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Comparison of Tissue Loading Before and After the Creation of a Continuous Density Gradient in Porcine Islet Purification

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Abstract

The purification step is one of the most important and difficult procedures in islet isolation for pancreatic islet transplantation. We previously reported that a purification method using large plastic bottles effectively achieved a high yield of islets from the porcine pancreas. In this study, we evaluated the impact of the timing of tissue loading on porcine islet purification using large plastic bottles. One method involved loading digested tissue after creating a continuous density gradient (tissue after gradient [TAG]). The other method involved loading digested tissue before creating a continuous density gradient (tissue before gradient [TBG]). There were no significant differences between TAG and TBG in terms of the islet yield, rates of viability and purity, score, and in the stimulation index after purification. Furthermore, there were no marked differences in the attainability or suitability of post-transplantation normoglycemia. Our study shows the equivalency of these two methods of islet purification.

Keywords

bottle purification, islet transplantation, islet isolation, islet purification, tissue loading

Introduction

Remarkable progress has been achieved in both the technical aspects of islet cell processing and the outcomes of clinical pancreatic islet transplantation[1–5]. The islet isolation process consists of pancreas distension, pancreas digestion, and islet purification. Islet purification is one of the most important steps in islet isolation for pancreatic islet transplantation. The most common method of islet purification is density gradient centrifugation based on the different densities between islets and acinar tissue[1,4,6,7]. We previously reported that purification using large plastic bottles substantially improved the efficacy in comparison to standard purification using a COBE 2991 cell processor[8]. Moreover, we evaluated methods for creating a continuous density gradient in a large plastic bottle[9], purification solutions with different osmolality[10], and a method of mixing digested tissue with a low-density solution and then creating a continuous gradient[11].

In this study, we evaluated the impact of the timing of tissue loading on islet purification using large plastic bottles. One method involved loading digested tissue after creating a continuous density gradient (tissue after gradient [TAG]; see supplemental movie TAG). The other method involved loading digested tissue before creating a continuous density gradient (tissue before gradient [TBG]; see supplemental movie TBG). We compared the effects of the purification methods on the outcomes of islet purification and transplantation.

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Materials and Methods

Porcine Pancreas Procurement and Pancreas Digestion

Pancreata from 3-year-old pigs (female, $n = 6$) were obtained from a local slaughterhouse. The operation was started approximately 10 min after the cessation of the heartbeat. All pancreata were procured using a standardized technique to minimize the warm ischemic time (WIT). After removing the pancreas, we immediately inserted a cannula into the main pancreatic duct. The pancreas was weighed, and 1 ml/g pancreas weight of modified Kyoto (MK) solution (Otsuka Pharmaceutical Factory Inc., Naruto, Japan) was infused through the intraductal cannula[12]. The pancreata were placed into the MK solution container at 4°C for approximately 18 h until the islet isolation procedure[13,14]. The "operation time" was defined as the time from the start of the operation until the removal of the pancreas. The WIT was defined as the time from the cessation of the heartbeat until the placement of the pancreas into the preservation solution. The cold ischemic time (CIT) was defined as the time from the placement of the pancreas into the preservation solution until the start of islet isolation.

Islet isolation was conducted as previously described[5], according to the standard Ricordi technique[15] with modifications that were later introduced in the Edmonton protocol[1,4,16,17]. After decontamination of the pancreas, the ducts were perfused in a controlled fashion with a cold enzyme blend of Liberase MTF (1.0 mg/ml) with thermolysin (0.075 mg/ml) (Roche Diagnostics Corporation, Indianapolis, IN, USA). The distended pancreas was then cut into 7–9 pieces, placed in a Ricordi chamber, and gently shaken. While the pancreas was being digested by recirculating the enzyme solution through the Ricordi chamber at 37°C, we monitored the extent of digestion with dithizone staining (2 mg/ml final concentration; Sigma Chemical Co., St. Louis, MO, USA) by taking small samples from the system. Once digestion was confirmed to be complete, the dilution solution (Center for Promotion of Education and Science, Hiroshima, Japan) was introduced into the system. The system was then cooled to stop further digestive activity. The digested tissue was collected in flasks containing 5% fetal bovine serum (FBS; GIBCO-Invitrogen, Carlsbad, CA, USA). The phase I period was defined as the time from the placement of the pancreas in the Ricordi chamber until the start of digested pancreas collection. The phase II period was defined as the time between the start and end of collection. After digestion, the tissue was collected and washed with fresh medium to remove the enzyme. The digested tissue was incubated in UW solution (ViaSpan, DuPont Pharmaceuticals, Wilmington, DE, USA) for 30 min before purification[18].

Islet Purification

Islets were purified with a continuous density gradient of iodixanol (Optiprep®; Sigma-Aldrich, St. Louis, MO, USA) and UW solution as previously reported[6,9–11,19]. We combined iodixanol with UW solution (IU solution) to generate purification solutions. Low-density (1.075 g/cm^3) and high-density (1.085 g/cm^3) solutions were produced by changing the volumetric ratio of iodixanol and UW solution, as reported previously[6]. Before purification by IU solution, we determined the density of the digested tissue. During this step, digested tissue (0.2 ml) (after incubation in UW solution and prior to purification) was added to six 5 ml test tubes (Corning Japan, Tokyo, Japan) of different densities (1.085, 1.090, 1.095, 1.100, 1.105, and 1.110 g/cm^3), and these tubes were centrifuged at 235 $\times g$ (1000 rpm) for 5 min. The density at which most of the digested tissue floated was defined as the density of the digested tissue. According to the outcome of the density determination step, we determined the necessary density of the high-density IU solution and added an appropriate amount of iodixanol to the high-density IU solution, as reported previously[6].

Islet purification was performed using digested tissue ($\leq$20 ml of tissue/run) with continuous gradients using low-density and density-adjusted high-density solutions in bottles (size 500 ml; NALGENE, Rochester, NY, USA). The gradient was produced with a gradient marker (Biorep Technologies, Miami Lakes, FL, USA) and candy cane-shaped stainless steel pipes (length 30 cm; UMIHIRA, Kyoto, Japan). One method involved creating a continuous gradient and then loading digested tissue in UW solution (50 ml) on top of the continuous density gradient (TAG). Another method involved loading digested tissue in UW solution (50 ml) and then creating a continuous gradient under the tissue (TBG) (Fig. 1A). The digested tissue was divided in half, so that equal amounts of tissue were used for each group. The bottles were centrifuged at 235 $\times g$ (1000 rpm) for 5 min at 4°C. After centrifugation, approximately nine fractions (50 ml each) were collected and examined for purity.

Islet Evaluation

The crude number of islets in each diameter class was determined by counting the islets after dithizone staining (final concentration, 2 mg/ml) using an optical graticule (Olympus, Tokyo, Japan). The crude number of islets was then converted to the standard number of islet equivalents (IEs) (diameter standardized to 150 μm)[20]. The gross morphology was qualitatively assessed by two independent investigators who scored the islets for shape (flat vs. spherical), border (irregular vs. well-rounded), integrity (fragmented vs. solid/compact), uniformity of staining (not uniform vs. perfectly uniform), and diameter (least desirable: all cells <100 μm/most desirable: more than 10% of cells >200 μm)[18]. Islet recovery was defined as the percentage of IEs recovered after purification divided by the IEs before purification. The islet viability after purification was assessed using double fluorescein diacetate/propidium iodide (FDA/PI; Sigma-Aldrich) staining to simultaneously visualize the living and dead islet cells[1,4,20]. Fifty islets were inspected, and their individual viability was determined visually. The average viability was then calculated[5].

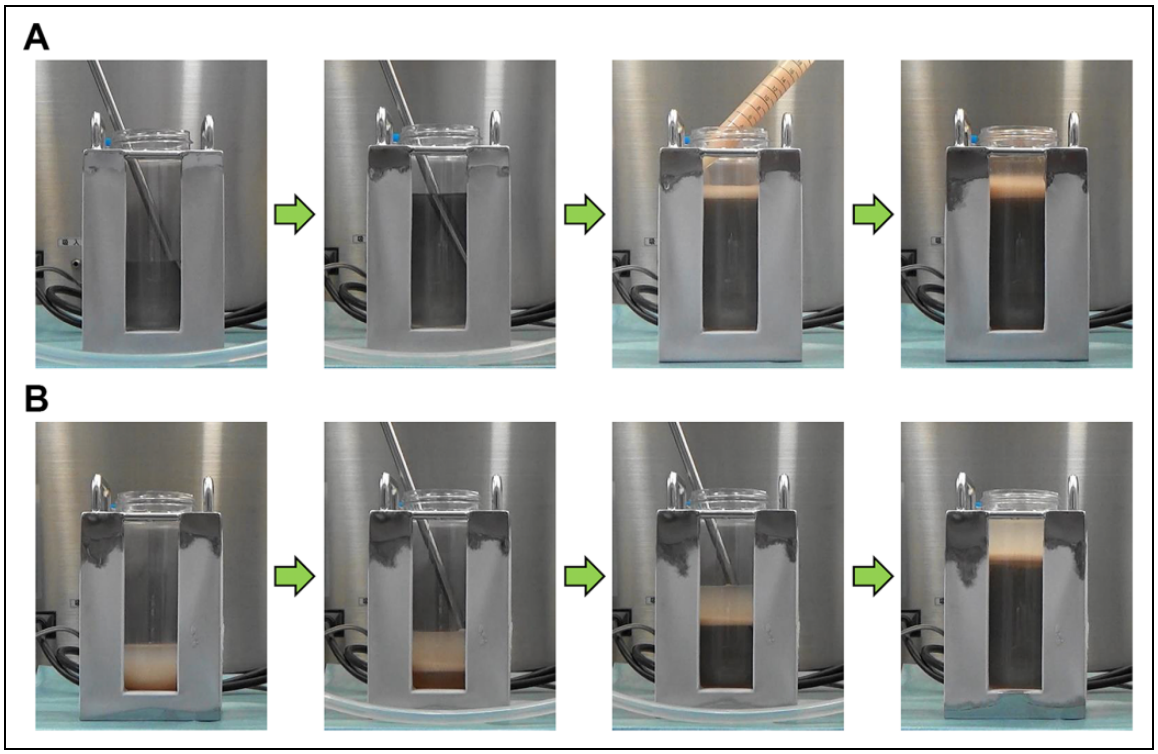

Fig. 1. Pictures of (A) TAG and (B) TBG purification. A cold block with a window was used for bottle cooling.

The islet function was assessed by monitoring the insulin secretory response of the purified islets during glucose stimulation using a procedure described by Shapiro et al.[1,4]. Briefly, 1200 IEs were incubated with either 2.8 or 25 mM glucose (Sigma-Aldrich) in Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich) for 2 h at 37°C and 5% CO_2. The supernatants were collected, and the insulin levels were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (ALPCO Insulin ELISA kit; ALPCO Diagnostics, Windham, NH, USA). The stimulation index was calculated by determining the ratio of insulin released from the islets in high-glucose media to the insulin released in low-glucose media. The data were expressed as the mean ± the standard error of the mean (SEM).

In-Vivo Assessment

Six-week-old nude mice (male: Charles River Laboratories Japan, Inc., Kanagawa, Japan) ($n = 24$) were rendered diabetic by a single intraperitoneal (i.p.) injection of 220 mg/kg of streptozotocin (STZ; Sigma-Aldrich). Hyperglycemia was defined by the detection of a glucose level of >350 mg/dl twice on four consecutive measurements after the administration of STZ**.** Then, 2000 IE porcine islets obtained from each group were transplanted into the renal subcapsular space of the left kidney of a diabetic nude mouse, as described previously[21–23]. During the 30-day post-transplantation period, the non-fasting blood glucose levels were monitored three times per week. Normoglycemia was defined by the detection of a blood glucose level of <200 mg/dl on two consecutive measurements. No statistically significant differences were observed between the two groups of mice with regard to the pre-transplantation blood glucose levels or the pre-transplantation body weight. The blood glucose level was measured using an ACCU-CHEK® Compact Plus kit (Roche Diagnostics K.K., Tokyo, Japan) in accordance with the manufacturer's instructions. All of the mouse studies were approved by the Institutional Animal Care and Use Committee of the University of the Ryukyus.

Statistical Analyses

The data are expressed as the mean ± SEM. Differences between the two groups were analyzed using either Student's *t*-test or the Kaplan–Meier log-rank test. *P* values of <0.05 were considered to indicate statistical significance.

Results

Characteristics of the Isolated Porcine Islets

Porcine islet isolation was performed using TAG or TBG, as shown in Fig. 1A and B, respectively. The characteristics of the porcine pancreas and islets before purification are shown in Table 1. There were no significant differences in the islet yield after purification (TAG group: 267,712 ± 38,018 IE, 2591 ± 402 IE/g; TBG group: 257,068 ± 40,766 IE, 2502 ± 426 IE/g) (Fig. 2), or in the post-purification recovery rate, viability, or score (Table 2).

Table 1. The Characteristics of the Tissue and Procedures Before Purification, $n = 6$.

Pancreas size (g) *	112.0 ± 3.9
Operation time (min)	4.8 ± 0.7
Warm ischemic time (min)	25.2 ± 1.2
Cold ischemic time (min)	1130.8 ± 16.1
Phase I period (min)	10.3 ± 0.3
Phase II period (min)	37.7 ± 0.9
Islet yield before purification (IE)	695,947 ± 120,052
Islet yield before purification (IE/g)	6718 ± 1178

The data are expressed as the means ± standard error of the mean.

Islet purity of high pure fraction (71–100%) was 84.2 ± 3.7% on TAG and 85.0 ± 5.5% on TBG. Islet purity of middle pure fraction (51–70%) was 57.1 ± 2.8% on TAG and 56.6 ± 2.3% on TBG. Islet purity of low pure fraction (30–50%) was 36.7 ± 4.2% on TAG and 34.2 ± 2.0% on TBG. There were no significant differences between the two groups (Table 2). In detail, islet purity of fraction 2 was 96.7 ± 2.1% on TAG and 93.3 ± 4.9% on TBG. Islet purity of fraction 3 was 77.5 ± 9.1% on TAG and 75.0 ± 5.6% on TBG. Islet purity of fraction 4 was 48.3 ± 7.9% on TAG and 55.0 ± 6.2% on TBG. Islet purity of fraction 5 was 35.0 ± 3.4% on TAG and 35.0 ± 2.2% on TBG. Islet purity of fraction 6 was 26.7 ± 3.3% on TAG and 31.7 ± 3.1% on TBG. Islet purity of fraction 7 was 18.0 ± 5.4% on TAG and 20.8 ± 4.5% on TBG. Islet purity of fraction 8 was 5.5 ± 1.0% on TAG and 10.5 ± 4.1% on TBG. Islet purity of fraction 9 was 3.3 ± 0.3% on TAG and 4.0 ± 0.4% on TBG. There were no significant differences between the two groups (Table 3). Thus, the two solutions achieved a similar level of efficiency in islet purification.

In-Vitro Assessment

To assess the islet quality in each group in vitro, the stimulation index of the isolated islets was measured. The stimulation index values of the two groups did not differ to a statistically significant extent (TAG group: 1.49 ± 0.18, $n = 6$; TBG group: 1.57 ± 0.19, $n = 6$) (Fig. 3). These data suggest that the islets in the two groups were of similar quality in vitro.

In-Vivo Assessment

To assess the islet graft function of each group in vivo, 2000 IEs from each group were transplanted below the kidney capsule of STZ-induced diabetic nude mice. The blood glucose levels of 10 of the 12 mice (83.3%) from the TAG group and 10 of the 12 mice (83.3%) from the TBG group decreased gradually after islet transplantation until they reached normoglycemia. The blood glucose levels remained stable thereafter (Fig. 4) and returned to the pre-transplantation levels after the removal of the islet-bearing kidneys (30 days post-transplantation). Post-transplantation normoglycemia was similarly attainable in the two groups. These data suggest that the islets of the two groups were of similar quality.

Discussion

In this study, we compared two purification methods. One involved loading digested tissue after creating a continuous density gradient (TAG); the other involved loading digested tissue before creating a continuous density gradient (TBG). Since the TBG method involved loading digested tissue and then creating a continuous gradient "under" the tissue, this method is essentially different from the so-called bottom-loading method[24]. Our data showed the equivalency of these two methods of islet purification. Although the TAG is the standard method for bottle purification, we have to load digested tissue gently on a continuous density gradient in order to avoid collapsing the gradient. Thus, more time is required to load tissue in the TAG method and the technical disparity affects the

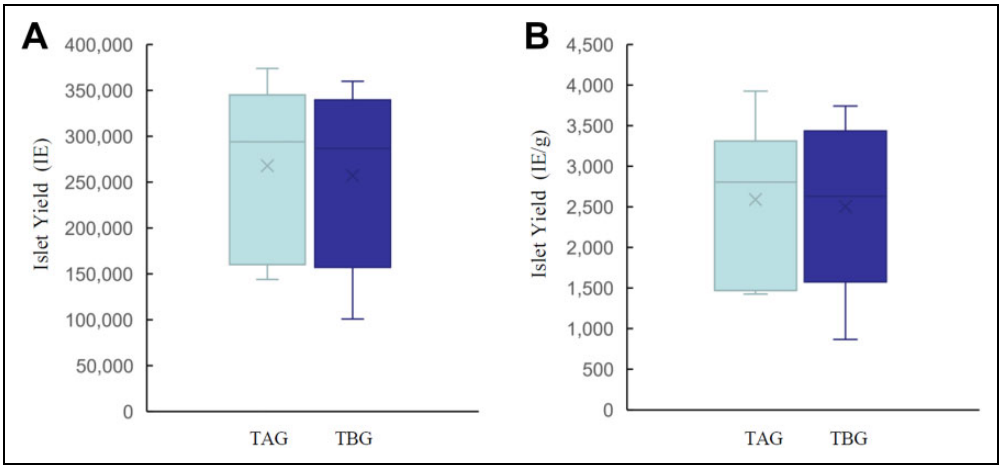

Fig. 2. The islet yields. (A) The islet yield after purification. (B) The islet yield per pancreas weight after purification. TAG; $n = 6$. TBG; $n = 6$. IE/g; islet equivalent per pancreas weight. The data are expressed as the mean ± standard error of the mean.

Table 2. The Islet Characteristics After Purification.

	TAG ($n = 6$)	TBG ($n = 6$)
Post-purification recovery (%)*	80.4 ± 5.9	75.6 ± 6.5
Viability (%)	95.2 ± 1.1	95.0 ± 0.9
Purity (%) (high + middle)	60.4 ± 3.4	59.6 ± 3.3
High purity (%) (71–100%)	84.2 ± 3.7	85.0 ± 5.5
Middle purity (%) (51–70%)	57.1 ± 2.8	56.6 ± 2.3
Low purity (%) (30–50%)	36.7 ± 4.2	34.2 ± 2.0
Score	9.5 ± 0.2	9.4 ± 0.2

The data are expressed as the means ± standard error of the mean.
* Post-purification recovery (%) = IE after purification/(IE before purification/2) × 100.

Table 3. Purity of Each Fraction.

	TAG ($n = 6$)	TBG ($n = 6$)
Fraction 1	(No tissue)	(No tissue)
Fraction 2	96.7 ± 2.1	93.3 ± 4.9
Fraction 3	77.5 ± 9.1	75.0 ± 5.6
Fraction 4	48.3 ± 7.9	55.0 ± 6.2
Fraction 5	35.0 ± 3.4	35.0 ± 2.2
Fraction 6	26.7 ± 3.3	31.7 ± 3.1
Fraction 7	18.0 ± 5.4	20.8 ± 4.5
Fraction 8	5.5 ± 1.0	10.5 ± 4.1
Fraction 9	3.3 ± 0.3	4.0 ± 0.4

The data are expressed as the means ± standard error of the mean.

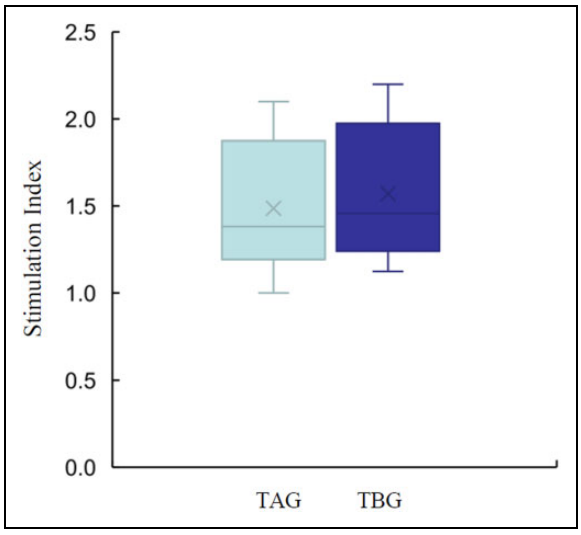

Fig. 3. The stimulation index of the isolated islets. The stimulation index was calculated by determining the ratio of insulin released from islets in high-glucose media to that released in low-glucose media. The data are expressed as the mean ± standard error of the mean ($n = 6$, each).

outcome of TAG purification. On the other hand, the collapse of the gradient is not an issue in the TBG method and the technical disparity and time spent loading tissue can be minimized. Although there were no marked differences

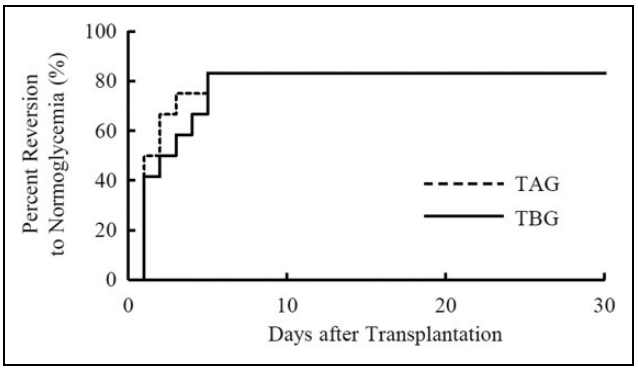

Fig. 4. Islet transplantation into diabetic nude mice. The percentage of streptozotocin (STZ)-induced diabetic nude mice, in which normoglycemia was achieved after islet transplantation, are depicted. A total of 2000 islet equivalents (IEs) were transplanted below the kidney capsule in the diabetic nude mice. Normoglycemia was defined as two consecutive post-transplant blood glucose levels of <200 mg/dl (TAG group, $n = 12$; TBG group, $n = 12$).

between the TAG and TBG methods, the TBG method has some advantages for bottle purification.

Porcine islets have been proposed as an alternative to human islets for clinical use. However, the successful isolation of significant yields of viable porcine islets is extremely difficult and requires extensive experience in the field. Several groups have described the technical pitfalls associated with the isolation of islets from juvenile and adult pigs[25–27]. As we used porcine pancreata in this study, islet purification using large plastic bottles may be useful for not only clinical islet isolation but also islet xenotransplantation.

Islet purification using a COBE 2991 cell processor is the gold standard method for clinical islet isolation. However, the high shear force involved in the standard purification method causes mechanical damage to the islets[28]. The shear stress can be minimized or eliminated using the bottle purification method. The size of the islets purified by the bottle method was significantly larger than that of the islets purified by COBE purification[8]. We used 500 ml bottles, a higher volume of the density than that of COBE purification because of elimination of the bottom solution. Moreover, we used less than 20 ml of tissue in one bottle. The condition of bottle purification was similar to that of COBE purification. Therefore, purification efficacy must be similar to that of COBE purification. The data in Table 3 seem to show a similar efficacy to the COBE purification method. We have experienced similar efficacy to COBE purification in not only pig but also human islet isolation[8,29].

One issue associated with bottle purification using the top-loading method (digested tissue on a continuous gradient) was the occasional clumping of pellets of digested tissue on the wall of the bottle. Although we previously attempted to use a mixed-loading method (mixing digested tissue with a low-density solution and then creating a continuous gradient) to overcome the issues associated with bottle purification, the purification efficacy of the top-loading and

mixed-loading methods was similar[11]. Since islet purification using large plastic bottles has some advantages over COBE purification, we will investigate more efficient methods to avoid the occasional clumping of pellets of digested tissue on the wall of the bottle in a future study.

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Ethical Approval

This study was approved by Institutional Animal Care and Use Committee of the University of the Ryukyus.

Statement of Human and Animal Rights

All of the mouse studies were approved by the Institutional Animal Care and Use Committee of the University of the Ryukyus.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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