Comparative efficacy of Belzer or Euro-Collins solutions for pancreatic preservation during cold ischemic storage in rats[1]

Rogério Renato Perez[I], Alberto Goldenberg[II], Alcides Augusto Salzedas Netto[III], Adriano Miziara Gonzalez[IV]

[I]Fellow Master degree, Postgraduate Program in Interdisciplinary Surgical Sciences, Sao Paulo Federal University(UNIFESP), Sao Paulo-SP, Brazil. Surgical procedures, acquisition and interpretation of data, manuscript writing.
[II]PhD, Associate Professor, Gastroenterological Surgery Division, Department of Surgery, Paulista School of Medicine (EPM), UNIFESP, Sao Paulo-SP, Brazil. Interpretation of data.
[III]PhD, Associate Professor, Pediatric Surgery Division, Department of Surgery, EPM-UNIFESP, Sao Paulo-SP, Brazil. Interpretation of data, critical revision.
[IV]PhD, Associate Professor, Gastroenterological Surgery Division, Liver and Pancreas Transplant Unit, Department of Surgery, EPM-UNIFESP, Sao Paulo-SP, Brazil. Conception and design of the study, manuscript writing, critical revision.

ABSTRACT

PURPOSE: To compare the efficacy of different types of solutions (Belzer or Euro-Collins) for the preservation of rat pancreas during cold ischemia.

METHODS: Thirty Wistar rats were divided into three groups according to the perfusion or storage solution: Group E (perfusion and storage in Euro-Collins solution); Group B (perfusion and storage in Belzer solution) and Group BE (Perfusion in Belzer solution and storage in Euro-Collins solution). After perfusion, the pancreas was excised and stored at 4˚C for 18 hours. Amylase was measured at 6, 12 and 18h, and histological analysis of the pancreas was performed after 18h of cold storage.

RESULTS: Amylase was elevated and comparable in Groups E and BE after 12 and 18 hours of ischemia ($p<0.05$). In the exocrine pancreas, histological differences in the amount of necrosis ($p=0.049$), lymphocytic infiltrate ($p<0.001$) and neutrophilic infiltrate ($p=0.004$) were observed, with more favorable features present in Group B. In the endocrine pancreas, Group B showed less edema ($p<0.001$), but other parameters were similar among all groups.

CONCLUSION: The Euro-Collins solution is inferior to the Belzer solution for the preservation of rat pancreas during cold ischemia.

Key words: Diabetes Mellitus, Type 1. Islets of Langerhans Transplantation. Organ Preservation Solutions. Rats.

Introduction

Type I diabetes is caused by an autoimmune reaction direct against pancreatic beta cells in Langerhans islets, leading to lifelong exogenous insulin dependence. Its prevalence is estimated to be around one million cases in Brazil[1], and the age of diagnosis peaks between eight and 12 years. Seven new cases are expected for 100,000 persons per year, but this incidence is increasing 3% annually. Half of these patients may develop severe damage to target organs, leading to loss in quality of life and restricting socioeconomic activities[1]. Conventional medical treatments with exogenous insulin prolong overall survival in diabetic patients, but do not control progression of chronic lesions in the long run[2].

William Kelly and Richard I. Lillehei performed the first successful kidney-pancreas transplant in 1966, demonstrating the feasibility of glycemic control without exogenous insulin[3]. Medical and technical advances in immunosuppressant drugs, surgical techniques and selection criteria for receptors and donors culminated in prolonged survival for these patients[4]. The kidney-pancreas transplant promotes perfect glycemic control and avoids progression of chronic lesion in target organs[5]. Such results lead the American Diabetes Association to accept the kidney-pancreas transplant as the treatment of choice for diabetic patients with end-stage renal-failure[6].

Crystalloid solutions, such as the Euro-Collins solution, were initially used for preservation of the organ after extraction until the late 1980s, but were later shown to be inferior to a more complex solution, such as Belzer's[7,8]. Others have shown that a crystalloid solution and the Belzer solution may be used sequentially for the cold perfusion and storage stages[9,10].

The average cost of a kidney-pancreas transplant in Brazil is US$ 20,000.00, including donor selection and hospitalization procedures[1], which may increase US$ 15,000.00 more in case of surgical complications[11]. Supplies, including storage solutions, account for 36% of that amount in our institution. The Belzer solution is ten times more expensive than the Euro-Collins solution (US$ 400.00 versus US$ 40.00 per liter) at our institution, and three liters are necessary for each transplant. Reducing costs by using a less expensive storage solution may, therefore, have a large impact promoting the use of kidney-pancreas transplant as a viable choice for diabetic patients with end-stage renal impairment.

Methods

Research approved by the Ethic Committee of Sao Paulo Federal University (CEP 1501-11) and followed the Council for International Organization of Medical Sciences (CIOMS) ethical code for animal experimentation[13].

Thirty female Wistar rats, three to four months old, were divided into three groups (10 rats per group) according to perfusion and storage solutions. In Group E, the pancreas was flushed and preserved with the Euro-Collins solution (Fresenius Kabi Brazil Ltda). In Group BE, the pancreas was flushed with the Belzer solution[12] and stored in Euro-Collins solution. In Group B, the whole procedure was performed with the Belzer solution.

Surgical procedures

Animals were kept without food 12 hours prior to surgery, which was performed by the same surgeon (R.R.P.) at the Development Experimental Models for Medicine and Biology (CEDEME), UNIFESP. On the day of surgery, the animals were weighed and anesthetized with a combination of 20% ketamine and 80% xylazine, 0.1ml/100g animal weight, intraperitoneally. The animal was positioned dorsally, contained by its four members. Asepsis was achieved with 70% ethanol.

The abdominal cavity was exposed by longitudinal section, and the duodenum was identified and ligated with a cotton 2-0 suture, right after the pylorus and at the duodenal-jejunal transition. The abdominal aorta was identified and dissected, and a 4-0-cotton suture positioned below the renal arteries. The right renal vein was injected with 0.1 ml of heparin with a 23needle. After 1 minute, the infra-renal aorta was punctured with a 22G Jelco® catheter, which was affixed with a 4-0 cotton suture.

A median thoracotomy was performed at this point, and the thoracic aorta was clamped close to the diaphragm. The thoracic inferior vena cava was sectioned to drain blood and perfusate. Perfusion with 20 ml of flushing solution at 4°C (Euro-Collins or Belzer) was initiated and maintained for 8 minutes, using a Samtronic, ST 670 model infusion pump (Samtronic Infusion Systems, Sao Paulo, Brazil), with a 150ml/hour infusion rate. Pancreas, duodenum and spleen were then dissected and maintained for 18h in 20ml of the selected storage solution in a closed storage flask.

Amylase detection

At six, 12 and 18 hours of storage, 2ml of storage fluid was collected in an Eppendorf tube and sent under refrigeration to Provet Moema Veterinary Clinic, Sao Paulo, Brazil. Amylase measurement was performed using a Vitros 250 Chemical Analyzer amylopectin kit (Johnson&Johnson, Brazil).

Histological analysis

After 18h in cold storage fluid, the dissected organs were fixated in 10% formaldehyde. The pancreas was separated from the duodenum and spleen and included in paraffin. Longitudinal 5 μm microtome cuts were performed and stained on glass slides with hematoxylin-eosin.

One veterinary pathologist at Provet Moema Veterinary Clinic, blinded for the assigned groups, was responsible for evaluating the histological parameters, using the classification described by Smith *et al*.[14] and later updated by Mayer *et al*.[15]. The exocrine and endocrine pancreas were evaluated separately for each parameter (edema, neutrophilic infiltrate, lymphocytic infiltrate, necrosis, apoptosis and vascular thrombosis) using a score of zero for absent, 1 for mild, 2 for moderate and 3 for severe.

Statistical analysis

Variables were described as mean, median, minimum and maximum values, standard deviation, and absolute and relative frequency. Inferential analysis was done using one-way ANOVA for weight and repeated-measures ANOVA, with the Tukey post-test, for amylase values at different times (6, 12 and 18h). The Kruskal-Wallis test was used for comparison of histological parameters. The level of significance α was set at 5%.

Excel 2010for Windows® was used for storing data, and statistical analyses were performed using Statistica v.12 for Windows® and R v.2.15.2.

Results

Animal weight

Results are shown in Table 1. Mean animal weight varied between 191 and 193g. There was no statistical difference among groups (p=0.961).

TABLE 1 - Animal weight for each group in grams (g).

Group*	Mean	Median	Min.Value	Max. Value	SD
B	193.0	190.0	170.0	230.0	19.5
BE	191.0	190.0	170.0	220.0	16.6
E	193.0	190.0	170.0	230.0	18.9

Group B, Belzer solution for perfusion and storage
Group BE, Belzer solution for perfusion, Euro-Collins solution for storage
Group E, Euro-Collins solution for perfusion and storage

Amylase

Amylase was collected from the storage solution six, 12 and 18h after placement of surgical specimen in the solution. A statistically significant interaction between time and amylase levels was observed (p<0.001), indicating that a difference was detected in amylase levels both when comparing groups and time.

After 12 and 18h, results from Group B showed lower amylase levels than either Group BE or Group E; furthermore, amylase levels did not change significantly during the storage period in Group B. Results are presented in Table 2. Meaningful statistical comparisons are presented in Table 3.

TABLE 2 - Amylase levels (UI/L).

	Groups	Mean	Median	Min.	Max.	SD
6h						
	B	43.8	32.5	29.0	76.0	18.7
	BE	219.2	182.5	54.0	459.0	130.2
	E	201.9	185.0	44.0	491.0	137.6
12h						
	B	74.9	70.5	29.0	182.0	47.1
	BE	356.4	272.5	133.0	635.0	176.3
	E	315.6	280.5	94.0	701.0	193.3
18h						
	B	113.7	108.0	35.0	214.0	60.7
	BE	514.7	489.0	265.0	827.0	207.3
	E	426.9	379.0	134.0	834.0	223.7

Group B, Belzer solution for perfusion and storage
Group BE, Belzer solution for perfusion, Euro-Collins solution for storage
Group E, Euro-Collins solution for perfusion and storage

TABLE 3 - Statistical analysis among groups and time for amylase values.

Type of comparison	Conclusions	p value**
Group B	6 hours = 12 hours	0.88
	6 hours = 18 hours	0.052
	12 hours = 18 hours	0,69
Group BE	6 hours < 12 hours	<0.001
	6 hours < 18 hours	<0.001
	12 hours < 18 hours	<0.001
Group E	6 hours < 12 hours	<0.001
	6 hours < 18 hours	<0.001
	12 hours < 18 hours	<0.001
6 hours	Group B = Group BE	0.224
	Group B = Group E	0.345
	Group BE = Group E	>0.999

12 hours	Group B < Group BE	0.006
	Group B < Group E	0.028
	Group BE = Group E	0.999
18 hours	Group B < Group SB	<0.001
	Group B < Group E	<0.001
	Group BE = Group E	0.922

Group B, Belzer solution for perfusion and storage. Group BE, Belzer solution for perfusion, Euro-Collins solution for storage. Group E, Euro-Collins solution for perfusion and storage. **Tukey post-test.

Histological analysis

Histological parameters were evaluated according to grade of severity (see Methods). For the exocrine pancreas, there were significant differences in the amount of necrosis (p=0.049), neutrophilic infiltrate (p=0.004) and lymphocytic infiltrate (p<0.001), all favoring Group B. There was no difference in the amount of edema among groups (p=0.368). No apoptosis or vascular thrombosis could be detected in any group (data not shown). Results are detailed in Table 4, and some examples are shown in Figure 1.

TABLE 4 - Histological parameters analysis for the exocrine pancreas, relative frequency (%).

	Group	B	BE	E	p**
Edema					0.368
	absent	0	0	0	
	mild	90	100	100	
	moderate	0	0	0	
	severe	0	0	0	
Necrosis					0.049
	absent	100	90	60	
	mild	0	10	40	
	moderate	0	0	0	
	severe	0	0	0	
Neutrophilic Infiltrate					0.004
	absent	50	0	10	
	mild	50	50	40	
	moderate	0	40	50	
	severe	0	10	0	
Lymphocytic Infiltrate					<0.001
	absent	80	0	10	
	mild	20	100	80	
	moderate	0	0	10	
	severe				

Group B, Belzer solution for perfusion and storage. Group BE, Belzer solution for perfusion, Euro-Collins solution for storage. Group E, Euro-Collins solution for perfusion and storage. **Kruskal-Wallis test.

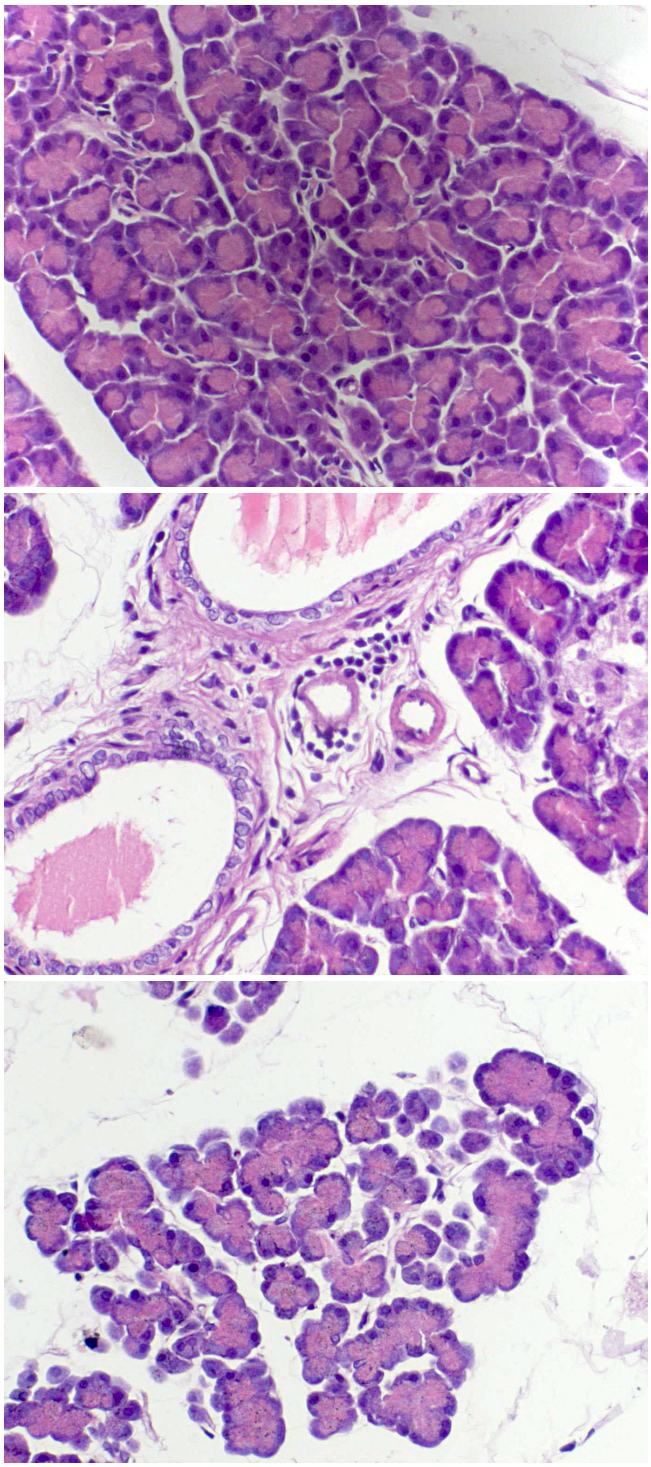

FIGURE 1 - Exocrine pancreas photomicrographs, HE staining, x200. A. Group B, normal glandular architecture. B. Perivascular lymphocytic infiltrate, Group BE. C. Mild necrosis and neutrophilic infiltrate, Group E.

For the endocrine pancreas, there were significant differences only in the amount of edema (p<0.001) with a much lower severity in Group B. There were no differences in necrosis or lymphocytic infiltrates among groups (p=ns). No neutrophilic infiltrate, apoptosis or vascular thrombosis could be detected in any group (data not shown). Results are detailed in Table 5 and example shown in Figure 2.

TABLE 5 - Histological parameters analysis for the endocrine pancreas, relative frequency (%).

	Group	B	BE	E	p
Edema					<0.001
	absent	20	0	0	
	mild	60	0	0	
	moderate	20	30	100	
	severe	0	70	0	
Necrosis					0.595
	absent	100	90	90	
	mild	0	0	10	
	moderate	0	10	0	
	severe	0	0	0	
Lymphocytic Infiltrate					0.342
	absent	100	90	80	
	mild	0	0	20	
	moderate	0	10	0	
	severe	0	0	0	

Group B, Belzer solution for perfusion and storage. Group BE, Belzer solution for perfusion, Euro-Collins solution for storage. Group E, Euro-Collins solution for perfusion and storage.**Kruskal-Wallis test.

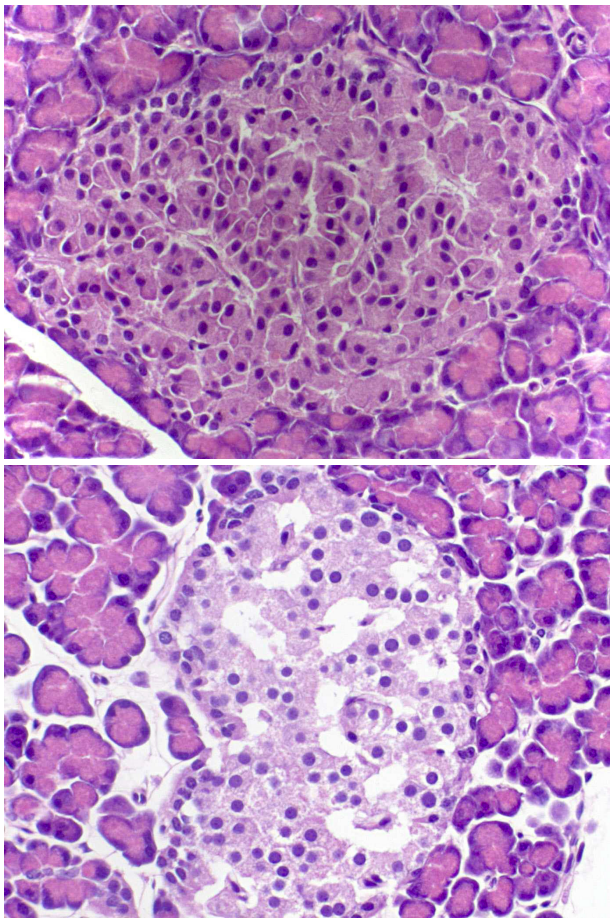

FIGURE 2 - Endocrine pancreas photomicrographs, HE staining, x400. **A.** Group B, normal glandular architecture. **B.** Severe edema in a Langerhans islet, Group BE.

Discussion

Type 1 Diabetes is a chronic disease with high socioeconomic impact due to its sequelae present in adult life. Its incidence is increasing, transforming this disease in an important public health issue[1].

Advances in pancreatic transplantation, especially when associated with kidney transplant, have produced encouraging results. Calne *et al*.[16] introduced cyclosporine use in clinical practice, improving immunosuppression considerably.

Advances in surgical technique[17], understandings of cellular events associated with organ rejection, better antibiotic treatments and developments in radiologic imaging[18] converted kidney-pancreas transplant into first choice treatment for patients with Type 1 Diabetes and end-stage kidney failure[5].

Nevertheless, the kidney-pancreas transplant continues to have very high complication rates compared to other types of transplant, impacting the total cost of the procedure[19]. Supplies, accounting for about 36% of costs in our hospital, are one of the most important components of the total price of the procedure. In particular, solutions for perfusion and storage of the donated organs, like the Belzer solution, can be very expensive (around 7% of total costs), prompting us to evaluate new options for this procedure.

Gonzalez *et al*.[10] described the first case series using 1 liter of Euro-Collins solutions, followed by 1 liter of Belzer solution during the perfusion of donated organs, demonstrating no differences in clinical results. Schraibman *et al*.[20], using the same technique in rats, also concluded that there were no differences in rates of pancreatic tissue preservation.

Although the feasibility of using a colloid solution like Euro-Collins for the perfusion phase of the procedure has been demonstrated, very few studies have examined whether the use of the Euro-Collins solutions for the storage phase is satisfactory.

Some groups have assessed the use of the Perfluorochemical Oxygen Carrier solution (PFC), expecting to minimize the effects of ischemia during cold storage in animals[21,22] or humans[23,24]. No differences were found compared to Belzer solution, suggesting that the kind of storage solution might be indifferent in terms of organ preservation.

Amylase values in the storage fluid are related to organ viability, with longer periods of ischemia showing greater release of this enzyme by damaged pancreatic tissue[25,26]. In our work, no statistically significant differences were observed at six hours of storage comparing three groups (Group B, Belzer solution for perfusion and storage; Group BE, Belzer solution for perfusion, Euro-Collins for storage and Group E, Euro-Collins solution

for perfusion and storage), suggesting that the amount of tissue injury was similar among groups. On the other hand, amylase values increased both at 12 and 18h in groups where the organs were stored in Euro-Collins solution (Groups BE and E). The organs stored in Belzer solution showed no statistically significant increases in amylase values, indicating that, for this group, no significant organ damage was present (Tables 2 and 3).

Furthermore, when analyzing histological parameters that correlate with tissue damage[14], we found important differences in the exocrine pancreatic tissue from organs stored with the Euro-Collins solution compared to Belzer solution (Table 4 and Figure 1). For the endocrine pancreas, edema was the only finding that was more evident in the Euro-Collins group (Table 5 and Figure 2). Preissler *et al*.[27] showed that Langerhans islets are more resistant to ischemia, which could explain why edema was the only histological parameter we found that was different among groups.

Conclusion

The Euro-Collins solution is inferior to the Belzer solution for the preservation of pancreatic tissue during the cold ischemic storage phase in rats.

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Correspondence:
Adriano Miziara Gonzalez
Alameda Casa Branca, 471/81
01408-001 São Paulo – SP Brasil
Tel.: (55 11)982229815
amgonzalez@uol.com.br

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