THE EFFECT OF DIFFERENT CONCENTRATIONS OF LIBERASE HI USED IN A NON AUTOMATED METHOD FOR HUMAN ADULT PANCREATIC ISLET ISOLATION

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Abstract - Liberase HI has many advantages over traditional collagenases. Liberase performs the rapid dissociation of human pancreata and allows for the preservation of the morphological and functional integrity of isolated islets. The aim of this study was to investigate the effect of different concentrations of Liberase HI (1.5, 2.5 and 3.5 mg/ml) on the yield, viability and function of islets isolated by a non-automated method. Based on our results, the Liberase HI concentration 1.5 mg/ml is recommended for this method of isolation.

Keywords: Liberase HI, yield, insulin secretion, human adult pancreatic islets

INTRODUCTION

Collagenase digestion is an important step in the isolation procedure of pancreatic islets and represents the main obstacle for the successful isolation of a sufficient number of good quality islets for transplantation. Collagenases are endopeptidases that digest collagen fibrils. Collagen consists of recurring polypeptide subunits called tropocollagen, arranged head to tail in parallel bundles. Boiling in water transforms collagen, which is insoluble, into gelatin, a soluble mixture of polypeptides. The resulting gelatin is sensitive to proteolytic enzymes, i.e. collagenases (Harper, 1980). Unlike animal collagenases, bacterial collagenase can degrade both water-insoluble native collagen and water-soluble denatured ones. During isolation it is necessary to break down the exocrine-endocrine connections between the cells (thus releasing the islets from the surrounding acinar tissue) and leave the endocrine-endocrine ones intact to maintain the integrity and compactness of the islets. Collagenase also acts on intrainsular bonds and prolonged exposure to the enzyme can destroy initially released islets. The

combination of specific enzymes and modification of the isolation conditions may provide a selectivity of digestion (Van Shilgfgaarde et al., 1994).

Traditional collagenase preparations are prepared from the bacteria Clostridium histolyticum. These preparations are heterogeneous and contain many different enzymes, pigments and endotoxin. The primary enzyme constituents are collagenases (classes I and II). The preparations also contain other proteases, including neutral protease, clostripain, trypsin and elastase. The preparations may contain non-proteolytic enzymes, such as phospholipase C. Traditional collagenases have many limitations, the most critical of which are high endotoxin levels and variability of composition.

Liberase consists of highly purified collagenase I and collagenase II which are blended in a precise ratio with each other and with neutral protease thermolysin. Liberase enzymes have many advantages over traditional collagenases, such as maximal tissue dissociation performance (including increased cell yields, improved cell viability and grea-

ter cell functionality), lot-to-lot consistency and lower toxicity.

The working concentration of collagenase depends on the type of pancreas as well as the isolation method. The recommended concentrations of collagenase for the isolation of the largest number of porcine pancreatic islets are 0.8-1 mg/ml (Vargas et al., 1996). The optimal concentrations for the successful isolation of human adult pancreatic islets are 1-3mg/ml (Botino et al., 2004). Higher concentrations (6 mg/ml) in a well-controlled digestion time using a non-automated method may provide a large number of viable islets. The recommended concentration for the isolation of human fetal pancreatic islets is 5 mg/ml, yielding 500-1500 islets and clusters with a viability of 80-90% (Nano et al., 2005).

Besides the collagenase concentration, an important factor is the duration of incubation established by frequent sampling for cytological analysis during the isolation procedure. The optimal duration of incubation for human adult pancreatic islets is 20 min, while a 30 min incubation leads to a reduction in islet number, a decrease in viability and an increase in defragmentation.

Collagenase catalyzes at a temperature range of 28 - 46°C. For adult islets, the optimal temperatures are from 39 - 41°C. During the isolation procedure a gradual warming of the enzyme to 39°C (3 C/min) is recommended. This allows for a larger number of islets, better viability and better preservation of structures (Cavanagh et al., 1994).

Collagenases from different manufacturers are used in human pancreatic islet isolation: Collagenase type P, Bohringer Mannheim, Germany (Basta et al., 1995), Liberase-HI, Roche, Indianapolis (Ricordi et al., 1998) or both types of collagenase in the case of isolation of islets for allotransplantation (Formby et al., 1988). One of the main disadvantages of collagenases is the presence of certain toxic substances which are the by-product of bacterial fermentation. These substances can damage the cells and diminish the functional ability of the iso-

lated islets. This is the reason why Liberase, as a highly purified enzyme blend, is recommended for the rapid dissociation of human pancreata with a complete recovery of unaltered islets with preserved functional ability (Warnoc et al., 1988).

Liberase was applied successfully in the isolation of human adult islets and experimentally in the isolation of adult porcine islets (Lakey et al., 1998; Bradhorst et al., 1999, 2003). Liberase was compared with traditional collagenase in the isolation of neonatal rat pancreatic islets. All results tended to be better when islets were isolated with liberase. However, no significant differences were found regarding islet yield, insulin and DNA content. The stimulation index was 1.49 in the collagenase-isolated islets and 1.55 for the liberase-isolated islets (Ayman, 2005).

The aim of this study was to evaluate the efficacy of 3 different concentrations of Liberase HI (1.5, 2.5 and 3.5 mg/ml) used in human adult pancreatic islet isolation. We measured the following parameters: yield, viability and functionality of isolated islets in short-term (7 day) cultures. Due to the small amount of pancreatic tissue, we used a non-automated method for islet isolation.

MATERIALS AND METHODS

Materials

Human adult pancreatic tissue was procured from the Surgical Clinic, Institute of Digestive Diseases, Clinical Centre of Serbia. Tissue samples were collected from live donors after total or subtotal pancreatectomy due to cysts or tumors (Steven et al., 2001). 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 the Ethical Committee of the Medical Faculty in Belgrade. Written consent was also obtained from the patients.

Methods

Pancreatic tissue was transported in physiological solution 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).

Isolation of the islets

Liberase HI was obtained from Roche Diagnostics GmbH, Lot 13497900, Cat. no. 11666720001: Collagenase 2093 Wunsch units/bt; Neutral Protease 50,251 units/bt Endotoxin 2 EU/mg.

The isolation of the adult pancreatic islets was performed in 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 into test tubes containing liberase (concentrations 1.5 mg/ml-L1, 2.5 mg/ml-L2 and 3.5 mg/ml-L3). The liberase solution was prepared by dissolving the enzyme in distilled water and maintained in the refrigerator for 30 min prior to use. The working concentration was adjusted by adding Hanks 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 400 x g, for 10 min at 15˚C. The supernatant was decanted and the remaining islets were rinsed several times with Hank's solution to eliminate the excess of lipids and liberase.

The separation of the islets from the surrounding acinar tissue was accomplished by centrifugation on Ficoll gradients (Sigma) prepared with HBSS solution (Nikolic et al., 2010). The islets were resuspended 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 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 a 25 min centrifugation at 550 x g and 22˚C, two fractions were collected: 1.045/1.075 and 1.075/1.085 interface layers. The islets were washed in HBSS medium by centrifugation (950 x g, 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 (Falcon 3013, volume 50 cm^3) in an incubator at 37˚C in a 5% CO2 and 95% humidity for 7 days.

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 the culture.

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

The viability of the islets was determined by dithizone (DTZ) staining on days 1, 3 and 7 after their isolation. Viability was expressed as the percentage of distinctly stained (red) islets in relation to the total number of islets in the 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 on a nylon filter of 0.20μm porosity. The samples (1 ml of each culture) were stained with 0.2 ml of DTZ solution and incubated in the incubator 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 with a stereo-light microscope in special counting micro-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.

Table 1. Biometrical parameters for pancreatic tissue digested with liberase HI

Pancreas number	Tissue weight (g)	Culture number	Warm ischemia (min.)	Cold ischemia (min.)
1	0.78	A33	83	97
2	0.63	A34	53	132
3	1.15	A36	110	60
4	0.63	A37	100	60
5	0.70	A38	109	90
mean ± SE	0.78±0.10		91.00±10.66	87.80±13.39

Table 2. Islet yield per gram of pancreatic tissue after isolation (day 1) with different concentrations of liberase HI.

Enzyme concentration (mg/ml)	Islet number/pancreatic tissue (g)	Standard error (SE)
liberase 1.5	3800.86	877.41
liberase 2.5	1816.32	416.58
liberase 3.5	2280.39	408.41

A static glucose stimulation assay was performed. The samples were incubated for 1 h in a low glucose (2.8 mM/L RPMI) medium, then 1 h in a high glucose (20 mM/L RPMI) medium and 1 h in a low glucose medium again. After each step of stimulation, the cultures were centrifuged at 400 x g for 10 min at 15˚C. The supernatant was decanted and stored at -18˚C for insulin quantification. Insulin content was determined by radioimmunoassay (RIA INSULIN PEG). The sensitivity of the assay was 0.60 mIU/L and detection range 0.6-300mIU/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 Mann-Whitney U test. Significance was determined at $P < 0.05$.

Abbreviations

L1 – islets isolated with Liberase HI concentration 1.5 mg/ml

L2 – islets isolated with Liberase HI concentration 2.5 mg/ml

L3 – islets isolated with Liberase HI concentration 3.5 mg/ml

RESULTS

We examined the efficacy of Liberase HI (L1, L2, L3) with regard to isolation, islet yield, viability and insulin secretion. Tissue from 5 human adult pancreata was digested with Liberase HI and the data (tissue weight, cold and warm ischemia) are presented in Table 1.

Islet Yield

We first examined 3 different concentrations of liberase: 1.5, 2.5 and 3.5 mg/ml, to establish which was the most effective in islet isolation. Isolation with Liberase yielded from 1816.324 to 3800.858 islets/tissue weight (Table 2 and Fig. 1). The smallest yield was in the L2 cultures and the highest yield was in the L1 cultures. However, no statistically significant differences were observed between the results for all the Liberase concentrations, ($P > 0.05$, Table 4).
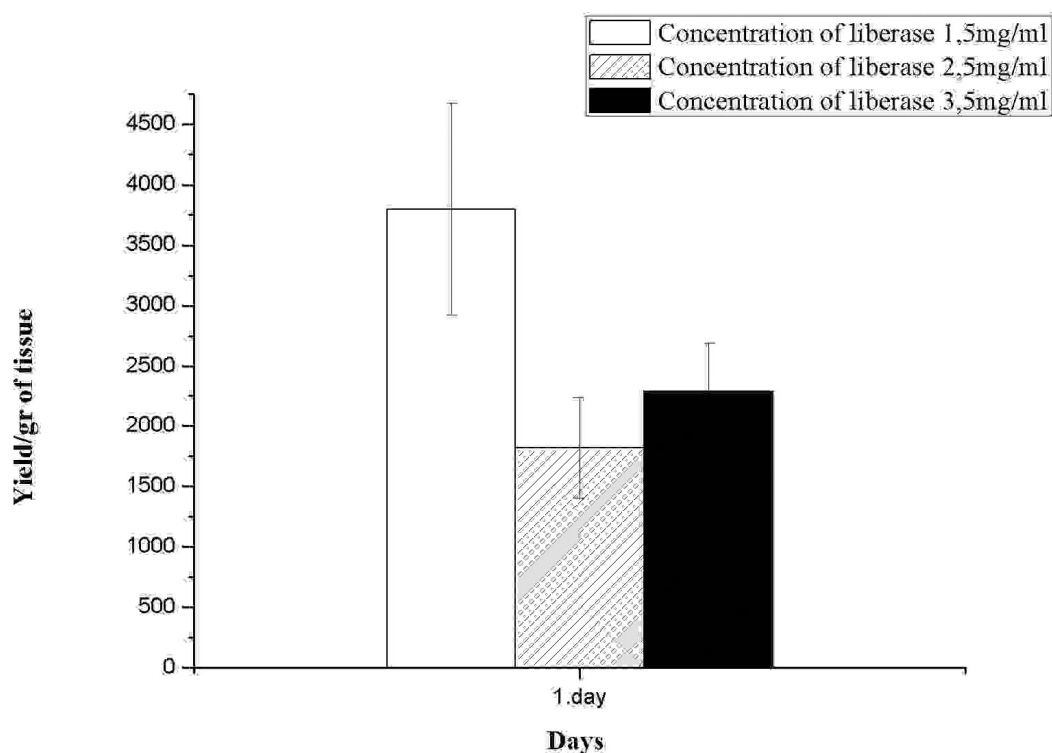
Figure 1. Islet yields obtained after isolation using different concentrations of Liberase HI (1.5, 2.5 and 3.5 mg/ml) expressed as number of islets per gram pancreatic tissue. Results are expressed as mean±SE. Statistical analysis by Mann-Whitney test revealed no significant differences between data (P>0.05).

Islet Viability per Culture

The next step was to establish the percentage of viable islets on the first, third and seventh culture days after islet isolation. The viability of the islets was determined by DTZ staining and the results are presented in Fig. 2. The viability of the islets on the first day was in the range from 21.63% (L2) to 59.87% (L1). Two liberase concentrations (L1 and L3) provided greater percentages of viable cells (59.87±6.79, 56.96±4.43, respectively) compared with L2 (21.63±2.73) and the difference was statistically significant (P<0.05).

On the third day of cultivation the number of distinctly stained islets declined in the L1 and L3 cultures, while the L2 cultures had the greatest percentage (77±3.93, 36.19±7.67 and 29.26±3.53, respectively) with a statistically significant difference (P<0.05). The viability of the islets isolated by L2 decreased on day 7 (30.92±3.35) compared with the results for L1 and L3 (77.34±3.16 and 60.13±4.05, respectively, P<0.05). Analysis of the results for L1, L2 and L3 during the 7-day cultivation shows that the percentage of viable cells isolated with L1 and L3 declined on day 3 and increased on day 7. The viability in the L2 cultures was the opposite, increasing on day 3 and declining on day 7.

Insulin secretion

To determine the functional capacity of the isolated islets, a static glucose test was performed on the first, third and seventh day of cultivation. The stimulation index (SI) values calculated for the different concentrations of the enzymes and days of cultivation are presented in Fig. 3. Statistical analysis of the results (P values) is given in Table 8.
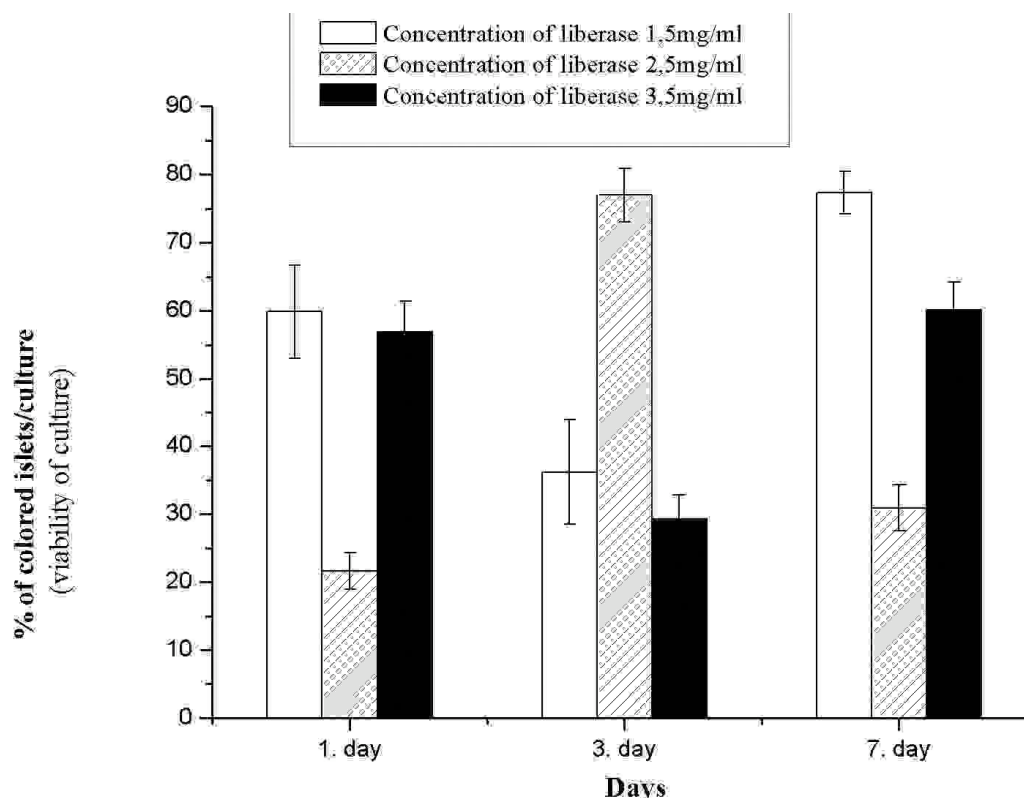
Figure 2. Number of viable islets expressed as percentage of clearly stained islets in relation to the total number of islets in culture. The results are given as mean±SE. Viability of the islets isolated either by liberase was determined by DTZ staining on the first, third and seventh day of cultivation.

In the case of liberase-isolated islets, the corresponding values for L1, L2 and L3 on day 1 were similar and were not statistically different (0.945±0.173, 0.832±0.119 and 0.764±0.049, P>0.05). The results for insulin secretion on day 3 were also similar and no significant differences were observed (0.738±0.121, 0.825±0.104 and 0.823±0.140). On day 7, the SI value for digestion with L3 was significantly higher than the SI values for L1 and L2 (1.047±0.158, 0.574±0.137 and 0.574±0.116, respectively, P<0.05). On the other hand, analysis of the results for each liberase concentration during the 7-day cultivation showed no statistically significant differences.

DISCUSSION

The outcome of islet isolation depends critically on the efficiency of the enzyme blend used for pancreas dissociation. During isolation, collagenase breaks down the exocrine-endocrine bonds while the endocrine-endocrine bonds between the cells inside the islet should be preserved. Collagenase is non-selective and acts on the intrainsular bonds also. Commercial collagenase preparations contain mixtures of enzymes whose function is not yet clarified (Wolters et al., 1990).

Preparations of collagenase contain certain toxic substances that may damage the cells and diminish their functional capacity. For this reason, Liberase, as a highly purified mixture of enzymes, is recommended for the rapid dissociation of human pancreatic tissue and it provides a complete recovery of the islets and the preservation of their functional ability.

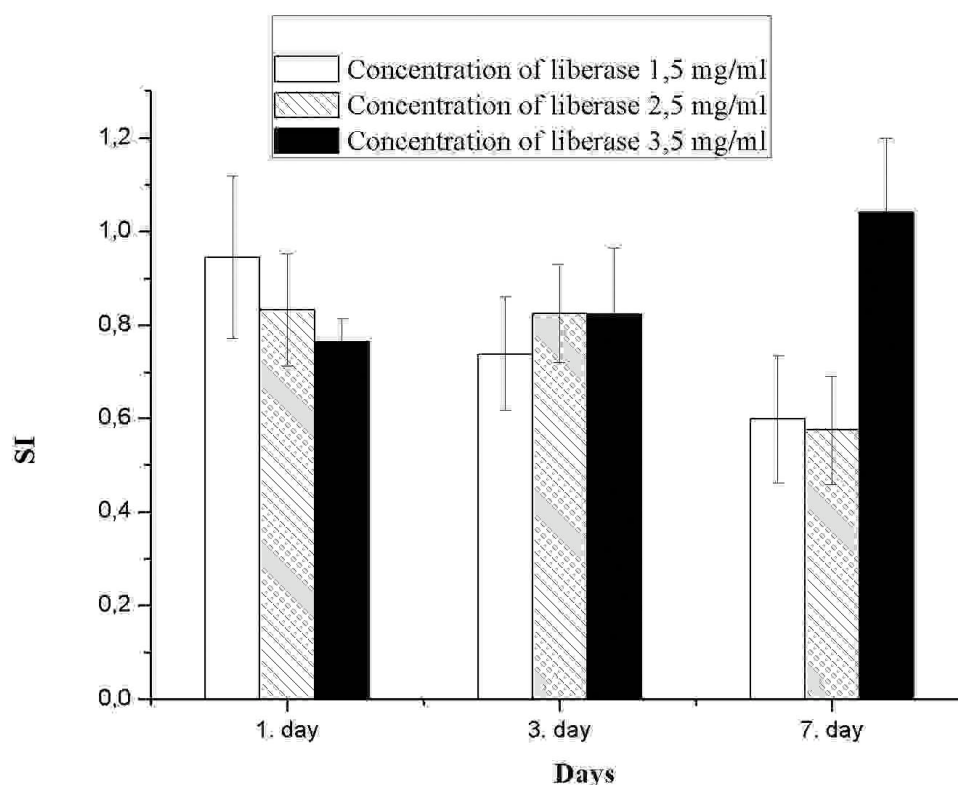We analyzed the efficacy of three different concentrations of liberase HI (L1, L2 and L3) When

Figure 3. The effect of a digestive enzyme (liberase) on glucose-stimulated insulin secretion. Values are presented as mean±SE. The stimulation index is the ratio of released insulin at low glucose (2.8 mM/L) and high glucose (20mM/L). Measurements were performed on the first, third and seventh day of cultivation.

measuring islet yields, no significant differences were found between the different concentrations of Liberase HI (Table 3, Fig. 1). The viability of the isolated islets was determined by DTZ staining on the first, third and seventh day of cultivation. On day 1, L2 had a significantly lower number of viable cells compared to the L1 and L3 cultures (Tables 5 and 6, Fig. 2). During cultivation (on day 3), the percentage of viable cells in the L2 cultures increased and had the greatest value compared to other 3 groups. On the other hand, the viability in the L1 and L3 cultures decreased and was smaller than in C cultures. All differences were statistically significant. At the end of the cultivation period the number of viable cells in L1 and L3 increased again and declined in the L2 cultures. The percentage of viable cells in the L1 and L3 cultures was greater on days 1 and 7 than in the L2 and C cultures, and smaller on the third day of cultivation. The number of viable

islets in the culture can be influenced also by apoptosis (Djordjevic et al., 2009). Besides islet yield and viability, the glucose-stimulated insulin secretion was measured as an indication of their function. In the case of liberase digestion, no significant differences were observed on days 1 and 3 of cultivation since the SI values were similar for all the examined liberase concentrations (L1, L2 and L3). But on day 7 L3 had significantly higher SI values than L1 and L2. Analysis of the results indicates that the functional ability of the liberase-isolated islets remains unaltered during 7-day cultivation.

Excluding the results for the first day of incubation, our results are in agreement with the results of the study of the efficacy of islet isolation in neonatal rats with Liberase (Ayman, 2005). The present study confirms previous reports about the superiority of Liberase HI in human islet isolation. During

liberase digestion, islets are exposed to less functional damage, resulting in the preservation of their morphological and functional integrity immediately after isolation and during cultivation. However, some studies demonstrated that apart from Liberase HI, other collagenase blends (such as recombinant and Sevac collagenase) could be successfully administered during the isolation procedure.

CONCLUSION

During digestion with Liberase HI islets are exposed to less functional damage, resulting in the preservation of their morphological and functional integrity immediately after isolation and during cultivation. Based on our results, the use of a liberase concentration of 1.5 for a non-automated method of human adult pancreatic islet isolation can be recommended.

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