Minami, K., Okuno, M., Miyawaki, K., *et al.* (2005). Lineage tracing and characterization of insulin-secreting cells generated from adult pancreatic acinar cells. Proc. Natl. Acad. Sci. USA 102, 15116–15121.

Naya, F.J., Stellrecht, C.M., and Tsai, M.J. (1995). Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. Genes Dev. 9, 1009–1019.

Naya, F.J., Huang, H.P., Qiu, Y., *et al.* (1997). Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. Genes Dev. 11, 2323–2334.

Noguchi, H., Xu, G., Matsumoto, S., *et al.* (2006). Induction of pancreatic stem/progenitor cells into insulin-producing cells by adenoviral-mediated gene transfer technology. Cell Transplant. 15, 929–938.

Noguchi, H., Ikemoto, T., Naziruddin, B., *et al.* (2009). Iodixanol-controlled density gradient during islet purification improves recovery rate in human islet isolation. Transplantation 87, 1629–1635.

Phillips, J.M., O'Reilly, L., Bland, C., *et al.* (2007). Patients with chronic pancreatitis have islet progenitor cells in their ducts, but reversal of overt diabetes in NOD mice by anti-CD3 shows no evidence for islet regeneration. Diabetes 56, 634–640.

Ramiya, V.K., Maraist, M., Arfors, K.E., *et al.* (2000). Reversal of insulin-dependent diabetes using islets generated *in vitro* from pancreatic stem cells. Nat. Med. 6, 278–282.

Ravassard, P., Hazhouz, Y., Pechberty, S., *et al.* (2011). A genetically engineered human pancreatic $\beta$ cell line exhibiting glucose-inducible insulin secretion. J. Clin. Invest. 121, 3589–3597.

Rosenberg, L. (1988). Induction of islet cell neogenesis in the adult pancreas: the partial duct obstruction model. Microsc. Res. Tech. 43, 337–346.

Russ, H.A., Ravassard, P., Kerr-Conte, J., *et al.* (2009). Epithelial-mesenchymal transition in cells expanded *in vitro* from lineage-traced adult human pancreatic beta cells. PLoS ONE 4, e6417.

Russ, H.A., Sintov, E., Anker-Kitai, L., *et al.* (2011). Insulin-producing cells generated from dedifferentiated human pancreatic beta cells expanded *in vitro*. PLoS ONE 6, e25566.

Seaberg, R.M., Smukler, S.R., Kieffer, T.J., *et al.* (2004). Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat. Biotechnol. 22, 1115–1124.

Shapiro, A.M., Lakey, J.R., Ryan, E.A., *et al.* (2000). Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N. Engl. J. Med. 343, 230–238.

Shapiro, A.M., Ricordi, C., Hering, B.J., *et al.* (2006). International trial of the Edmonton protocol for islet transplantation. N. Engl. J. Med. 355, 1318–1330.

Shimoda, M., Chen, S., Noguchi, H., *et al.* (2010a). Neurogenic differentiation 1 directs differentiation of cytokeratin 19-positive human pancreatic non-endocrine cells into insulin producing cells. Transplant. Proc. 42, 2071–2074.

Shimoda, M., Chen, S., Noguchi, H., *et al.* (2010b). *In vivo* non-viral gene delivery of human vascular endothelial growth factor improves revascularisation and restoration of euglycaemia after human islet transplantation into mouse liver. Diabetologia 53, 1669–1679.

Sutherland, D.E., Gruessner, A.C., Carlson, A.M., *et al.* (2008). Islet autotransplant outcomes after total pancreatectomy: a contrast to islet allograft outcomes. Transplantation 86, 1799–1802.

Suzuki, A., Nakauchi, H., and Taniguchi, H. (2004). Prospective isolation of multipotent pancreatic progenitors using flow-cytometric cell sorting. Diabetes 53, 2143–2152.

Takita, M., Naziruddin, B., Matsumoto, S., *et al.* (2011). Body mass index reflects islet isolation outcome in islet autotransplantation for patients with chronic pancreatitis. Cell Transplant. 20, 313–322.

Todorov, I., Omori, K., Pascual, M., *et al.* (2006). Generation of human islets through expansion and differentiation of non-islet pancreatic cells discarded (pancreatic discard) after islet isolation. Pancreas 32, 130–138.

Yamamoto, T., Yamato, E., Taniguchi, H., *et al.* (2006). Stimulation of cAMP signaling allows isolation of clonal pancreatic precursor cells from adult mouse pancreas. Diabetologia 49, 2359–2367.

Yatoh, S., Dodge, R., Akashi, T., *et al.* (2007). Differentiation of affinity-purified human pancreatic duct cells to beta-cells. Diabetes 56, 1802–1809.

Zhang, D., Jiang, W., Liu, M., *et al.* (2009). Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. Cell Res. 19, 429–438.

Zhao, M., Amiel, S.A., Christie, M.R., *et al.* (2005). Insulin-producing cells derived from human pancreatic non-endocrine cell cultures reverse streptozotocin-induced hyperglycaemia in mice. Diabetologia 48, 2051–2061.

Address correspondence to:
*Dr. Shinichi Matsumoto*
*Diabetes Research Center*
*Research Institute*
*National Center for Global Health and Medicine*
*1-21-1 Toyama, Shinjuku-ku*
*Tokyo 162-8655*
*Japan*

*E-mail:* shinichi41@mac.com

*Dr. Paul A. Grayburn*
*Baylor University Medical Center*
*621 North Hall Street, Suite H030*
*Dallas, TX 75226*

*E-mail:* paulgr@baylorhealth.edu

Received for publication June 16, 2013;
accepted after revision May 7, 2014.

Published online: May 19, 2014.

*Send Orders for Reprints to reprints@benthamscience.net*

 *Current Tissue Engineering*, 2014, 3, 34-38

# Islet Purification for Clinical Islet Transplantation

Hirofumi Noguchi*

*Department of Surgery, Chiba-East National Hospital, National Hospital Organization, Chiba 260-8712, Japan*

**Abstract:** In clinical islet transplantation, islet purification reduces the amount of tissues to be transplanted by removing the acinar tissue, therefore minimizing the risks associated with intraportal infusion. On the other hand, the purification procedure may result in decreased numbers of islets recovered from digested tissue and may be traumatic for the islets. Ficoll-based density gradients are widely used in islet purification in clinical trials. Recently, purification with iodixanol was reported in an islet transplantation series with successful clinical outcomes. This review describes current advances in islet purification for clinical islet transplantation.

**Keywords:** Density, islet isolation, islet transplantation, osmolality, regular purification, supplemental purification.

## INTRODUCTION

Pancreatic islet transplantation has been shown to be a successful and effective strategy to achieve tight glucose control in patients with type 1 diabetes who experience hypoglycemic unawareness despite maximal care [1-5]. Clinical trials have shown that insulin-independence can be consistently achieved when a sufficient number of islets (>10,000 islet equivalents (IE)/kg of recipient body weight) are implanted. However, current isolation techniques usually recover fewer than half of the islets from a given pancreas, and barely half of the processed pancreata effectively reach the threshold for clinical transplantation in most centers. Although islet isolation techniques have been gradually improving [6-11], islet transplantation from two or more donors is usually required to achieve euglycemia.

One of the most important steps for islet isolation is islet purification. The purification step allows for separation of the islets, which represent only 2-5% of the pancreas, and reduces the volume of tissue necessary for implantation. This procedure therefore minimizes the risks associated with islet infusion through the portal vein such as increased portal pressure and thrombosis. However, islet purification might result in a decreased number of islets recovered due to various stressors such as exposure to cytokines/chemokines. The minimum number of final islets required for clinical islet transplantation is 5,000 IE/kg of recipient body weight [1, 2]. Even when the prepurification counts are adequate, the postpurification islet yield sometimes falls below the required minimum number, resulting in a lower transplant rate. In some cases, a substantial proportion of islets may be found in less pure fractions after density gradient centrifugation.

This review describes current advances in islet purification for clinical islet transplantation.

*Address correspondence to this author at the Department of Surgery, Chiba-East National Hospital, National Hospital Organization, 673 Nitonacho, Chuo-ku, Chiba 260-8712, Japan; Tel: +81-43-261-5171; Fax: +81-43-268-2613; E-mail: n.hirofumi@cehpnet.com

2211-5439/14 $58.00+.00

## CONTINUOUS DENSITY GRADIENTS IN FICOLL SOLUTIONS WITH A COBE 2991 CELL PROCESSOR

The most common method of islet purification is density gradient centrifugation due to the differences in density between islets and acinar tissue. Continuous Ficoll-based density gradient purification using a semiautomated computerized COBE-2991 cell processor is considered to be the gold standard method at present [1, 2]. Standard human islet purification is performed by top loading digested tissue (≤20 mL of tissue/run) in University of Wisconsin (UW) solution [12] on high-density (1.100 g/cm$^3$) and low-density (1.077 g/cm$^3$) Ficoll-based density gradients in doughnut-shaped COBE purification bags. After five minutes of centrifugation approximately 10-20 fractions (25-50 ml each) are collected and examined for purity.

It has been reported that the percentage of islets recovered from a standard Ficoll purification is 55-65% [7, 13, 14]. The density of islets/acinar tissue is influenced by differences in donor characteristics, the secretory status of exocrine cells, pancreas procurement, preservation protocols that affect cellular swelling and tissue edema and the islet isolation procedure, which determines the extent of tissue dissociation and the ultimate size of the aggregates [15-18]. Since the purification gradients used are fixed using the gold standard method, significant exocrine tissue contamination of islet fractions leading to lower postpurification purity often results when the exocrine density is less than 1.100 g/cm$^3$. Conversely, when islet densities are more than 1.100 g/cm$^3$, as may be seen with embedded islets, most of the islets form sediment in the COBE bag, resulting in significant islet loss and diminished postpurification recovery. Our group investigated the density of digested tissue (>95% acinar tissue) in 28 human islet isolations [12]. The tissue densities in a solution in which the osmolality was 411 mOsm were 1.085 g/cm$^3$ (14.3% of the isolations), 1.090 g/cm$^3$ (32.1%), 1.095 g/cm$^3$ (46.4%), 1.100 g/cm$^3$ (3.6%) or 1.105 g/cm$^3$ (3.6%), indicating that the density varies with the individual isolation. This has profound implications for difficulty in performing islet purification.

It has been reported that the density gradient itself is likely harmful for islets [19-21]. Exposure to the sucrose-based Ficoll density gradient as well as enzymes and endotoxins during isolation may cause release of inflammatory mediators *in vitro* [22, 23]. This may contribute to the generation of inflammation, apoptosis and immunologic attack after islet implantation *in vivo*. Therefore, minimizing the stress to islet cells during purification is important for improving islet yield and quality.

## CONTINUOUS DENSITY GRADIENTS IN IODIXANOL SOLUTIONS

The use of iodixanol for islet purification has been recently reported in clinical islet transplantation at a limited number of centers [4, 6, 12, 24, 25] and is associated with a high rate of success evaluated based on clinical outcomes [5]. Ficoll is a neutral, highly branched, high mass, hydrophilic polysaccharide that readily dissolves in aqueous solutions. Iodixanol is a nonionic, iso-osmolar contrast medium used in patients for intravenous administration and has a lower viscosity than Ficoll; therefore, islets should suffer less force. Mita *et al.* compared the effects of the purification methods using iodixanol-based and Ficoll-based density gradients in terms of the efficiency of purification, islet yield, islet quality, cellular composition and anti-inflammatory aspects of purified islet preparations [25]. Although islet purity, postpurification islet equivalents, the islet recovery rate, islet viability and fractional β-cell viability were comparable, the β-cell mass after a 48-hour culture significantly improved in the iodixanol group when compared to that observed in the Ficoll group. The production of cytokines/chemokines, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-6, IL-8, macrophage inflammatory protein 1β (MIP-1β), monocyte chemoattractant-1 (MCP-1) and regulated upon activation, normal T cell expressed and secreted (RANTES), from the iodixanol group was significantly lower during the 48-hour culture after isolation than that observed in the Ficoll group and was associated with improved β-cell survival during the pretransplant culture. IL-1β, IFN-γ and TNF-α induce apoptosis in human islet cells [8, 26]. A low level of MCP-1 production from human islet preparations is associated with better clinical outcomes in islet transplantation [27]. Therefore, regulating cytokine/chemokine production may favor islet transplantation outcomes. These data suggest that the purification method using iodixanol gradient media may be helpful for improving the rate of successful islet transplantation.

## IODIXANOL-CONTROLLED DENSITY GRADIENT DURING ISLET PURIFICATION

The tissue density before purification varies with the individual isolation, which has profound implications for the difficulty of performing islet purification. A test gradient approach using multiple discontinuous gradients has been suggested to enhance islet purification recovery by optimizing the range of density gradients [12, 28, 29]. We recently demonstrated the effectiveness of controlled-density gradients using iodixanol [12] (Fig. **1A**). In our study, iodixanol was combined with a preservation solution (Kyoto solution) to generate a new purification solution (iodixanol + Kyoto (IK) solution). Before performing purification with the IK solution, we calculated the density of the digested tissue (named "density determination"). According to the outcome of the density determination step, the density of the purification solutions was controlled (high density; 1.085-1.110 g/cm$^3$) by changing the volumetric ratio of iodixanol and the purification solutions. The purification procedure used for the IK solution was the same as that used for the Ficoll solution, with the exception that the speed of centrifugation was lower than that of Ficoll solution since the IK solution has a lower viscosity. The islet yield after purification and the

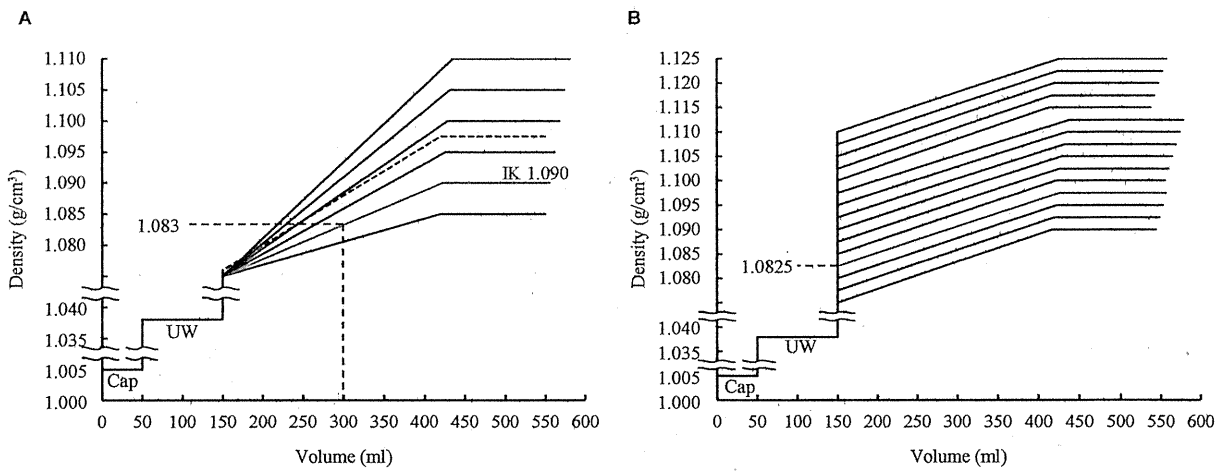


**Fig. (1). Islet purification. A**, Regular purification. The standard purification procedure for human islet preparations is performed by making a Ficoll-based density gradient between high density (1.0975 g/cm$^3$) and low density (1.0760 g/cm$^3$) (dotted line) or by IK solution-based density gradients between high density (1.085–1.110 g/cm$^3$) and low density (1.0750 g/cm$^3$). This is followed by top loading the digested tissue in 100mL UW solution followed by 50mL of capsolution and a 5-min centrifugation. **B**. Supplemental purification. Densities for SP were decided based on the outcome of RP. For example, when 1.090 g/cm$^3$ of high density solution was used in RP (Fig **1A**, dotted line) and islets in 50% of upper bottles were collected for pure fraction, it means that islets with a density of up to 1.083 g/cm$^3$ were collected. Therefore, we selected 1.0825 g/cm$^3$ for the low density solution for SP (dotted line).

postpurification recovery rate were significantly higher in the controlled density gradient purification (84.9%) than in the standard continuous gradient purification using the Ficoll solution (55.6%). These data suggest that using an iodixanol controlled-density gradient improves the islet recovery rate in human islet isolation [12].

Some of the initial test gradients, including that used in our study, employ many separate conical tubes with different densities, requiring more tissue and more time to determine the islet and acinar densities before the actual purification. Anazawa *et al.* developed an analytical test gradient system (ATGS) to determine the density distribution of exocrine and islet tissue from human pancreata before the actual COBE purification [6]. The ATGS uses a single conical tube with a continuous gradient. Using ATGS-guided continuous density gradients maximizes islet recovery for successful transplantation by reducing acinar contamination in allograft preparations and reducing sedimentation of islets in the COBE bag in autograft preparations.

## OSMOLALITY OF PURIFICATION SOLUTIONS

The osmolality of the purification solution affects the density of the islets and the acinar tissue as well as the purification itself. Our group previously reported that the purification efficacy of Ficoll with high osmolality (1.0975 g/cm$^3$ for a high-density solution) is similar to the 1.090 g/cm$^3$ of the iodixanol-based solution for a high-density solution rather than 1.095 g/cm$^3$ [12]. This is due to the difference in osmolality between the iodixanol-based solution (approximately 400 mOsm) and Ficoll with high osmolality (approximately 500 mOsm). These data suggest that tissue density increases in Ficoll solution more so than in iodixanol-based solution because the higher osmolality Ficoll solution leads to greater shrinkage of digested tissue than the iodixanol-based solution. We investigated the effects on tissue density of three different osmolalities (400, 450 and 500 mOsm/kg) of iodixanol-based purification solution [30]. The density of both islets and acinar tissue increased relative to the increase of the osmolality of the purification solutions. The density of the digested tissue increased by approximately 0.005 g/cm$^3$ relative to the 50 mOsm/kg increase in the purification solutions. There were no significant differences among the three groups in islet yield, the rate of postpurification recovery after density-adjusted purification, or the quality of the islets.

Osmolality is known to be one of the most critical variables in human islet purification [31]. It has been reported that endocrine and exocrine tissues of pancreata have distinct osmotic sensitivities [32, 33]. However, in our study, the density differences between islets and acinar tissue were similar among the three groups. We speculate that poorly preserved pancreata resulted in edematous changes due to hypoxia-induced cell swelling during cold storage. Since exocrine tissues are more susceptible than islets to cold ischemic injury [34], exocrine tissues should be more edematous than islets. In such cases, the effects of high osmolality solution are more apparent in exocrine tissue. In contrast, all pancreata used in our study were preserved with ductal injection followed by the oxygen-charged static two-layer method. Therefore, our pancreata were not edematous at all, and the ratio of shrinkage was thus similar between the islets and the exocrine tissue.

In summary, the densities of both islets and acinar tissue are observed to change due to the osmolality of the purification solution. Therefore, it should be taken into consideration that the osmolality of the purification solution affects the tissue density and may affect the efficacy of purification without the use of density adjustment.

## SUPPLEMENTAL PURIFICATION

Iodixanol controlled-density gradients improve the islet recovery rate in human islet isolation [12]. However, the percentage of islets recovered from the controlled-density solution is approximately 80% [12], and 20% of islets are still lost during purification. We and other groups recently reported the effectiveness of performing an additional purification step (supplemental purification/rescue purification) after regular purification [14, 35, 36] (Fig. **1B**). In our study of supplemental purification (SP), we designed the densities of low- and high-density solutions based on the outcome of regular purification (RP). For example, when 1.090 g/cm$^3$ of high-density solution was used in RP and islets in 50% of the upper bottles were collected for pure fraction, then islets with a density of up to 1.083 g/cm$^3$ were collected. Therefore, we selected 1.0825 g/cm$^3$ for the low-density solution for SP (Fig. **1A, B**). We set the high-density solution as a 0.015 gm/cm$^3$ increase of the low-density solution for SP. Moreover, both a continuous density and a continuous osmolality gradient were used for the supplemental purification. Eckhard *et al.* showed that a continuous density and continuous osmolality gradient induces differences in density between exocrine cells and endocrine cells (the difference in density is 0.013 g/cm$^3$), which is better than a continuous density, but not a continuous osmolality, gradient (0.004 g/cm$^3$) and results in increased islet purity and recovery [16]. The addition of SP using a continuous density and osmolality gradient increased islet recovery by approximately 8%. Therefore, the usage of a combined continuous density and continuous osmolality gradient for SP can efficiently improve islet equivalents in the final preparation.

Ichii *et al.* showed that performing SP (which they called "rescue purification") using a discontinuous gradient (1.037, 1.096, 1.108 and 1.132 g/cm$^3$) with high osmolality maximizes the number of islet preparations [35]. Using higher density solutions (1.108 and 1.132 g/cm$^3$) rather than a regular solution (1.100 g/cm$^3$) with high osmolality, the SP contributed 28% of islet equivalents to the final preparation, which is a higher percentage than that observed in our study. However, the authors only performed the rescue purification when the RP procedure resulted in relatively low islet yields compared to the prepurification counts or when a considerable number of islets were still observed in the impure layers generally not considered for transplantation. Since the percentage of islet recovery after RP was 80% in our study, we could theoretically purify up to 20% of the remaining islets using SP. Both their and our data clearly suggest that the use of supplemental purification after regular purification could be of assistance in maximizing the number of islet preparations used for islet transplantation. Their data also suggest that using higher density solutions occasionally improves the

islet recovery rate after purification because the density of islets (especially embedded islets) is sometimes near or higher than 1.100 g/cm$^3$.

## CONCLUSION

Currently, the use of continuous density gradient centrifugation on the COBE 2991 cell processor is the only method that has been shown to be consistently successful and is used clinically for large-scale human islet purification [1-5, 12]. Significant progress in islet purification techniques has been made in recent years, including the development of gradient media [7, 12, 25, 37], cooling systems during purification [38], storage of pancreatic digest in the preservation solution before purification [39] and supplemental/rescue purification [35, 36]. Several other purification techniques have been investigated, including tissue culture [40], filtration [19], magnetic microspheres coated with antiacinar cell monoclonal antibodies [41] or magnetic retraction [42], the use of antiacinar cytotoxic antibodies [43] and hypotonic lysis [44].

The success and long-term outcomes of clinical islet transplantation are limited by obstacles such as a nonoptimal transplantation site and severe inflammatory and immunological responses to the transplant. Tissue engineering strategies are poised to combat these challenges [45]. Improving the efficacy of islet transplantation seems to be the most realistic and tissue engineering strategies will help to establish cell-based therapies for diabetes.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

## ACKNOWLEDGEMENTS

This work was supported in part by the Japan Society for the Promotion of Science and the Ministry of Health, Labour and Welfare.

## ABBREVIATIONS

| | | |
|---|---|---|
| ATGS | = | analytical test gradient system |
| HBSS | = | hanks balanced salt solution |
| IE | = | islet equivalents |
| IFN | = | interferon |
| IK | = | iodixanol + Kyoto |
| IL | = | interleukin |
| MCP-1 | = | monocyte chemoattractant-1 |
| MIP | = | macrophage inflammatory protein |
| RANTES | = | regulated upon activation, normal T cell expressed and secreted |
| RP | = | regular purifications |
| SP | = | supplemental purification |
| TNF | = | tumor necrosis factor |
| UW | = | University of Wisconsin |

## REFERENCES

[1] Shapiro AM, Lakey JR, Ryan EA, *et al.* Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N. Engl J Med 2000; 343(4): 230-8.

[2] Shapiro AM, Ricordi C, Hering BJ, *et al.* International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006; 355: 1318-30.

[3] Froud T, Ricordi C, Baidal DA, *et al.* Islet transplantation in type 1 diabetes mellitus using cultured islets and steroid-free immunosuppression: Miami experience. Am J Transplant 2005; 5: 2037-46.

[4] Hering BJ, Kandaswamy R, Ansite JD, *et al.* Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. JAMA 2005; 293: 830–35.

[5] Noguchi H, Iwanaga Y, Okitsu T, Nagata H, Yonekawa Y, Matsumoto S. Evaluation of islet transplantation from non-heart beating donors. Am J Transplant 2006; 6: 2476-82.

[6] Anazawa T, Matsumoto S, Yonekawa Y, *et al.* Prediction of pancreatic tissue densities by an analytical test gradient system before purification maximizes human islet recovery for islet autotransplantation/allotransplantation. Transplantation 2011; 91: 508-14.

[7] Barbaro B, Salehi P, Wang Y, *et al.* Improved human pancreatic islet purification with the refined UIC-UB density gradient. Transplantation 2007; 84: 1200-03.

[8] Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG, Eizirik DL. Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. Endocrinology 1997; 138: 2610-14.

[9] Noguchi H, Matsushita M, Okitsu T, *et al.* A new cell-permeable peptide allows successful allogeneic islet transplantation in mice. Nat Med 2004; 10: 305-09.

[10] Noguchi H, Naziruddin B, Jackson A, *et al.* Comparison of ulinastatin, gabexate mesilate, and nafamostat mesilate in preservation solution for islet isolation. Cell Transplant 2012; 21: 509-16.

[11] Noguchi H, Naziruddin B, Jackson A, *et al.* Fresh islets are more effective for islet transplantation than cultured islets. Cell Transplant 2012; 21: 517-23.

[12] Noguchi H, Ikemoto T, Naziruddin B, *et al.* Iodixanol-controlled density gradient during islet purification improves recovery rate in human islet isolation. Transplantation 2009; 87: 1629-35.

[13] Ichii H, Wang X, Messinger S, *et al.* Improved human islet isolation using nicotinamide. Am J Transplant 2006; 6: 2060-68.

[14] Kin T, Mirbolooki M, Salehi P, *et al.* Islet isolation and transplantation outcomes of pancreas preserved with University of Wisconsin solution versus two-layer method using preoxygenated perfluorocarbon. Transplantation 2006; 82: 1286-90.

[15] Chadwick DR, Robertson GS, Rose S, *et al.* Storage of porcine pancreatic digest prior to islet purification. The benefits of UW solution and the roles of its individual components. Transplantation 1993; 56: 288-93.

[16] Eckhard M, Brandhorst D, Brandhorst H, Brendel MD, Bretzel RG. Optimization in osmolality and range of density of a continuous ficoll-sodium-diatrizoate gradient for isopycnic purification of isolated human islets. Transplant Proc 2004; 36: 2849-54.

[17] Hering BJ. Repurification: rescue rather than routine remedy. Am J Transplant 2005; 5: 1-2.

[18] London NJ, Swift SM, Clayton HA. Isolation, culture and functional evaluation of islets of Langerhans. Diabetes Metab 1998; 24: 200-7.

[19] Salvalaggio PR, Deng S, Ariyan CE, *et al.* Islet filtration: A simple and rapid new purification procedure that avoids ficoll and improves islet mass and function. Transplantation 2002; 74: 877-9.

[20] Scharp DW, Kemp CB, Knight MJ, Ballinge WF, Lacy PE. Use of Ficoll in preparation of viable islets of Langerhans from rat pancreas. Transplantation 1973; 16: 686-9.

[21] Warnock GL, Ellis D, Rajotte RV, Dawidson I, Baekkeskov S, Egebjerg J. Studies of the isolation and viability of human islets of Langerhans. Transplantation 1988; 45: 957-63.

[22] Brandhorst H, Brandhorst D, Brendel MD, Hering BJ, Bretzel RG. Assessment of intracellular insulin content during all steps of human islet isolation procedure. Cell Transplant 1998; 7: 489-95.

[23] Jahr H, Pfeiffer G, Hering BJ, Federlin K, Bretzel RG. Endotoxin-mediated activation of cytokine production in human PBMCs by collagenase and Ficoll. J Mol Med 1999; 77: 118-20.

[24] Hering BJ, Kandaswamy R, Harmon JV, *et al.* Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. Am J Transplant 2004; 4: 390-401.

[25] Mita A, Ricordi C, Messinger S, *et al.* Antiproinflammatory effects of iodixanol (OptiPrep)-based density gradient purification on human islet preparations. Cell Transplant 2010; 19: 1537-46.

[26] Hanley S, Liu S, Lipsett M, *et al.* Tumor necrosis factor-alpha production by human islets leads to postisolation cell death. Transplantation 2006; 82: 813-8.

[27] Piemonti L, Leone BE, Nano R, *et al.* Human pancreatic islets produce and secrete MCP-1/CCL2: Relevance in human islet transplantation. Diabetes 2002; 51: 55-65.

[28] Anderson J, Deeds MC, Armstrong A, Gastineau D, Kudva Y. Utilization of a test gradient enhances islet recovery from deceased donor pancreases. Cytotherapy 2007; 9: 630-6.

[29] Lake SP, Chamberlain J, Walczak K, Bell PR, James RF. A test gradient system for optimizing density gradient isolation of pancreatic islets. Transplantation 1989; 48: 354-7.

[30] Noguchi H, Naziruddin B, Shimoda M, *et al.* Evaluation of osmolality of density gradient for human islet purification. Cell Transplant 2012; 21: 493-500.

[31] Vincent R, Nadeau D. Adjustment of the osmolality of Percoll for the isopycnic separation of cells and cell organelles. Anal Biochem 1984; 141: 322-8.

[32] Chadwick DR, Robertson GS, Rose S, *et al.* Islet purification: optimisation of islet isolation solutions. Transplant Proc 1994; 26: 816-7.

[33] London NJ, Toomey P, Contractor H, Thirdborough ST James RF, Bell PR. The effect of osmolality and glucose concentration on the purity of human islet isolates. Transplant Proc 1992; 24: 1002.

[34] Noguchi H, Ueda M, Hayashi S, *et al.* Ductal injection of preservation solution increases islet yields in islet isolation and improves islet graft function. Cell Transplant 2008; 17: 69-81.

[35] Ichii H, Pileggi A, Molano RD, *et al.* Rescue purification maximizes the use of human islet preparations for transplantation. Am J Transplant 2005; 5: 21-30.

[36] Noguchi H, Naziruddin B, Shimoda M, *et al.* A combined continuous density/osmolality gradient for supplemental purification of human islets. Cell Med 2012; 3: 33-41.

[37] Huang GC, Zhao M, Jones P, *et al.* The development of new density gradient media for purifying human islets and islet-quality assessments. Transplantation 2004; 77: 143-5.

[38] Swift S, Kin T, Mirbolooki M, Wilson R, Lakey JR. Comparison of cooling systems during islet purification. Cell Transplant 2006; 15: 175-80.

[39] Robertson GS, Chadwick D, Contractor H, *et al.* Storage of human pancreatic digest in University of Wisconsin solution significantly improves subsequent islet purification. Br J Surg 1992; 79: 899-902.

[40] Matas AJ, Sutherland DE, Steffes MW, Najarian JS. Short-term culture of adult pancreatic fragments for purification and transplantation of islets of Langerhans. Surgery 1976; 80: 183-91.

[41] Fujioka T, Terasaki PI, Heintz R, *et al.* Rapid purification of islets using magnetic microspheres coated with anti-acinar cell monoclonal antibodies. Transplantation 1990; 49: 404-7.

[42] Töns HA, Baranski AG, Terpstra OT, Bouwman E. Isolation of the islets of Langerhans from the human pancreas with magnetic retraction. Transplant Proc 2008; 40: 413-4.

[43] Soon-Shiong P, Heintz R, Fujioka T, Terasaki P, Merideth N, Lanza RP. Utilization of anti-acinar cell monoclonal antibodies in the purification of rat and canine islets. Horm Metab Res Suppl 1990; 25: 45-50.

[44] Liu C, McGann LE, Gao D, Haag BW, Critser JK. Osmotic separation of pancreatic exocrine cells from crude islet cell preparations. Cell Transplant 1996; 5: 31-9.

[45] Giraldo JA, Weaver JD, Stabler CL. Tissue engineering approaches to enhancing clinical islet transplantation through tissue engineering strategies. J Diabetes Sci Technol 2010; 4: 1238-47.

**Received: November 07, 2013 Revised: December 20, 2013 Accepted: December 27, 2013**

TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

# Improving allogeneic islet transplantation by suppressing Th17 and enhancing Treg with histone deacetylase inhibitors

Koji Sugimoto,[1,6] Takeshi Itoh,[1] Morihito Takita,[1,2] Masayuki Shimoda,[3] Daisuke Chujo,[4] Jeff A. SoRelle,[5] Bashoo Naziruddin,[2] Marlon F. Levy,[1,2] Mitsuo Shimada[6] and Shinichi Matsumoto[1]

1 Baylor Research Institute Fort Worth Campus, Fort Worth, TX, USA
2 Baylor Regional Transplant Institute, Dallas, TX, USA
3 Division of Cardiology, Department of Internal Medicine, Baylor University Medical Center, Baylor Heart and Vascular Institute, Dallas, TX, USA
4 Baylor Institute for Immunology Research, Dallas, TX, USA
5 Institute of Biomedical Studies, Baylor University, Waco, TX, USA
6 The Departments of Surgery, Tokushima University, Tokushima, Japan

**Keywords**
donor-specific transfusion, histone deacetylase inhibitor, islet transplantation, regulatory T cell, T helper 17 cell.

**Correspondence**
Dr Koji Sugimoto, 3-18-15 Kuramoto cho, Tokushima 7708503, Japan.
Tel.: +81 88 633 3179;
fax: +81 88 631 9698;
e-mail: ko2moto23@hotmail.com

**Conflicts of interest**
The authors of this manuscript have no conflict of interests.

Received: 20 May 2013
Revision requested: 1 July 2013
Accepted: 7 January 2014
Published online: 10 February 2014

doi:10.1111/tri.12265

## Summary

Islet transplantation is a new treatment for achieving insulin independence for patients with severe diabetes. However, major drawbacks of this treatment are the long graft survival, the necessity for immunosuppressive drugs, and the efficacy of transplantation. Donor-specific transfusion (DST) has been shown to reduce rejection after organ transplantation, potentially through enhanced regulatory T-cell (Treg) activity. However, recent findings have shown that activated Treg can be converted into Th17 cells. We focused on histone deacetylase inhibitors (HDACi) because it was reported that inhibition of HDAC activity prevented Treg differentiation into IL17-producing cells. We therefore sought to enhance Treg while suppressing Th17 cells using DST with HDACi to prolong graft survival. To stimulate Treg by DST, we used donor splenocytes. In DST with HDACi group, Foxp3 mRNA expression and Treg population increased in the thymus and spleen, whereas Th17 population decreased. qPCR analysis of lymphocyte mRNA indicated that Foxp3, IL-10, and TGF-b expression increased. However, interleukin 17a, Stat3 (Th17), and IFN-g expression decreased in DST + HDACi group, relative to DST alone. Moreover, DST treated with HDACi prolonged graft survival relative to controls in mice islet transplantation. DST with HDACi may therefore have utility in islet transplantation.

## Introduction

### Beneficial effects and unsolved issues of islet transplantation

The Edmonton protocol of 2000 opened a new age of clinical islet transplantation research for the treatment of type 1 diabetes. In their report, seven patients with type 1 diabetes became insulin independent after islet transplantation with glucocorticoid-free immunosuppression [1]. Approximately 80% of study subjects had islet function as indicated by the presence of C-peptide at 5-year follow-up, although only 10% of patients maintained insulin independence [2]. That study demonstrated that while there are several issues to be solved, islet transplantation holds promise as a treatment for severe diabetes.

Islet transplantation still faces several challenges, including the requirement for immunosuppressants to prevent rejection. Immunosuppressants cause side effects and hinder beta cell regeneration, and incomplete immunosuppression can lead to autoimmune recurrence or allorejection [3]. Eliminating the need for immunosuppressants is therefore a major goal for islet transplantation and would significantly improve its efficacy.



 

### Donor-specific blood transfusion for immunological tolerance

Donor-specific blood transfusion (DST) has been shown to reduce rejection after organ transplantation [4]. A possible mechanism of DST is stimulation of regulatory T cells that have potent immunosuppressive effects. It has also been shown that the simultaneous infusion of islets and regulatory T cells reduces the rejection and prolongs islet survival in a mouse model [5].

### Histone deacetylase inhibitors (HDACi) for enhancing Treg

We focused on HDACi for promoting the generation of Treg [6–8]. Histone deacetylases (HDACs), in conjunction with histone acetyltransferases, control the level of acetylation on lysine residues in histones. Treatment of cells with HDACi, such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), leads to hyperacetylation of histones, resulting in a more open chromatin architecture and increased access for transcription factors [9]. HDACi regulates gene expression as well as the functions of more than 50 transcription factors and nonhistone proteins [10]. Treg is a target of HDACi. Studies on Foxp3, a key gene of Treg, reveal that histone H4 is hyperacetylated when the gene is activated [11]. Another study revealed that acetylated Foxp3 is upregulated in $CD4^+$ $CD25^+$ Treg cells [12]. Moreover, TSA induced Treg production from naïve $CD4^+$ CD25-T-cell populations following epigenetic modification [13]. These results suggest that HDACi treatments altered CpG island methylation sites that allow FOXP3 to enter the space between DNA and histone proteins, allowing transcription. Both methylated and acetylated FOXP3 within $CD4^+$ CD25-T cells induced Treg phenotypes *in vitro*. To summarize, HDACi treatment may enhance Treg expression by methylation and acetylation of Foxp3.

### HDACi for anti-Th17 effect

Studies suggest that activated Treg promotes Th17 cell differentiation from CD4 T cells, through production of TGF-b. In addition, transfer of Treg enhanced IL17 production in a mouse model, and enhanced IL17 is associated with systemic autoimmune disease [14]. Therefore, expansion of Th17 cells may disrupt Treg and immunological tolerance.

Importantly, treatment of HDACi may help address this problem. Interestingly, differentiation of Treg into IL17-producing cells depended on HDAC activity, and inhibition of HDAC activity prevented differentiation into IL17-producing cells, yet sustained Foxp3 expression [15]. Based on these data, we hypothesized that HDACi could be critical for increasing Treg growth and preventing Treg from becoming Th17 cells.

## Materials and method

*In vitro* experiments

Balb/c mice were used as donors, and C57BL/6 mice were used as recipients. To stimulate Treg by DST, we used donor splenocytes. Splenocytes ($1.0 \times 10^8$ cells) derived from Balb/c mice were injected into C57BL/6 mice (day 0, i.v.). TSA, a HDACi, was also injected (1.0 mg/kg/day, day 0–6, i.p.). On day 7, thymic and splenic lymphocytes were isolated and analyzed by flow cytometry (CD4, CD25, Foxp3, and IL17a) as *in vitro* experiments. In addition, mRNA expressions in thymic and splenic lymphocytes were analyzed by qPCR (Foxp3, TGF-b, IL-6, IL-10, IL-17a, IL-21, Stat3, and IFN-g) (SABiosciences, Frederick, MD, USA) as *in vitro* experiments.

*In vivo* experiments

Streptozotocin (180 mg/kg, i.v.)-induced diabetic C57BL/6 mice were used as recipients. Donor splenocytes ($1.0 \times 10^8$ cells, day 0, i.v.) and TSA (1.0 mg/kg/day, day 0–6, i.p.) were injected, and on day 7, 400 islets from donor mice were transplanted into the left renal capsule of recipient mice as *in vivo* experiments. Graft survival was observed by checking the blood glucose level three times a week.

### Immunohistochemistry

Frozen sections were made with Cryostat (CM 3050S, Leica, Wetzlar, Germany) from the left renal capsule of DST + TSA recipient mice 60 days after islet transplantation and control recipient mice after graft rejection. Sections were stained with anti-mouse insulin antibody (Abcam, Cambridge, MA, USA).

### Statistics

Statistical significance was determined by one-way ANOVA and Tukey/Kramer *post hoc* test. All statistical analyses were performed using STATVIEW 5.0 (SAS Institute Inc, Cary, NC, USA). Differences were considered significant if $P < 0.05$.

## Results

### DST and HDACi induced Treg expression

In *in vitro* and *in vivo* models, mice were divided into four groups ($n = 5$): (i) control, (ii) DST, (iii) TSA (HDACi), and (iv) DST + TSA. In preliminary data, we observed that Treg did not significantly increase on day 3 after DST. However, Treg significantly increased on day 7 after DST.

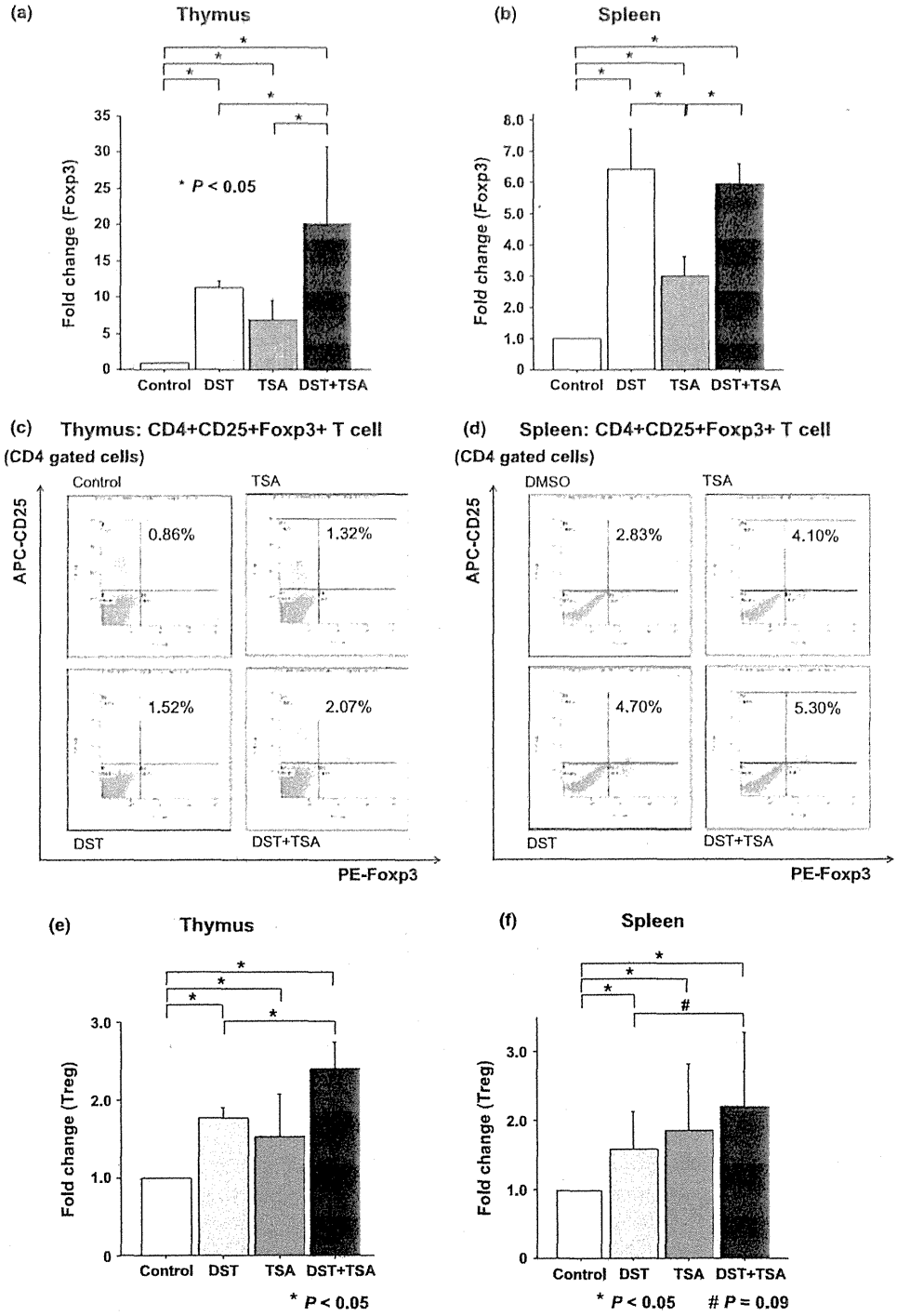


**Figure 1** Donor-specific transfusion (DST) and HDACi induced Treg expression. (a) Foxp3 mRNA expression in thymus (day 7). Foxp3 mRNA expression following DST + TSA treatment is significantly higher than with DST or TSA alone ($P < 0.05$). (b) Foxp3 mRNA expression in spleen (day 7). There is no significant difference between DST and DST + TSA groups. (c) Flow cytometry chart of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in thymus (day 7). The representative data of FACS analysis in thymus were shown. (d) Flow cytometry chart of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in spleen (day 7). The representative data of FACS analysis in spleen were shown. (e) FACS analysis of the fold change in Treg in thymus. The fold change of Treg in DST + TSA was significantly higher than that in the DST group ($P < 0.05$). (f) FACS analysis of the fold change of Treg in spleen. Treg increase observed for DST + TSA tended to be higher than that in the DST group ($P = 0.09$). TSA, trichostatin A.



 

So we analyzed the immune response on day 7. In *in vitro* model, we made the four groups, and on day 7, Foxp3 mRNA expression in thymus was significantly increased in DST, TSA, and DST + TSA, relative to controls. Moreover, Foxp3 mRNA expression in DST + TSA was significantly higher than DST or TSA alone (Fig. 1a). On the other hand, splenic Foxp3 mRNA expression was significantly increased in DST, TSA, and DST + TSA relative to controls. However, there was no significant difference between the DST and DST + TSA groups (Fig. 1b). As determined by FACS analysis, the fold change of Treg in thymus was significantly higher in DST, TSA, and DST + TSA than in controls. Moreover, the Treg increase observed for DST + TSA treatment was significantly higher than that observed in DST-only treatment (Fig. 1e). In spleen, the fold increase in Treg was significantly higher for DST, TSA, and DST + TSA against controls. However, the Treg increase in DST + TSA group tended to be higher than that for DST treatment ($P = 0.09$) (Fig. 1f). The representative data of FACS analysis of lymphocytes in thymus and spleen are shown in Fig. 1c and d.

### HDACi decreased Th17 expression

We next focused on Th17 expression. In *in vitro* model, we used four groups ($n = 5$): (i) control, (ii) DST, (iii) TSA (HDACi), and (iv) DST + TSA. We made the four groups, and on day 7, splenocytes were taken and analyzed for mRNA expression and flow cytometry. IL-17, a major Th17 cytokine, increased (not significantly) mRNA expression when treated with DST. However, IL-17 mRNA expression decreased significantly in DST + TSA group in comparison with DST-alone group (Fig. 2a). FACS analysis indicated that the population of $CD4^+$ $IL17^+$ cells in DST + TSA treatment group was significantly lower than that in DST group (Fig. 2c). The representative data of FACS analysis of $CD4^+$ $IL17^+$ cells in spleen are shown in Fig. 2b.

### DST and HDACi changed mRNA expression associated with Treg and Th17

We measured various mRNAs associated with Treg and Th17 from splenocytes of each of the four groups (Fig. 3). Treg secretes IL-10 and TGF-b, and they suppress the immunological reaction. We measured IL-10 and Tgfb1, mRNA of IL-10 and TGF-b, and the expression of those cytokines in recipient splenocytes was significantly higher in DST + TSA treatment relative to TSA only, or control (Fig. 3a and b). Th17 secretes IL-17 family, and they involve in inducing and mediating proinflammatory responses. We measured IL-17a, a member of IL-17 family, and IL-17a mRNA expression in recipient splenocytes was significantly

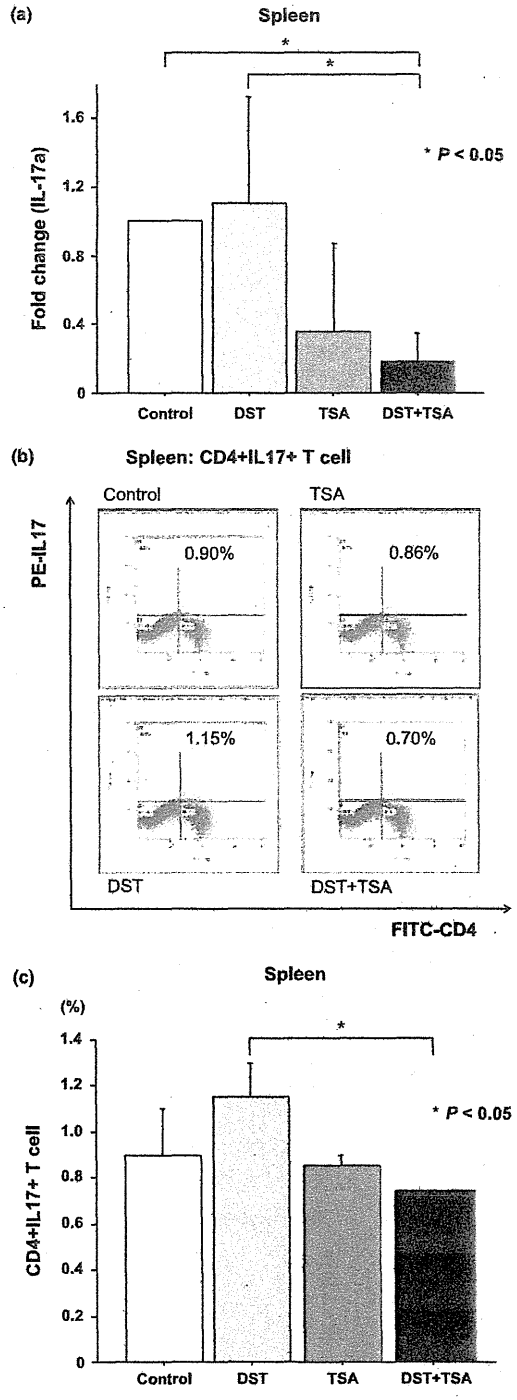


**Figure 2** Histone deacetylase inhibitors (HDACis) decreased Il-17 expression. (a) IL-17 mRNA expression decreased significantly in DST + TSA group ($P < 0.05$). (b) Flow cytometry chart of $CD4^+$ $IL17^+$ T cells in spleen (day 7). The representative data of FACS analysis in spleen were shown. (c) FACS analysis of $CD4^+$ $IL17^+$ cells following DST + TSA treatment IL-17 was significantly lower than that of DST only ($P < 0.05$). DSA, donor-specific transfusion; TSA, trichostatin A.

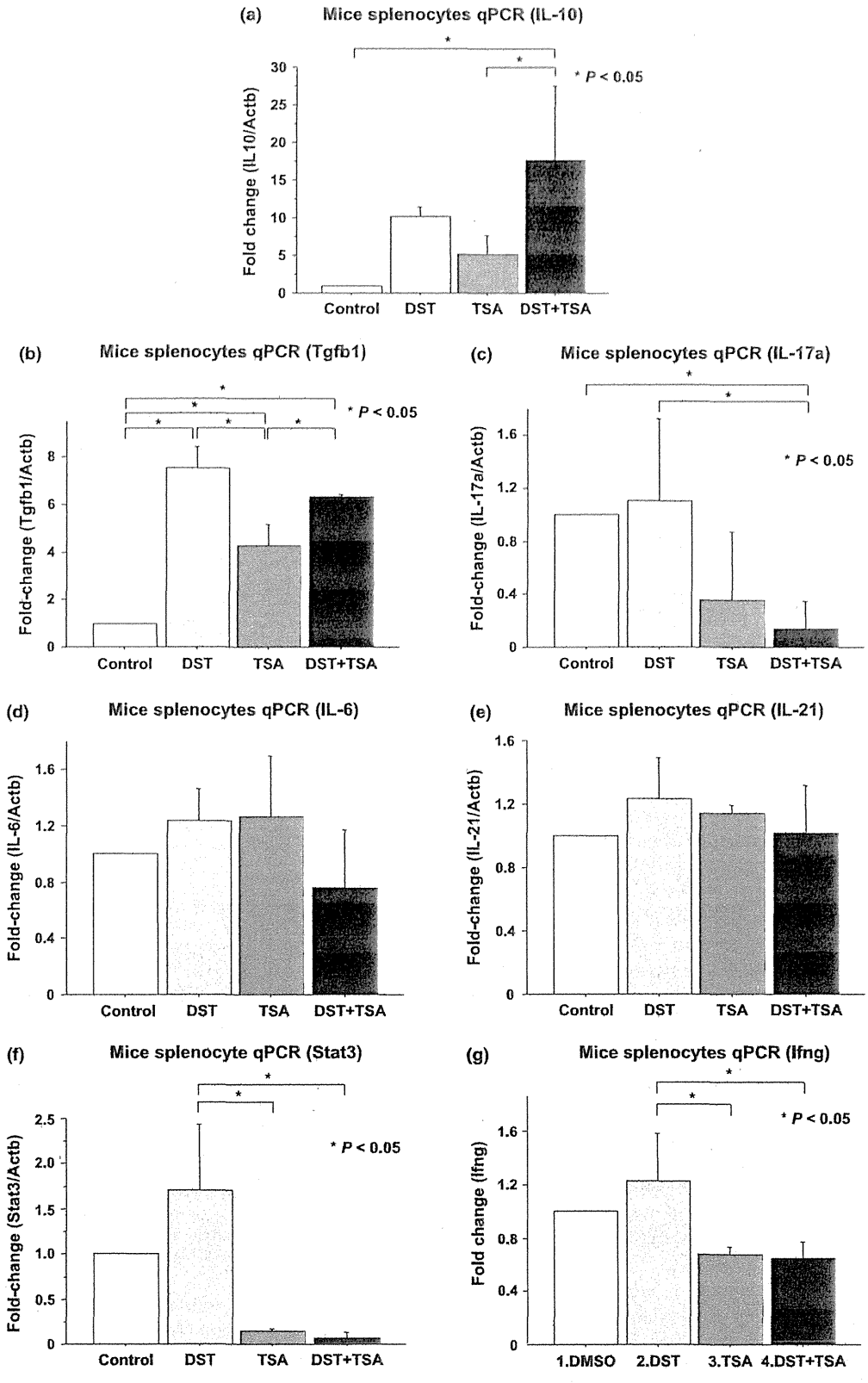
(a)
Mice splenocytes qPCR (IL-10)
Fold change (IL10/Actb)
* P < 0.05
Control DST TSA DST+TSA
(b)
Mice splenocytes qPCR (Tgfb1)
Fold-change (Tgfb1/Actb)
* P < 0.05
Control DST TSA DST+TSA
(c)
Mice splenocytes qPCR (IL-17a)
Fold-change (IL-17a/Actb)
* P < 0.05
Control DST TSA DST+TSA
(d)
Mice splenocytes qPCR (IL-6)
Fold-change (IL-6/Actb)
Control DST TSA DST+TSA
(e)
Mice splenocytes qPCR (IL-21)
Fold-change (IL-21/Actb)
Control DST TSA DST+TSA
(f)
Mice splenocyte qPCR (Stat3)
Fold-change (Stat3/Actb)
* P < 0.05
Control DST TSA DST+TSA
(g)
Mice splenocytes qPCR (Ifng)
Fold change (Ifng)
* P < 0.05
1.DMSO 2.DST 3.TSA 4.DST+TSA

**Figure 3** Donor-specific transfusion (DST) and HDACi changed mRNA expression associated with Treg and Th17. Various mRNAs associated with Treg and Th17 from lymphocytes of the four groups were measured by qPCR. (a) IL-10 mRNA expression in spleen. IL-10 mRNA expression following DST + TSA treatment is significantly higher than following control or TSA alone. (b) Tgfb1 mRNA expression in spleen. Tgfb1 mRNA expressions following DST + TSA treatment or DST only are significantly higher than following control or TSA alone. (c) IL-17 mRNA expression in spleen. IL-17 mRNA expression following DST + TSA treatment is significantly lower than following DST only. (d) IL-6 mRNA expression in spleen. There is no significant difference in IL-6 mRNA expression. (e) IL-21 mRNA expression in spleen. There is no significant difference in IL-21 mRNA expression. (f) Stat3 mRNA expression in spleen. Stat3 mRNA expression following DST + TSA treatment is significantly lower than following DST only. (g) Ifng mRNA expression in spleen. Ifng mRNA expression following DST + TSA treatment is significantly lower than following DST only. TSA, trichostatin A.

decreased in DST + TSA treatment group in comparison with control and DST-alone groups (Fig. 3c). However, IL-6 and IL-21 mRNA expression was not significantly different between groups (Fig. 3d and e). Moreover, we measured Stat3, a major Th17 transcription factor, to evaluate the activity of Th17 cells. Stat3 was expressed significantly higher following DST treatment relative to control. However, Stat3 expression did not differ between DST + TSA and control. In addition to Treg and Th17 system, IFN-g was measured because it was critical for innate and adaptive immunity and produced from natural killer cells, natural killer T cells, CD4 Th1 cells, and CD8 cytotoxic T cells. Ifng mRNA expression in TSA and DST + TSA was significantly lower than that following DST only.

### DST + TSA improved the graft survival in mouse islet transplantation

We examined islet graft survival in a mouse model ($n$ = 5). There was no difference in graft survival between mice in the control, DST, and TSA groups. However, with DST + TSA treatment, graft survival was significantly improved. Moreover, we observed extended survival (over 60 days) in the DST + TSA group (Fig. 4). Insulin staining

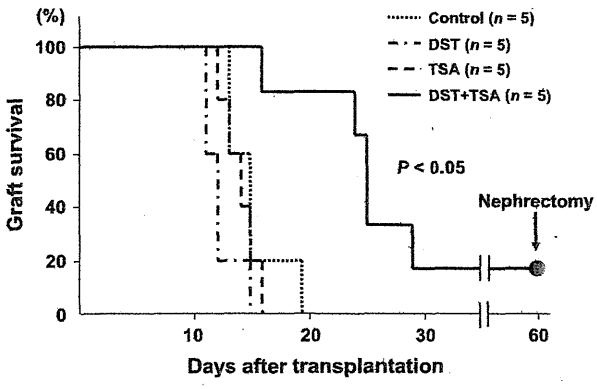


**Figure 4** DST + TSA improved graft survival in a mouse islet transplantation model. A total of 400 islets from donor mice were transplanted into the left renal capsule of recipient mice, and graft survival was observed by checking blood glucose level three times a week. Following DST + TSA treatment, graft survival was significantly improved ($P$ < 0.05). DSA, donor-specific transfusion; TSA, trichostatin A.

indicated that islets secrete insulin in the transplantation site 60 days after transplantation (Fig. 5a and b). On the other hand, in control group there was no islet secreting insulin, and we recognized the fibrillization and inflammation cells under the renal capsule after rejection (Fig. 5c and d). We observed the similar changes in DST or TSA group.

## Discussion

Islet transplantation is a promising treatment for diabetes. However, there are several problems to be solved. These problems include length of graft survival, the reduction of immunosuppressive drugs, and transplantation efficacy. Treg is likely a key regulatory cell type that needs to be managed to solve these problems. Sakaguchi *et al.* originally identified this cell population as a regulator for autoreactive T cells [16]. Treg can strongly regulate other T cells depending on cell-associated molecules such as CTLA-4 and GITR, as well as soluble mediators including IL-10 or TGF-b, and cytotoxic $CD8^+$ T cells. Graft

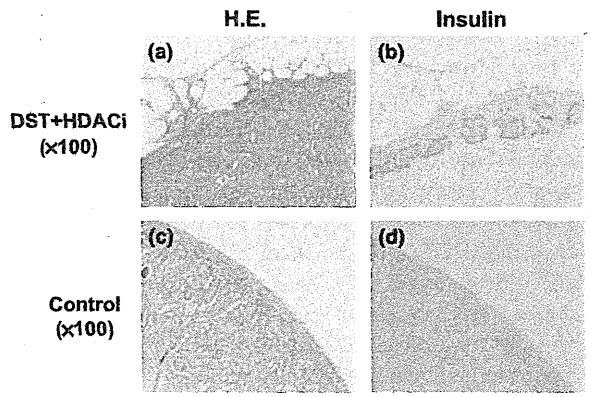


**Figure 5** Insulin staining of transplantation site in a mouse islet transplantation model. (a) H.E. staining (×100). H.E. staining identified islets under the renal capsule of recipient mice. (b) Insulin staining (×100). Insulin staining identified islets that secrete insulin in the transplantation site 60 days following transplantation. (c) H.E. staining (×100). H.E. staining identified no islets under the renal capsule of recipient mice after rejection. We recognize the fibrillization and inflammation cells under the renal capsule after rejection. (d) Insulin staining (×100). Insulin staining identified no islets that secrete insulin in the transplantation site.

rejections are T-cell-mediated immunoreactions, making Tregs a natural target for researchers to consider in controlling graft rejection. Indeed, it is known that Treg increases in patients with immunological tolerance [17,18] and that increasing the number of Tregs in recipients prevents both acute and chronic rejection in several animal models [19]. Treg can be increased in several experimental models, such as multiple blood transplantations [20], blocking of CD40-CD154 or CD80/CD86-CD28 costimulatory interactions [21–23], anti-CD28 antagonist [24,25], and ex vivo Treg expansion [26]. In this study, we used donor-specific transfusion (DST). DST is a classic and empirical method. However, we still sought to evaluate the utility of this approach. It is reported that anergy [27,28], clonal deletion [29,30], regulation of cytokine production [31,32], microchimerism [33,34], generation of soluble MHC antigen [35], or a combination of these mechanisms may mediate DST. However, the specific mechanism of DST is still unknown [36,37].

Our data show that DST increased the Treg population and increased Foxp3, IL-10, and Tgfb1 mRNA expression. In this respect, it may be thought that DST has utility by itself. However, other data suggest that DST is not effective for graft survival in an islet transplantation model [38], indicating that DST has limits.

We focused on Th17 cells because previous studies showed that Treg enhancement also increases Th17 cells via TGF-b and IL-6 induction. Treg differentiates into Th17 cells. A GAD vaccination study showed that GAD vaccination enhanced not only Treg but also Th1 and Th17 cells, which failed to prevent type 1 diabetes. Moreover, in a mouse syngeneic islet transplantation model, blockade of IL-17 resulted in extended graft survival [39]. Therefore, we hypothesized that DST with blockage of anti-inflammatory drugs could improve graft survival.

We therefore used HDACi to inhibit inflammatory cytokines (Th1 and Th17). HDACi was also known to increase Treg and decrease Th17 differentiation by sustaining Foxp3 expression and inhibiting IL-6. Moreover, it has been shown that HDACi blocks IL-23 production and inhibits Th17 differentiation [40]. Our study showed that TSA increased Treg expression and regulatory cytokines (IL-10 and Tgfb1). Moreover, DST + TSA induced expansion of Tregs and IL-10 and significantly decreased Th17 (IL-17a) and Th1 (Ifng), compared with DST only. In the mouse islet transplantation model, DST + TSA improved graft survival, and we observed extended survival (over 60 days after transplantation).

Cytokine analysis indicated that IL-10 and Tgfb1 mRNA were significantly increased by DST + TSA and improving, in principle, Treg function. IL17a and Ifng mRNA expression was decreased by TSA. However, IL-6 and IL-21 expression was not significantly decreased.

In conclusion, HDACi increased Treg expression and inhibited Th17 differentiation, accompanied with Treg induction. These results suggest certain therapeutic strategies that may be useful for improving graft survival.

## Authorship

KS: designed and performed research, analyzed data, wrote paper. TI: performed research, analyzed data. MT: performed research, analyzed data. MS: analyzed data. DC: analyzed data. JASR: analyzed data. BN: analyzed data. MFL: analyzed data. MS: analyzed data. SM: designed research, analyzed data.

## Funding

The authors have declared no funding.

## Acknowledgements

The authors thank Ms. Yoshiko Tamura and Zehra Tekin for technical support. This work was supported in part by the Japan IDDM network and the All Saints Health Foundation.

## References

1. Shapiro AM, Lakey JR, Ryan EA, *et al.* Islet transplantation in seven patients with Type 1 Diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 2000; **343**: 230.
2. Ryan EA, Paty BW, Senior PA, *et al.* Five-year follow-up after clinical islet transplantation. *Diabetes* 2005; **54**: 2060.
3. Morales AE, Thrailkill KM. GAD-alum immunotherapy in Type 1 diabetes mellitus. *Immunotherapy* 2011; **3**: 323.
4. Sengar DP, Rashid A, Jindal SL. Effect of blood transfusions on renal allograft survival. *Transplant Proc* 1979; **11**: 179.
5. Velasquez-Lopera MM, Eaton VL, Lerret NM, *et al.* Induction of transplantation tolerance by allogeneic donor-derived CD4+ CD25+ Foxp3+ regulatory T cells. *Transpl Immunol* 2008; **19**: 127.
6. Tao R, de Zoeten EF, Ozkynak E, *et al.* Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat Med* 2007; **13**: 1299.
7. Wang L, Tao R, Hancock WW. Using histone deacetylase inhibitors to enhance Foxp3+ regulatory T cell function and induce allograft tolerance. *Immunol Cell Biol* 2009; **87**: 195.
8. Tao R, de Zoeten EF, Ozkaynak E, *et al.* Histone deacetylase inhibitors and transplantation. *Curr Opin Immunol* 2007; **19**: 589.
9. Maclaughlin F, La Thangue NB. Histone deacetylase inhibitors open new doors in cancer therapy. *Biochem Pharmacol* 2004; **67**: 1139.
10. Lucas JL, Mirshahpanah P, Stapleton EH, *et al.* Induction of Foxp3+ regulatory T cells with histone deacetylase inhibitors. *Cell Immunol* 2009; **257**: 97.

11. Mantel P-Y, Oakud N, Ruckert B, *et al.* Molecular mechanisms underlying Foxp3 induction in human T cells. *J Immunol* 2006; **176**: 3593.
12. Li B, Samanta A, Song X, *et al.* Foxp3 interactions with histone acetyltransferases and class II histone deacetylases are required for repression. *Proc Natl Acad Sci* 2007; **104**: 4571.
13. Moon C, Kim SH, Park KS, *et al.* Use of epigenetic modification to induce FOXP3 expression in Naïve T cells. *Transplant Proc* 2009; **41**: 1848.
14. Lohr J, Knoechel B, Wang JJ, Villarino AV, Abbas AK. Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. *J Exp Med* 2006; **203**: 2785.
15. Koenen HJPM, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17 producing cells. *Blood* 2008; **112**: 2340.
16. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; **155**: 1151.
17. Li W, Kuhr CS, Zheng XX, *et al.* New insights into mechanisms of spontaneous liver transplant tolerance: the role of Foxp3-expressing $CD25^+CD4^+$ regulatory T cells. *Am J Transplant* 2008; **8**: 1639.
18. Li Y, Zhao X, Cheng D, *et al.* The presence of Foxp3 expressing T cells within grafts of tolerant human liver transplant recipients. *Transplantation* 2008; **86**: 1837.
19. Joffre O, Santolaria T, Calise D, *et al.* Prevention of acute and chronic allograft rejection with $CD4^+CD25^+Foxp3^+$ regulatory T lymphocytes. *Nat Med* 2008; **14**: 88.
20. Bushell A, Karim M, Kingsley CI, Wood KJ. Pretransplant blood transfusion without additional immunotherapy generates CD25+ CD4+ regulatory T cells: a potential explanation for the blood-transfusion effect. *Transplantation* 2003; **76**: 449.
21. Verbinnen B, Billiau AD, Vermeiren J, *et al.* Contribution of regulatory T cells and effector T cell deletion in tolerance induction by costimulation blockade. *J Immunol* 2008; **181**: 1034.
22. Blazar BR, Taylor PA, Linsley PS, Vallera DA. In vivo blockade of CD28/CTLA4: B7/BB1 interaction with CTLA4-Ig reduces lethal murine graft-versus-host disease across the major histocompatibility complex barrier in mice. *Blood* 1994; **83**: 3815.
23. Lin H, Bolling SF, Linsley PS, *et al.* Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. *J Exp Med* 1993; **178**: 1801.
24. Azuma H, Isaka Y, Li X, *et al.* Superagonistic CD28 antibody induces donor-specific tolerance in rat renal allografts. *Am J Transplant* 2008; **8**: 2004.
25. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol* 2002; **2**: 116.
26. Chai JG, Coe D, Chen D, Simpson E, Dyson J, Scott D. In vitro expansion improves in vivo regulation by $CD4^+CD25^+$ regulatory T cells. *J Immunol* 2008; **180**: 858.
27. Margenthaler JA, Kataoka M, Flye MW. Donor-specific antigen transfusion-mediated skin-graft tolerance results from the peripheral deletion of donor-reactive CD8+ T cells. *Transplantation* 2003; **75**: 2119.
28. Lombardi G, Sidhu S, Batchelor R, Lechler R. Anergic T cells as suppressor cells in vitro. *Science* 1994; **264**: 1587.
29. Manilay J, Pearson D, Sergio J, Swenson K, Sykes M. Intrathymic deletion of alloreactive T cells in mixed bone marrow chimeras prepared with a nonmyeloablative conditioning regime. *Transplantation* 1998; **66**: 96.
30. Burlingham WJ, Grailer A, Sondel PM, Sollinger HW. Improved renal allograft survival following donor-specific transfusions. III. Kinetics of mixed lymphocyte culture responses before and after transplantation. *Transplantation* 1988; **45**: 127.
31. Kang HG, Zhang D, Degauque N, Mariat C, Alexopoulos S, Zheng XX. Effects of cyclosporine on transplant tolerance: the role of IL-2. *Am J Transplant* 2007; **7**: 1907.
32. Demirkiran A, Bosma BM, Kok A, *et al.* Allosuppressive donor CD4+ CD25+ regulatory T cells detach from the graft and circulate in recipients after liver transplantation. *J Immunol* 2007; **178**: 6066.
33. Miller DM, Thornley TB, Pearson T, *et al.* TLR agonists prevent the establishment of allogeneic hematopoietic chimerism in mice treated with costimulation blockade. *J Immunol* 2009; **182**: 5547.
34. Pree I, Wekerle T. Inducing mixed chimerism and transplantation tolerance through allogeneic bone marrow transplantation with costimulation blockade. *Methods Mol Biol* 2007; **380**: 391.
35. Nicholls S, Piper KP, Mohammed F, *et al.* Secondary anchor polymorphism in the HA-1 minor histocompatibility antigen critically affects MHC stability and TCR recognition. *Proc Natl Acad Sci USA* 2009; **106**: 3889.
36. Yang L, Du Temple B, Khan Q, Zhang L. Mechanisms of long-term donor-specific allograft survival induced by pretransplant infusion of lymphocytes. *Blood* 1998; **91**: 324.
37. Quezada SA, Fuller B, Jarvinen LZ, *et al.* Mechanisms of donor-specific transfusion tolerance: preemptive induction of clonal T-cell exhaustion via indirect presentation. *Blood* 2003; **102**: 1920.
38. Wojtusciszyn A, Andres A, Morel P, *et al.* Immunomodulation by blockade of the TRANCE co-stimulatory pathway in murine allogeneic islet transplantation. *Transpl Int* 2009; **22**: 931.
39. Emamaullee JA, Davis J, Merani S, *et al.* Inhibition of Th17 cells regulates autoimmune diabetes in NOD mice. *Diabetes* 2009; **58**: 1302.
40. Bosisio D, Vulcano M, Del Prete A, *et al.* Blocking TH17-polarizing cytokines by histone deacetylase inhibitors in vitro and in vivo. *J Leukoc Biol* 2008; **84**: 1540.

# Pancreatic Ductal Perfusion at Organ Procurement Enhances Islet Yield in Human Islet Isolation

*Morihito Takita, MD, PhD,* Takeshi Itoh, MD, PhD,* Masayuki Shimoda, MD, PhD,* Mazhar A. Kanak, MS,† Rauf Shahbazov, MD, PhD,* Faisal Kunnathodi, PhD,* Michael C. Lawrence, PhD,* Bashoo Naziruddin, PhD,‡ and Marlon F. Levy, MD, FACS‡*

**Objective:** Pancreas preservation is a major factor influencing the results of islet cell transplantation. This study evaluated the effects of 2 different solutions for pancreatic ductal perfusion (PDP) at organ procurement.

**Methods:** Eighteen human pancreases were assigned to 3 groups: non-PDP (control), PDP with ET-Kyoto solution, and PDP with cold storage/purification stock solution. Pancreatic islets were isolated according to the modified Ricordi method.

**Results:** No significant differences in donor characteristics, including cold ischemia time, were observed between the 3 groups. All islet isolations in the PDP groups had more than 400,000 islet equivalence in total islet yield after purification, a significant increase when compared with the control ($P = 0.04$ and $P < 0.01$). The islet quality assessments, including an in vivo diabetic nude mice assay and the response of high-mobility group box protein 1 to cytokine stimulation, also showed no significant differences. The proportion of terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive cells showing apoptosis in islets in the PDP groups was significantly lower than in the control group ($P < 0.05$).

**Conclusions:** Both ET-Kyoto solution and cold storage/purification stock solution are suitable for PDP and consistently resulted in isolation success. Further studies with a larger number of pancreas donors should be done to compare the effects of the PDP solutions.

**Key Words:** organ preservation, pancreatic islet isolation, islet potency assay, islet transplantation, apoptosis

(*Pancreas* 2014;00: 00–00)

Pancreatic islet cell transplantation is a promising treatment option, with allogeneic transplants used for patients with brittle type 1 diabetes and autologous transplants after total pancreatectomy used for patients with refractory chronic pancreatitis.[1,2] In 2000, the Edmonton protocol opened a new era of allogeneic islet transplantation, achieving insulin independence in all islet recipients; however, there are still major barriers to its wide use, such as the need for multiple infusions with multiple donors, failed islet isolation, difficulty in maintaining long-term graft function, and the use of strong immunosuppression.[3]

A major element in improving islet isolation outcomes and, in turn, clinical results is pancreas preservation.[4] To that end, pancreatic ductal perfusion (PDP) at organ procurement was originally examined and shown to be effective in rodent models, where it was called *prestorage ductal flush* and *intraductal distension* and involved the use of collagenase-containing Hanks solution or University of Wisconsin solution (UWS).[5,6] These techniques allowed sufficient distribution of the collagenase solution in the entire pancreas, preserving pancreatic ducts and inhibiting cold ischemia injury in ductal epithelium.[7] Sawada et al[7] showed that ductal perfusion using UWS without collagenase could significantly improve islet yield and quality. The UWS can prevent hypothermia-induced cell swelling during cold ischemia time.[8] Contradictory effects have also been reported, such as the fact that the UWS inhibited collagenase activity and has high viscosity, possibly resulting in poor isolation outcomes.[9,10] Another disadvantage in the use of UWS for PDP is the β cell exhaustion caused by its high potassium level.[11] Thus, extracellular fluid-like solution with a low potassium level should be considered for PDP.

The ET-Kyoto solution (ETKS; Otsuka Pharmaceutical Factory Inc, Naruto, Japan) was originally developed as an organ-preservation solution for lung transplantation and has an extracellular fluid-like electrolyte composition with sodium and potassium levels of approximately 100 and 44 mmol/L, respectively (Supplemental Digital Content Table S1, http://links.lww.com/MPA/A320).[12,13] The potassium concentration in ETKS was designed at a lower level than UWS but higher than extracellular fluid because 40 mmol/L of potassium had a benefit in keeping vascular resistance lower in preclinical lung transplantation model when compared with specially prepared ETKS with much lower potassium level (20 mmol/L) and Euro-Collins solution with higher potassium (115 mmol/L).[14,15] The ETKS includes unique ingredients of trehalose and gluconate, which help stabilize the cell membrane and prevent cell swelling.[16,17] The ETKS showed less inhibition of collagenase activity than UWS did but had comparable benefits in islet isolation.[18] Recently, PDP with ETKS coupled with the 2-layer method was shown to contribute to highly successful islet isolation.[19,20]

The cold storage/purification stock solution (CSPS) (Mediatech, Inc, Manassas, Va) has a sodium-potassium composition similar to that of extracellular fluid (Supplemental Digital Content Table S1, http://links.lww.com/MPA/A320) and contains histidine, allowing a robust buffering capacity.[21] Histidine-lactobionate–based preservation solution has been reported to improve the viability of purified islets up to 48 hours.[22]

Both ETKS and CSPS have an electrolyte composition of higher sodium and lower potassium levels compared with UWS, which should be beneficial for PDP, although each solution has its own unique ingredients to improve pancreas or islet preservation. No reports have directly compared ETKS and CSPS solution for

From the *Islet Cell Laboratory, Baylor Research Institute, Dallas, †Institute for Biomedical Studies, Baylor University, Waco, and ‡Baylor Annette C. and Harold C. Simmons Transplant Institute, Baylor University Medical Center, Dallas, TX.

Received for publication November 8, 2013; accepted June 19, 2014.

Reprints: Marlon F. Levy, MD, FACS, Baylor Annette C. and Harold C. Simmons Transplant Institute, 1400 8th Ave, Fort Worth, TX 76104 (e-mail: Marlon.Levy@BaylorHealth.edu); Bashoo Naziruddin, PhD, Baylor Annette C. and Harold C. Simmons Transplant Institute, 3410 Worth St, Suite 950, Dallas, TX 75246 (e-mail: BashooN@baylorhealth.edu).

This study was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (1R21DK090513-019 to M.F.L.) and the Juvenile Diabetes Research Foundation (no. 3-2011-447 to M.T.). The ET-Kyoto solution was provided as a gift by Otsuka Pharmaceutical Factory, Inc (Naruto, Tokushima, Japan) under a previous collaborative agreement.

The authors declare no conflict of interest.

Authors Takita and Itoh equally contributed to this article.

Supplemental digital contents are available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.pancreasjournal.com).

PDP. Hence, we designed a prospective study to investigate the impact of the 2 PDP solutions on islet isolation outcomes, comparing with control group without ductal perfusion at organ procurement. Total islet yield after purification and other islet quality parameters were used for the primary and secondary end points. We also evaluated apoptosis in islets immediately after cold ischemia time as an ancillary study to elucidate the influence of PDP solutions on islets before the isolation.

## MATERIALS AND METHODS

### Study Design and Donor Criteria

This study was designed as a prospective trial, and the donor criteria listed in Supplemental Digital Content Table S2, http://links.lww.com/MPA/A320 were defined before the study initiation according to the international trial of the Edmonton protocol.[23] The recovered pancreas was assigned to 1 of 3 groups, which are no PDP (control), PDP with ETKS, or PDP with CSPS, with the goal of avoiding significant differences in donor characteristics among the groups, particularly for age and body mass index.

A power analysis was completed to determine the appropriate number of donors for this study (Supplemental Digital Content Fig. S1, http://links.lww.com/MPA/A320). On the basis of our previous observation,[19] we expected a difference between the control group and the PDP groups of $2.5 \pm 1.4 \times 10^5$ IEQ (mean ± SD) for the primary end point. The analysis revealed that a minimum of 6 donors per group should be evaluated to detect the anticipated difference with a statistical power of $\beta = 0.80$ and $\alpha = 0.05$.

The primary end point was total islet yield after purification, represented by a standard number of islet equivalents (IEQ).[24] The secondary end points included islet yield per pancreas weight postdigestion and postpurification (IEQ/g) and islet quality evaluated by viability, stimulation index of the glucose-stimulated insulin release assay, and in vivo nude mice assay. As an ancillary study to explain the effect of PDP, we evaluated apoptosis by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay in the pancreas immediately after cold preservation and measured high-mobility group box protein 1 (HMGB1) level with 10 islet cultures in each group to assess islet damage after the isolation.[23,25,26]

### Human Pancreas Procurement

Research-grade human pancreases from brain-dead donors were provided through the local organ procurement organizations (Southwest Transplant Alliance, Dallas, Tex, and LifeGift, Houston, Tex). The pancreases were removed en bloc with a standard procedure after vascular perfusion with UWS or histidine-tryptophan-ketoglutarate solution. Immediately after the pancreas procurement, PDP was performed, wherein an 18- or 20-G cannula was inserted into the main pancreatic duct from the pancreatic head after removing the spleen and duodenum.[19] Approximately 1 mL/g of either ETKS or CSPS was then injected intraductally.[25] After completion of PDP, the pancreas was preserved with the 2-layer method using the corresponding preservation solutions of ETKS or CSPS and perfluorocarbon.[26,27] No PDP was performed for the control group, but the recovered pancreas was preserved with the 2-layer method with CSPS and perfluorocarbon ($n = 6$).

### TUNEL Assay with Pancreas Tissue Before Islet Isolation

The TUNEL assay was performed with an ApopTag fluorescein in situ apoptosis detection kit (Millipore, Billerica, Mass).[28] Pancreas tissue was taken from the pancreas body and prepared for paraffin sectioning. Digoxigenin-labeled nucleotides were added to DNA fragments by terminal deoxynucleotidyl transferase, and then fluorescein-labeled antidigoxigenin antibodies were bound to the digoxigenin. Apoptotic cells, those that appeared fluorescently green in the islet area stained by anti-insulin antibody (Sigma-Aldrich, St Louis, Mo), were manually counted. Similarly, manual counts were made of nuclei stained by the blue signal of 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Sigma-Aldrich, St Louis, Mo). The proportion of TUNEL-positive cells in an islet or acinar cell area was evaluated.

### Pancreatic Islet Isolation

Islet processing was performed on the basis of the modified Ricordi method in accordance with current good manufacturing practice at Baylor Research Institute's Islet Cell Laboratory (Dallas, TX) as previously described.[29,30] Briefly, after decontaminating the pancreas surface with 1% povidone-iodine and 1 g of cephalosporin, the chilled collagenase enzyme solution was perfused into the pancreatic duct for 10 minutes. Then, the distended pancreas was cut into approximately 10 pieces. The pancreas pieces were put in the Ricordi chamber and digested by circulating the collagenase solution at 37°C. After dilution and recombination of digested tissue, the islets were purified with continuous density gradient using iodixanol, followed by adjustment of islet density.[30] The proportion of undigested tissue weight was calculated by undigested tissue weight divided by trimmed pancreas weight. The recovery rate in the final preparation was calculated by the total islet yield (IEQ) after purification divided by that after digestion.

### In Vitro Islet Evaluations

Viability was evaluated with fluorescein diacetate (10 μM)/propidium iodide (15 μM) staining.[31] The average viability in 50 islets was calculated. Islet yield was assessed using dithizone staining (Sigma Chemical Co, St Louis, Mo) (2 mg/mL) and converted to a standard number of IEQ (diameter standardizing to 150 μm).[24]

The glucose-stimulated insulin release assay was performed as follows. After overnight culture, triplicates of 150 IEQ islets were incubated with low (1.67 mM) and high (16.7 mM) concentrations of glucose in functionality/viability medium CMRL1066 (Mediatech Inc, Manassas, Va) for 1 hour at 37°C. Insulin concentrations were measured with an enzyme-linked immunosorbent assay kit (ALPCO Diagnostics, Salem, NH) and a spectrophotometer (BioTek Instruments, Inc, Winooski, Vt). The stimulation index was calculated by dividing the insulin concentration in the high-glucose solution by that in the low-glucose solution on the basis of 3 independent measurements.

### Islet Culture with Cytokines and HMGB1 Measurement

Isolated human islets were cultured in CMRL1066 (Mediatech, Inc, Manassas, Va) at 37°C in 95% air and 5% $CO_2$ for 24 hours after islet isolation. The islets were washed twice with culture medium after initial culture and then cultured again under the same conditions without adding cytokines for 48 hours (nonstimulation group). In the stimulation group, the islets were cultured with a cytokine cocktail consisting of 20 ng/mL of recombinant human interferon γ, tumor necrosis factor α, and interleukin 1β (Sigma-Aldrich Co, St Louis, Mo) for 48 hours after medium exchange.[32,33] The amount of HMGB1 in the medium after culturing for 48 hours was measured with the HMGB1 enzyme-linked immunosorbent assay kit (IBL International GmbH, Hamburg, Germany). The amounts of HMGB1 in media

TABLE 1. Donor Characteristics

| Variables | Control (n = 6) | PDP | | P |
|---|---|---|---|---|
| | | ETKS (n = 6) | CSPS (n = 6) | |
| Age, y | 51 [20–68] | 42 [26–54] | 50 [27–51] | 0.62 |
| Sex: female, n (%) | 3 (50) | 4 (67) | 3 (50) | 0.80 |
| Body weight, kg | 93.8 [63.0–115.6] | 81.1 [72.5–99.8] | 97.9 [72.7–125.7] | 0.52 |
| Body mass index, kg/m$^2$ | 30.5 [20.5–44.5] | 29.3 [25.0–36.6] | 32.8 [24.4–38.1] | 0.83 |
| Body surface area, m$^2$ | 1.98 [1.77–2.37] | 1.93 [1.76–2.10] | 2.13 [1.86–2.45] | 0.20 |
| Cause of death, n (%) | | | | 0.70 |
| Cerebrovascular stroke | 3 (50) | 3 (50) | 4 (67) | |
| Head trauma | 3 (50) | 2 (33) | 1 (17) | |
| Other | 0 (0) | 1 (17) | 1 (17) | |
| Mechanism of death, n (%) | | | | 0.53 |
| Intracranial hemorrhage or stroke | 3 (50) | 4 (67) | 4 (67) | |
| Trauma without abdominal injury | 3 (50) | 2 (33) | 1 (17) | |
| Cardiovascular | 0 (0) | 0 (0) | 1 (17) | |
| Length of hospitalization, d | 4 [3–9] | 3 [2–10] | 3 [1–9] | 0.44 |
| Cold ischemia time, min | 282 [153–390] | 196 [159–394] | 167 [82–220] | 0.17 |

Median [range] or numbers (percentage) are shown.
Numerical and categorical values were evaluated with the Kruskal-Wallis test for independent samples and the Pearson $\chi^2$ square test, respectively.

were normalized to the total DNA of cultured islets (dsDNA Assay Kit; Molecular Probes, Inc, Eugene, Ore).

## In Vivo Assessment

Nude male mice (Harlan, Houston, Tex) were used as the recipients. A single dose (180 mg/dL) of streptozotocin (Sigma-Aldrich Co, St Louis, Mo) was administered intravenously on day 2, and hyperglycemia greater than 300 mg/dL was confirmed twice in each mouse before transplantation. An islet mass of 2500 IEQ as curable dose was injected into the kidney capsule.[34] Nonfasting blood glucose levels were measured using Accu-Chek Aviva (Roche Diagnostics, Indianapolis, Ind) 3 times a week in all the recipients for 30 days after islet transplantation. *Normoglycemia* was defined as 2 consecutive blood glucose levels reading less than 200 mg/dL.[32,33] This study was approved by the institutional animal care and use committee at Baylor Research Institute (Dallas, Tex).

## Statistical Assessments

Statistical evaluations were performed with GraphPad Prism version 6.03 (GraphPad Software, Inc, San Diego, Calif). Numerical and categorical values between the 3 groups were evaluated with the Kruskal-Wallis test, followed by post hoc Dunn method for independent samples and the Pearson $\chi^2$ square test, respectively. Numerical and categorical data were expressed as median [range] and number (percentage), respectively. Survival curves of curative rate were evaluated with the Mantel-Cox log-rank test. Results were considered statistically significant when a 2-sided $P$ value was less than 0.05.

# RESULTS

## Donor Characteristics

Eighteen human islet isolations, 6 in each study group, were performed for this study between October 2007 and May 2013. There were no significant differences in donor characteristics among the 3 groups (Table 1), and none of the donors had any warm ischemia time.

## Primary Outcome: Total Islet Yield

The total islet yield postpurification was 304 [168–554] × $10^3$ IEQ in the control group, 674 [481–975] ×$10^3$ IEQ in the ETKS group, and 742 [624–1057] ×$10^3$ IEQ in the CSPS group (Fig. 1). Statistically significant differences were found between

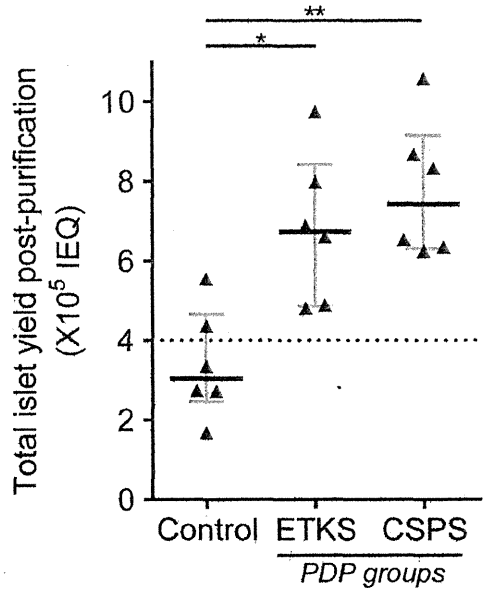


FIGURE 1. Effect of PDP using ETKS or CSPS compared with a control group for the study's primary outcome: islet yield after purification. Dot plots with median (bold bars) and interquartile range (gray bars) are shown. Significant differences were observed between the control and the ETKS and CSPS groups (*$P < 0.05$ and **$P < 0.01$). The dotted line shows 400,000 IEQ; both the ETKS and CSPS groups resulted in greater than 400,000 IEQ of final islet yield.

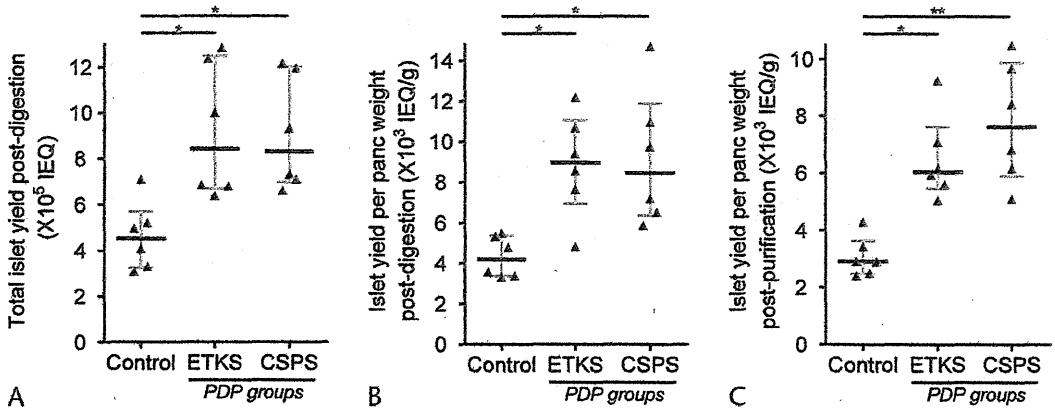


**FIGURE 2.** Effect of PDP using ETKS or CSPS compared with a control group for the study's secondary outcomes: total islet yield postdigestion (A), islet yield per trimmed pancreas weight postdigestion (B), and islet yield per trimmed pancreas weight postpurification (C). Dot plots with median (bold bars) and interquartile range (gray bars) are shown. *$P < 0.05$ and **$P < 0.01$.

the control and both the ETKS and CSPS groups ($P = 0.04$ and $P < 0.01$) but not between the ETKS and CSPS groups ($P = 1.0$). All islet isolations in the PDP groups had more than 400,000 IEQ.

## Secondary Outcomes for Islet Isolation

Significant differences between the control and PDP groups were found for the secondary outcomes for the isolated islet mass: total islet yield postdigestion, islet yield per trimmed pancreas weight postdigestion, and islet yield per trimmed pancreas weight postpurification (Fig. 2). No significant differences, however, were detected between the ETKS and CSPS groups.

The trimmed pancreas weight was not significantly different between the 3 groups (Table 2), but significant differences were seen in digestion time, undigested tissue weight, and the proportion of undigested tissue ($P = 0.03$, $P = 0.01$, and $P = 0.006$, respectively).

## Islet Quality Assessment

No significant differences were observed in the islet purity assessment and stimulation index between the 3 groups, but there was a marginally significant difference in viability between the control and CSPS groups (Table 2). As shown in Figure 3, there was no significant difference in the groups' curative rate on the basis of an in vivo nude mice assay using human islet preparations

**TABLE 2.** Islet Isolation Results

| Variables | Control (n = 6) | PDP | | P |
|---|---|---|---|---|
| | | ETKS (n = 6) | CSPS (n = 6) | |
| Pancreas digestion | | | | |
| Trimmed pancreas weight, g | 111 [58–150] | 111 [68–142] | 100 [83–125] | 0.75 |
| Digestion time, min | 19 [14–24] | 14 [12–16] | 14 [9–16] | 0.03* |
| Dilution time, min | 50 [34–58] | 50 [26–78] | 49 [28–55] | 0.83 |
| Undigested tissue weight, g | 29 [9–44] | 5 [1–11] | 9 [2–28] | 0.01* |
| Proportion of undigested tissue in trimmed pancreas weight, % | 24 [9–39] | 6 [1–8] | 9 [2–27] | 0.006* |
| Tissue volume postdigestion, mL | 38 [20–50] | 43 [35–60] | 35 [24–45] | 0.22 |
| Total islet yield postdigestion, $\times 10^3$ IEQ | 451 [308–709] | 841 [638–1284] | 830 [661–1215] | 0.01 |
| Islet yield per pancreas weight, $\times 10^3$ IEQ/g | 4.19 [3.32–5.50] | 8.97 [4.81–12.2] | 8.45 [5.87–14.7] | 0.006 |
| Final preparation | | | | |
| Islet yield per pancreas weight, $\times 10^3$ IEQ/g | 2.90 [2.40–4.29] | 6.04 [5.04–9.24] | 7.61 [5.09–10.5] | 0.002 |
| Tissue volume, mL | 15 [3–27] | 10 [2–12] | 10 [5–21] | 0.80 |
| Recovery rate, % | 73 [52–100] | 74 [56–100] | 91 [70–100] | 0.44 |
| Embedded islets, % | 20 [8–90] | 20 [0–33] | 20 [7–51] | 0.97 |
| Average purity, % | 58 [33–74] | 69 [46–75] | 64 [52–78] | 0.50 |
| Viability, % | 98 [95–100] | 96 [94–98] | 95 [93–96] | 0.05* |
| Stimulation index | 2.1 [1.2–6.9] | 8.0 [1.0–22.0] | 5.4 [1.8–13.2] | 0.12 |

Medians [ranges] are shown.

The Kruskal-Wallis test was performed for statistical evaluation.

*Results on the pairwise comparison are shown in Supplemental Digital Content Fig. S2, http://links.lww.com/MPA/A320.

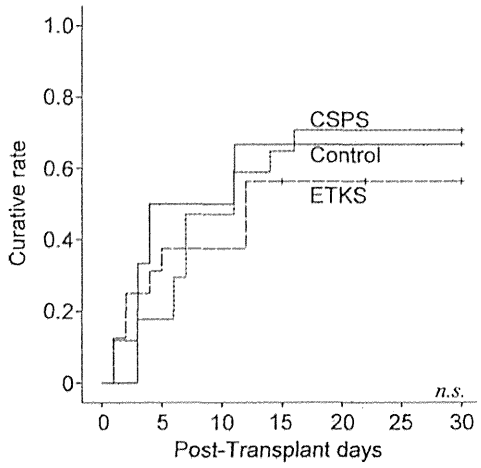


**FIGURE 3.** Effect of PDP in an in vivo nude mice assay. The curative rates for the control group (solid line, n = 6), ETKS PDP group (long dotted line, n = 16), and CSPS PDP group (short dotted line, n = 17) are shown. No significant difference in the curative rate was observed in the 3 groups ($P = 0.57$).

($P$ with the Mantel-Cox log-rank test = 0.57). Furthermore, HMGB1 levels on the static and cytokine-stimulated conditions and the fold changes did not show any statistically significant differences between the 3 groups (Fig. 4). The proportion of TUNEL-positive cells in an islet was less than 10% in both PDP groups, but significant differences were seen between the control as well as the ETKS and CSPS groups ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 5). Similar results were observed in the acinar cell area; the proportions of TUNEL-positive cells in PDP groups were significantly lower than those in the control ($P < 0.05$: Fig. 5B).

## DISCUSSION

Preservation of the pancreatic duct is essential in human islet cell transplantation because wide distribution of collagenase solution in the pancreatic duct is a critical procedure in pancreas digestion.[35] We examined 2 different solutions of ETKS and CSPS with higher sodium and lower potassium levels, compared with UWS, for PDP and found no significant differences in islet yield or islet quality between the 2 groups but a significant increase in islet yield compared with the control with no intraductal pancreatic duct preservation solution delivery. The final islet preparations in all isolations in the PDP groups had greater than $4.0 \times 10^5$ IEQ, which is a higher yield than the isolation success defined in previous reports.[36,37] Thus, both ETKS and CSPS can be used for PDP in pancreas preservation and are likely to result in isolation success. Islet investigators can implement PDP using either ETKS or CSPS on the basis of what is more accessible to them; CSPS has been used for islet purification in a multicenter trial on allogeneic islet transplantation in the United States and Europe, and ETKS has been applied in Japan.[18,38,39] In the United States, ETKS is not commercially available, whereas CSPS is extensively used in major islet isolation centers. Hence, on the basis of results from this study, CSPS can be a solution of choice for PDP.

No statistically significant differences between the ETKS and CSPS groups were found in the variables related to islet isolation and ancillary studies as well. The stimulated HMGB1 levels, the fold change, and the proportion of TUNEL-positive cells were slightly lower in the CSPS than in the ETKS group, supporting feasibility of PDP with CSPS. The differences between the 2 solutions include higher level of potassium, containing gluconate and trehalose and higher osmolality in ETKS versus that containing lactobionate, raffinose, and histidine in CSPS (Supplemental Digital Content Table S1, http://links.lww.com/MPA/A320). Because the 2 solutions have been developed and investigated with different background, ETKS was originally designed for lung preservation and CSPS was for islet preservation after pancreas digestion, it is very difficult to mention the effect of individual component in PDP and islet isolation outcomes.

The effect of PDP in the outcome of pancreatic islet isolation is still controversial. Nakanishi et al[40] showed that PDP was effective in a rodent model of islet isolation but observed no additive effect when vascular perfusion was simultaneously performed. In present study, we obtained islet yields with PDP similar to those of our previous report, showing significantly higher islet mass of approximately $6.0 \times 10^5$ IEQ when compared with the control.[19]

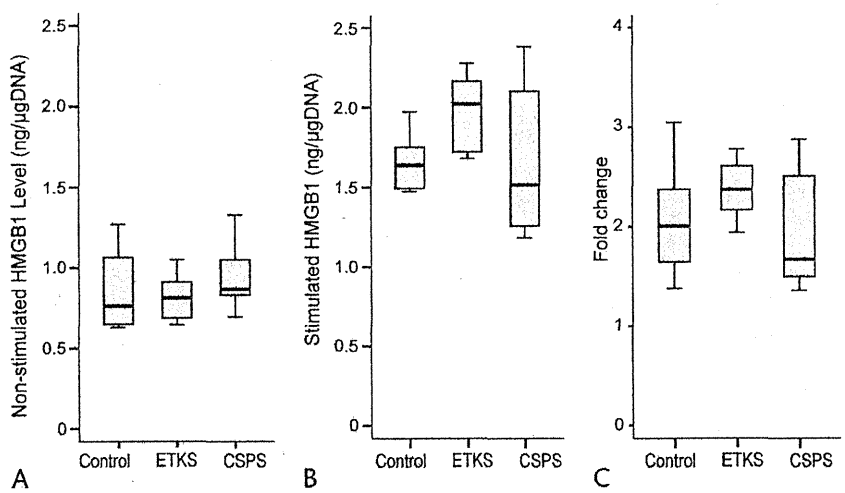


**FIGURE 4.** Effect of PDP in an HMGB1 assay assessing islet damage after isolation. No significant differences were seen in the HMGB1 levels between the control group, ETKS PDP group, and CSPS PDP group in a static culture (no stimulation) ($P = 0.31$) (A); a culture with cytokine stimulation ($P = 0.15$) (B); or fold change ($P = 0.32$) (C). The bar, box, and bold line indicate range, interquartile range, and median, respectively.

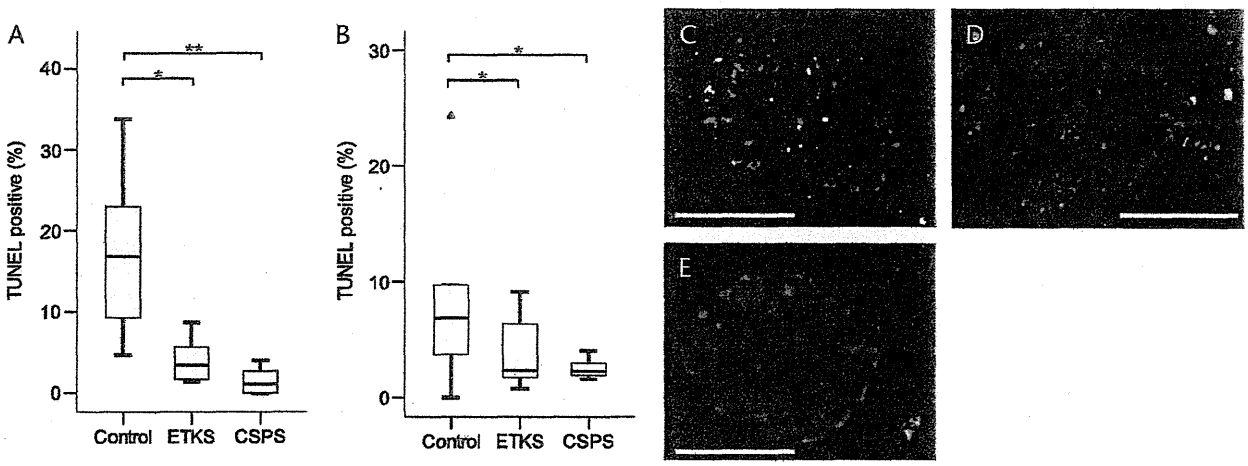


**FIGURE 5.** Effect of PDP evaluated through a TUNEL assay to count the number of apoptotic cells. A, The proportion of TUNEL-positive cells in the islet area was significantly lower in the PDP groups than in the control group. B, Similarly, those in the nonislet area were significantly lower in the PDP groups than in the control group. Representative fluorescent stains of insulin (red), nuclei (blue), and TUNEL (green) are shown for the ETKS PDP group (C), the CSPS PDP group (D), and the control group (E). $^{*}P < 0.05$, $^{**}P < 0.01$. **Editor's note:** A color image accompanies the online version of this article.

We believe that additional benefits of PDP are possible in human islet isolation because vascular perfusion with UWS or histidine-tryptophan-ketoglutarate solution was performed in both control and PDP groups and we consistently obtained higher islet yield with PDP in this study. Previously, it was shown that PDP significantly improved the viability of pancreatic duct cells as well as acinar and islet cells, using morphological evaluation with Trypan blue and TUNEL assay, along with increased islet yield.[7,18] Pancreatic ductal preservation was able to inhibit trypsin activity during pancreas digestion.[18] In addition, we reported improved distribution of collagenase enzyme in human pancreas preserved with PDP, where the collagenase was successfully detected into islet surface area without islet damage.[41] Therefore, PDP can contribute to maintaining higher viability of duct cells as well as acinar and islet cells but also to keeping pancreas microstructure intact, resulting in significantly higher islet yield. Clinically applicable test(s) such as biomarkers in pancreatic duct fluid or biopsy using very tiny tissue before islet isolation would provide more reliable information on the effect of PDP, and this is an important area for future study. Of note, pancreas preservation in all 3 groups was performed with a 2-layer method in the present study; thus, all groups should have benefits of the 2-layer method in islet isolation outcomes as well as prevention of cell swelling and apoptosis during pancreas preservation.[42,43]

Several limitations should be noted in this study. The minimum number of pancreas donors was calculated by power analysis using our previous observation.[19] A larger number of donors ($n \geq 52$ per group for statistical $\beta = 0.8$ and $\alpha = 0.05$ as well as the 2-tailed test), however, would be required if the anticipated difference in total islet yield postpurification between the 2 PDP groups is $1.0 \pm 1.8 \times 10^5$ IEQ, as shown here. Thus, the power in this study was not sufficient to detect smaller differences and further studies with larger cohorts are needed. Also, randomization with categorized donor information should be planned to obtain robust results. This study did not aim to investigate islet isolation using pancreases with a prolonged cold ischemia time. Specific clinical biomarker to indicate viability of pancreatic duct cells should be developed and validated in a large study, which would provide a link between PDP and islet isolation outcomes because it was demonstrated that HMGB1 was specifically expressed in pancreatic islets, not duct cells.[28]

In conclusion, no significant differences in islet isolation outcomes were observed between the extracellular fluid-like PDP solutions, whereas we consistently achieved isolation success when PDP was implemented. Both ETKS and CSPS can be safely used for PDP. Further study with a larger number of pancreas donors and well-planned randomization should be undertaken to compare the effect of PDP solutions.

## ACKNOWLEDGMENTS

*The authors thank Ana M. Rahman, Nofit Borenstein, and Yoshiko Tamura (Baylor University Medical Center, Dallas, Tex) for their technical support as well as Cynthia Orticio for professional editing.*

## REFERENCES

1. Fiorina P, Shapiro AM, Ricordi C, et al. The clinical impact of islet transplantation. *Am J Transplant.* 2008;8:1990–1997.
2. Bramis K, Gordon-Weeks AN, Friend PJ, et al. Systematic review of total pancreatectomy and islet autotransplantation for chronic pancreatitis. *Br J Surg.* 2012;99:761–766.
3. Mineo D, Pileggi A, Alejandro R, et al. Point: steady progress and current challenges in clinical islet transplantation. *Diabetes Care.* 2009;32: 1563–1569.
4. Iwanaga Y, Sutherland DE, Harmon JV, et al. Pancreas preservation for pancreas and islet transplantation. *Curr Opin Organ Transplant.* 2008;13: 135–141.
5. van der Vliet JA, Meloche RM, Field MJ, et al. Pancreatic islet isolation in rats with ductal collagenase distention, stationary digestion, and dextran separation. *Transplantation.* 1988;45:493–495.
6. Field MJ, Sutherland DE, Munn SR. Experimental pancreatic preservation prior to islet isolation and transplantation. *J Surg Res.* 1989;46:474–478.
7. Sawada T, Matsumoto I, Nakano M, et al. Improved islet yield and function with ductal injection of University of Wisconsin solution before pancreas preservation. *Transplantation.* 2003;75:1965–1969.
8. Southard JH, van Gulik TM, Ametani MS, et al. Important components of the UW solution. *Transplantation.* 1990;49:251–257.
9. Robertson GS, Chadwick D, Thirdborough S, et al. Human islet isolation—a prospective randomized comparison of pancreatic vascular

 