Allotransplantation of Sulphate Glucomannan-Alginate Barium (SGA)-Microencapsulated Rat Islets for the Treatment of Diabetes Mellitus

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To offer a more effective microencapsulation technique of islets for the treatment of diabetes, we have developed a new type of microcapsule comprising sulphate glucomannan-alginate barium (SGA). We compared it with traditional microencapsulated APA (alginate-poly-L-lysine-alginate) and ABa (Ba²⁺-alginate) microencapsulated islets. These three types of microencapsulated islets were prepared and cultured in vitro and we studied their morphology and activity. To determine their effects on insulin secretion and cytokine production (MCP-1, IL-1, IFN-γ, TNF-α) the islets were transplanted into diabetic rats. There was no difference in the morphologies of the three types of microencapsulated islets or their insulin secretory capacity in vitro. However, the SGA microencapsulated islets had higher activity and produced more insulin than the APA and ABa microencapsulated islets after transplantation. Normoglycemia was maintained for longer in the SGA-transplanted group than in the other two groups. The concentrations of cytokines in the peritoneal fluid were significantly decreased in the SGA group, as was the infiltration of inflammatory cells around the microcapsules. In conclusion, the novel SGA microencapsulated islets can maintain normoglycemia in diabetic rats without immunosuppression for longer than APA and ABa microencapsulated islets.

Keywords SGA microcapsule, Islet transplantation, Diabetes mellitus.
INTRODUCTION

The success of the Edmonton Protocol (Shapiro et al., 2000) has made islet transplantation an effective method to treat diabetes mellitus. However, the approach has two barriers: the adverse effects of chronic immunosuppression and the shortage of donors (Shapiro et al., 2000; Ryan et al., 2005). The introduction of microencapsulation technology, which immunologically isolates the islet from the host, could help to overcome both barriers. In the 1980s, Lim and Sun (1980) first used APA to encapsulate islets and transplanted them to treat diabetic rats. This method has many advantages (Sandler et al., 1997; Lopez-Avalos et al., 2001) including little manipulation, a high level of safety, and significantly prolonged graft survival. However, further research has shown that the APA microcapsule is antigenic and very permeable, which enables inflammatory factors to aggregate within the microcapsules.

This prompted further developments, whereby Ba\(^{2+}\) replaced Ca\(^{2+}\) for the synthesis of the adhesive, and the poly-lysine encapsulation step was omitted to yield ABa microcapsules (Jork et al., 2000; Omer et al., 2003). ABa has similar mechanical strength to APA and, because it does not contain poly-lysine, it has lower antigenicity, thus reducing the risk of rejection. However, its permeability was found to be even greater, with detrimental effects on the long-term survival of islet grafts (Omer et al., 2005). Moreover, the traditional microcapsules have other limitations (Vos et al., 1999), including fibrosis of the semi-permeable membrane, which causes the microencapsulated islets to lose their function, poor nutrient supply, which leads to starvation of the cells, and activation of host antibodies and immune cells, which leads to host rejection of the microencapsulated islets. These properties have limited the clinical application of microencapsulated islet transplantation.

In this study, we describe the development of a new capsule material for the microencapsulation of islet grafts. We used sulphate glucomannan, which has properties similar to heparin, to prepare SGA microencapsulated islets, which we evaluated in vivo in diabetic rats and compared their efficacy with APA and ABa microencapsulated islets.

MATERIAL AND METHODS

Animals

One-hundred-and-fifty male Sprague–Dawley rats (SD; weighing 250–350 g, Experimental Animal Center of Harbin Medical University, China) were used as the transplantation donors. Seventy-two male Lewis rats (LEW RT1\(^1\), weighing 196 ± 15 g, Beijing Weitong Lihua Experimental Animal Technology Co. Ltd, China) were used as the transplant recipients. All rats were housed under specific pathogen-free conditions.
Islet Isolation

According to the modified Wahoff's method (Wahoff et al., 1994), the SD rats were anesthetized with intraperitoneal injection of 1% sodium pentobarbital (Shanghai Chemical Reagents Inc., China). Thereafter, 5 mL of pre-chilled collagenase V (1.0 mg/mL; Gibco) solution was quickly injected through the pancreatic duct. The pancreas was subsequently removed and statically digested in a water bath for 10 to 15 min at 38°C. The pancreases were shaken slowly until they were fully digested, and pre-chilled Hanks' balanced salt solution (HBSS) containing 10% fetal bovine serum was added to terminate the digestion. Islets were purified by the discontinuous Ficoll (Sigma) density gradient method (1.084, 1.077, 1.068 and 1.038 g/mL). The islets were collected at the 1.068/1.077 and 1.038/1.068 interfaces, observed under an inverted microscope, and counted.

Microencapsulation

SGA Microcapsule Preparation. Chlorosulfonic acid, formamide and glucomannan powder (Wuhan Boshide Company, China) were mixed (500 rpm/min) at 40°C for 5 h. Then, 95% ethanol (volume fraction) was added to the mixture for full sedimentation and filtration, cleaned with water to obtain sulfated glucomannan, and mixed with sodium alginate (Sigma) at a 1:4 ratio to prepare a mixture of 3% gel fluid, which was evenly mixed with the separated and purified islet cells. Subsequently, the mixed islet cells and gel solution were poured into a pressurized microcapsule generator (SPA-00282-type, Nisco Engineering) at 2.6 kPa in a dual-chamber nozzle of 0.3 mm diameter for pressurized spraying into microspheres. The microspheres were then agglutinated in 120 mM BaCl₂ solution for 8 min, and 55 mM sodium citrate solution was added to liquefy the core.

ABA Microcapsule Preparation. The mixed gel solution was substituted with 2% sodium alginate gel solution for agglutination into microspheres with Krebs-Ringer bicarbonate buffer (Sigma) containing 120 mM BaCl₂ and 13 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid buffer (HEPS). After the steps described above, the microcapsules were cleaned in a solution containing 260 mM BaCl₂ and Krebs-Ringer buffer (KRBB).

APA Microcapsule Preparation. The microspheres prepared as described above were agglutinated in 120 mM BaCl₂ for 8 min, coated with 0.05% polylysine (Sigma) solution for 15 min and were immersed in a 0.3% gel solution of sodium alginate for 8 min to coat the outer membrane.

Establishment of the Diabetic Rat Model

The Lewis rats were rendered diabetic by a single intravenous injection of streptozotocin (Sigma) freshly dissolved in saline (50 mg/kg). Only rats with
blood glucose concentrations exceeding 16.8 mM for more than 3 consecutive days were selected as diabetic rats for islet transplantation.

**Islet Transplantation**

Lewis rats were randomly divided into four treatment groups and received SGA microencapsulated islets (SGA group), ABa microencapsulated islets (ABa group), APA microencapsulated islets (APA group) or no islets (C group) \( (n = 18 \text{ per group}) \). All rats were anesthetized by intraperitoneal injection of 1% pentobarbital, and a small incision was placed in the abdomen to inject 15 mL of the microencapsulated islet vehicle (saline), which contained approximately 3000 IEQ islets. Rats were then given intramuscular injection of 20,000 U penicillin. Rats in the control group received 15 mL of microcapsule liquid without islets. After transplantation, six rats from each group were randomly selected for determination of glucose levels in blood samples taken from the caudal vein every other day; islets would be considered functional if the fasting blood glucose concentration was below 11.1 mmol/L. The recipients did not receive any immunosuppression before or after transplantation.

**Pathology and Cytokine ELISA**

Pathological analysis of the transplanted microencapsulated islets was conducted by H&E staining. At week 3 after transplantation, the abdominal microencapsulated islets were collected from six randomly selected rats in each group. The islets were stained with H&E to observe the infiltration of inflammatory cells. Peritoneal fluid from the transplant site was also collected to assay for cytokines including MCP-1\(\text{(Rat MCP-1 Instant ELISA, BMS631INST)}\), TNF-\(\alpha\)\(\text{(Rat TNF-alpha ELISA BMS622)}\), IL-1\(\beta\)\(\text{(Rat IL-1beta ELISA BMS630)}\), and IFN-\(\gamma\)\(\text{(Rat IFN-gamma ELISA BMS621)}\) using ELISA kits (Bender MedSystems). The concentrations of the cytokines were calculated from the standard curve of the appropriate recombinant cytokine.

**Islet Function**

Islet function was determined based on the insulin release index test, which was performed before and after transplantation. After culturing the islets in vitro for 14 days, 30 SGA, APA and ABa microencapsulated islets underwent the insulin release index test. In brief, the islets were incubated in 1 mLDMEM (Invitrogen) supplemented with different amounts of glucose (low glucose, 5.6 mmol/L; high glucose, 16.7 mmol/L; high glucose + 10 mmol/L theophylline [Sigma]) for 4 h. The culture solution was collected, insulin levels were determined by ELISA, and results were obtained as means of three
determinants. Eight weeks after transplantation, 50 abdominal microencapsulated islets were surgically removed from six randomly selected rats in each group and their function was studied via the insulin release index test as above. The activity of the microencapsulated islets was studied as follows. The islets in microcapsules were incubated with anti-pancreatic islet EB-FDA (Sigma, ethidium bromide-dual luciferase acetate) and observed under a fluorescent microscope (Optiphot-2 type, Nikon). Dead cells were stained red and active cells were stained green using this method.

**Statistical Analysis**

All data are expressed as means ± standard deviation. Student’s t-test was used to determine statistical significance of differences between groups. For all comparisons, p-values of less than 0.05 were considered significant and p-values less than 0.01 were considered highly significant.

**RESULTS**

**Isolation and Purification of Rat Islets**

The high yield and purity of rat islet isolation is essential for subsequent experiments. Using the modified Wahoff method, we obtained islets with high yield and purity. The morphology of the isolated islets was round or elliptical of varying diameters, with a tightly bound and compact structure. The diameter of most of the isolated islets was 150–250 μm. The average number of islets isolated was 950 ± 80 IEQ per pancreas.

**Morphology and Insulin Secretory Function in vitro**

The prepared SGA microcapsules did not differ morphologically from the APA or the ABa microcapsules (Figure 1). The microencapsulated islets were 300–400 μm in diameter and contained an average of 1–2 islets/microcapsule.

![Figure 1: Morphology of microcapsules after preparation. A, SGA microencapsulated islets. B, APA microencapsulated islets. C, ABa microencapsulated islets.](image-url)
All of the microencapsulated islets had a round morphology and a smooth surface with good light transmission properties.

After preparation of the microencapsulated islets, they were cultured \textit{in vitro} for 14 days and their insulin secretory function was evaluated. Thirty microencapsulated islets of each type were randomly selected for the stimulated insulin release test. Figure 2A shows that the three types of microencapsulated islets had similar insulin secretory responses to glucose stimulation.

\textbf{Function of Microencapsulated Islets \textit{in vivo}}

The activity and insulin secretory capacity of the microencapsulated islets were tested by transplanting 3000 IEQ islets into diabetic allogeneic rats. Eight weeks after transplantation, the insulin-secretion index was determined and insulin secretion from SGA microencapsulated islets was higher than that from the ABa or APA microencapsulated islets. The insulin secretion increased with increasing glucose stimuli for all three types of microencapsulated islets. The insulin secretion under the high glucose + theophylline condition was 1.5 times higher than that with high glucose alone and was 2.8 times higher than the low glucose condition, indicating that the islets were functional after preparation of the microcapsules (Figure 2B).

The \textit{in vivo} activity of microencapsulated islets after transplantation was analyzed by EB-FDA staining. Fifty microencapsulated islets were selected at
random from 6 rat recipients from each group. The activity of the SGA microencapsulated islets was significantly higher than the other types of islets. The percentage of active islets was analyzed by image analysis software. The green areas indicate active cells. Fifty microencapsulated islets were randomly selected from six rats in each group. The average percentage of active islets was calculated from 10 fields of view. The percentage of active islets was $72.1 \pm 16.0$, $51.8 \pm 10.2$ and $34.5 \pm 9.0\%$ in the SGA, ABa and APA groups, respectively.

**Peritoneal Cellular Infiltration and Cytokine Secretion in vivo**

The immune isolation of microcapsules is demonstrated by its role in shielding the contents from harmful cytokines in vivo. Microencapsulation can prevent diffusion of harmful cytokines both out of the islet into the host and from the host into the islet. To compare the immune isolation of the three types of microcapsules, we measured the concentrations of a variety of cytokines related to immune rejection in peritoneal fluid using ELISA. Compared with ABa and APA, the SGA microcapsules significantly reduced the secretion of MCP-1 ($P < 0.05$), TNF-$\alpha$, IL-1$\beta$ and IFN-$\gamma$ in the host peritoneal cavity (Figure 3).

The presence of inflammatory cells after transplantation is a valuable marker for evaluating the antigenicity of microencapsulated islets. Three weeks after transplantation, microencapsulated islets were removed from the peritoneal cavity from 6 randomly selected rats and were stained with H&E. The infiltration of inflammatory cell around the SGA microcapsules was less than that observed around the other two types of microcapsule (Fig. 3). The APA microcapsules showed the greatest degree of inflammatory cell activation.

**Restoration of Euglycemia in Diabetic Rats Transplanted with Microencapsulated Islets**

To determine the function of the three types of microencapsulated islets on diabetes status, blood glucose concentrations were monitored after transplantation. Before transplantation, the rats were hyperglycemic but, within 1 week after transplantation, the blood glucose levels were normalized in the three groups of rats that received islets. The blood glucose levels remained within normal ranges ($<11.1$ mmol/L) for more than 2 weeks in the three treatment groups, indicating that the microencapsulated islets were functional. Of particular interest, rats in the SGA group remained normoglycemic until week 13, and developed marked hyperglycemia at week 17, with glucose levels $>16.8$ mmol/L. Rats in the ABa and APA groups were normoglycemic until weeks 7 and 5, respectively. The control group were hyperglycemic for the entire study duration (Figure 4).
DISCUSSION

The novel capsule material SGA is a derivative of glucomannan and contains crystallized and amorphous regions in its structure, which gives excellent biocompatibility, and film-forming and gelling properties (Douglas et al., 1997). Of note, there are many sulfanilamides with heparin-like properties that form bonds at the C2, C3 and C6 positions in their molecular structures, which are able to bind to various cytokines (Xiao et al., 2002). Because of these properties, SGA is suitable for the production of microcapsules to circumvent the
disadvantages of traditional microcapsules. Disadvantages of the traditional microcapsules include excessive antigenicity, as seen in APA microcapsules, and excessive permeability, as seen in ABa microcapsules.

The mixed SGA-alginate gelling solution was used in a one step process to prepare the smooth-surfaced SGA microcapsules, which show high elasticity and have good light transmittance. The diameter of the aperture of the SGA microcapsule was around 400 microns, which restricted the release of inflammatory cytokines from the microcapsules and allowed unhindered uptake of nutritional molecules into the microcapsules. The SGA microcapsule functioned better as an immune shield than the classical APA microcapsules, for which the diameter of the aperture is approximately 700 microns) (Vos et al., 2006).

After transplantation, the SGA microcapsules showed better biocompatibility in vivo than the APA and ABa microcapsules. The insulin activity of the SGA microcapsules was significantly higher than that of the APA and ABa microcapsules, which may be attributable to the ability of the SGA components to bind harmful cytokines. Moreover, SGA microencapsulated islets secreted less MCP-1, thus prohibiting macrophage infiltration and subsequently prevented the production of IL-1β, IFN-γ and TNF-α by host immune system. Another possible cause of the enhanced biocompatibility of the SGA microcapsules is that they have heparin-like properties, which inhibit the aggregation of inflammatory cells, as demonstrated by the H&E staining. Comparison of the blood glucose levels in the three different islet groups showed that the SGA microcapsules maintained normoglycemia for a
longer time and reversed the initial hyperglycemia more rapidly than that achieved with APA and ABa microcapsules.

SGA microcapsules offer an extra level of protection for allogeneic islet grafts and, with appropriate immunosuppressive or immunomodulatory strategies, could make xenogeneic islet transplantation a clinical reality. In addition, our experiments showed that SGA microencapsulated islets with immune isolation survived without immunosuppression for 17 weeks \textit{in vivo}.

Although the biochemical profiles were significantly better in the SGA group than in the ABa and APA groups, 28\% of the cells in the transplanted SGA microencapsulated islets had died by the week 8, and the SGA group developed marked hyperglycemia (blood glucose $>16.8$ mmol/L) at week 17. We speculated this decline in islet function over time was due to hypoxia because the isolation of transplanted microcapsules prevented revascularization of the islets, and the oxygen supply was obtained by passive diffusion from the abdominal cavity rather than directly from the blood supply. Accordingly, the efficiency of the oxygen supply was inadequate, which led to the death of islet cells. However, it may be possible to overcome hypoxia by altering the transplant site or by increasing the tolerance of the islet cells. For example, the survival time of islet cells could be extended if the microcapsules were injected directly into the liver through the portal vein and transplanted into the hepatic sinusoid, which has an abundant blood supply. However, the microcapsules would need to be small (less than 300 microns in diameter), or the recipient will be exposed to increased risk of complications such as obstruction and fibrosis of the blood vessels (Leblond et al., 1999).

In summary, compared with APA and ABa microcapsules, the SGA microcapsules showed higher biocompatibility and more pronounced anti-hyperglycemic effects in diabetic rats, offering potential for clinical applications. Further studies should focus on optimizing the structures of the SGA microcapsules, and identifying optimal positions of the transplantation to prolong the survival time \textit{in vivo}. Future successful studies with SGA microcapsules are needed to demonstrate the beneficial clinical utility of transplantation of SGA microencapsulated islets and, potentially, other fields of cell transplantation.

\textbf{ACKNOWLEDGMENTS}

This work was supported by the National Natural Science Foundation of China (No. 30600606) and the Second Affiliated Hospital, Harbin Medical University (BS2008-37) and the Natural Science Foundation of Heilongjiang province (D2004-30).

\textbf{Declaration of Interest:} The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.
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