough to be stained with calcium orange from $34 \pm 6.9$ to $46 \pm 9.4$ after 4 days falling back to $38 \pm 6.0$ after 7 days. The percentage of calcium orange positive sperm increased more slowly in CFSCM and changed very little in CCSCF (Table I). CCSCF did not induce ARs, but the CFSCM and BWW conditions increased the AR (Table I), following the trend of the intracellular calcium (Table I).

Capacitation and induction of AR

After 7-days culture in CCSCF, the percentage of sperm AR was about 27%. We found that the sperm were still able to capacitate and to...
undergo AR since they responded to progesterone stimulus by increasing AR from 23.4 ± 4.9 to 55.9 ± 7.5, P < 0.007, t-test, n = 10). We did not examine the sperm obtained from the other two experimental conditions (CFSCM and BWW) since the results of AR evaluation showed a very high AR positive sperm after 7-day culture.

### Discussion

A major problem in ART is the rapid deterioration of sperm viability in culture, which makes them unsuitable for fertilization after 48 h. Since SCs constitute a mechanical and functional support for developing spermatozoa, we cultured spermatozoa on a SCs mono-layer acting as a feeder (CCSCF), and compared the results with those derived from sperm cultured in SCs medium only (CFSCM) or in BWW, the latter being one of the media suggested by the WHO manual (Cooper et al., 2010; World Health Organization, 2010). Prepubertal porcine SCs were selected due to technical problems encountered in retrieval of SCs from the adult testis as well as ethical/legal issues burdening the retrieval of donor adult human testes. The potential risk from known xenotic agents in xenotransplantation can be managed by providing a pig source free of such agents, and carefully testing the transplanted materials. With respect to a major issue in pig tissue transplantation, such as potential PERV transmission, despite many attempts, using cells that express these viruses or the viruses themselves, no productive infection to man (Paradis et al., 1999) has ever been transmitted, even when the recipient was immune-incompetent or immune-suppressed (Specke et al., 2009). Moreover, the risk, if real, could be managed (Auchincloss et al., 1999). New proliferating human SCs cultures (Chui et al., 2010) are now becoming available and may make the use of porcine tissue unnecessary.

Our results pinpoint that sperm can preserve their properties in culture for 7 days, although it requires physical contact with the SCs, rather than only with the specific SCs medium. The nursing function of SCs is well known and it is due not only to production of defined factors but also to specific intracellular links between SCs and developing spermatozoa, as well as to signalling transduction pathways involving membrane interactions (Wong and Cheng, 2009).

The explanation of the observed data about prolonged viability and maintained mitochondrial functional integrity, as induced by CCSCF, could be explained by the Sertoli-related growth (IGF-1, IGF-2, PDGF, VEGF) and anti-apoptotic (Bcl-2) (Fujikawa et al., 2001) factors.

As far as significant increase in sperm motility after CCSCF treatment is concerned, we postulate a crucial role of Sertoli-released VEGF, as demonstrated by lybokurt et al. (2009). Indeed in the past, several mammal cell-feeder layers have successfully been employed to culture germinal cells (Seandel et al., 2007). Moreover, we confirmed that BWW is not the appropriate medium for long-term sperm culture maintenance.

We are developing a new series of experiments to test the survival and quality of the sperm in the presence of conditioned media (SC medium obtained from SC feeder culture flask with or without the addition of FSH, LH and/or Testosterone) or in co-culture with SCs embedded in alginate-based microcapsules, but we cannot speculate, at this time, about the outcome of these studies. By the way, very recent data in our laboratory (not shown) seem to document a better performance of cell contact over SC-conditioned medium, suggesting a pivotal role of cell versus humoural factors.

In conclusion, we found a method to maintain human sperm in good condition for at least 7 days, which could be used advantageously to either keep the spermatozoa viable while waiting for maturation of the oocytes during ARTs, or to transfer sperm between two distant Centres without cryopreservation.
**Authors’ roles**

M.M., D.Z., G.L., R.C., C.F. were involved in research design, performing research, analysis of the data, manuscript drafting and critical discussion. A.F. played a role in analysis of the data, manuscript drafting and critical discussion. M.C. and F.M. took part in performing research, analysis of the data, manuscript drafting and critical discussion.

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