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The thyroid hormone T3 improves function and survival of rat pancreatic islets during in vitro culture

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Ex vivo islet cell culture in the presence of stimulating factors prior to transplantation is considered a good strategy in contrast to the short conclusion of islets transplantation. Previously, we demonstrated how T3 can increase β -cell function via specific activation of Akt; therefore we hypothesized that thyroid hormone T3 can be considered a promising candidate for the in vitro expansion of islet cell mass. Rat pancreatic islets have been isolated by the collagenase digestion and cultured with or without the presence of the thyroid hormone T3 10^{-7} M. Islets viability has been evaluated by the use of two different dyes, one cell-permeable green fluorescent dye and propidium iodide, and by the analysis of core cell damage upcoming. Moreover, islets function has been evaluated by insulin secretion. The ability of β -cells to counteract apoptosis induced by streptozotocin has been analyzed by TUNEL assay. We demonstrated that treatment of primary cultures of rat pancreatic islets with T3 results in augmented β -cell vitality with an increase of their functional properties. In addition, a sensible reduction of the core cell damage has been observed in the T3 treated islets, suggesting the preservation of the β -cells integrity during the culture period. Nonetheless, the insulin secretion is sensibly augmented after T3 stimulation. The strong increment shown in Akt activation suggests the involvement of this pathway in the observed phenomena. In conclusion we indicate T3 as a good factor to improve ex vivo islets cell culture.

Introduction

β -cell replacement by transplantation of whole organ or islet cells are currently regarded as acceptable therapeutic options for patients with type 1 diabetes and both have shown benefits in achieving and maintaining good glycaemic control.¹ Islet cells transplantation has clinical indications similar to whole organ procedure and it is a more current and highly specialized technique. It is a less invasive protocol but it requires multiple donor organs per recipient in order to transplant an adequate number of islets. This represents a major handicap to widespread application because of the shortage of donor organs.² In addition, an immunosuppressive regimen is required to avoid graft rejection. Apart from immune rejection, a critical problem is how islet cells can survive in a new environment. In fact, shortly after implantation, islet grafts function poorly and many transplanted β -cells undergo apoptosis prior to full engraftment.³ It is estimated that only 30% of the islet mass is stably engrafted, despite the administration of a large amount of islets per diabetic recipient. Therefore, instead of increasing the number of islets implanted, a more desirable strategy is to improve islet graft survival and

proliferation potential during a pre-transplantation culture period and during the days immediately following transplantation.⁴ In addition, some major concerns regard the isolation procedure and the possibility that a great percentage of islets might suffer from the isolation phase. Even after the recent improvements of the isolation technique, such as the use of the two-layer method and a less toxic iodixanol gradient, many skilled groups are able to isolate enough islets for transplantation in only 50% of the procedures performed.² Furthermore, a significant number of cells are damaged by the apoptotic and necrotic processes occurring in the post-isolation immediately period of time.^{5,6} In this context the identification of molecules able to promote both β -cell survival and function would be of relevance in the design of new therapeutic strategies aimed at improving β -cell function and increasing the amount of islets for transplantation.^{7,8}

Thyroid hormones have widely been known for their versatile ability to influence many physiological and cellular processes.⁹ Although the link between thyroid and pancreatic function has not been clearly documented yet, our recent studies evidenced how T3 action might influence pancreatic β -cells. Previously, we had demonstrated¹⁰ that thyroid hormone T3 induces a

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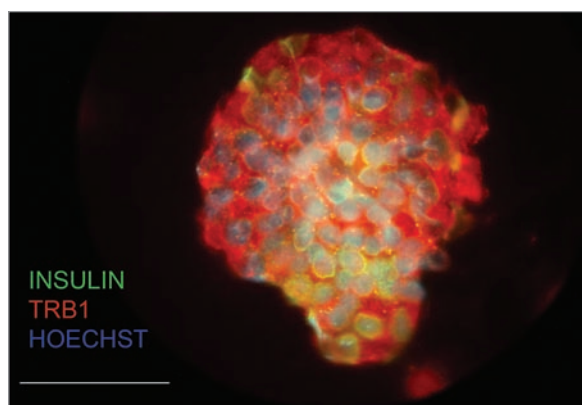


Figure 1. TRB1 Immunofluorescence. Islets were cultured for 24 h and then indirect immunofluorescence was performed on islets for Thyroid Receptor β 1 (red) and insulin (green). Nuclei were counterstained with Hoechst (blue). Bar: 100 μ m.

transdifferentiation towards a β -cell like phenotype in pancreatic ductal cells, which are considered β -cell precursors. In addition our studies on pancreatic islet cells¹¹⁻¹³ evidenced the ability of the said thyroid hormone to act as a mitogenic and protective factor in pancreatic β -cells undergoing apoptosis; we also demonstrated that the thyroid hormone T3 can directly activate the kinase Akt thus influencing cellular processes strictly related to β -cell function such as cell proliferation and survival, cell size regulation, protein synthesis and insulin production. The insulin receptor substrate (insulin receptor 2/phosphoinositide 3 kinase) pathway plays a crucial role in regulating β -cell mass and function.¹⁴ The serine-threonine kinase Akt is a major downstream target of PI3K and it has been implicated in cell cycle progression and survival of pancreatic β -cells.^{15,16}

It has been demonstrated that immediately following isolation, human islets display low levels of Akt phosphorylation.^{6,17} However, following overnight culture Akt becomes highly phosphorylated in a PI3K-dependent manner;¹⁸ this increased phosphorylation affords islets a certain degree of protection against insults.

Considering the relevant role that has been demonstrated for Akt phosphorylation levels in pancreatic β -cell survival, both in vitro and in vivo, and our observation that thyroid hormone T3 can specifically upregulate PI3K/Akt signaling in pancreatic β -cells, the goal of the present study was to evaluate the effect of T3 treatment on primary rat islets and to elucidate the molecular mechanisms involved.

The present study demonstrates that thyroid hormone T3 treatment of freshly isolated rat islets has a beneficial effect on islet survival after transplantation, by increasing their vitality and their function, and in addition their insulin secretion. Moreover, T3 has potent antiapoptotic effect in rat islets exposed to streptozotocin. The observed effects are mediated through the Akt pathway activation.

Results

Thyroid receptor β 1 is highly expressed in the cytoplasm of rat islets β -cells. To evaluate if the thyroid receptor β 1, which

resulted to be the main mediator of T3 action on pancreatic β -cells (Falzacappa et al. 2009), is present in the rat islets, immunofluorescence experiments have been performed. As shown in **Figure 1**, the staining for TRB1, revealed that the receptor is highly expressed in the islets cells and that it is mainly located in the cytoplasm. In addition, when the islets were counterstained for insulin, it was possible to observe that the two signals were superimposable, indicating that the rat islets beta cells do express the thyroid receptor β 1.

Islets viability in vitro is augmented by T3 treatment. In order to demonstrate a pro-survival role of Thyroid hormone T3 in freshly isolated rat islets, the islets were cultured in with and without the presence of T3 (10^{-7} M) for 72 h and 96 h. Our previous works on cell lines (hCM ad rRINm5F) indicated that the 10^{-7} M dose of thyroid hormone was able to influence β -cell viability, proliferation, survival and function in vitro; considering that data we decided to utilize the same dose for our ex vivo experiments. Fluorescence microscopy was utilized to determine the proportion of dead cells within the islets. As shown in **Figure 2A**, already at 72 h of culture the viability of islets culture without T3 was reduced as evident by the considerable number of PI positive cells. T3 treatment sensibly improved the percentage of viable islets from 50% to 65% (**Fig. 2A**). Importantly, after 96 h culture without T3, 80% of islets were decomposed, compared to 50% of islet cells cultured in the presence of T3.

In accordance with the microscopic observations, MTT assay (panel B) confirmed that the T3 treated islets have cell viability values higher than untreated islets, confirming that the hormone treatment could preserve islets vitality.

Core cell damage is reduced by T3 treatment. During in vitro culture, necrosis of the cells occurs within the center of the islets; core islets are primarily constituted by β -cells. In this experiment we investigated whether T3 could contrast the core cell damage in isolated islets. Under light microscopy, freshly isolated pancreatic islets from rat had a smooth appearance with compact spherical shapes in varied sizes (**Fig. 2C**). After 48 h of in vitro culture, the islets began to present some cell damage. It was usually located in the center of the islets and characterized by a zone of dark cells that was separated from the surrounding viable tissue. By 72 h of culture an extensive damaged area appeared in the center of the islets. The damaged area was even larger, being extended throughout the islet. The observed results were consistent with the live/death cell analysis, whether they were different from the TUNEL assay. In the TUNEL assay, in fact, apoptotic cell death of single damaged cells within the core of a 24 h cultured islet could be identified; however, many cells within the damaged area were also TUNEL negative, indicating that both necrotic and apoptotic cell deaths were involved in the process of core cell damage in cultured isolated rat islets. Strikingly, when the islets were exposed to T3 the damaged area resulted sensibly reduced. As shown in the **Figure 2**, after 72 and 96 h of in vitro tissue culture, T3 treated group (right) indicated a significant higher recovery of islets than control group (left). Islets treated with T3 exhibited excellent morphology and did not lead to core cell damage. These data demonstrate that T3 treatment was effective to reduce core cell damage of islets during the in vitro culture period.

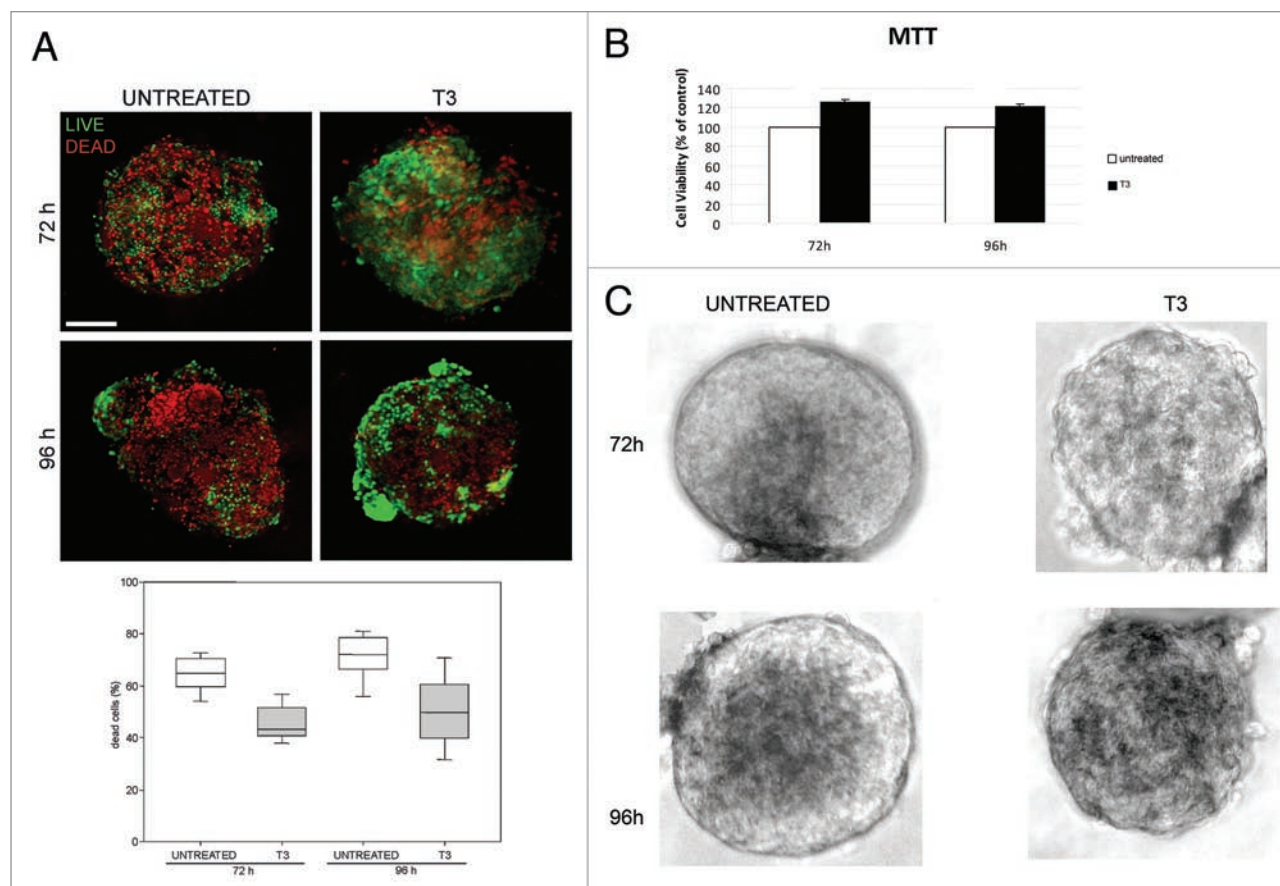


Figure 2. (A) Islets viability. Islets were cultured with and without the presence of T₃ (10⁻⁷ M). Assessment of islet cell viability using fluorescence microscopy with propidium iodide (dead cells, red) and Live cell dye* (MBL) (living cells, green) staining. Representative merged pictures are shown. Islets cultured without T₃ showed the presence of numerous dead cells at the center of the islets; whether islets cultured with T₃ showed fewer dead cells. The percentage of green and red cells was calculated by counting up to a minimum of 200 cells for ten optical fields (200X) for each sample, randomly taken from two different experiments. p-value was calculated using a Kruskal-Wallis test. The boxplot showed the median and the 10th, 25th, 75th and 90th percentile. Bar: 50 μm; p < 0.005. (B) MTT assay. Cell viability has been evaluated by MTT assay performed on islets cultured in the presence or the absence of T₃ for the indicated time. Data presented (B) are the OD values (570 nm) expressed as percentage of control on the y axis, as means ± SD, and are the results of at least five independent experiments. Control has been taken as 100%. A comparison of the individual treatment was conducted by using one-way ANOVA followed by Dunnett post-hoc test p < 0.005. (C) Core cell damage. Islets were cultured in the presence or not of T₃ (10⁻⁷ M). The core cell damage was visualized under light microscopy and representative images are shown. Islets cultured in the presence of T₃ showed a reduced core cell damage compared with islets cultured without T₃. Magnification x100.

T₃ induces BrdU incorporation in rat islets cells. Our previous observations in β-cell lines revealed a mitogenic role for T₃; although it is well known that the proliferation rate in islets is slight, we decided to investigate the T₃ effect on islets cell proliferation via BrdU incorporation. Surprisingly, as shown in **Figure 3**, in the core of the islets some BrdU positive nuclei were detectable in both the untreated and the treated samples. The T₃ treated BrdU nuclei number was increased. As shown, the counterstaining for insulin (green fluorescence) confirmed that the core of the islets is mainly populated by β-cells, which were the ones positive for the BrdU staining.

T₃ protects rat islets from STZ induced apoptosis. To assess whether T₃ could also affect the survival of the islets exposed to proapoptotic agents, the apoptotic process was induced by Streptozotocin treatment in the islets exposed to T₃ or to vehicle alone. Islets were treated with Streptozotocin 2 or 5 mmol/L for 2 h. As shown in the **Figure 4**, for the 5 mmol/L dose, the

percentage of TUNEL positive cells was high in the islets treated only with STZ, which demonstrates the presence of apoptosis (80%), while in the islets treated with T₃ the TUNEL positive cells were highly reduced (30%), indicating that the hormone T₃ is able to counteract the proapoptotic action of the drug. To evidence the β-cells inside the islets, counterstaining with insulin has been performed. As shown the insulin signal was mainly present in the core of the islets and it was superimposable with the TUNEL positivity, indicating that Streptozotocin could induce apoptosis specifically in the β-cells. As shown, the untreated islets also show a low number of TUNEL positive cells, indicating that a physiological apoptosis was anyway present in the cultured islets.

T₃ improves islet function. The thyroid hormone treatment preserved basal glucose responsiveness and insulin secretory function in rat islets. Isolated islets were incubated with or without T₃ for 72 and 96 h. A static glucose challenge assay (**Fig. 5**)

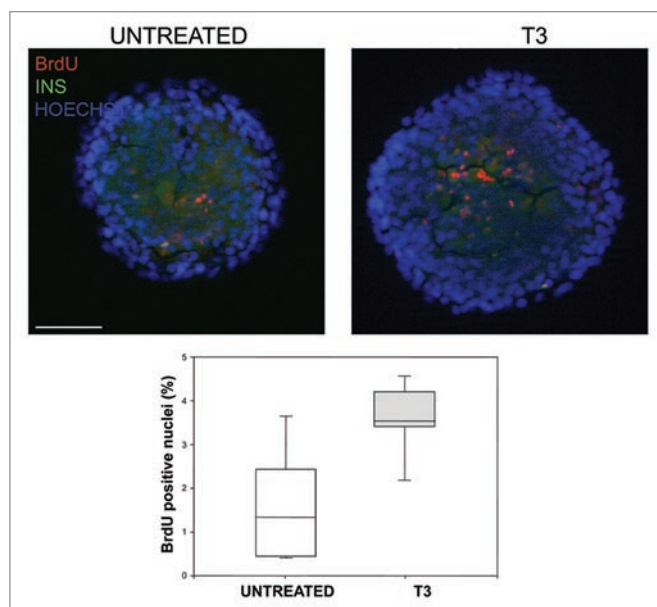


Figure 3. BrdU labeling. Islets were cultured in the presence or the absence of T3 for 48 h and exposed to BrdU (10 μ M) for the last 48 h. BrdU incorporation was evidenced with indirect immunofluorescence for BrdU (red). Islets were counterstained for Insulin (green) and nuclei were counterstained for Hoechst (blue). The percentage of BrdU positive cells was calculated by counting up to a minimum of 200 cells for ten optical fields (200X) for each sample, randomly taken from two different experiments. p-value was calculated using a Mann-Whitney U test. The boxplot showed the median and the 10th, 25th, 75th and 90th percentile. Bar: 50 μ m; p = 0.002.

was performed and indicated that T3 was able to preserve β -cell glucose responsiveness and insulin secretion in both basal (2.8 mmol/L) and stimulated (28 mmol/L) glucose condition. Moreover a significant increase in insulin secretion was observed in both the utilized glucose condition, thus suggesting that the thyroid hormone treatment improves the islets ability to secrete insulin.

T3 upregulates Akt activation. Thyroid hormone treatment can induce the Akt phosphorylation in rat islets. As shown in the **Figure 6**, western blot for pAkt (Ser 473) clearly indicated that T3 treatment (24 h) was able to induce the activation of the kinase of 8 fold (4 R.D.U. in T3 cells vs. 0.2 in control).

Discussion

The present studies were undertaken to determine the effects of thyroid hormone T3 on the survival and function of primary islets and resulted in four major findings.

First, the addition of T3 to the culture medium can per se enhance islets viability and counteract the ongoing of core cell damage. Second, we demonstrated that thyroid hormone protects rat islets from apoptosis that occurs after streptozotocin exposure. Third the insulin secretion of islets is augmented in the islets cultured in the presence of T3.

Fourth, T3 induced a significant increment in the activation of Akt in rat islets.

The ability of thyroid hormone T3 to influence pancreatic β -cells has recently been investigated by our group. Our evidences^{11,13} clearly demonstrated that T3 can be considered a mitogenic and survival factor for pancreatic β cells in vitro.

The major obstacles for successful clinical islet transplantation are the isolation of sufficient mass of islets together with the management of graft rejection.¹⁹⁻²¹ The fatal outcome which is not related to immune rejection, has been thought to be due to insufficient or non-established vascularization of transplanted islets.²²⁻²⁵ It has been demonstrated that during the first two days after transplantation, islets are avascular, leading to processes that impairs the central β -cell mass of the islets.^{26,27} As demonstrated^{28,29} the main causative mechanisms involved in core cell damage might be necrosis or apoptosis. Necrotic cell death may depend on the limitation of nutrition diffusion, while apoptosis is generally caused by pathological atmosphere arising from the isolation procedure. In this study we evidenced the presence of a relevant core cell damage, which occurs mainly in the first days of culture, and is predominantly due to necrosis, as clearly demonstrated by TUNEL assay. Thyroid hormone T3 was able to counteract the ongoing of this process, thus preserving islets vitality. Although at the present moment it is not precisely known which molecular mechanisms are involved in the core cell damage, and no evidences exist about the link between thyroid hormones and necrosis, our previous works have unambiguously evidenced that T3 can promote the β -cell proliferation, viability and survival by regulating mainly Akt pathway. We thus can hypothesize that a general impulse from T3 to improve islets status might be due to its ability of regulating the main β -cell features via Akt.

An additional advantage of the pre-transplantation culture consists in practicing interventional strategies to prevent the profound β -cell loss occurring via apoptosis, which has been estimated to cause up to 70% of the transplanted β -cell mass destruction.³⁰⁻³² Hence, another approach has been to directly inhibit the apoptotic cascade, thus improving the survival capability of the islets.⁴ Wide spread apoptosis in the implanted tissue may also have long-term deleterious consequences in islets transplantation, since the recipient's immune system is challenged with a large amount of apoptotic tissue, possessing both allo- and auto-antigens from two or more donors.

Considering these indications, today the utilization of protective factors to enhance β -cell survival and prevent islets apoptosis is widely explored.⁴ In accordance with our previous finding about T3 prosurvival effect, we herein demonstrated that the apoptotic process, induced by Streptozotocin, could be counteracted by the T3 presence in a relevant manner. We previously¹¹ showed the survival properties of T3 against STZ induced apoptosis even in a rat insulinoma cell line and elucidated the molecular mechanisms underlying this effect, which involved, once again, the Akt pathway;¹³ it is conceivable that T3 might exert its survival action on STZ induced apoptosis mainly involving the same mechanisms.

Treatments that activate Akt during islets culture might improve graft survival,¹⁷ indicating that Akt activity could render islets less susceptible to injury during the immediate post-transplantation period. Therefore, it is plausible that adding T3 to the

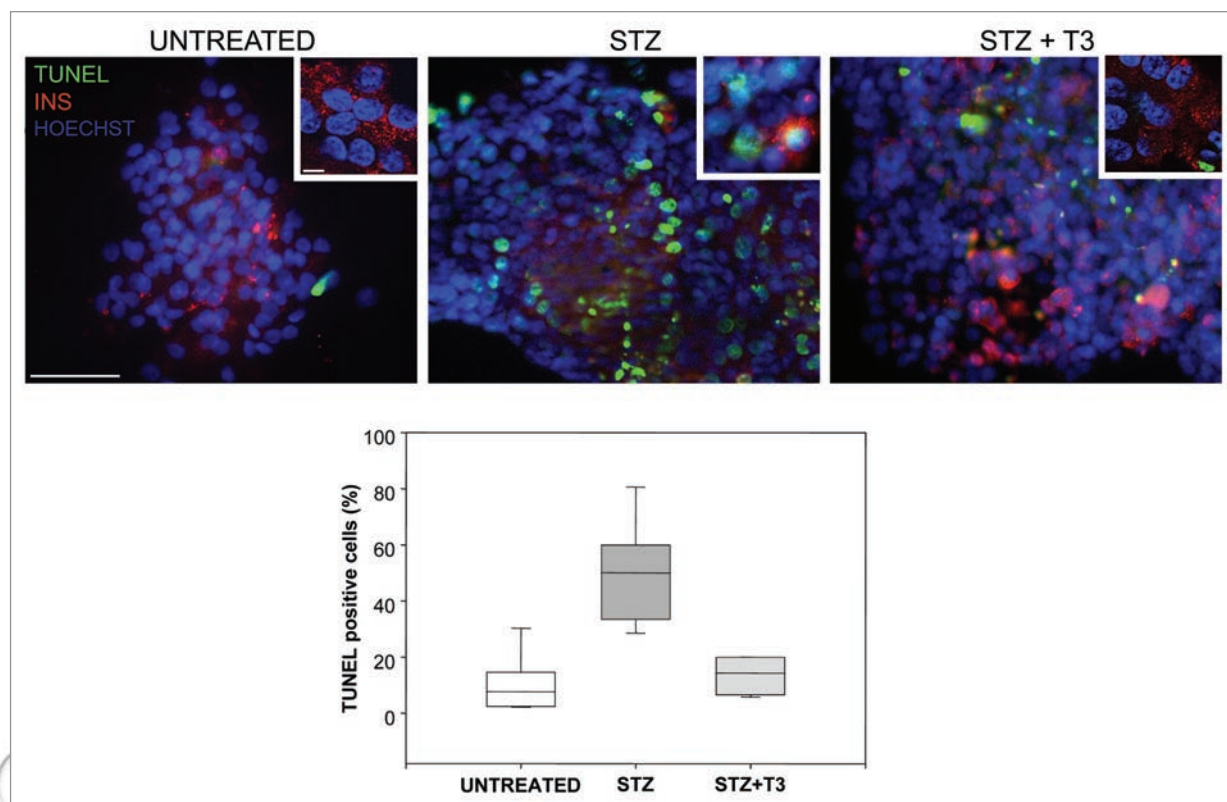


Figure 4. TUNEL assay. Islets were exposed to two different doses (2 mmol/L and 5 mmol/L) of Streptozotocin or not (untreated) and cultured in the presence (T3) or the absence (untreated) of T_3 10^{-7} M, as described in Materials and Methods. Apoptotic nuclei were detected as TUNEL-positive, nuclei were counter-stained with Hoechst and merged images from a representative field (5 mmol/L dose) are shown. The percentage of TUNEL positive cells was calculated by counting up to a minimum of 200 cells for ten optical fields (200X) for each sample, randomly taken from two different experiments. p-value was calculated using a Kruskal-Wallis test. The boxplot showed the median and the 10th, 25th, 75th and 90th percentile. Bar: 50 μ m (10 μ m in the inset); $p = 0.0005$.

islets culture medium and thus activating Akt during an in vitro culture period prior to transplantation could yield islets that are more likely to survive the insults encountered immediately after transplantation. In this study we sought how T3 is able to induce a 60% increment in Akt activation. We have previously deeply examined the key role that Akt plays in T3 action on pancreatic β -cells.¹¹⁻¹³ It is well established that Akt signaling, involving PI3K, is implicated in cell cycle progression and survival of pancreatic β -cells, thus rendering the link between this molecule and the modulation of β -cell mass, function and plasticity a critical subject to study for the intervention against diabetes. T3 is able to induce cell proliferation and survival; this hormone can moreover increase pancreatic β -cell size and protein synthesis and that insulin secretion is also augmented by T3 treatment. All the cited effects appeared to be Akt mediated, thus confirming the cruciality of this molecule in pancreatic β -cells.¹³ In this study we evidenced that the thyroid hormone treatment can indeed induce islet survival and function. In fact, as shown, T3 can also increase insulin secretion. It has been demonstrated an autocrine effect of insulin on Akt activation, which results in an increment of survival and vitality of islets in culture.³³ Here we demonstrated that T3 can increase insulin secretion and we also made evidence that the hormone caused an increment of about eight-fold

in Akt activation already after 48 h of treatment. Considering our previous evidences, we proposed that the Akt activation we observed is directly dependent on T3 presence, however, considering the data about insulin autocrine action, we can speculate that Akt phosphorylation resulted from both a direct and an indirect action of T3 on the signaling, thus involving also the insulin action. It has been demonstrated^{33,34} that addition of exogenous insulin immediately following isolation was not able to improve short-term islet survival, while autocrine regulation via Akt was. In particular, exogenous insulin can upregulate Akt during the first days of culture, but the effect is lost later. It is thus plausible that stimulating insulin secretion via Akt with T3 might promote islet survival together with function involving the already sought mechanism,¹³ mainly through direct Akt activation. However, given the increment in insulin secretion due to thyroid hormone presence, we can hypothesize that also the autocrine insulin action might play a role in the survival effect of T3 on the islets.

To our opinion these observations propose thyroid hormone T3 as a suitable factor to optimize and stimulate recovery and subsequent function of islets during in vitro tissue culture, indicating that thyroid hormone could play an important role in the biological function of pancreatic islets.

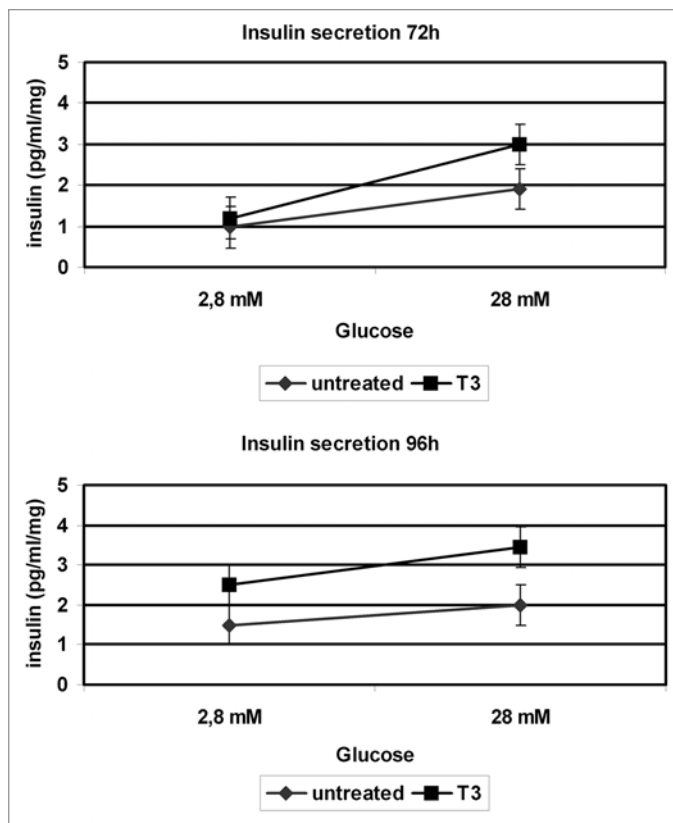


Figure 5. Insulin secretion. Islets were cultured in the presence or not of T3 (10^{-7} M). Insulin content of medium from islets cultured in the presence or the absence of T3 and exposed to basal (2.8 mmol/L) and stimulating (28 mmol/L) glucose concentration for 1 h was assessed by ELISA assay. Results represent the mean \pm SE of three separate experiments.

Whether T3 treatment of islets during an in vitro culture period could enhance the chances of graft survival needs to be investigated.

Materials and Methods

Chemicals. Crude collagenase type 4 was obtained from Worthington Biochemicals Corporation (Lakewood, NJ); 3,5,3'-Triiodothyronine (T3), Polysucrose 400 and Streptozotocin (STZ) and 5-Bromo-2'-deoxyuridine (BrdU) were obtained from Sigma-Aldrich (Saint Louis, MO).

Animals. *Wistar* rats male adult (about 12 weeks old) were used as islets donors. The animals had free access to tap water and pelleted food throughout the course of the study. The local animal ethics committee approved all experiments.

Isolation and culture of rat islets. Pancreatic islets were isolated from 300 g weighting male adult *Wistar* rats by standard surgical procurement followed by intraductal collagenase distension, mechanical dissociation and Euroficol purification. In brief animals anesthetized with ketamine 70 mg/Kg + domitor 0.5 mg/Kg injected intraperitoneally, were sacrificed by CO_2 inhalation. For the exposure of the whole pancreas, the abdominal wall was opened via a midline incision and the pancreas ductal connection to the intestine clamped. The pancreas was cannulated in situ via

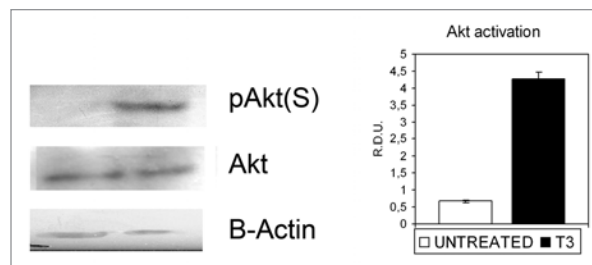


Figure 6. Akt activation. Western blot analyses were performed as described in Materials and Methods and a specific band corresponding to the phosphorylated Akt (Ser 473) was detected. The expression of total Akt was analyzed as a control for gel loading. (Left line: untreated; right line: treated) At least three different experiments were performed, and a representative one is shown here. Densitometric absorbance values from three separate experiments were averaged (\pm SD), after they had been normalized to Akt for equal loading. Data relative to each protein are presented on the right of the western blot panel in the histogram as Relative Densitometric Units (y axis). The different experimental groups are indicated on the x axis. A comparison of the individual treatment was conducted by using Student's t test. $p = 0.003$.

the common bile duct using a polyethilen tube (BD, Franklin Lakes, NJ) and distended by pumping a cold solution of collagenase (0.2%) prepared in a specific isolation medium KRHB, containing (in mmol/l) NaCl 134, KC 4,7, CaCl_2 1, MgSO_4 1.2, KH_2PO_4 1.2, HEPES 10, BSA 0.5% pH 7.35. The whole pancreas was excised and transferred to a centrifugal tube and incubated for 20 min with gentle tumbling, at 37°C . Islets were purified on a discontinuous Euroficol gradient, handpicked under a light stereomicroscope, pooled and then separated into study group and control group for the subsequent culture period.

The islets were cultured in CMRL 1066 medium (GIBCO, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum, L-Glutamine 2 mmol/L and Penicillin 100 $\mu\text{g/ml}$ -Streptomycin 50 $\mu\text{g/ml}$ in not coated plates (BD) with or without T3 (10^{-7} M) at 37°C in a humidified atmosphere of 5% CO_2 .

Immunofluorescence. Immunofluorescence analysis was performed in the various experimental condition (specifically described within this section) to detect insulin signal. In addition the thyroid receptor β 1 was detected by the same procedure. Islets were stained with primary antibodies, rabbit anti-insulin (Cell Signaling Technology, Inc., 3 Trask lane, Dansvers, MA) and anti-TR β 1 (Santa Cruz Biotechnology Inc., San Diego, CA). after washing in PBS 1x (Lonza), islets were incubated with secondary antibodies fluorochrome conjugated (Alexafluor 488 anti rabbit; Alexafluor 546 anti mouse). Hoechst dye (1 $\mu\text{g/ml}$) was used for nuclear detection. Fluorescence was detected with an epifluorescence microscope (Leica, Germany), images were captured by a Canon digital camera and images were processed with ImageJ software (Wayne Rusband, National Institute of Health), where no differently specified.

Microscopic monitoring of cultured islets. Morphological changes of isolated islets were monitored during 96 hours of culture. Images were recorded by a Canon digital camera and processed by Image J software. To evaluate the core cell damage

of isolated islets, light microscopic analyses were performed at different time points (72 and 96 h) during the culture.

Islets viability. Batches of 15 islets were cultured in the presence or not of fresh aliquots of T3 (10^{-7} M) added every day for 72 h and 96 h. Islets viability was tested by using *live-dead cell viability test* (MBL International, 4 H Constitution Way Woburn, MA, US) under manufacturer's instruction. The assay utilizes two different dyes which can differently pass through cell membrane, the Propidium Iodide (PI, red Fluorescence) can only pass damaged membrane (death cells), while the Green Dye can pass the intact membrane (live cells). Images were visualized with a Leica (D-35606 Burgsolms, Solms, Germany) epifluorescence microscope and taken by a Canon digital Camera. Secondly images were processed by ImageJ software. Nuclei were counted and percentages of live vs. death cells were visualized on histograms.

MTT assay. Islets were cultured in 96 multiwells for 72 and 96 h and treated as previously described. A solution of a tetrazolium salt was added to the culture medium and, after 4 h, the metabolic formazan product was solubilized in an organic solution. After 1 h of solubilization, the absorbances at 570 and 630 nm were recorded by using a 96 well plate reader.

BrdU labeling. Cell proliferation was determined additionally by BrdU staining.

Islets were cultured for 48 h in the presence and not of T3 (10^{-7} M); during the last 24 h of culture BrdU $10 \mu\text{M}$ (Sigma-Aldrich) was added. The islets were cytospun on polarized slides. Slides were then washed in PBS 1x (Lonza) and incubated with HCl 3N for 25 min at RT; the reaction was then neutralized with borax-borate buffer (pH 9.1), and slides were washed in PBS 1x. Slides were incubated sequentially with PBS 1x, Goat serum 15% (Sigma-Aldrich), Triton X100 0.3% (Sigma) for 15 min at RT, and then with mouse monoclonal antibody anti-BrdU (Roche Diagnostic) 1:200, for 1 h at room temperature. After three washes in PBS 1x, slides were incubated with the secondary antibody Alexafluor 546 anti-mouse (Invitrogen) 1:1,000, for 1 h at room temperature in dark. Slides were then washed in PBS 1x twice and stained with Hoechst $1 \mu\text{g}/\text{ml}$ for nuclear detection. Localization and intensity of fluorescence were evaluated by optical sections obtained using an Axiovert 200M microscope (Zeiss, Oberkochen, Germany) with ApoTOME[®] device connected with a CCD camera Zeiss AxioCam. Negative controls including omission of the primary antibody were also performed.

Measurement of apoptosis. Groups of 15 islets were cultured for 48 h in medium with and without T3 (10^{-7} M) and exposed to STZ (5 mmol/L and 2 mmol/L), to H_2O_2 (100 μM) for the last 2 h of treatment (before assessment of apoptosis) and cultured in serum-free CMRL medium for 48 h after a sensitization period (24 h) with CMRL completed medium. At the end of the treatment period, islets were fixed for 1 h with 4% paraformaldehyde, washed with cold PBS, incubated in Triton 0.1% in Sodium Citrate 0.1% for 2 min. on ice. After washes, apoptotic cells within islets were detected by the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method using an *in situ Cell Death detection kit* (Roche, D-68305 Waldhof, Mannheim,

Germany) for 1 h, at RT, in dark, in according to manufacturer's procedures. Islets were counterstained for Insulin revealed by indirect fluorescence and nuclei were counter-stained with $1 \mu\text{g}/\text{ml}$ Hoechst dye diluted in PBS. TUNEL positivity signal was evaluated by optical sections obtained using an Axiovert 200M microscope (Zeiss, Oberkochen, Germany) with ApoTOME[®] device connected with a CCD camera Zeiss AxioCam.

Insulin secretion. Islets¹⁵ cultured in presence or in absence of T3 were stimulated with two different glucose concentrations ranging from 2.8 to 28 mmol/L in isolation medium and incubated for 45 min at 37°C . Insulin releasing was measured on supernatants by Mercodia Ultrasensitive Rat Insulin Elisa (Mercodia AB, Sylveniusgatan 8A, SE-754 50 Uppsala, Sweden) under manufacture's protocol. Results were presented in pg/ml after normalization with total protein content.

Western blot analyses. Islets cultured in the presence or not in the absence of T3 were collected (100 per group), washed in PBS 1x, and lysed for 10 min in ice-cold lysis buffer containing 1% Tween 20, 10% glycerol, 150 mmol/L NaCl, 50 mmol/L HEPES pH 7, 1 mmol/L MgCl_2 , 1 mmol/L CaCl_2 , 1 mmol/L NaF, 10 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 2 mmol/L NaVO_3 , 1 mmol/L phenylmethylsulfonylfluoride, protease inhibitors. The lysates were sonicated and centrifugated at 12,000 rpm for 30 min. and the total cellular protein content was measured using Bradford method (Bio-rad, Richmond, CA). $40 \mu\text{g}$ of total extract per sample were loaded onto a 10% SDS-polyacrilammol/Lide gel, electrophoresed, and then blotted onto PVDF membranes (Bio-Rad). Filters were blocked for non specific reactivity by incubation for 1 h at RT in 5% non-fat dry milk dissolved in PBS 1X, Tween 20 0.1% and then incubated for 16 h at 4°C with pAkt Ser473 (Santa Cruz 1:200) and β -actin (Sigma-Aldrich, 1:1,000) Akt (Santa Cruz 1:500) diluted in 5% milk, PBS 1X, Tween 20 0.1%. After three washes in PBS 1X, Tween 20 0.1% the membranes were incubated for 45 min with the secondary HRP antibodies (anti-mouse, anti-rabbit; Sigma-Aldrich) 1:4,000 in milk 5%, PBS 1X, Tween 20 0.1% for 45 min at RT.

Immunoreactivity was visualized by the ECL immune-detection system (Amersham Corp., Arlington Heights, IL) in according to manufacturer's instructions. The relative band intensity was evaluated by densitometric analysis (Image J, Wayne Rusband, National Institute of Health) and normalized to total Akt.

Statistical analysis. Different statistical analyses were performed depending on the experimental type and are indicated in the relative Figure legends.

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