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Improved Function of Rat Islets upon Co-Microencapsulation with Sertoli's Cells in Alginate/Poly-L-Ornithine

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ABSTRACT

The purpose of this study was to assess whether Sertoli's cells would improve functional performance of homologous pancreatic islets within microcapsules. Purified rat Sertoli's cells were co-enveloped with islets in microcapsules that had been fabricated with alginic acid and poly-L-ornithine. Confocal laser microscopy was used to determine any mitogenic effects of Sertoli's cells on islets ß-cells. Insulin secretion from islets, with or without Sertoli's cells, was examined, and grafts of Sertoli's cells with islets in microcapsules into diabetic mice were carried out. Co-incubation of Sertoli's cells with islets resulted in a significant increase in the islet ß-cell mitotic rate, which was coupled with significantly higher insulin release under glucose stimulation, as compared to controls. Grafts of co-microencapsulated Sertoli's cells with islets resulted in prolongation of the achieved normoglycemia in the animals receiving Sertoli's cells with islets as compared to controls that received islets only. Sertoli's cells do promote mitogenic activities upon in vitro co-incubation with islets, whose in vitro functional and in vivo post-transplant consequences were evident. Sertoli's cells could, therefore, be co-microencapsulated with islets for transplantation in diabetic recipients.

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INTRODUCTION

Transplantation of islets in patients affected by type I diabetes mellitus has been recently demonstrated to be a good alternative to exogenous insulin administration [1], although at least three major issues are still pending: 1) the restricted availability of donor pancreatic islets; 2) the necessity of the host's general immunosuppression; and 3) the minimal proliferation capacity of adult islet ß-cells.

Due to restricted availability of cadaveric human donor pancreases, it may be possible to use adult and neonatal pig islets, although the use of xenogeneic animal tissue sources in humans is questionable.

To protect pancreatic islet xenografts, islets were microencapsulated in highly biocompatible and selective permeable membranes [2,3].

Sodium alginate/poly-L-ornithine (ALG/PLO) microcapsules were prepared by a semi-automated procedure developed in our laboratories [4]. This semi-automated procedure allows preparation of microbeads with uniform size (400-500 µm) and shape. However, adult pancreatic ß-cells, putatively deriving from exocrine duct stem cells [5], have a very low replication capacity. In fact, the ß-cell proliferation capacity depends on animal age (from 10% in the fetal period to 1%-2% in adulthood) [6,7,8,9].

Sertoli's cells, considered in the past to have a simple mechanical architectural function, have been recently re-evaluated. In particular, it has been recently demonstrated that they play a critical role in different pathophysiological activities, such as the synthesis of many partially known growth factors (IGF-1, IGF-2, EGF) [10], immuno-modulatory properties (mainly

related to TGF-1ß secretion) [11], and anti-apoptotic function (production of Bcl-w).

Recent reports have shown that Sertoli's cells may improve both the in vitro and in vivo functional competence of different cells. In particular, it has been demonstrated that Sertoli's cells improve in vitro functional competence of islets [12] while in vivo; they may also prolong islet allograft survival in animal models of diabetes mellitus [13]. Finally, intracerebral transplantation of Sertoli's cells relieved symptoms in rats with pharmacologically induced hemiparkinsonism [14].

To investigate the reasons for in vitro improvement of the functional performance of the co-incubated islets, we evaluated the islet ß-cell mitotic rate versus that of control islets alone. In vivo diabetes-correction rat studies are also planned.

MATERIAL AND METHODS

Rat pancreatic islet source and isolation

Rat islets were isolated—according to original methods [15] modified in our laboratory—from male Sprague/Dawley (S/D) rats, weighing approximately 120-130 g each. Briefly, after anesthesia and total laparotomy, the rats were injected with a collagenase solution in the common bile duct, which was cannulated by an indwelling polyethylene catheter after duct ligation at the merging point with the duodenum to permit retrograde pancreatic distention.

The pancreas was detached from the small and large intestine, stomach, spleen and lymph nodes and removed. Pancreas digestion was performed under gentle shaking at 37°C until the pancreatic tissue became very fine. After many washes, the digested tissue was centrifuged against Eurocollins/Ficoll density gradients. The islets were collected at the 1.096-1.060 interface. The resulting pancreatic islets obtained were intact, viable, and pure.

Sertoli's cells isolation

Sertoli's cells were separated from the testes of prepubertal S/D male rats (weighing approximately 50 g) according to previously described methods [13,16] recently upgraded in our laboratory [17]. Briefly, upon removal of their fibrous cap, the testes were finely chopped until a fine homogeneous tissue was obtained; this tissue was treated by double enzymatic digestion with collagenase P (Roche Diagnostics, Milan, Italy), trypsin, and DNAse (Sigma, St Louis, MO). The digestion continued until a white connective tissue-like matter formed, which, upon removal and washing, was eventually passed through a 500-µm pore-size stainless mesh filter, collected and seeded in 100 x 15 mm Petri dishes, and incubated at 37°C. Then the preparation was treated with 20 mM TRIS at pH 7.2 for 7 minutes to eliminate the residual germinal cells [16]. The isolated Sertoli's cells were viable and highly purified (90%) (Figure 1).

ALG/PLO microcapsules fabrication

Isolated islets combined with Sertoli's cells were diluted in LVCR (pharmaceutical grade) 1.6% sodium alginate High M (mannuronic acid: 75%; guluronic acid: 25%) (Kelco-Monsanto, San Diego, CA) and thoroughly, but gently, mixed in order to obtain uniform suspension. The suspension was continuously aspirated at a fixed flow rate by a peristaltic pump and extruded through a single jet-head connected with air (4.5 L/min). The resulting microdroplets, each containing a single islet, were collected in a 1.2% CaCl2 bath and immediately turned into calcium alginate gel microspheres. The gel microbeads were washed twice in saline and then incubated with 0.12% PLO (coat I) (Sigma). After washing in saline, the beads underwent a second incubation with 0.06% PLO (coat II). After a final washing in saline, the beads underwent a final incubation with 0.04% sodium alginate. The purpose of PLO coats I and II was to thoroughly saturate the COO radicals of the calcium alginate microbeads by displacement of calcium by PLO. The outer low-concentration (0.04%) sodium alginate coat served only to increase the capsule's biocompatibility. The average diameter of these microcapsules ranged from 400 µm to 500 µm.

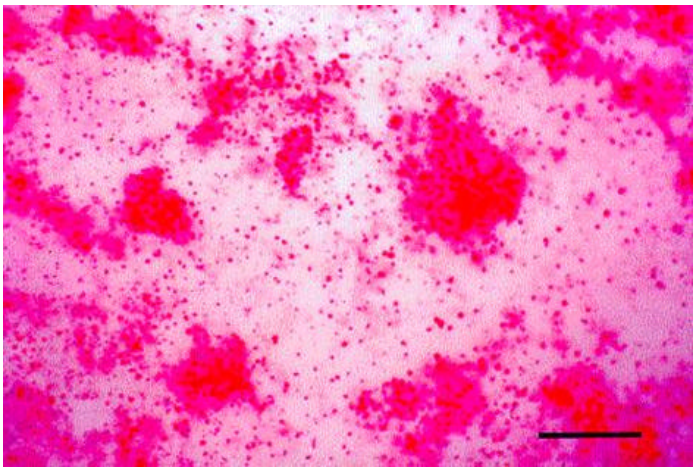


Figure 1. Photomicrograph of freshly isolated Sertoli's cells after staining with Sudan III (bar = 100 mm).

In vitro studies

Batches of 20 islets were incubated with either 10 000 or 20 000 Sertoli's cells with islets, in HAM F-12, in triplicate. Isolated islets or Sertoli's cells batches were used as controls. Tissue culture medium was changed every 3 days. During 12 days of in vitro culture maintenance, insulin levels were determined in the supernatant (IRI) by RIA. To assess the functional performance of the islets, the co-cultures were exposed (day 9) to different glucose concentrations (50-300-50 mg/dL) in a 2-hour static incubation system. On day 12, the tissue pellets underwent fixation and were double stained with bromo-deoxyuridine (BrdU) and fluorescein isothiocyanate (FITC)-conjugate anti-BrdU MoAb as a cell mitosis marker, green fluorescence and TRITC-conjugate anti-insulin as a ß-cell marker, and red fluorescence to detect images of ß-cell proliferating activities under confocal laser microscopy (CLM) examination. Briefly, BrdU 10 µm was added to the culture medium for 24 hours [18]. After washings, the tissue was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) for 30 minutes at room temperature.

The BrdU-labeled islets, upon denaturation of DNA by acid hydrolysis (2 M HCl) and washing, were incubated at room temperature with a 1:25 dilution of a mouse anti-BrdU MoAb (Boehringer Mannheim, Indianapolis, IN) in PBSwith 0.3% Triton X-100 (PBS/T) for 18 hours on a rotating plate. The islets were washed, incubated with a 1:25 dilution of FITC-conjugated goat anti-mouse immunoglobulin G (Sigma Aldrich, St. Louis, MO) in PBS/T for 18 hours on a rotating plate, washed again, and finally examined under CLM, having previously been incubated with mouse anti-insulin Ab (Sigma) for 18 hours at 4°C and with anti-mouse TRITC for 18 hours at 4°C, as a secondary Ab. The confocal laser microscope used was an 25 mW multiline argon ion LSM410 (Zeiss, Oberckochen, Germany). The batches were examined with a x100, 1.3 numerical aperture PlanNeofluar objective lens. Images were acquired frame by frame with a scanning mode format of 512 x 512 pixels. The HeNe laser, which produces a major line at 543 nm, was employed for double labelling experiments. To this end, FITC and TRITC signals were separated by a secondary dichroic mirror (565 nm) and simultaneously detected by two photomultiplier tubes. A band pass filter (520 ± 15 nm for FITC) and a barrier filter (590 nm for TRITC) were placed before the two photomultiplier tubes to avoid overlapping between the two signals, as previously described [19].

Digitalized optical sections (ie, Z series of confocal data, or "stacks") were transferred from the CLM to the graphics workstation Indigo Irix XS24 (Silicon Graphics, Mountain View, CA) and stored with a scanning mode format of 512 x512 pixels and 2567 gray levels. The image processing was performed using the ImageSpace software (Molecular Dynamics, Sunnyvale, CA), as previously described [20]. All in vitro data were analyzed by repeated analysis of variance (ANOVA) measures.

In vivo studies

Streptozotocin (STZ)-induced CD-1 diabetic mice were intraperitoneally transplanted with a unit dose of 1000 islets plus 6000 Sertoli's cells with islets containing MSM ALG/PLO microcapsules (Figure 2) (group 1, 5 animals) or alternatively with 1000 islets containing MSM ALG/PLO microcapsules (Figure 3) (Group 2, 5 control animals). Blood glucose level was measured by a reflectometer.

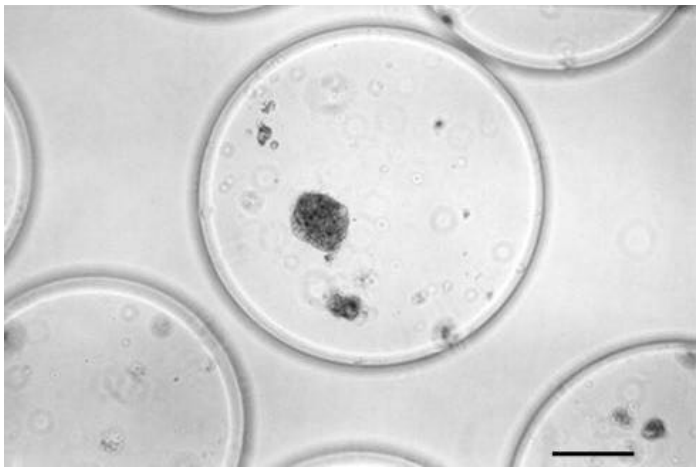


Figure 2. Microcapsules containing 6000 Sertoli's cells with islets (bar = 100 µm).

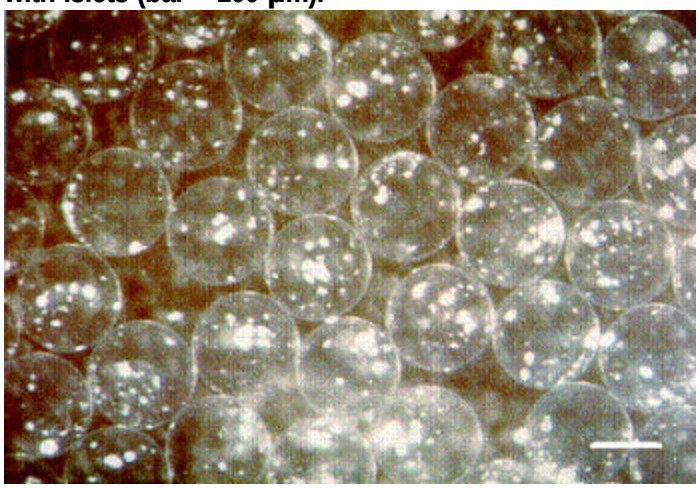


Figure 3. Microcapsules containing rat islets alone (bar = 200 µm).

RESULTS

In vitro studies

CLM examination was associated with a significant number of ß-cell mitotic figures when the islets were co-incubated with Sertoli's cells as compared to controls (Table 1) ($p < 0.01$).

At day 12, we observed a significant increase in endogenous insulin output from the islets that were co-cultured with Sertoli's cells, in comparison with either islets or Sertoli's cells alone ($p < 0.05$) (Figure 4). The enhanced insulin secretion deriving from the Sertoli's cells with islets complexes was significantly higher than that derived from islets alone. Interestingly, although insulin released from Sertoli's cells with islets co-cultures under static glucose incubation increased on day 9, the statistical significance was reached only when the Sertoli's cells with islets ratio was 1:20 000 ($p < 0.05$) (Figure 5). This observation indicated that only certain Sertoli's cells with islets ratios should be employed for in vitro as well as in vivo application. Static glucose incubation performed at day 3 and day 6 showed exactly the same pattern (data not shown).

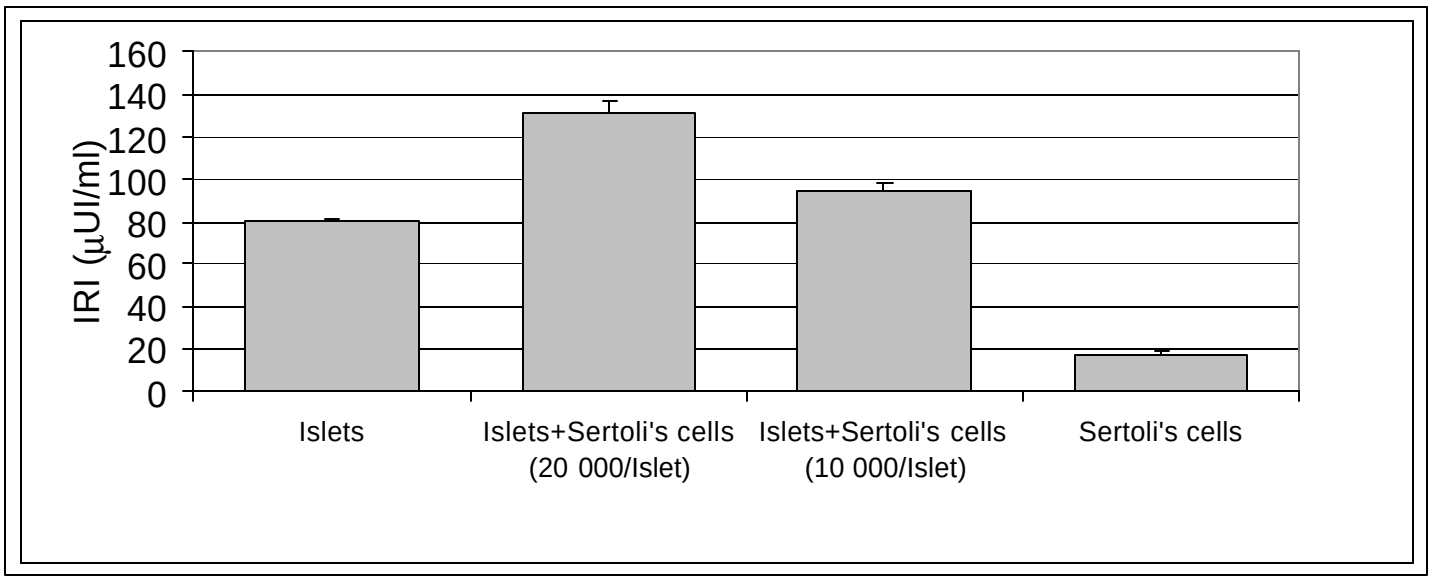
In vivo studies

In vivo reversal of hyperglycemia was fully achieved in both experimental groups and was sustained in 100% of the recipients at 45 days post-transplantation, when some animals of the islets group (but not the Sertoli's cells with islets group) started failing. One hundred percent of the animals belonging to the Sertoli's cells with islets group were associated with full graft survival through 80 days post-transplantation. At that time, no animals grafted with islets were still euglycemic, but two animals transplanted with Sertoli's cells with islets were (Figure 6).

Table 1: Percentage of ß-cells that were positive to immunostaining with 5'-bromo-deoxyuridine (BrdU) at 12 days. For each experiment, 300 cells were counted. Data are expressed as mean ± SD from three separate experiments. p values < 0.01 were significant.

Sertoli's cells	% viable cells	% 5'-BrdU+ ß cells
None	91 ± 5	1.0 ± 1
10 000/islet	93 ± 3	5.8 ± 1.3
20 000/islet	92 ± 4	8.1 ± 1
*p < 0.01		

Figure 4. In vitro endogenous insulin output (mU/mL) from islets alone vs. Sertoli's cells with islets at different concentrations (20 000/islet vs. 10 000/islet) at day 12.



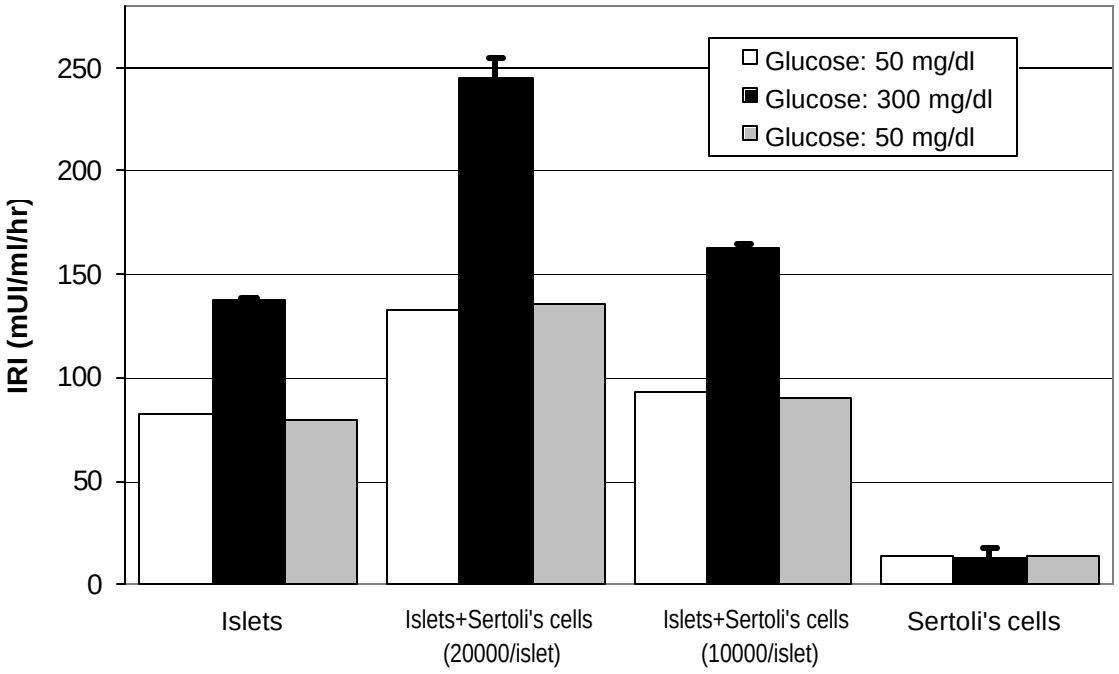


Figure 5. Insulin release under static glucose incubation on day 9. Insulin release was significantly higher ($p < 0.05$) only in the group Sertoli's cells with islets 20 000.

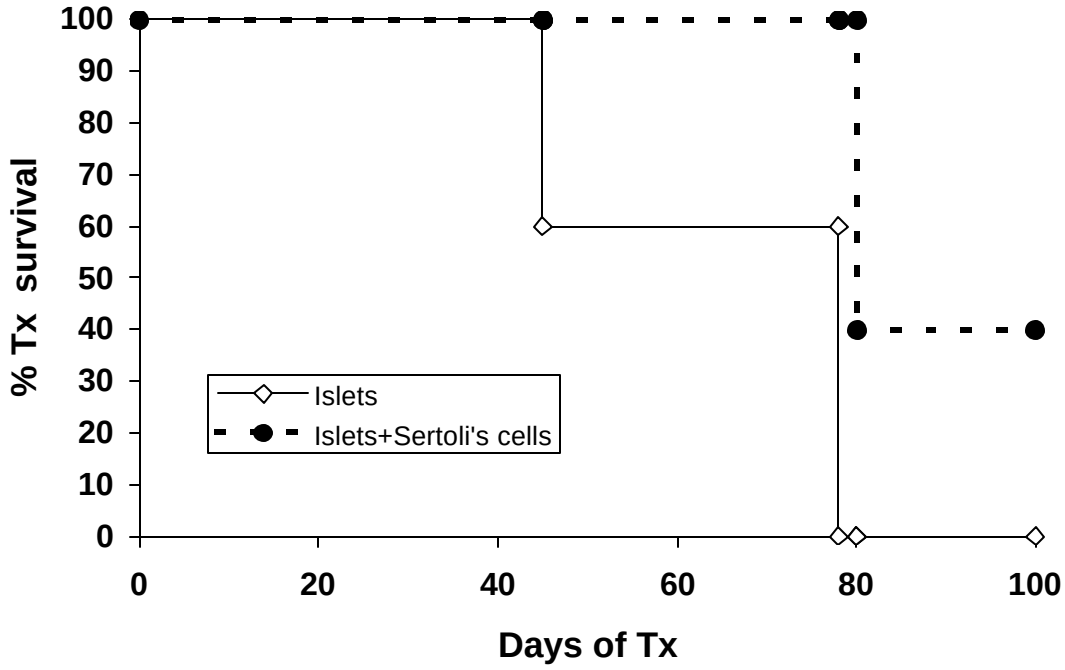


Figure 6. Graft survival of microencapsulated Sertoli's cells with islets vs. islets alone in diabetic mice. TX = transplantation.

DISCUSSION

Although the host's immune response represents a major problem for transplantation of pancreatic islets, the problem of the longevity of the grafted islets, which is unrelated to the immune system, may also prevent successful islet cell transplantation. Although the immune system issue remains the highest priority on the islet transplantation progress schedule, evidence can now be marshalled for the fact that islet autografts, where non-immune destruction would occur, are associated with finite life-span. Therefore, improvements in islet cell graft survival could indirectly help the islet transplantation's functional performance, within an immunologically active set-up such as an islet allograph or xenograft.

ALG/PLO microcapsules, while securing adequate biochemical exchange, would deny access to humoral as well as cellular mediators of the immune system. The microcapsules' uniformity of size and shape, coupled with the high purity of the constituent polymers, accounted for the absolute biocompatibility.

Transplantation of cerebral Sertoli's cells relieved symptoms in rats with pharmacologically induced hemiparkinsonism [14]. Moreover, allograft of unpurified Sertoli's cells, in combination with islets, beneath the kidney capsule of rats with STZ-induced diabetes significantly prolonged the achieved normoglycemia, as compared to controls receiving islet transplantation alone [13].

The aim of this work was to assess if Sertoli's cells with islets co-cultures could i) increase, in vitro, adult rat pancreatic islet ß-cell proliferation capacity, ii) sustain normoglycemia in the recipients, and iii) confirm the in vitro results by comparing them with those of controls receiving islet transplantion alone.

We have preliminarily demonstrated that Sertoli's cells, in vitro, may significantly stimulate the mitogenicity of homologous rat islet ß-cells (as confirmed by insulin output basally and under static glucose stimulation). This effect seems to be dose-dependent. Moreover, in vivo normoglycemic state in mice with STZ-induced diabetes was substantially more prolonged by microencapsules containing Sertoli's cells with islets, as compared to those with islets alone, thereby supporting the in vitro results. Of course, the in vivo data are just preliminary and warrant the confirmation studies that are in progress.

CONCLUSION

Sertoli's cells preliminarily seem to promote islet ß-cell proliferation as confirmed by morphological as well as in vitro functional studies. Furthermore, Sertoli's cells improve the functional performance of microencapsulated homologous rat islet grafts in non-immunosuppressed, diabetic mice, thus supporting the in vitro data. Implications of these preliminary results could be relevant for providing new means to improve life-span and functional performance of the islets grafts as a final cure for type I diabetes mellitus.

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