**Functional properties and in vivo biocompatibility of microencapsulated Sertoli's cells.**

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## Introduction

Recently, Sertoli Cells (SC) have been revisited with respect to their functional competence (Jégou B. et al. 1992, Emerich D.F. et al. 2003). SC have been recognised to play important roles at different levels, including regulation of syntesis of androgen binding protein (ABP) (Jégou B. et al. 1992), the paracrine action on peritubular and Leydig cells (Weinbauer G.F. et al. 1999). In addition, SC provide an appropriate microenvironment for the development of germ cells (Jégou B. et al. 1992, Emerich D.F. et al. 2003).
Moreover, recent reports have shown that these cells may provide for nutrients, immunomodulatory and trophic factors that are able to ameliorate survival and development of different cell types and improve functional competence in different cell types (Emerich D.F. et al. 2003, Korbutt G.S. 1997). Infact, many papers have reported on the ability of SC to protect the tissue allografts (mainly pancreatic B-cells) (Emerich D.F. et al. 2003, Korbutt G.S. 1997) by forming a testis-like immunoprivileged environment, which obviates no need for immunosuppression therapy regimens.
With respect to the possible use of "free" SC (with no polymer coating) in xenograft protocols, at least two points deserve attention. First, use of "free" cells could result in serious problems with regard to eventual need for cell retrieval, an important regulatory issue.
Second, in long-term transplantation protocols, the well known immunomodulation-immunoprevilege-oriented activity of SC could not suffice to preserve long-term functional integrity of the transplant.
Bearing in mind this point, in the present paper we report on: (a) efficient strategy to encapsulate SC in alginate based microcapsules; (b) in vitro characterization and in vivo biocompatibility of microencapsulated SC upon graft in NOD mice.

## Materials and Methods

*Isolation of Sertoli cells*
Neonatal pre-pubertal "Large-White" pigs, aging 7-15 days, were used as SC donors. SC were isolated, according to modified previously established methods, modified in our laboratory (Korbutt G.S. et al. 1997, Luca G. et al. 2005).

*In vitro assessment of isolated Sertoli cells*
After isolation and purification, in vitro cultured SC were characterized and examined for viability and function (α-aromatase activity and IGF-1 secretion).
Viability. Immediately after isolation, at day 2 of culture and before microencapsulation (after trypsin treatment) SC viability was assessed by staining the preparations with ethidium bromide (EB) (Sigma) and fluorescein-diacetate (FDA) (Sigma).
Functional competence (α-aromatase activity and IGF-1 secretion).
SC were examined for α-aromatase activity. Briefly, $10^6$ free or encapsulated SC, were treated for 3 days with 1 μg/ml Follicular Stimulating Hormone (FSH) (Serono, Rome, Italy), thereafter, 0.2 μg/ml testoterone enantate (SIT, Pavia, Italy) was added for 8 h. At the end of the incubation, the supernatant was used to determine 17-β-estradiol (E2) concentration by direct chemiluminescence (ADVIA Centaur, Estradiol-6 III, Bayer Diagnostics, Germany). Insulin-like growth factor 1 (IGF-1) secretion from free and encapsulated SC was determined by RIA.

*Preparation of alginate based microcapsules*

SC were embodied in alginate microcapsules according to our method (Luca G. et al. 2005). Briefly, the AG/SC suspension was continuously aspirated by a peristaltic pump and thereafter extruded through a mono air-jet device under sterile conditions. The microdroplets were collected on a gelling bath, whereby they immediately turned into alginate gel microbeads and let incubate. After the different incubation times, the obtained microcapsules were washed twice in saline and directly used, in the case of Ba based microcapsules (AG-Ba MC) or sequentially incubated, in the case of Ca based microcapsules (AG-Ca MC) with aqueous solutions of low molecular weight poly-L-ornithine (PLO) (Sigma). Microparticles morphology, size and size distribution were assessed under inverted phase and stereomicroscopy, by counting at least 300 particles/batch.

*In vivo biocompatibility of encapsulated SC*

At 8 months of transplant, the microcapsules were retrieved from 6 animals to evaluate biocompatibility by peritoneal lavage. General characteristics of the retrieved microcapsules, with special regards to extent of the fibrotic overgrowth under phase contrast microscopy, (examining 200 capsules/animal), and morphological features such as microcapsules sphericity, surface smoothness and finally viability by EB/FDA, were examined.

**Results and Discussion**

After 24 hours of TRIS treatment, SC preparation were characterized as far as purity, morphology and functional competence were concerned (see Fig. 1).

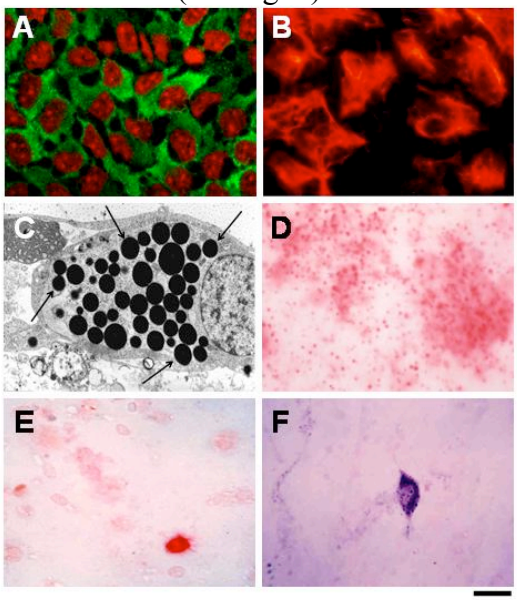


**Fig. 1**: Morphological characterization of SC preparation by optical (A, D, E, F), fluorescence (B) and electron (C) microscopy. A: SC after immunostaining with specific Ab for the Mullerian inhibiting substance (MIS). B: SC after immunostaining with anti-vimentin Ab. C: transmission elecron microscopy (TEM) showing the typical lipid bodies (see arrows) present in the SC cytoplasm. D: SC after positive staining with Sudan III. Within the non-SC cell populations, eliminated during the purification of SC, peritubular cells constituted a minimal fraction out of the isolated cell mass (E), Leydig cells were detected at negligible concentrations (F). Bar corresponds to 10, 5, 0.2, 5, 10, 5 μm in panel A, B, C, D, E and F, respectively.

*Sertoli cells encapsulation*

Immediately before encapsulation, SC were detached from culture dish after trypsin treatment and characterized for viability (by EB-FDA staining, Fig. 2 A) and functional competence, and finally evaluating the α-aromatase activity (by RIA test) that is specific for SC.

SC functional competence was clearly documented by a significant raise in E2 concentration in the supernatant upon incubation with FSH that, in presence of the SC localized α-aromatase, converted testosterone into E2.

Moreover we assessed secretion of IGF-1, that represents one of the growth factors secreted by SC, by RIA test, demonstrating that SC are functionally active as showed by the high output of IGF-1 (60-80 ng/ml/20x$10^6$ cells).
Alginate microcapsules were prepared by a dripping method resulting in the production of ‘‘medium size’’ microcapsules that were extremely monodisperse.
The isolated SC pellet was passed through a droplet generator based on a air-driven jethead, which resulted in the continuous formation of spherical droplets.
The resulting droplets were, in a first set of experiments, gelled upon contact with a $CaCl_2$ solution and let to react for 10 minutes (“long incubation”). The resulting AG-Ca MC were then incubated in a solution of poly-L-ornithine (PLO). The obtained microcapsules were fully satisfactory in terms of size, morphology, sphericity and coalescence as clearly appreciable from the photomicrographs reported in Fig. 2 B-C.
In addition, the microcapsules resulted elastic and translucent thus facilitating the microscopic observation of the SC viability and morphology during the following in vitro studies, Fig. 2 D.
Unfortunately, the EB-FDA viability staining reported in Fig. 2 D, performed at 2 days of the encapsulation, demonstrated the unsatisfactory viability of the encapsulated SC in AG-Ca-PLO-AG MC (32±4%).

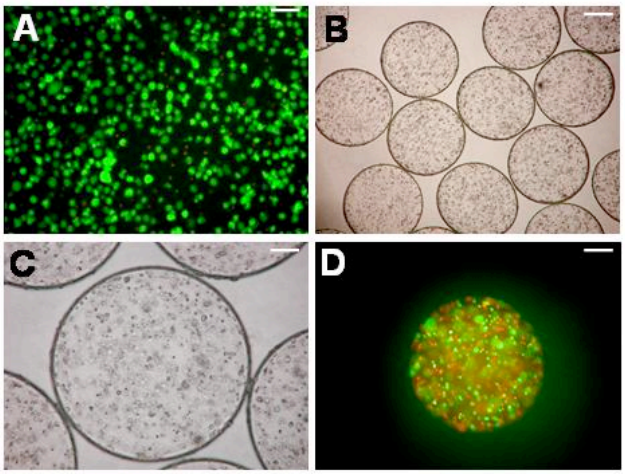


**Fig. 2**: Fluorescence (A, D) and optical (B, C) photomicrographs of SC. Viability after trypsin detachment (immediately before the encapsulation procedure) (A) and alginate microencapsulation (B-D). SC were encapsulated in Ca-alginate-PLO microcapsules as reported in the experimental section (“long incubation”). The cell viability of encapsulated SC was determined by EB-FDA staining after 2 days from the encapsulation (D). Bar corresponds to 80, 200, 80 and 120 μm in panel A, B, C and D, respectively.

Aiming to find the optimal $Ba^{+2}$ ion concentration for the encapsulation of SC without compromising the morphology of the microcapsules and the cell viability, we examined different concentration of $BaCl_2$ (Fig. 3).
These experiments demonstrated that low $Ba^{+2}$ ion concentrations (0.5%) resulted in the production of microcapsules with unsatisfactory morphologic and mechanical properties. In fact, these microcapsules were unable to withstand the mechanical stress associated to the normal procedures of cell culture, transfer and microcapsule manipulation (Fig. 3 B). In fact, the microcapsules produced with the lower concentration of barium ions (0.5% $BaCl_2$) were (a) imperfectly spherical, mainly ellyptical, (b) partially broken with an irregular surface and finally (c) the microcapsules exhibited many exposed cells in the periphery of the microcapsular structure.
On the contrary, using 1 or 1.5% $BaCl_2$ gelling solutions, the resulting AG-Ba MC showed much better morphological characteristics (Fig. 3 A, C-D), comparable to those associated with the well known AG-Ca-PLO-AG MC.[5] Ba-based microcapsules appeared only slightly larger in size (see Fig. 4), as proven by microscopic examination (Fig. 3 A, C-D). In the case of AG-Ba microcapsules, viability of the encapsulated SC, at 9 days of encapsulation, exceeded 90% of the

counted cells. Within the different concentrations of $Ba^{+2}$, the best results were obtained using 1.0% $BaCl_2$, as proved by the higher viability and cell density of SC at 9 days of the encapsulation.

*Evaluation of encapsulated SC*

As a further control, mandatory to possibly apply the encapsulated SC system to in vivo experiments, we determined the functional properties of the SC encapsulated in AG-Ba MC.

These two tests were performed since they allow to verify SC functional competence. In fact, α-aromatase activity was studied since biochemical and histochemical studies have identified several enzymes that are associated with specific cell types of testis and are referred to as marker enzymes. α-aromatase activity is considered as important markers of SC function since SC cultures actively synthesize estradiol from androgens (catalyzed by aromatase) and secrete it in the medium.

Finally, in order to evaluate the biocompatibility of AG-Ba MC ($BaCl_2$ at 1.0%), microencapsulated SC were transplanted in the peritoneal cavity of NOD mice as described in the experimental plan section.

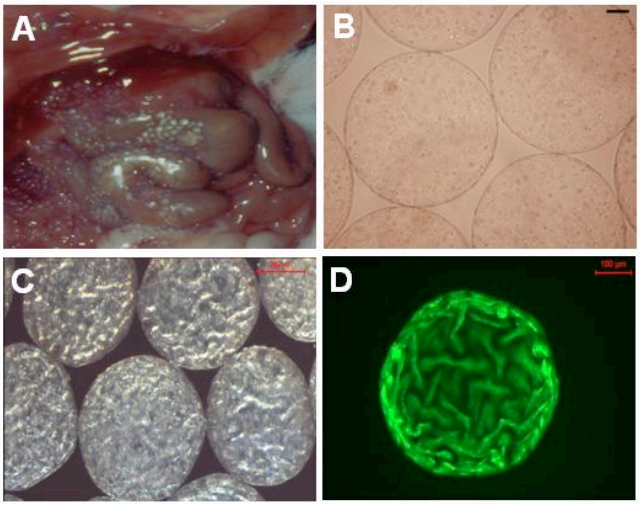


**Fig. 7:** Biocompatibility of AG-Ba 1.0% MC containing SC after implantation in the peritoneal cavity of NOD mice. Panel A: general aspect of the peritoneal cavity after having sacrified a mouse showing the free flowing of the capsules; Panels B-D: Examination of a typical batch of microcapsules containing SC upon retrieval after 4 (B) or 8 months (C-D) from the peritoneal cavity, freshly (Panels B-C) and after staining with EB+FDA for SC viability (Panel D) respectively. Bar corresponds to 80, 200 and 100 μm in panels B, C and D, respectively.

The obtained results, summarized in Fig. 3, demonstrated that the capsules were freely floating in the peritoneal cavity (Fig. 3 A) and morphologically intact, with the majority of the microcapsules (86 % ±10) appearing free of fibrotic tissue overgrowth at 4 (Fig. 3 B) and 8 months from transplant (Fig. 3 C). In addition viability (Fig. 3 D) of the explanted AG-Ba 1.0% MC, containing SC showed, at 8 months of transplantation, that the embodied SC were extraordinarily very viable and formed a tubules-like structures in the capsules.

**References**

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