Influence of the purification of human adult pancreatic islets on insulin secretion

Uticaj purifikacije ostrvaca humanog adultnog pankreasa na insulinsku sekreciju

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Abstract

Background/Aim. The most effective method for human adult pancreatic islets purification is density-gradient centrifugation. The aim of this study was to analyze the effects of non-automated purification on preservation of functional capacity of human adult pancreatic islet cells. Methods. Human pancreata were obtained after pancreatectomy in the patients with chronic pancreatitis or benign tumors. Pancreatic islets were purified by non-automated method in discontinuous Ficoll density gradient. The samples were divided in 2 fractions: purified (P) and non-purified (NP) cultures. Islets were stained with diphenyl-thiocarbazone. The efficiency of separation was determined by comparing percentage of stained cells in P and NP cultures on day 1, 3 and 7 of short-term cultivation. Glucose-stimulated insulin secretion was expressed as stimulation index (SI). Results. The results obtained showed a statistically significant difference (p < 0.01) between P and NP cultures. P cultures had higher percentages of stained cells (70.43 ± 3.97%, 73.77 ± 4.22% and 71.34 ± 4.69% on the first, third and seventh day of cultivation, respectively) than NP cultures (53.68 ± 1.71%, 57.14 ± 3.94% and 43.97 ± 4.56%, respectively). P cultures had higher values of SI for the first, third and seventh day of cultivation than NP cultures (0.45 ± 0.08, 0.80 ± 0.21, 1.28 ± 0.15 and 0.46 ± 0.10, 0.752 ± 0.16, 0.76 ± 0.11 for P and NP cultures respectively). The difference was statistically significant on day seven (p = 0.01). Conclusion. Although during purification process islets were exposed to a number of insults that might result in cellular damage and functional impairment, our assessments showed that islets in P cultures preserved their functional capacity better than islets in NP cultures, since they had greater insulin secretion.

Key words: islets of langerhans transplantation; insulin; secretory pathway.

Apstrakt

Uvod/Gilj. Najefikasnija metoda purifikacije ostrvaca humanog adultnog pankreasa jeste gustinsko-gradijentno centrifugiranje. Cilj ovog rada bio je analiza efekta neautomatske purifikacije na očuvanost i funkcionalni kapacitet ostrvaca humanog adultnog pankreasa. Metode. Tkivo humanog pankreasa dobijano je resekcijom kod bolesnika oboljelih od hroničnog pankreatitisa ili benignog tumora. Ostrvca pankreasa su purifikovana neautomatskom metodom u diskontinuiranom Fikol gustinskom gradijentu. Uzorci su bili podeljeni na dve frakcije: purifikovane (P) i nepurifikovane (NP) kulture. Ostrvca su bojena difenil-thiocarbazonom. Efikasnost prečišćavanja određivana je opoređivanjem procenta obojenih čelija kod kultura P i NP 1, 3. i 7. dana kratkotrajne kultivacije. Glukozo-stimulativna insulinska sekrecija predstavljena je kao stimulacijski indeks (SI). Rezultati. Dobijeni rezultati pokazuju statistički značajnu razliku (p < 0,01) između kultura P i NP. P kulture sadrže veći procenat obojenih čelija, (70,43 ± 3,97%, 73,77 ± 4,22% i 71,34 ± 4,69% prvo, treće i sedmog dana kultivacije) u odnosu na NP kulture (53,68 ± 1,71%, 57,14 ± 3,94% i 43,97 ± 4,56%), i veći nivo SI, prvo, treće i sedmog dana kultivacije (0,45 ± 0,08, 0,80 ± 0,21, 1,28 ± 0,15) u odnosu na NP kulture (0,46 ± 0,10, 0,752 ± 0,16 i 0,76 ± 0,11). Postoji značajna statistička razlika između njih sedmog dana (p = 0,01). Zaključak. Mada su u toku purifikacije ostrvca bila izložena dejstvu mnogih štetnih faktora koji mogu da dovedu do oštećenja čelija i funkcijalnih poremećaja, naši rezultati pokazuju da su ostrvca u P kulturama sačuvala svoju funkcijsku sposobnost i da imaju veću insulinsku sekreciju nego ostrvca kod NP kultura.

Ključne reči: transplantacija langerhansovih ostrvaca; insulin; proces sekrecije.

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O R I G I N A L  A R T I C L E

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Introduction

The most effective method for human islet purification is density-gradient centrifugation. Even the development of large-scale purification by using continuous gradient in COBE 2991 cell processor has not obscured the significant loss during purification step. This cell processor represents a closed system for islet isolation which is much faster, more effective and less expensive than the traditional islet preparation. Another crucial factor is temperature. Some studies showed that cooling a COBE 2991 density gradient and pancreatic tissue is important for reducing the less pure fractions after centrifugation. During isolation, while a substantial proportion of islets may be found in relatively small proportion of islets in the higher purity fractions, the density-gradient centrifugation results in enhanced density differences between acinar tissue and islets, therefore favoring separation.

Purification procedure may result in the recovery of a relatively small proportion of islets in the higher purity fractions, while a substantial proportion of islets may be found in the less pure fractions after centrifugation. During isolation and purification, islets are exposed to a number of insults that may result in cellular damage and functional impairment, which ultimately lead to a reduction of the viable islet mass recovered. Furthermore, most of islet insulin is lost during purification. This seems to be caused rather by an amplified insulin release than by the loss of islets itself. Previous studies showed that osmolality is one of the most critical variables in isopycnic purification. Cells behave like osmometers showing volume and density changes dependent on the osmotic pressure. It is conceivable that exposure to high-density polysucrose solutions can be toxic for islet cells. On the other hand, exposure to high osmolality media may decrease acinar tissue swelling and edema, resulting in enhanced density differences between acinar tissue and islets, therefore favoring separation.

Because of an increased complication after transplantation of unpurified islets preparations (increased portal pressure and thrombosis), purification is still recommended prior to portal infusion. The aim of the present study was to analyze the effects of non-automated purification in discontinuous density-gradient (Ficoll) on preservation of functional capacity of human adult pancreatic islet cells. This is measured as adequate insulin response to glucose stimulation in short-term (7 days) cultivation.

Methods

Human adult pancreata were obtained after pancreatectomy in the patients with chronic pancreatitis or benign tumors. Operative procedures were performed in the Institute for Gastrointestinal Diseases, Clinical Center of Serbia. In case of tumors, we took exclusively healthy tissue by line of resection. Islet cultures were divided into two groups, 10 purified (P) and 10 non-purified (NP). Cold ischemia lasted 70.2 ± 28.33 min; warm ischemia lasted 117.2 ± 17.4 min and 192.8 ± 21.98 for NP and P cultures, respectively. Warm ischemia is the time measured from the beginning of isolation procedure to the moment when the islets were placed in culture medium. Average weight of pancreatic tissue was 5.032 ± 0.57 g.

Islets were isolated under aseptic conditions by non-automated method using collagenase IX, 5mg/mL (Sigma-Aldrich). After semi digestion for 30 min at 37° C, supernatant was decanted and cells were washed in HBSS (Aplichem-GmbH) solution containing 20 mM Hepes, 0.2% FCS (fetal calf serum), pH 7.4.

Separation of the islets from the surrounding acinar tissue was accomplished by centrifugation on Ficoll gradients (Sigma) prepared with HBSS solution. Islets were resuspended in 4 ml RPMI followed by addition of 16.7 mL of stock Ficoll medium (density 1.125 g/mL) in conic test tubes with 50 mL volume. The sample was carefully stirred with medium to remove interphase. The discontinuous gradient was obtained by applying subsequently Ficoll solutions of the density 1.085, 1.075 and 1.045 g/mL (5 mL each). After 25-min centrifugation at 550 g and 22° C, two fractions were collected, 1.045/1.075 and 1.075/1.085 interface layers. Islets were washed in HBSS medium by centrifugation (950 g, 5 min, at 4° C). Islet yield was 2–4000 IEQ/g pancreas.

Then the islets were resuspended in culture medium RPMI 1640 (Sigma-Aldrich) containing Ca(NO₃)₂ × 4H₂O 0,1 g/L, MgSO₄ (anhyd) 0,048 g/L, KCl 0,4 g/L, NaHCO₃ 2 g/L, NaCl 6 g/L, Na₂HPO₄ (Anhyd) 0,8 g/L, Glutamine 0,3 g/L, D-Glucose 1 g/L, 25 mM/L HEPES, 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin and transferred to plastic culture flasks (Falcon 3013, volume 50 cm³) and incubated at 37° C in a 5% CO₂, 95% humidity atmosphere for 7 days. To determine the functional capacity of isolated islets static glucose stimulation was performed. Islets were incubated with either 2.8 mM or 20 mM glucose in HEPES-buffered Hank’s balanced salt solution for one hour at 37° C on the first, third and 7th day of cultivation. Supernatant of each sample was collected and stored at -18° C. The insulin content was measured by radioimmunoassay (RIA INSULIN PEG, Inep, Zemun, Belgrade). The sensitivity of the assay was 0.60 mIU/L and detection range 0.6–300 mIU/L. Glucose-stimulated insulin release was expressed as stimulation index (SI), calculated as the ratio of insulin released during exposure to high glucose over the insulin released during low glucose incubation (20 mM and 2.8 mM, respectively).

Islets were stained with dithizone (diphenylthiocarbazone), as described previously (Figures 1 and 2). These studies were approved by the Ethical Committee, Clinical Center, Belgrade, Serbia and written consent obtained from the patients.

Statistical analysis: Results are expressed as mean ± SE. Comparisons between stimulation indexes of islets in P and NP cultures were performed by Student’s t test; p value of less than 0.05 was considered to be statistically significant.

Results

The efficiency of separation of the islets from acinar tissue by purification on a density gradient was established by comparing percentage of stained and non-stained cells in P and NP cultures on the day 1, 3 and 7.

Fig. 1 – Pancreatic islet stained with dithizone in a non-purified culture; small amount of exocrine tissue (unstained) is present (magnification bar = 100 µm).

Fig. 2 – Viable islet in purified culture stained bright red with dithizone (magnification bar = 100 µm).

Comparison of P and NP cultures (Figure 3) showed a statistically significant difference ($p < 0.01$) since the percentage of stained cells in P cultures was greater than in NP cultures for each analysis. On day one, percentage of stained cells in P cultures was $70.43 \pm 3.97\%$ and $53.68 \pm 1.71\%$ in NP cultures ($p < 0.01$). On the day 3, the percentage of stained cells in P cultures was also greater than in NP cultures ($73.77 \pm 4.22$ and $57.14 \pm 3.49$, respectively; $p = 0.01$). The difference existed throughout cultivation: $71.34 \pm 4.69\%$ of stained cells in P cultures and $43.97 \pm 4.56\%$ in NP cultures on the day 7 ($p < 0.01$).

The insulin secretion capacity of both cultures was determined by SI values on the first, third and seventh day of cultivation (Figure 4). SI for P cultures were $0.45 \pm 0.08$, $0.80 \pm 0.21$, $1.28 \pm 0.15$ respectively, and for NP cultures SI $= 0.46 \pm 0.10$, $0.752 \pm 0.16$, $0.76 \pm 0.11$ respectively. The difference was statistically significant only on the day seven ($p = 0.01$).

Fig. 3 – Percentage of the stained cells in purified and non-purified cultures on the first, third and seventh day of cultivation.

Fig. 4 – Insulin stimulation indexes (SI) of purified and non-purified cultures on the first, third and seventh day of incubation.

Discussion

Recent studies demonstrated that insulin-dependence can be achieved by implanting $> 10000$ IEQ/kg of recipient body weight. Pre-transplantation and isolation procedures may cause significant loss of pancreatic islet cells which represents only 1–2% of the total pancreatic tissue. By purification is not possible to obtain absolutely pure culture, but by manual isolation and in this case the loss of the islets is the greatest. Beside the loss of islets, purification may cause mechanical damages which ultimately result in declining of their functional ability during cultivation.

In the present study we analyzed effects of non-automated method of purification in discontinuous Ficoll gradient on functional capacity of islets determining insulin response to glucose stimulation. We observed that the percentage of stained cells in P cultures were greater by 20% than in NP cultures on the first and seventh day of cultivation (Figure 3). The differences are statistically significant, particularly on the seventh day. The number of the stained cells in P cultures is almost constant, while that number decline in NP cultures by 10% ($53.68\%$ on the first and $43.97\%$ on the seventh day of cultivation).

We agree with the findings of some authors that during purification cells are mechanically and chemically damaged resulting in the loss of cell insulin. Since dithizone binds directly to insulin in the islet cells, the number of stained cells should be lower on the day one than on the day seven, which is not the case in this study. The question is if the cells can recompense this insulin loss during overnight culture. The number of stained cells in NP cultures decreases, indicating that the presence of exocrine tissue may provoke and speed

up the apoptosis of the islet cells. Based on the results in Figure 3 (the percentage of the stained cells) we can conclude that P cultures are more stable than NP ones. This feature could be important after transplantation in patients. To determine the preservation of the functional capacity of the isolated cells, static glucose assay was performed. Glucose stimulated insulin release was expressed as SI. Functional capacity of both P and NP cultures showed no significant difference on the first and the third day of cultivation (Figure 4). But on the seventh day of cultivation, the difference was statistically significant (SI = 0.76 and 1.28, respectively; p = 0.01). On the first day of cultivation, insulin secretion of the cells in P and NP cultures was similar, indicating that purification did not have any toxic effects. During cultivation, functional capacity of both cultures increases. This can be explained as adaptation of the cells to conditions of in vitro cultivation and to a certain medium. But on the day seven, cells in P cultures showed greater response to glucose stimulation suggesting that exocrine tissue in NP cultures has negative effects on the cells during cultivation. Apoptosis and necrosis of exocrine tissue followed by release of certain endotoxins, probably cause decline of the functional capacity of the islets. Another factor contributing to these facts is frequency of the replacement of the medium. First three days medium was replaced daily and then every second day. This means that prior to an assessment on the seventh day of cultivation, the cells were on the same medium for 48 hours. These data suggest that cultivation medium should be replaced daily.

Non-automated method of purification in Ficoll density-gradient can increase probability of infections and prolong the warm ischemia compared to NP cultures, resulting in smaller islet yields. But it does not affect the functional capacity of the cells, since after overnight culture, insulin secretion of the cells in P cultures were similar to insulin secretion of the cells in NP culture.

Conclusion

This method of purification can be used in experimental researches, especially when an amount of an available tissue is relatively small. The loss of the cells does not affect the results, since the stimulation index values do not depend on the number of the cells. Because of the extensive cell loss during purification, this method is not suitable for clinical and transplantation use.

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REFERENCES


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