EXTENDED TIME OF COLD ISCHEMIA AND ITS INFLUENCE ON THE PHYSIOLOGICAL FUNCTION OF HUMAN ADULT PANCREATIC ISLETS

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Abstract - In this study we compared the effects of duration of cold ischemia (longer and shorter ischemia) on the yield, viability and preservation of the physiological function and insulin secretion of adult human pancreatic islets in short-term (seven days) culture. Based on the tested parameters, we established that there are no major differences between these two test groups and that the storage and transport of pancreatic tissue in physiological solution at 4ºC gives quite satisfactory results.

Key words: Extended cold ischemia time, islet preservation, human adult pancreatic islets

INTRODUCTION

In type-1 diabetes mellitus (T1DM), the destruction of pancreatic beta cells causes insulinopenia, consequently leading to hyperglycemia and ketoacidosis unless the lack of endogenous insulin production is balanced by multiple exogenous insulin injections, which today remains the primary treatment for T1, together with regular monitoring of blood glucose levels. While exogenous insulin therapy has dramatically improved the quality of life, chronic diabetic complications develop in a substantial proportion of subjects with diabetes and generally show a progressive worsening over time. Intensive insulin therapy has proven effective to delay and sometimes prevent the progression of complications such as nephropathy, neuropathy or retinopathy. However, it is difficult to achieve and maintain long-term in most subjects, either for compliance issues or due to the increased risk of severe hypoglycemic episodes which are generally associated with intensification of exogenous insulin therapy (Hirochito et al., 2009). Despite the efficacy of insulin therapy as a treatment for T1DM there are numerous clinical complications. These are the reasons for the extensive research into pancreatic tissue transplantation as an alternative for insulin therapy during the last decade (Ricordi, 2003).

Transplantation of isolated human islets has the advantage of being a relatively simple surgical technique, together with the possibility of *in-vitro* modulation, which allows for a significant reduction of postoperative immunosuppressive treatment (Bredzel et al., 1995). Human adult islets can be collected from multiple cadaveric donors, but optimal results are obtained by isolating sufficiently large numbers of islets from a single pancreas with good tissue compatibility (Lacy, 1995). In this case, insulin independence lasting over a 6-year period can be established (Aleyandro et al., 1997). One of the problems encountered in the preparation of islets for transplantation is the distance of the place where the pancreas is taken from cadaveric donors and its transport to the laboratory where pre-transplantation preparation of islets is undertaken. It is preferable that the pancreas reaches the laboratory as soon as possible, so that it can be maintained in the corresponding solution and at the appropriate temperature to avoid pathological changes, and to preserve as many normal viable functioning islets. One of the problems during transport is the possible appearance of viral, bacterial and fungal infections of tissue (Ylipaasto et al., 2004; Bucher et al., 2004). The pancreas is transported in special containers and preserved in the University of Wisconsin solution (UW) (Dalesandro et al., 1989; Mun et al., 1989). Reducing pancreatic injury, particularly cold ischemic injury, has been recognized to be increasingly more important for human islets over the past several years. The two-layer method in which the pancreas is stored at the interface of UW solution and oxygenated PFC (perfluorochemical) is effective for pancreatic preservation (Tsujimura et al., 2002). Porcine pancreas is transported in an ice-cold saline solution (Braun, Melsungen, Germany) to the isolation laboratory, within 1.5-2.0 h of cold ischemia (Bradhorst et al., 1999a), or in Euro Collins solution (Fresenius, Bad Homburg, Germany) cooled at 4°C. (Bradhorst et al., 1999b). The mean cold ischemia time before the isolation procedure can be 6 h (2-9 h) (Oberholzer et al., 2000), or 6 h and ranging from 1to17 h (Nano et al., 2005). Cold storage time significantly and negatively impacts on the successful recovery of viable islets and significantly reduces isolation success. Endogenous proteolytic enzymes released during prolonged cold storage can become activated during enzymatic dissociation, leading to destruction and damage of the the islets (Jonathan et al., 2001).

The aim of this research was to determine the extent to which prolonged ischemia affects the physiological functions of the islets, manifested as adequate insulin secretion after stimulated by glucose during short-term cultivation of seven days.

MATERIALS AND METHODS

Materials

Human adult pancreatic tissue was procured from the Institute for Gastrointestinal Diseases, Clinical Center of Serbia. Tissue samples were collected from live donors, after total or subtotal pancreatectomy due to cysts or tumors (16). In the case of tumors, healthy tissue was obtained near the line of the resection. All procedures were performed in accordance with the rules of Ethical Committee of the Medical Faculty in Belgrade. Also, written consent was obtained from the patients.

Methods

Pancreatic tissue was transported in physiological solution (Natrii chloridi infundibile, 0.9%, Zdravlje-Actavisy, Serbia) in sterile vessels (in a volume of 75 ml), from the Institute for Gastrointestinal Diseases to the Laboratory for Pancreatic Islet Culture in the Institute for Endocrinology, Diabetes and Metabolic Diseases. The material was kept in the refrigerator at 4°C (cold ischemia). Warm ischemia encompasses the period from the time measured at the beginning of anthe isolation procedure to the moment when the islets are placed in a culture medium. Bio-parameter data of the examined group A (shorter cold ischemia) and group B (prolonged cold ischemia) are shown in table 1 and 2.

Isolation of the islets

Isolation of the adult pancreatic islets was per-

formed under aseptic conditions in a laminar chamber. The tissue was transferred to Hank's solution (Sigma-Aldrich) and mechanically chopped. The material was collected with a pipette and placed in test tubes containing liberase (Liberase HI, Roche Diagnostics GmbH: Collagenase, 2093 Wunsch units/bt; Neutral Protease, 50,251 units/bt; Endotoxin, 2 EU/mg). The liberase solution (1.5 mg/ml) (described in: Nikolic et al., 2010a), was prepared by dissolving the enzyme in distilled water and it was maintaining in the refrigerator for 30 min prior to use. The working concentration was adjusted by adding Hank's solution. The liberase solutions were sterilized using a cellulose acetate membrane filter (0.22μm porosity). The duration of the incubation was 30 min at 37˚C with occasional mechanical stirring. After incubation, the content of the test tubes was centrifuged at 400xg, for 10 min at 15˚C (Nikolic et al., 2010b). The supernatant was decanted and the remaining islets were rinsed several times with Hanks solution to eliminate excess lipids and liberase.

The islets were separated from the surrounding acinar tissue by centrifugation in Ficoll gradients (Sigma) prepared with HBSS solution (Nikolic et al., 2010c). The islets were re-suspended in 4 ml RPMI followed by the addition of 16.7 ml of stock Ficoll medium (density 1.125 g/mL) in 50 ml conical test tubes. The sample was carefully stirred with medium to remove the interphase. The discontinuous gradient was obtained by subsequently applying Ficoll solutions with densities of 1.085, 1.075 and 1.045 g/mL (5 mL each). After 25 min centrifugation at 550x g at 22°C, two fractions were collected: a 1.045/1.075 and 1.075/1.085 interface layers. The islets were washed in HBSS medium by centrifugation at 950xg for 5 min at 4 C.

After rinsing, the islets were re-suspended in RPMI medium 1640 (Sigma-Aldrich), supplemented with 0.1% L-glutamine, 5.5 mM glucose, 25 mM Hepes, 100 U/ml penicillin, 100μg/ml streptomycin and 10% fetal calf serum (FCS, Sigma). The islets were incubated in plastic flasks at 37˚C in a 5% CO_2, 95% humidity atmosphere for 7 days.

The warm ischemia time is the time measured from the beginning of the mechanical mincing of the tissue, including the isolation and the purification procedures, to the moment of placing the islets in culture.

Determination of the number and viability of the isolated human adult islets per culture

Viability of the islets was determined by dithizone (DTZ) staining on days 1, 3 and 7 after their isolation. Viability was expressed as a percentage of distinctly stained (red) islets in relation to the total number of the islets in culture.

Preparation of the dithizone solution

Fifty milligrams of DTZ was dissolved in 10 ml of DMSO and 10 ml of Hank's solution. The solution was filtered through a nylon filter (0.20μm). Samples (1 ml of each culture) were stained with 0.2 ml of DTZ solution and incubated for 30 min. The stained islets were rinsed in Hank's solution and resuspended in 1 ml of RPMI medium. The number of cells was determined using a stereo-light microscope and counting chambers.

Determination of the functional capacity and insulin secretion

To determine the preservation of the functional capacity of the isolated islets, the glucose-stimulated insulin secretion was measured on the first, third and seventh days of cultivation.

The static glucose stimulation assay was performed by incubating the samples for 1 h in low glucose (2.8 mM glucose in RPMI) medium, followed by 1 h incubation in high glucose (20 mM glucose in RPMI) medium and for 1 h in the low glucose medium. After each step of stimulation the cultures were centrifuged at 400xg for 10 min at 15˚C. The supernatant was decanted and stored at -18˚C for insulin quantification. The insulin content was determined by radioimmunoassay (RIA INSULIN PEG). Sensitivity of the assay is 0.60 mIU/L and the detec-
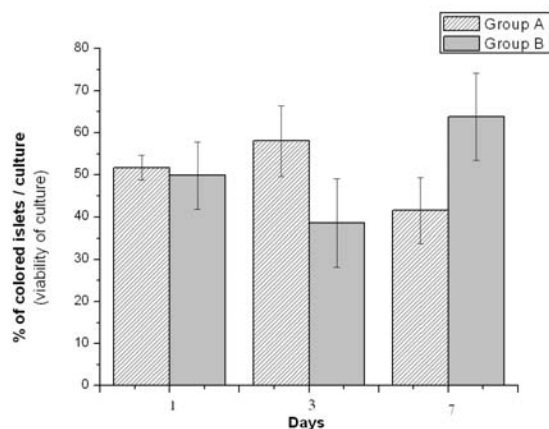
Fig. 1. The number of viable islets expressed as the percentage of clearly stained islets in relation to the total number of islets in culture. The results are given as the mean ± SE. Viability of the islets isolated from group A and group B was determined by DTZ staining on the first, third and seventh days of cultivation.
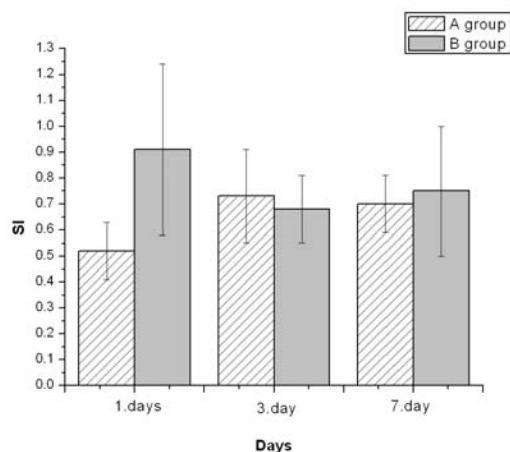

Fig. 2. The effect of the duration of extended cold ischemia on glucose-stimulated insulin secretion. Values are presented as the mean ± SE. The stimulation index is the ratio of released insulin at low glucose (2.8 mM) and high glucose (20mM). Measurements were performed on the first, third and seventh days of cultivation.

tion range is 0.6-300 mIU/L. The relative insulin release was expressed as a stimulation index (SI) and calculated as the ratio of insulin release during high glucose stimulation to insulin release during low glucose stimulation.

Statistical analysis

All values are expressed as means±SE. Comparisons of data were carried out by Student's *t* test; *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

In this study we examined the effects of the duration of cold ischemia on islet yield, viability of islets and preservation of the physiological function of the islets in two groups, A (shorter cold ischemia) and B (longer cold ischemia).

The islet yield after the first isolation did not exhibit any statistically significant differences between the groups (P=0.460). The islet yield was 2,536.63±422.17 (group A) and 4,891.34±913.27 (group B)

Viability of the cultures was determined as the percentage of distinctly colored islets in both groups A and B. On the first day of incubation, the viability of the islets was 51.72±2.88% and 49.82±7.94% for groups A and B, respectively (P=0.05). On the third day of incubation the viability in group A was 58.03±8.37% and ,in group B it was 38.63±10.47%. On the seventh day of the incubation the percentage of viable islets was 41.55±7.83 in group A, and 63.77±10.39 in group B (Fig. 1). The differences between the two groups on the third and seventh days were not statistically significant (P> 0.05).

Preservation of the physiological capacity of the islets, reflected as an adequate response to high and low glucose stimulation, was determined by the stimulation index (SI).On the first day of incubation, the SI value for group A was 0.52±0.11 while for group B it was 0.91±12.33; ,the difference was not statistically significant (P> 0.05). On the third day of incubation the SI values were 0.73±0.18 and 0.68±0.13 for groups A and B, respectively (P=0.05), while on the seventh day of incubation the SI values were 0.70±0.11 (group A) and 0.75±0.25 (group B) (Fig. 2), and the difference was statistically significant (P=0.05).

Table 1. Biometrical parameters for pancreatic tissue in Group A

Pancreas number	Tissue weight (g)	Culture number	Warm ischemia (min.)	Cold ischemia (min.)
1	6.09	A17	143	60
2	6.67	A18	152	55
3	3.90	A19	77	21
4	4.00	A20	74	35
mean ± SE	5.16±0.71		111.5±20.87	42.75±9.04

Table 2. Biometrical parameters for pancreatic tissue in Group B

Pancreas number	Tissue weight (g)	Culture number	Warm ischemia (min.)	Cold ischemia (min.)
1	4.5	B21	140	180
2	0.78	B33	83	97
3	0.63	B34	53	132
4	0.69	B38	109	90
mean ± SE	1.65±0.94		96.25±18.54	124.75±20.58

DISCUSSION

In this experiment we followed two groups of cultures obtained from a human adult pancreas: group A which was exposed to a short period of cold ischemia and group B which was exposed to greater and longer effects of cold ischemia (Table 1).

Regarding the yield of islets per gram of tissue, there was no difference between both groups (P>0.05). The viability of the cultures (percentage of clearly stained cells) and preservation of insulin secretion were examined on the first, third and seventh days of incubation of the islets. The viability of the cultures on the first day of incubation showed a small difference (about 2%) in favor of the group with the shorter cold ischemia (group A). However, on the third day of incubation the percentage of clearly stained cells in group A was greater by about 20%. On the other hand, on the seventh day of incubation, the viability of the islets in group B was higher by about 20%. However, these differences were not statistically significant. The cultures with the shorter cold ischemia time were more stable on the first and the third day of incubation, while on the seventh day the viability of the islets declined, which was the

expected result. On the other hand, the viability of the islets in group B declined on the third day of incubation and increased on the seventh day. On the first day of cultivation, the differences between both groups were negligible.

The stimulation index (SI) was determined as the ability of the pancreatic islets to respond adequately to low and high glucose stimulation, which actually represents the preservation of normal physiological functions. On the first day of stimulation, the culture of group B showed a higher value of SI: however, this difference was not statistically significant. On the third and seventh days of stimulation the difference in SI values were very small and statistically significant. Excluding the great differences in the first day of incubation, we can say that there is no difference in the preservation of the functional capacity of the pancreatic islets between both groups (with group A exposed to having the shorter cold ischemia time and group B exposed to cold ischemia that was 4 times longer). Given the closeness of the institute where the pancreatic tissue was procured, even longer cold ischemia times were significantly lower than the values presented by other authors, ranging from 6 to 12 h (Jonathan, 2004; Yang et al., 2004). Analyzing

the results of other authors (Shapiro et al., 2000), a longer duration of cold ischemia does not condition the lower islet yield and lower SI values immediately before transplantation.

One of the authors used a rat model of cold preservation of the pancreas to assess its effects on islets stored in UW solution. The authors, found that the pancreatic islets obtained from a group with a shorter cold ischemia (3 h) showed greater viability, higher islet yields as well as greater recovery after overnight culture compared to another group with longer cold ischemia (18 h). Cold ischemia of the pancreas significantly affects both islet cell yield and potency (viability and functions) paralleled increased phosphorylation of stress kinases (Pilegi et al., 2009).

Since the general consensus among transplant centers is that a cold ischemia time (CIT) lasting more than 8 h (Hanley et al., 2008; Toso et al., 2002) results in reduced yields and lower quality of human islets, one group of researchers tried to reduce the harmful effects of CIT by prolonged incubation of the islets in a different medium instead of the standard one (Kuhtreiber et al., 2010). We disagree with the findings of other authors (Tsujimura et al., 2002) that useding an oxygenated solution for storage and transportation of the pancreas because it is known that the presence of oxygen can increase the metabolism of cells and thus accelerate apoptotic processes. Most researches use UW solution with greater distances and times of transport. This significantly extends the time of cold ischemia. The tissue was obtained mainly from cadaveric donors.

In our study we obtained pancreatic tissues from living donors. These were small amounts of tissue (weight of 1-5g). Storage and transport of small amounts of tissue in a sterile container volume of 75 ml in a physiological solution has given very good results, considering that the differences were negligible.

CONCLUSION

Based on the presented results we concluded that there are no significant differences between the two groups of cultures: one with a shorter CIT and the other with a prolonged CIT, with regard to islet yield, cell viability and SI values during short-term (7 days) incubation.

REFERENCES

Alejandro, R., Lehnann, R., Ricordi, C., Kenyon, N.S., Angelico, M.C., Burke, G., Esquenazi, V., Nery, J., Betancourt, A.E., Kong, S.S., Miller, J. and *D.H. Mintz* (1997). Long-term function (6 years) of islet allograft in type 1 diabetes. *Diabetes* **46**, 1983-1989.

Bradhorst, D., Bradhorst, H., Hering, B., and *R. Bretzel* (1999). Long term survival, morphology and *in vitro* function of isolated pig islets under different culture conditions. *Transplantation* **67**, 1533-1541 (a).

Bradhorst, H.; Bradhorst, D.; Hering, B.; and *R. Bretzel* (1999). Significant progress in porcine islet mass isolation utilizing liberase HI for enzymatic low temperature pancreas digestion. *Transplantation* **68**, 355-361 (b).

Brezel, R., Hering, B., and *K. F. Federlin* (1995). Assessment of adult islet preparation, In: *Methods in cell transplantation* (Eds. C. Ricordi, R. G. Landes), 345-351. Company Austin.

Bucher, P, Math, Z., Bosco, D., Oberholzer, J., Toso, C., Andres, A., Buhler, L., Morel, P., and *T. Berney* (2004). Microbial surveillance during human pancreatic islet isolation. *Transplantation Proceedings* **36**, 1147-1148.

Dalesandro, A.M., Stratta, R.J., Sollinger, H.F., Kalayoglu, M., Pisch, J.D., and *F.O. Belzer* (1989). Use of UW solution in pancreas transplantation. *Diabetes* **38**, 586-591.

Hanley, S.C., Paraskevas, S., and *L. Rosenberg* (2008). Door and isolation variables predicting human islets isolation success. *Transplantation* **85**, 689-694.

Hirochito, I., and *C. Ricordi* (2009). Current status of islet cell transplantation. *J. Hepatobiliary Pancreat. Surg.* **16**, 101-112.

Jonathan, R.T.L., Lisa, M.H., Tatsuya, K., Gregori, S., Ray, V., Shapiro, J., and *G. Warnock* (2001). Serine protease inhibition during islet isolation increases islet yield from human pancreases with prolonged ischemia. *Transplantation* **72**, 565-570.

Kuhtreiber, W.M., Hoa, L.T., Kamireddya, A., Yacouba, J.A.W., and *D.W. Scharpa* (2010). Islet isolation from human pan-

creas with extended cold ischemia time. *Transplantation Proceedings* **42**, 2027-2031.

Lacy P (1995). Islet cell transplantation for insulin dependent diabetes. *Hospital Practice* **30**, 41-45.

Mun, S.R., Kaufman, D.B., Field, M.J., Vieste, A.B., and D.E. Sutherland (1989). Cold-storage preservation of the canine and rat pancreas prior to islet isolation. *Transplantation* **47**, 28-32.

Nano, R.; and B.Clissi (2005). Islet isolation for allotransplantation: variables associated with successful islet yield and graft function. *Diabetologia* **48**, 906-912.

Nikolic D. M., Djordjevic P.B, Dimitrijevic-Sreckovic V., Dzingalasevic M., Belij S, and N. Kalezic (2010). Influence of the purification of human adult pancreatic islets on insulin secretion. *Vojnosanitetski Pregled* **67**, 128-132 (a).

Nikolic, D. M., Djordjevic, P. B., Dimitrijevic-Sreckovic, V., Paunovic, I., Kalezic, N., Popovic, S., Stefanovic, D., and M. Dzingalasevic (2010). The effect of different concentrations of Liberase HI used in a non automated method for human adult pancreatic islet isolation. *Arch. Biol. Sci.*, *Belgrade*, **62**, 833-840 (b).

Nikolic, D. M., Djordjevic, P. B., Dimitrijevic-Sreckovic, V., Paunovic, I., Kalezic, N., Popovic, S., Nikolic, B., and D. Stefanovic (2010). Comparative analysis of Collagenase XI and Liberase H1 for the isolation of human pancreatic islets. *Hepato-Gastroenterology* **58**, 1573-1578 (c).

Oberholzer, J, Tripones, F., Mage, R., Anderegen, E., Buhler, L., Cretin, N., Fournier, B., Goumaz, C., Lou, J., Phillipe, J., and P. Morel (2000). Human islet transplantation: Lessons from 13 Autologous and 13 Allogenic Transplantations (Clinical Transplantation). *Transplantation* **69**, 1115-1123.

Pilegi, A., Ribeiro, M.M., Hogan, A.R., Molano, R.D., Embury, J.E., Ichi, H., Cobianchi, L., Fornononi, A., Ricordi, C., and R.L. Pastori (2009). Effects of Pancreas Cold Ischemia on islet Function and Quality. *Transplantation Proceedings* **41**, 1808-1809.

Ricordi, C (2003). Perspectives in Diabetes. Lilly Lecture 2002. Islet Transplantation: A Brave New World: *Diabetes* **52**, 1595-1611.

Shapiro, A. M., Lakey, J. R.; Ryan, E. A., Korbutt, G. S., Toth, E.; Warnock, G. L., Kneteman, N. M., and R. Rajotte (2000). Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med.* **343,** 230–238.

Steven, A.; Joane, E.; Cris, P.; Sue, M.; Heather, A.; Chris, D.; Simon, W.; Patrick, P.; David, P.; and R. Ashley (2001). Pancreas Resection and Islet Autotransplantation for End Stage Chronic Pancreatitis: *Annals of Surgery* **223**, 423-431.

Toso, C., Oberholzer, J., and F. Ris (2002). Factors affecting human islet of Langerhans isolation yields. *Transplantation Proceedings* **34**, 826-701.

Tsujimura, C., Kuroda, Y., Kin, T., Avila, J., Kin, T., Oberholzer, J,.; Shapiro, A. M. J.; and J. Lakey (2002). Human islet transplantation from pancreases with prolonged cold ischemia using additional preservation by two layer (UW solution/ perfluorochemical) cold storage method. *Transplantation* **74**, 1687-1692.

Yang, Z., Chen, M., Deng, S., Ellet, D.J., Wu, R., Langman, L., Carter, J.D., Fialkow, L.B., Markmann, J., Nadler, J,L., and K. Brayman (2004). Assessment of human pancreatic islets after long distance transportation. *Transplantation proceedings* **36**, 1532-1533.

Ylipaasto, P., Klingel, K., Lindberg, M., Otonkoski, T., Kandolf, R., Hovi, T., and M. Roivainen (2004). Enterovirus infection in human pancreatic islet cells, islet tropism *in vivo* and receptor involvement in cultured islet beta cells. *Diabetologia* **47**, 225-239.