
EFFECT OF CYCLOSPORIN-A AS AN IMMUNOSUPPRESSIVE DRUG ON ISLET CELLS TRANSPLANTATION IN MICE

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Abstract

Transplantation of normal isolated islets of langerhans for the treatment of diabetes remains an elusive goal in clinical practice. Perhaps the major problem preventing clinical islet transplantation has been limited by the inability to prevent islet allograft or xenograft rejection. Cyclosporin A is an immunosuppressive agent that improves survival of transplant. This exciting immunosuppressive agent was first used clinically in renal transplantation in 1978 (**Ferguson and Fidlus 1982**). The aim of this study was the isolation , purification and transplantation of hamster pancreatic islet as xenograft transplantation for the treatment of diabetic mice. It is also aimed to study the effect of cyclosporin A as an immunosuppressive agent on some biochemical parameters e.g blood glucose and blood insulin levels to induce maximum suppression of mice immune system and consequently realizing maximum survival of transplanted islets. A total of 30 streptozotocin induced mice were randomized to receive islets xenografts from golden syrian hamsters by three different approaches ,the first group of ten mice received islets only in the renal subcapsular space, the 2nd of ten mice received islets in the renal subcapsular space and was given cyclosporin A in a dose of 15 mg/kg/day for three days. The 3rd group of ten mice were received islets in the renal subcapsular space and were given cyclosporin A orally every day at a dose of 15 mg/kg/day for three days which was gradually decreased to 5 mg/kg/day. Non of the mice of group 1 became normoglycemic. All mice of group 2 became normoglycemic for 11 ± 4 (range 6-18 days) . only mice of group 3 enjoyed normoglycemic as long as cyclosporin-A was administrated. Consequently, prolonged survival of islets xenografts may be achieved with administration of cyclosporin-A.

Introduction:

Transplantation of normal isolated islets of langerhans for the treatment of diabetes remains an elusive goal in clinical practice. Perhaps the major problem in preventing clinical islet transplantation has been limited by the inability to prevent islet allograft or xenograft rejection. Various approaches and different sites have been suggested to prevent islet graft rejection and maintain long-term islet cell function (**Lacy, 1983**).

Immunosuppression by pharmacological agents such as cyclophosphamide, azathioprine and corticosteroids was of minor effectiveness. However, cyclosporin

A (CyA) is an immunosuppressive agent that improves survival of transplant. (Calne et al, 1962).

The structure cyclosporin was established by chemical degradation together with an X-ray crystallographic cyclic peptide composed of eleven amino acids residues, all having the L- configuration of the natural amino acids except for the D-alanine (D-Ala) in position 8 and the non chiral sarcosine **fig (1)**. This exciting immunosuppressive agent was first used clinically in renal transplantation in 1978 (Ferguson and Fidler 1982).

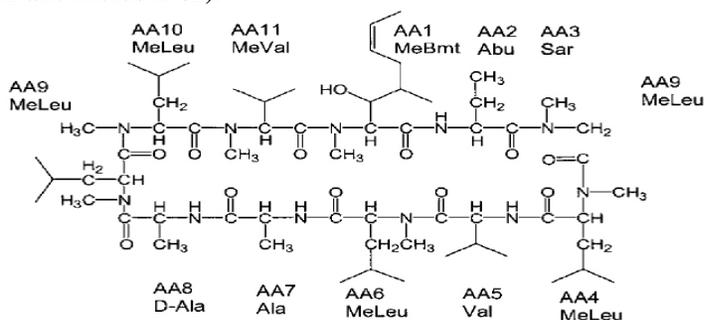


Fig (1). Structure of cyclosporine

Our study was conducted to evaluate the role of cyclosporin-A on the biochemical parameters such as blood glucose, Cyclosporin A level in a trial to determine the minimum level of cyclosporin which cause maximum suppression of immune system and minimal nephrotoxicity to attain maximum survival of transplanted islets

Materials And Methods:-

Animals:

Golden Syrian hamsters, weighing 100-120 g were used as islet donors. Swiss mice were the recipients. Non fasting plasma glucose levels of the recipient mice were determined before the induction of diabetes. Blood sugar level was monitored via orbital sinus blood samples with Aqua trend sensor. The mice were made diabetic by a single intraperitoneal injection of streptozotocin (150 mg/ Kg body weight) and only those mice with serum glucose levels more than 350 mg/ dl were used for transplantation.

Islet Isolation:

Hamster islet was isolated according to the method previously described **Getoh et al.,1985 and Avila et al.,2003** by intraduct injection of collagenase solution

followed by digestion and extensive purification on Ficoll gradients. Hand-picked islets were cultured in CMRL-1066 medium containing 10% heat inactivated fetal calf serum (FCS) and antibiotic-antimycotic solution (1 ml/ 100 ml). The islets were then incubated at 37 °C for 3 days in a humidified atmosphere of 5% CO₂. Islets were stained for viability by diphenylthiocarbazone (DTZ).

In Vitro Insulin Secretion of Islets:

Insulin secretion in response to glucose was tested according to the method described by **Gotoh et al, 1987**. Freshly isolated or 24- hour cultured islets were incubated for 30 minutes at 37 °C in a modified Krebs Ringer bicarbonate buffer containing 10 mM HBSS , 0.5 mM NaHCO₃, 1mM CaCL₂ and 05% bovine serum albumin{KRB-HEPES,OPH 7.4} The basal KRB - HEPES used for incubation also contained low (2.8 mM) glucose or high {16.7 mM} glucose content . After incubation, the supernatant was immediately removed and stored separately at -20°C until assayed for insulin content.

Transplantation of Islets:

At transplantation, Islets were maintained for 3-4 days and checked for viability by diphenyl thiocarbazon (DTZ) staining .Streptozotocin- induced diabetic mice were used as recipients with at least two consecutive blood glucose analysis of 350 mg/dl A total of 30 mice were divided into 3 different treatment groups. A small incision was made in the left lumbar region to expose the left kidney. Countered islet were suspended in 0.3 ml CMRL-1066 medium and implanted underneath the renal capsule using 27 gauge needle without making an incision into the capsule. Each diabetic mice received a total of 10 islet per gram of body weight

Cyclosporin-A treatment:-

Group 2,3 were transplanted with islets and treated with Cyclosporin-A was administered orally using stomach canula every day from the day of transplantation. The first dose was 15 mg/kg/day for three days (group 2), and 15mg/kg/day for three days gradually decreased to 5 mg/kg/day for one month (group3).

Post-transplantation Follow-up:

After transplantation, the mice were transferred to metabolic cages for daily examination. Non-fasting blood glucose levels of the recipients were monitored 3 times weekly for the first 3 weeks, then twice weekly thereafter. The mice were considered cured if exhibited the following criteria: a random plasma glucose less

than 200 mg/ dl, a glucosuria with steady weight gain. Rejection was considered when blood sugar concentration exceeded 200 mg /dl on two consecutive bleedings.

Glucose Tolerance Test:

Glucose tolerance test was carried out in transplanted mice that maintained a normoglycemia state for at least 30 days as previously described (**Iwata et al.,1994**). For comparison the glucose tolerance test was also performed with 5 normal non-transplanted mice and 5 diabetic non-transplanted mice. Glucose solution 1 mg/ kg body weight was infused endogastrically through a polyethylene tube into mice that had been fasted overnight. Blood glucose levels were determined at 0, 30, 60, 90 and 120 min of glucose injection. The results were expressed as average blood glucose level \pm SE. The degradation in glucose per minutes (k value) was calculated.

Determination of Cyclosporin A level:-

Cyclosporin A (CyA) was determined post-transplantation and after I week and 14 days according to the method of (**Pesco et al , 1990**) as follow:

Whole blood sample (150 μ L) , 50 μ L solublizing reagent and 300 μ L precipitating reagent (Supplemented in the CyA kit) were mixed together , vortex for 10 seconds and centrifuged for 5 minutes at 10900 rpm. The supernatant was then analyzed on the TDx analyzer using Cy A monoclonal whole blood kit . The Cyclosporin A level was obtained in ng/ml .

Where:- The constituents of the solublizing reagent are surfactant in water with 0.1% Sodium Azide as preservative . The constituents of the precipitating reagent are zinc sulphate solution in methanol and ethylene glycol.

Histological Examination

The grafts of transplanted mice were surgically removed at various intervals following transplant. the graft tissues were fixed in 10% formalin for 24 hours. dehydrated in a series of ethanol and transferred to xylene for two hours. After infiltration in paraffin at 60°C for one hour, the tissue was embedded in paraffin block until hardening. Sections of 4 μ m were cut and routinely stained with haematoxylin.

Result

The mean number of islets obtained from one pancreas using 0.7 mg/ml collagenase type XI was 414 \pm 48 islets. The islets were spherical or oval, some of them had exocrine tissue attached to their surface. Morphologically the cultured

islets were characterized by golden brown with a distinct outer intact membrane **Fig (2)**. The insulin release from fresh islets compared with cultured islets in response to low (2.8 mM) and high (16.7 mM) glucose concentration is shown in **fig (3)**.

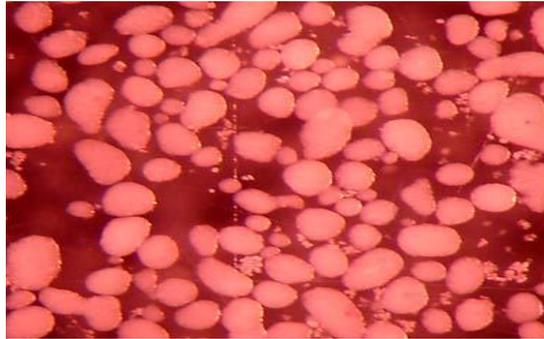


Fig (2). The microscopic appearance of hamster isolated islets

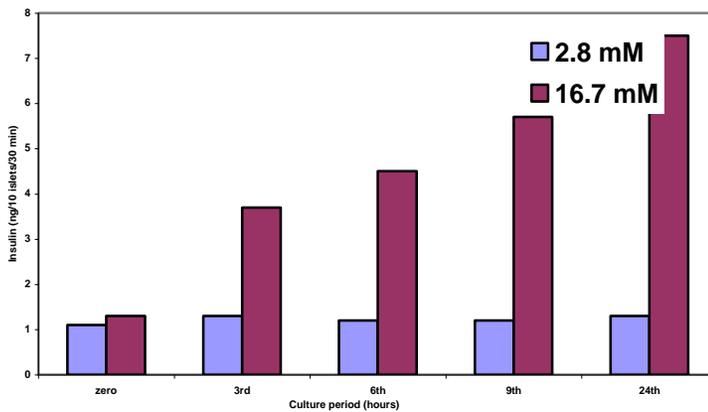


Fig (3) Insulin release from fresh and cultured islets in presence of 2.8 mM (low) and 16.7 mM (high) glucose concentration.

The result of islet transplantation are summarized in **table 1**. None of mice transplanted with islet only under the kidney capsule (Group 1) without administration of cyclosporin-A became normoglycemic. Mice receiving islets only in the renal subcapsular space and treated with cyclosporin-A 15 mg/kg/day for three days (Group 2) became normoglycemic for a mean of 11 ± 4 . on the other hand mice receiving islets only in the renal subcapsular space and treated with cyclosporin-A 15 mg/kg/day for three days. gradually decreased to 5 mg/kg/day for

one month (group 3) became normoglycemic for a mean of 30 ± 6 and exhibit normoglycemia as long as cyclosporin-A was administrated.

Table1. Results of islet transplanted in the renal subcapsular space with cyclosporine –A administration

No	Site of transplantation	Treatment	Duration of normoglycemia	Mean graft survival
1	Kidney capsule	-----	-----	-----
2	Kidney capsule	Cy-A	6,9,7,11,8,16,9,16,12,18	11 ± 4
3	Kidney capsule	Cy-A	26,34,27,36.36,24,29,32,29,26	30 ± 6

Glucose Tolerance Test:

Glucose tolerance tests were carried out in transplanted mice that had been maintained normoglycemic state for more than 30 days. Five mice from each group were infused with 1 g /kg glucose after 30 days of transplantation. The mice were able to normalize their blood glucose. In normal mice and mice transplanted with islet microcapsules, Plasma glucose peaked at 30 minutes. And returned to base line levels by 120 minutes. Diabetic mice showed higher glucose levels after 120 minutes. Compared to the normal mice with transplanted mice, there was no significant difference in blood glucose levels at 0,30,60,90 and 120 minutes, **fig (4).**

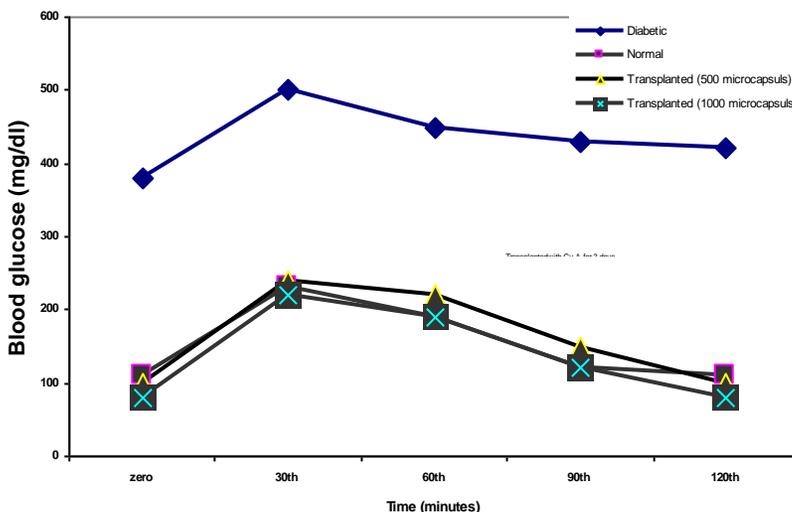


Fig (4) Blood glucose levels in response to orally glucose (1g/kg) at 30 days following transplantation.

Histological Examination :-

The histological examination of islets grafted under the kidney capsules revealed that is The islets were immediately beneath the kidney capsule and appeared normal (fig 5)

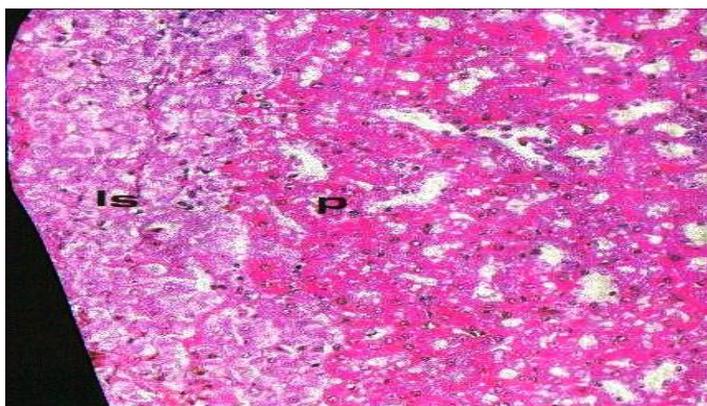


Fig (5) Light photograph showing hamster islets transplanted (Is) under the kidney capsule (KC) , The kidney parenchyma (P) appears normal.

Discussion

All streptozotocin injected mice exhibits diabetic criteria including hyperglycemia , polyurea and progressive weight loss. Diabetes induction in this study is shown to agree with the results of **Harlan , et al., (1995) and Leow, et al., (1995)**.

In the present study, the using inductal collagenase technique, approximately 414 ± 48 intact islets of each adult rat pancreas. The viability of the isolated islets with this method were comparable with or even better than those in previous reports by others (**Faustman et al., 1983; Yasunami et al., 1983; Gotoh, 1987. and Wolters et al., 1995**).

The insulin release from the islet gradually increased during 24 hours of culture, and the insulin content of cultured islets was relatively lower than that of freshly isolated (fig 10). This in agreement with some previous reports by other investigators (**Lacy, et al., 1976; Robinovitsh, et al., 1978**). which showed that fresh islets isolated by conventional agitation with collagenase solution responded to

a glucose stimulus immediately after isolation, and that responsiveness of the islets was better than that of the cultured islets and **Gotoh, et al., 1987**.

Islets of Langerhans have been considered extremely immunogenic sometimes surviving only for one or more days if transplanted across a strong histocompatibility barrier **Barker, et al., 1980**.

The data indicated that mice transplanted with islets under the kidney capsule and treated cyclosporine-A enjoy normoglycemia for a maximum eleven days , Moreover, a daily administration of cyclosporine-A causes the graft survival to be prolonged and enjoy normoglycemia as long as cyclosporin-A is given.

It is clear that the introduction of cyclosporin A (CyA) has resulted in significant improvement in transplant results and has external transplantation a routine procedures. The survival rate of islet transplantation xenografts was significantly improved under cyclosporin A immunosuppression, primarily due to the ability of this agent to prevent rejection of histocompatible grafts (**Opelz, 1992**).

It was suggested that CyA may either directly inhibits the function of unclear proteins critical to the T - lymphocytes activation (**Ream, 1992**) or may prevents the transduction of the mitogenic signal resulting from receptor ligation, to the nucleus at a stage after the rise in intracellular calcium levels (**Angela, 1990 and Riesbeck et.al., 1994**). CyA may also function as an immune inhibitor by impairing the ability of activated helper T - cells to respond to IL - 2, probably by limiting interleukin -2- receptor (IL-2R) expression in which IL-2 is an essential cofactor in the activation of both cytotoxic T - lymphocytes and B - cells in acute rejection episodes (**Winkelstein, 1994**).

While cyclosporin A has remarkable immunosuppressive properties, it also produces acute nephrotoxicity which may be purely functional or both functional and morphological. This kidney toxicity represents a serious limitation to its use as an immunosuppressive agent, but the benefits of CyA clearly outweigh the adverse effects of kidney (**First, 1993 and Cardoso et al., 1996**).

In summary, It may concluded that , it is possible to create an immunoprivileged site under the kidney capsule in xenograft transplantation of pancreatic islet cells by administration of cyclosporine-A. It is hoped that the results of the present study could be applied to human of both sexes especially when the use of cyclosporine-A gave encouraging results.

References:-

1. ANGELA G.P.(1990). Lymphokine gene expression in vivo is inhibited by cyclosporin A. *J.Exp. Med.*, 171:533-544.
2. AVILLA,J.; TSUJUMURA,T.; OBERHALZER,J.; CHURCTELL,T.; SALIHI,P.; JANES,A.M.; LAKEY,J.R.T. (2003): Improvement of pancreatic islet isolation outcomes using Glutamine perfusion during isolation procedures. *Cell Transplant.*, 12: 1230
3. BARKER, C.F., NAJI, A. AND SUIVS,W,K. (1980). Immunologic problems in islet transplantation. *Diabetes*,29: 86 – 92.
4. CALNE, R. Y. ; ALEXANDER, G. P. AND MURRAY, J. E.(1962). A Study of the Effect of Drugs in Prolonging Survival of Homologus Renal Transplantation in Dogs. *Ann. NY. Ascand. Sci.*, 99 : 743 – 761.
5. CARDOSO.L.R.,SANTOS, O.F.P., BIOM, M.A., BARROS, E.G., AJZEN,H. AND SCHOR.N.(1996). Fructose -1,6diphosphate: potential protection in cyclosporine induced renal impairment. *Nephron*,72: 67-71.
6. FERGUSON, R. M. AND FIDELUS, G. R. (1982). Immunosuppressive Action of Cyclosporin in Man. *Transplantation Proceeding.* 15 : 2485 – 2489.
7. FIRST,M.R.(1993).Renal allograft survival after 1 and 10 years: comparison between pre-cyclosporin and cyclosporin data. *Nephrol.Dia. Transplant.*, 9: 90-97.
8. FUSTMAN, D., HAUPTFELD, V., LACY, P. AND DAVIE, J. (1983). Autograft prolongation by immunization with donor blood depleted of Ia-bearing cells. *Transplant. Proc.*, 15: 1341 – 1343.
9. GETOH, M.; MAKI, T. AND KIYOIAMI, T.(1985): An improved method for isolation of mouse pancreatic islet. *Transplantation*, 40(4):437.
10. GETOH, M.; MAKI, T.; SALOMI, S.; PORTER, J.; WEIR,S.D.; O*HARA,C.J. AND MONACCO,,A.P. (1987): Reproducible high yield of rat islets by stationary in vitro digestion following ductal or portal venous collagenase injection. *Transplantation*, 43: 725.
11. HARLAN, D.M., BARNETT,M.A., ABE,K., PACHOLD,K.,PATTERSON,N.B.AND GRAY,G.S.(1995). Very low dose streptozotocin induced diabetes in insulin promoter-mB7-1 transgenic mice *Diabetes*, 44:816-823.
12. IWATA, H.; KOBAYASHI, T.; TAKAGI, T.; OKA, T.; YONG, H.; AMMIYA, H.; TSUJI, T. AND ITO, F. (1994): Feasibility of agarose microbeads with xenogeneic islet as a bioartificial pancreas. *J. Biomed Mater. Res.*,28(9):1003.
13. LACY, P. E. (1993): Status of islet cell transplantation. *Diabetes Rev.*, 1 (1):76.
14. LACY, P.E., MOSKAIIEWSKI, M.(1976). Method for the isolation of intact islets of langerhans from the rat pancreas. *Diabetes*, 16:35.

15. LEOW, C.K., GRAY, D.W.R. AND MORRIS, P.J. (1995). The long term metabolic fun of intraportal and renal subcapsular islet isograft and the effect on glomerular basement membrane thickness in rats. *Diabetologia*, 38:1014-1024.
16. OPLES, G. (1992). Collorative study: 10 years report. *Transplantation Proceedings*, 24: 2342-2355.
17. PESCE, A.J.; SCHROEDER, T.J. AND FIRST, M.R. (1990): Evaluation of cyclosporin monitoring by nonselective fluorescence polarization immunoassay. *Transplantation proceedings*, 22: 1171-1174.
18. REAM, G.H. (1992). Molecular mode of action of cyclosporine and FK-506 in human thymocyte. *J. Autoimmun.*, 5: 159.
19. RIESBECK, K., GULLBERG, M. AND FORSGREN, A. (1994). Evidence that the antibiotic ciprofloxacin counteracts cyclosporin independent suppression of cytokine production. *Transplantation*, 57(2):267-272.
20. WINKELSEIN, A. (1994). Immunosuppressive therapy. Cited from: *Basic and clinical immunology*, ed. by Slites, O.P., Terr, A.J. and Parslow, T.G., Large medical book, Ltd., USA. Pp. 765-780.
21. WOLTERS, G.H.J., VOSCHPORKEUTER, G.H., LIN, H.C. AND SCHLFIGAOIDE, R.V. (1995). Different rules of class II clostridium histolytica collagenase in rat pancreatic islets isolation, *Diabetes*; 44:227-233.
22. YASUNAMI, Y., LACY, P.E., AND FINKE, E.H. (1983). A newsite for islet transplantation – a peritoneal-omental pouch, *Transplantation*, 36:181.