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THE TONGUE AS A SITE FOR PANCREATIC ISLET TRANSPLANTATION: AN IMMUNOHISTOCHEMICAL AND FUNCTIONAL EVALUATION

By

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INTRODUCTION

In recent years there has been a resurgence of interest in transplanting the pancreatic islets for the treatment of diabetes mellitus. Several transplantation sites have previously been examined such as intrahepatic (**Kemp et al., 1973-a & 1973-b; Pipeleers et al., 1975; Alejandro et al., 1986; Warnock and Rajotte, 1988; Hiller and Klempnauer, 1989; Scharp et al., 1992; Van Suylichem et al., 1994**), intrasplenic (**Warnock et al., 1983; Hesse et al., 1986; Scharp et al., 1992; Van Suylichem et al., 1994**) and renal subcapsular (**Reece-Smith et al., 1981; Toledo-Pereyra et al., 1984; Amiel et al., 1987; Mendola et al.,1994**). Such studies used sites requiring an invasive operative procedure; however an easily accessible and suitable site for transplantation of the pancreatic islets has yet to be determined.

The present study was initiated to investigate the suitability of the tongue as a site for transplanting the pancreatic islets in streptozotocin-diabetic rats and to determine the ability of the islets to survive and function. The tongue was selected as a transplantation site in view of its being a highly vascularized organ that can provide the implanted islets with a rich blood supply needed for their survival and normal function. Furthermore, the tongue contains numerous serous and mucous glands existing in symbiosis with the skeletal muscles that form the main mass of the tongue. There have been reports indicating the successful survival of fragments of mature rat submandibular salivary glands autografted into the tongue (**Sharawy and O'Dell, 1981; O'Dell et al., 1983 & 1987**). Moreover, the tongue

was found to be capable of accommodating the thyroid tissue in cases of lingual thyroid. The thyroid gland or part of it is housed in the tongue, and the thyroid was found viable and normally functioning (**Moore, 1983; Umehara and Okuyama, 1985; Haddad et al., 1986**).

MATERIALS AND METHODS

Isolation of the pancreatic islets: The pancreatic islets were isolated from adult male inbred Lewis rats (230-280 g body weight) using the intraductal perfusion method of **Sutton et al. (1986)**. This method is based on the original collagenase digestion technique of **Lacy and Kostianovsky (1967)**, that has previously been described in detail (**El-Naggar et al., 1993**). The isolated islets were counted under the dissecting microscope immediately after purification and the counting was confirmed by phase contrast microscopy. The phase contrast microscope was equipped with a calibrated grid in its eyepiece to estimate the purity of the islet preparation that represents the relative volume of the islets to the total islet preparation. This was calculated as the percentage number of the intersections that overlie islets to the number of the intersections that overlie islet and non-islet tissues. Aliquots from the isolated islet preparations were functionally evaluated by measuring their adenine nucleotide content (**Lamprecht and Trautschold, 1974; Jaworek et al., 1974**) and their ability to secrete insulin in response to challenges with glucose and theophylline (**Henquin and Meissner, 1984**) as described previously (**El-Naggar et al., 1993**).

Induction of diabetes: Adult male inbred Lewis rats (280-340g body weight) were made diabetic by intravenous injection of a single dose of streptozotocin (STZ). It was freshly prepared immediately before use by dissolving STZ (30 mg/ml) in phosphate-buffered saline (PBS). Then it was injected into the penile vien of the recipient rats, under light ether anesthesia, at a dose of 40 mg/kg body weight.

Rats were housed individually in metabolic cages one week before injection of STZ and then throughout the period of the experiment. They were maintained with free access to standard diet and water *ad libitum*, without any attempt to control diabetes. The metabolic status of the rats was assessed by daily measurement of body weight, water intake, urinary output, urinary glucose and ketone bodies. Urinary glucose and ketone bodies were determined by Medi-Test glucose 3 strips (Macherey-Nagel, Düren, Germany). Blood glucose levels were measured using Aimes Glucometer II (Miles Inc., Elkhart, IN, USA).

Animals were considered diabetic if they showed the following: 24 h water intake being greater than 40 ml, 24 h urine output being greater than 40 ml, persisting 3+ urinary glucose, and non-fasting blood glucose level being above 16.7 mmol/L.

Transplantation of the islets into the tongue: A total number of 2500-3000 islets, harvested from 8 donor rats, was used for each tansplantation procedure. The islets were resuspended into a small amount of RPMI medium with L-glutamine and 25 mM HEPES, pH 7.4 (Sigma Chemical Co., St. Louis, MO, USA) and aspirated with 1 ml plastic syringe. The total amount of fluid in the syringe was adjusted to be 0.2-0.3 ml, and a 23-gauge needle was used to inject the islets into the tongue.

Rats that were confirmed diabetic for at least one week, were used as recipients for islet transplantation. Rats were anaesthetized with intraperitoneal injection of chloral hydrate (300 mg/kg). The mouth was widely opened and the tongue was pulled out. The needle was introduced into the dorso-lateral aspect of the tongue at the junction of its anterior and middle thirds. Then the needle was pushed backwards for about one cm and the islets were slowly injected into the tongue muscles close to the sulcus terminalis. As the needle was withdrawn from the tongue, the site of injection was clamped by an artery forceps for 3-5 min to prevent bleeding.

In the control group, STZ-diabetic rats were injected with RPMI medium without islets into the tongue using the same procedure described above.

Histological and immunohistochemical examination of the transplanted islets: Rats with transplanted islets were killed at 2, 24 h, or 2,4 and 8 weeks after transplantation (4 rats in each group). The tongue was excised, fixed in buffered neutral formalin and processed for paraffin impregnation. Serial sections were cut at 5 μm thickness and stained with aldehyde fuchsin and immunoperoxidase to demonstrate the insulin-containing cells. Histological sections were also prepared from the pancreas of the recipient rats and were stained immunohistochemically to evaluate the toxic effect of the STZ on the insulin-producing beta-cells.

The indirect immunohistochemical technique was used to localize the insulin-producing beta-cells **(Sternberger, 1979)**. The primary antibody used was guinea pig anti-swine insulin serum diluted in PBS with 1% (v/v) normal rabbit serum. Different dilutions were tested to obtain the optimal one that gave the best staining with the least background effects. This was found to be 1:500 and was used for staining the sections thereafter. Sections were incubated in the primary antibody for overnight in a humidity chamber, at 4°C. The secondary antibody was rabbit anti-

guinea pig immunoglobulin conjugated with peroxidase (dilution 1:200). Sera and antisera were obtained from Dako Corporation (Carpenteria, CA, USA). The chromogen substrate used was 3, 3-Diaminobenzidine Tetrahydrochloride (Sigma) and the sections were counterstained with Harris' hematoxylin to facilitate the nuclear identification. Specificity controls for the immunohistochemical staining included omitting the primary antibody, replacing it by non-immune serum, and absorption of the primary antiserum with purified crystalline insulin.

Morphometric analysis: Morphometric study was performed on the sections that were stained immunohistochemically for insulin. Ten randomly selected sections from each specimen were quantitated at a magnification of x400. Beta-cells were counted per islet section using the nucleus as the counting base. Islet area was represented by the profile islet diameter and was estimated as if each islet is an ellipse. The profile diameter (d) of each islet was calculated from the equation $d=\sqrt{ab}$, where a and b are the major, and major at right angle semi-axis, respectively **(Williams, 1977)**.

Functional evaluation of the transplanted islets: The functional metabolic effects of the transplanted islets were assessed by measuring the body weight, urinary output, and non-fasting blood glucose levels. These values were assessed twice per week. Fasting plasma glucose concentrations were measured weekly. Rats were fasted overnight and blood was obtained from the tail vien. Plasma was separated and the glucose level was measured using glucose oxidase method in Clinical Chemistry Analyzer (Beckman Instruments, Inc., Palo Alto, CA, USA).

Plamsa insulin and C-peptide concentrations were measured in blood samples collected from rats at the time of their killing, by direct cardiac puncture under ether anesthesia. Blood was also collected from control non-diabetic age-matched rats. Plasma was prepared by centrifugation of blood, and was stored at -20°C until assayed. Plasma insulin and C-peptide concentrations were measured by radioimmunoassay procedure using Coat-A-Count and Double Antibody methods, respectively (Diagnostic Products Corporation, Los Angeles, CA, USA). Radioactivity was determined using gamma counter, model 5500 (Beckman).

Statistical analysis: The results are presented as mean ± SEM. Student's *t*- test for non-paired observations was used for statistical evaluation of the data. Values of $P< 0.05$ were considered significant.

RESULTS

Evaluation of the isolated islets: The final purity of preparations of the isolated islets obtained in the present work was 83.4±2.3%. The non-islet tissues consisted mostly of pancreatic ducts and exocrine acini and were difficult to separate from the islets. The concentrations of ATP and the total adenine nucleotides of the isolated islets were found to be similar (not significantly different at $P< 0.05$) to those of the freeze-clamped intact pancreas. The ability of the islets to secrete insulin after being challenged with glucose (15 and 30 mmol/L) resulted in highly reproducible response (Table 1). Maximal secretion of insulin could be elicited by a final challenge of glucose (15 mmol/L) together with theophylline (15 mmol/L), (see Table 1).

Histological and immunohistochemical examinations: Histological examination of the tongues of the recipient rats, 2 h after transplantation, showed the presence of grafted islets among the muscle fibers of the tongue. The islets could be detected with ordinary stains and were histologically intact. Immunohistochemical reaction for insulin revealed that most of the islet cells were positively stained for insulin (Fig.1). Few pancreatic acini could also be seen in between the grafted islets. Non-specific cellular reaction at the site of the graft was detected 24 h after transplantation, which rendered the identification of the islets to be difficult with ordinary stains. Islet cells could, however, be identified by the immunoperoxidase reaction for insulin, which revealed positively stained masses scattered inside the area of the cellular reaction (Fig. 2). Moderate cellular reaction was also noticed in the diabetic control rats.

Two weeks after tansplantation, the grafted islets could easily be detected in the tongue with ordinary stains. Immunohistochemical staining of these islets revealed insulin-positive cells with degranulated cytoplasm (Fig. 3). Some of these cells were enlarged and exhibited vascular degeneration and hyperchromatic nuclei. The tongues of the diabetic control rats showed mild cellular reaction at the site of injection of the RPMI medium. Insulin-positive beta-cells could also be detected in the tongues of the rats 4 weeks (Fig. 4) and 8 weeks (Fig. 5) after transplantation. The cells were present in aggregates, suggestive of islets, among the muscles of the tongue and could easily be detected with ordinary stains. They reacted weakly positive to anti-insulin serum, indicating that they were degranulated.

The pancreas of the recipient rat showed islets that were composed entirely of non beta-cells and showed negative reaction to the immunoperoxidase for insulin (Fig. 6). Few scattered beta-cells could, however, be detected in some islets, where

the beta-cells were hypertrophied, partially or completely degranulated, with prominent nuclei and nucleoli.

Morphometric analysis: The results of the morphometric study are summarized in Table 2. It showed that the number of cells per islet section and the profile diameter of the islets at 2 and 24 h after transplantation were significantly lower compared to those of the originally tansplanted islets. This indicates that there had been some loss of the islet mass following transplantation. The loss of the islet mass continued with advancing transplantation time, as indicated by the lower number of cells per islet section and smaller profile diameter of the islets at 2,4 and 8 weeks post-transplantation (Table 2).

Functional evaluation: The body weight of the rats that received transplanted islets could be maintained approximately at the pre-transplantation value for 8 weeks after transplantation. The diabetic control rats showed continuous weight loss during the same time (Fig. 7). The difference in the body weight between the two groups was statistically significant at 6-8 weeks after transplantation ($P<0.05$). The volume of urine per 24 h in the rats that received tansplanted islets was slightly lower than that of the corresponding diabetic control rats (Fig. 8). The difference was statistically significant during the first week after transplantation ($P< 0.05$).

There was gradual fall in the non-fasting blood glucose concentration following transplantation (Fig. 9). Blood glucose levels at 4 to 7 weeks after transplantation were significantly lower than those of the corresponding diabetic control rats ($P< 0.05$). In addition, plasma glucose concentrations of the overnight-fasted rats were lower in the group of the transplanted islets than that of the corresponding diabetic control group. Significant difference ($P< 0.05$) between the two groups was recorded at 1 and 2 weeks post-transplantation (Fig. 10).

Plasma insulin concentrations at 2 and 8 weeks post-transplantation were higher than those of the diabetic control rats. It did not, however, reach that of the age-matched non-diabetic control rats (Table 3). C-peptide concentrations at 2 and 8 weeks post-transplantation were also higher than those of the diabetic control rats, but did not reach that of the age-matched non-diabetic control rats (Table 3).

Table (1): Insulin release from aliquots of isolated islets, in response to challenge with glucose and theophylline*

Glucose concentration (mmol/L)	Insulin release (μU/h/mg protein)
5.5	61.3 ± 2.9 (20)
15.0	142.0 ± 3.3 (20)
30.0	168.0 ± 3.5 (20)
15.0 + 15.0 mmol/L theophylline	308.9 ± 13.8 (20)

* Values are expressed as mean ± SEM. Numbers in parenthesis are numbers of specimens examined.

Table (2): Number of cells per islet section and profile diameter of isolated and transplanted islets.*

Specimen	Number of cells per islet section	Profile diameter of islets (μm)
Isolated islets	73.0±2.7 (272)	76.3±1.5 (272)
2 hours after transplantation	39.8±3.5 (340)	64.6±3.0 (340)
24 hours after transplantation	35.5±3.4 (296)	46.6±1.8 (296)
2 weeks after transplantation	10.7±1.8 (108)	35.5±3.8 (108)
4 weeks after transplantation	5.0 ± 1.0 (60)	24.0±2.6 (60)
8 weeks after transplantation	4.0 ± 0.8 (38)	25.3±2.7 (38)

* Values are expressed as mean ± SEM . Numbers in parenthesis are numbers of islets examined.

Table (3): Effect of islet transplantation into the tongue on serum insulin and C-peptide concentrations*.

	Transplantation	Diabetic Control	Non-diabetic Control
Insulin (μU/ml)			
2 W	20.7 ± 3.2 (5)	13.5 ± 4.0 (5)	35.77 ± 2.4 (5)
8 W	17.7 ± 2.7 (5)	11.6 ± 2.2 (6)	34.35 ± 4.1 (5)
C-peptide (ng/ml)			
2 W	0.302±0.032 (5)	0.298±0.005 (5)	0.334±0.029 (5)
8 W	0.275±0.019 (5)	0.208±0.054 (6)	0.312±0.027 (5)

* Values are expressed as mean ± SEM. Numbers in parenthesis are numbers of rats examined.