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TREATMENT OF STREPTOZOTOCIN INDUCED DIABETES IN MALE RATS BY IMMUNOISOLATED TRANSPLANTATION OF ISLET CELLS

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ABSTRACT

Insulin injection is the main way to combat against insulin-dependent diabetes mellitus effects. Today in some laboratories in the world, the investigators are trying to find some treatments for this disease with insulin-secreting pancreatic islet cells transplantation. Donor tissue in each step of work was prepared from 36 adult male wistar Rats weighted 250-300 grams (75-90 days). Transplantation was done in rats after 2-4 weeks induction of diabetes with 60mg/kg of streptozotocin injection by intravenous method. Encapsulation of pancreatic islet cells allows for transplantation in the absence of immunosuppression. This technique that is called "immunoisolation" is based on the principle that transplanted tissue is protected for the host immune system by an artificial or natural membrane. In this study, the levels of insulin, C-peptide and glucose in diabetic rats have been reached to normal range as compared to un-diabetic rats in 20 days after transplantation of islet cells, so that testis is immunoisolated place for islet cells transplantation. Inside the testis subcutaneously and intrapretoneally implantation of pure islet cells graft, that is a natural immunoisolation method, rapidly and permanently normalized the diabetic state of streptozocin-administered animals.

KEY WORDS

Immunoisolation, Islet cells, Transplantation, Diabetes

INTRODUCTION

Diabetes mellitus is a syndrome that disturbs the metabolism of carbohydrates, fat and protein and results from shortage or lack of insulin secretion or reduced sensitivity of the tissues to insulin. There are two types of diabetes mellitus (1). 1. Diabetes Type I, which is also known as insulin-dependent diabetes mellitus (IDDM) and results from lack of insulin (2). 2. Diabetes Type II, which is also known as non-insulin-dependent diabetes mellitus (NIDDM) and results from reduced sensitivity of the target tissues to the metabolic effects of insulin (3). This reduced sensitivity to insulin is often referred to as 'resistance to insulin'.

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In both types of diabetes mellitus, the metabolism of all foodstuffs changes. The basic effect of lack of insulin or resistance to insulin on the metabolism of glucose is the prevention of effective reception and consumption of glucose by a major part of the body cells, except for cerebral cells (4). As a result, blood glucose concentration increases, cellular consumption of glucose falls increasingly to lower quantities and use of fats and proteins increases. Diabetes Type I is the lack of production of insulin by pancreatic beta cells (5). Damage of pancreatic beta cells or diseases that disturb the production of insulin can also result in Diabetes Type I. Viral infections or autoimmune disorders may be involved in the destruction of beta cells in many patients infected with diabetes Type I although heredity also plays a major role in aptitude of beta cells for destruction by these offensive agents. In some cases, a hereditary tendency to degeneration of beta cells may exist even without any infection or autoimmune disease.

The usual beginning of Diabetes Type I may be at the age of

14 in human being and, therefore, is mainly referred to as juvenile diabetes mellitus (6). Diabetes Type I may appear very abruptly or in a phase of few days or weeks with three effects: very high increase of blood sugar up to 1200-1300 mg in 100 ml, excretion of glucose in urine or dehydration. The long-term effects of insulin-dependent diabetes mellitus has become a major problem to the health of the patients and it has become known today that control of the effects of improper blood sugar homeostasis is possible by injecting insulin. However, its strict control by injection of insulin is difficult. The symptoms of interrupted secretion of insulin in rats having been injected, with diabetes chemically or by streptozotocin can be removed by transplantation (7), which is either transplantation of the entire pancreas or transplantation of pancreas components while the latter is carried out much more easily than the former. Transplantation of pancreas components can be in one of the following forms: 1. Transplantation of dissociated Langerhans islets; 2. Transplantation of mass of the Langerhans islets cells; 3. Transplantation of embryonic tissues; 4. Transplantation of neonatal tissues, which has been carried out successfully in different areas such as the liver, kidneys, spleen, testes subcutaneously. However, transplantation of the Langerhans islets as a logical solution for treating these patients is still argued (8-9). Transplantation of the Langerhans islets cells is a new method for treating diabetes. Standardizing and optimizing separation and purification conditions of Langerhans islets cells is one of the most important phases of the transplantation. It is only after achieving and stabilizing this method that the researchers will be able to carry out studies for solving the transplantation problems. Factors such as the number of implanted cells, capacity of performance of the new medium and the size of cell groups are effective in the relative control of the metabolism after transplantation (9) .In this research, the transplantation of the Langerhans islets cells in a group of diabetic samples to underneath the inside of the testis subcutaneously and, in another group, to the peritoneal space and restoration of the blood glucose level to the normal range.

MATERIALS AND METHODS

Collagenase, Crystalline Trypsin, Bovine Pancreatic DNase, 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-Ethan-sulfonic acid (HEPES), silicon dicholorodimethylsilan and bovine serum albumin V were supplied by Merck , German Company. Percol is a commercial solution made up of silicon particles, coated with polyvinyl pyrolidone. Silicon coated with polyvinyl pyrolidone is used for sterilized cellular separation. The zanosar or streptozotocin (STZ) was supplied by Pharmacia, Swedish Company. Ethylene glycol-bis (β-amino ethyl ether)-N, N, N', N',-tetra acetic acid is a product of Sigma.

Mediums : All media were sterilized by 0.22 micrometer filters and the materials were autoclaved or they were purchased as sterilized single use materials. The glass containers used for collecting Langerhans islets cells were siliconized with silicon. Silicon-coating consists of 30 min. incubation of the containers to be used with sterilized solution of 10 ìg/ml silicon after washing with distilled water. Separation of the islet and cellular purification were carried out in an EH buffer medium consisting of 123 mM NaCl; 1.8 mM $CaCl_2$; 0.8 mM $MgSO_4$; 5.4 mM KCl; 1.0 mM NaH_2PO_4; 4.2 mM $NaHCO_3$; 2.8 mM Glucose and 10 mM HEPES.

The medium was completed with 2.5% and 5% solutions from V fraction BSA (Bovine Serum Albumin), its pH was controlled to 7.3 with 5% CO_2 in room temperature and the final volume reached to one liter.

Animals Used : The donor tissues were taken from 38 male adult Wistar rats weighting 250-300 gr. (75-90 days old). The Islet cells have been purified from pancreas of 38 rats and transplanted to 19 rats and other 19 rats have taken as control. Transplantation was carried out in rats that had been afflicted with diabetes 2-4 weeks before transplantation by intravenous injection of 60 mg/kg dose of streptozotocin. Streptozotocin induces diabetes within three days by destroying the beta cells. Transplanted animals and diabetic and non-diabetic control groups were kept individually and separately in metabolic cages and were controlled in terms of feeding and metabolism. The amount of food (gr), water (ml) and urine (ml) were measured volumetrically daily and, every 2-4 weeks, the levels of C-peptide, insulin and glucose in blood serum were measured so that chemical diabetes was confirmed in rats that had undergone injection of streptozotocin and their insulin had been minimized and also signs of recovery were approved in rats receiving the transplantation.

Separation of the Langerhans islets and Preparation : The pancreatic Langerhans islets were separated from the donor rats with the modified collagenase digestion method. Then the cells were changed to single cell suspension. Finally, the cellular suspension was dissolved in physiological serum (9-11).

Measurement of Glucose, Insulin and C-Peptide in Blood : Normal, diabetic and donor rats were anesthetized with ether (two min. contact with ether does not affect blood glucose, insulin or C-peptide concentrations). Each time 1ml of blood

was taken from them in order to measure glucose, insulin and C-peptide. Blood was taken from the heart. The samples were collected in sterilized tubes and kept at 4°C and, after separating the clot, the serum was separated by centrifuging. Blood glucose was measured by glucose oxidase method and serum insulin, C-peptide by radioimmunoassay method. This phase of the work was carried out once every 2-4 weeks in diabetic and donor rats as well as in their control counterparts.

Flow Cytometery : Our aim in flow cytometery is to obtain information on the homogeneity of the beta cells and the percentage of homogeneity of these cells in cellular suspension obtained at the end of purification of the Langerhans islets cells so as to determine the percentage of beta cells in the suspension for transplantation. In view of the considerable difference in the sizes of types of Langerhans islets cells, a sample cellular suspension solution can be injected into the flow cytometery system and the appropriate graph can be prepared, which indicates the types of cells and their percentage in the suspension. (11, 12).

Transplantation of Langerhans Islets Beta Cells : In brief the purification of Langerhans islets beta cells was carried out as follows: The Langerhans islets suspension was first kept in room temperature for 8 min. and then aspired by a 9 inch length siliconized Pasteur pipette. Then, trypsin with final concentration of 25 μg/ml and DNase with final concentration of 1.5μg/ml were added to it. The degree of enzymatic differentiation and dissociation were regularly examined with phase-contrast microscope and, when 50-60% of the cells converted into single units, the work was stopped. This condition often occurs after 10 min. The suspension of the Langerhans islets cells was diluted immediately with 40 ml of EH buffer and the whole collection was put in ice bath and filtered by passing through a 63μ diameter nylon sheet .Thus , the undigested materials and the big cell masses were eliminated. The resulting product, which contains single cells, was centrifuged for 6 minutes in 300 g. The sediment was further changed to suspension and centrifuged. In this stage, the cellular sediment was suspended in isotonic Percol solution with density of 1.040 g/ml, and was put in ice bath for 10 min. so that the cellular suspension was layered and, thus, the dead and destroyed cells and cell pieces obtained in consecutive centrifuging were eliminated. Finally, in the cellular suspension layer, the healthy cells were dissolved in the physiological serum (13). The purified cells of the Langerhans islets were transplanted to diabetic rats stimulated with STZ in a group of diabetic samples inside the testis subcutaneously and, in another group, to the peritoneal space. Transplantation of the cellular suspension in the physiological serum was carried out by using needle no. 20 in specified areas in each injected rat.

Biopsy and Histology : Two months after transplantation, the transplanted areas were vivisected in order to identify the Langerhans islets cells grown in the transplant receptor. For this purpose, the testes of the recipient rats were removed, stabilized in 10% formalin buffer and given to the Electronic Microscope Department for light microscopic examination. After framing in paraffin, thin 3-micron tissue cuts were created. Staining was carried out by hematoxylene and eosin stain in order to recognize the transplanted islets (Fig. 1 with Leitz microscope).

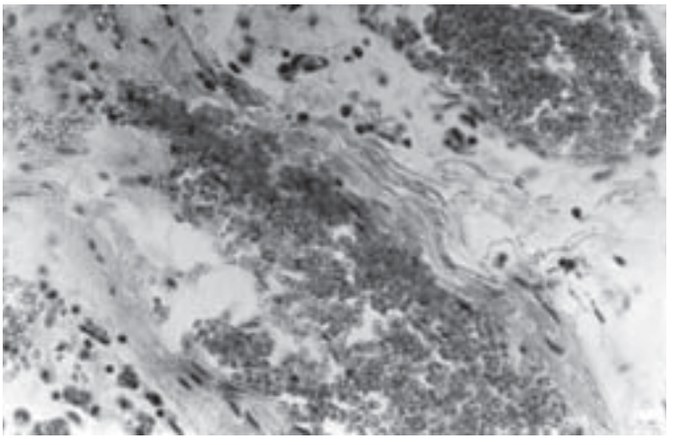
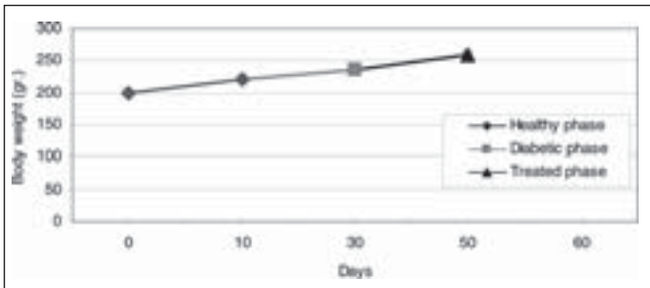


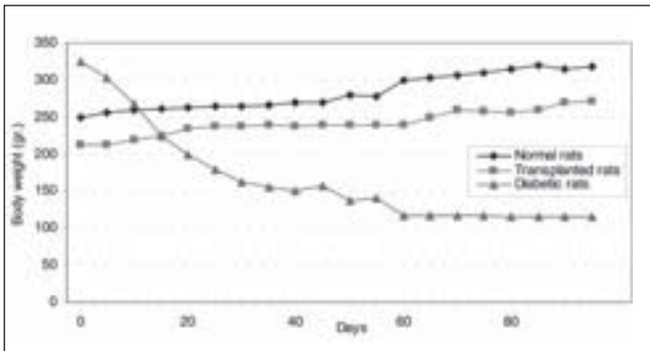
Fig. 1: The existing transplanted Langerhans islet cells inside the testis subcutaneous of the diabetic rat which has been treated via transplantation of Langerhans islet cells. Observed after taking a tissue from the testis and stabilizing in 10% formaldehyde solution

RESULTS

Normal levels of glucose, insulin and C-peptide in healthy adult rats measured 134.6±4.96 mg/dl, 2.1±0.19 mIU/L and 0.056±0.003 ng/ml, respectively. Daily consumption of water and food in healthy adult rats measured 30±5ml and 10±2 gr, respectively. Daily urine volume in healthy adult rats measured 10±1ml. In diabetic rats the levels of glucose, insulin and C-peptide measured 500±19.6 mg/dl, 1.5±0.17 mIU/L and 0.042±0.002 ng/ml, respectively. Daily consumption of water and food in them measured 145±5 ml and 45±4 gr. Daily urine volume in diabetic rats measured 130±5ml. Changes of weight in adult and non-adult diabetic rats vary. Since the non-adult rats are in the growing age, diabetic loss of weight is not seen in them and they even show a slight weight gain. (Graph 1), shows continuous changes in average of body weight in 8 non-adult rats in three healthy, diabetic and treated phases and a slight increase in the weight of non-adult rats in the



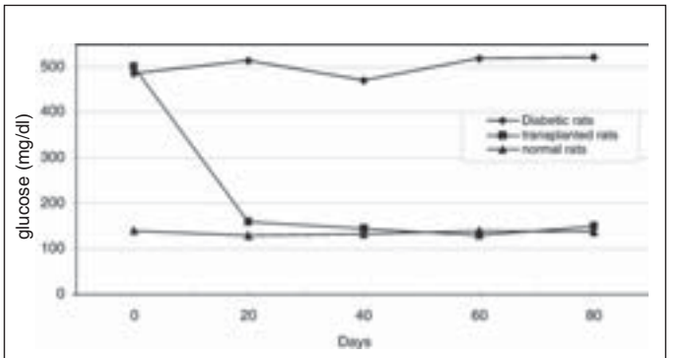
Graph 1 : shows continuous changes in average of body weight is 8 non-adult rats in three healthy, diabetic and treated phases



Graph 2 : Shows a comparison of the curves relating to the average changes in 19 body weight in the three phases of healthy, diabetic and transplanted rats. These curves reveal loss of weight and thinness due to streptozotocin used for diabetes induction in adult rats and elimination of these effects after transplantation of pancreatic Langerhans islets cells.

three healthy, diabetic and treated phases. In adult rats, however, diabetes is accompanied by loss of weight. 2-4 weeks after diabetes induction and observing its effects, transplantation of purified Langerhans islets cells was carried out in collagenase method with 91% beta cells in the cellular suspension for treatment of diabetes. (Graph 2), shows a comparison of the curves relating to the average changes in body weight in the three groups of healthy, diabetic and transplanted rats. This diagram reveals the loss of weight and thinness due to streptozotocin used for diabetes induction in adult rats and elimination of these effects after transplantation of pancreatic Langerhans islets cells. By analyzing of variance (ANOVA) with SPSS.12 program, the standard error mean (S.E.M.) is found to be equal to 8.19, F=40.87, df=2, 57, p<0.001, which well indicates the weight loss in diabetic adult rats. By carrying out this operation, signs of recovery were gradually observed in the rats. So that the levels of glucose, insulin and C-peptide in transplanted rats were 145±11.2 mg/dl, 1.98±0.25 MIU/L and 0.042±0.008 ng/ml, respectively. (Diagram 3), shows the average of the changes in the level of glucose in blood serum of 19 diabetic rats treated by transplantation of Langerhans islets cells and normal ones during 80 days, that by using of ANOVA on transplanted and diabetic rats, we found the standard error mean (SEM) is equal to 48.1, F=903.18, df=2,11, p<0.001. (Diagram 4), shows the average of the changes in the level of insulin in blood serum of 19 diabetic rats treated by transplantation of Langerhans islets cells and normal ones during 80 days, in which the ANOVA on transplanted and diabetic rats shows the standard error mean (SEM) is equal to 0.088, F=8.53, df=2,12, p<0.005.

(Diagram 5), shows the average of the changes in the level of C-peptide in blood serum of 19 diabetic rats treated by



Graph 3 : shows the average changes in the level of glucose in blood serum of 9 diabetic rats treated by transplantation of Langerhans is lets cells and normal ones during 80 days.

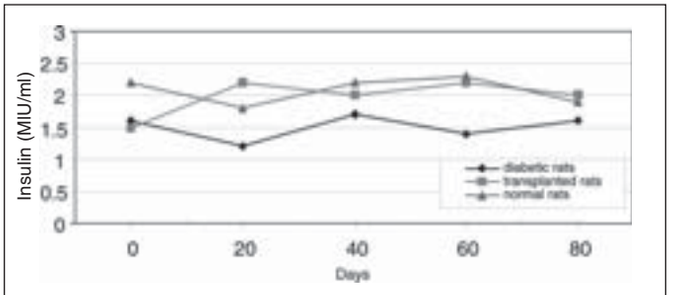


Diagram 4 : Shows the average changes in the level of insulin in serum of 19 diabetic rats treated by transplantation of Langerhans islets cells and normal ones during 80 days

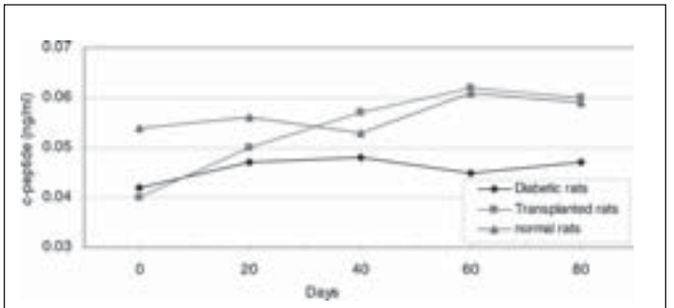


Diagram 5 : shows the average changes in the level of C-peptid in serum of 19 diabetic rats treated by transplantation of Langerhans islets cells and normal ones during 80 days

transplantation of Langerhans islets cells and normal ones during 80 days, in which the ANOVA on transplanted and diabetic rats shows the standard error mean (SEM) is equal to 0.002, F=4.85, df=2,12, p<0.029. Consequently, data analysis of glucose, insulin and C- peptide show the high significant difference between transplanted and diabetic rats serum and confirm the success of the transplantation project. Moreover, the daily consumption of water and food reached the relatively normal limit of 40±5 cc and 30±5 gr, respectively and daily urine in treated rats measured 35±5 cc.

DISCUSSION

With the transplantation of the cellular suspension obtained, it was expected that, due to secretion of insulin by transplanted beta cells, the level of blood serum glucose would fall to the normal, healthy limit and the amount of insulin and C-peptide of the Blood would increased and the clinical manifestations of the disease, such as polyuria, polyphagia and polydipsia would be eliminated. All these were clearly seen immediately and in full, the day after transplantation (12). The considerable point in this research is that inbred rats were not used as receptor or donor of the transplantation. In spite of this, however, no sign of rejection of the transplantation was observed (14). To explain this, one must say that the phenomenon of immuno-isolation due to the effect of transplantation of Langerhans islets cells, in an immunity-quarantined space, prevented access of the immunity cells to the external transplanted cells and rejection of the transplantation (15). Transplantation of Langerhans islets cells is generally used for treating a type of diabetes that results from the autoimmune destruction of beta cells of the islets. Therefore, as expected, this autoimmune process also continues with respect to the transplanted beta cells. In this research, by carrying out transplantation of Langerhans islets cells in parts of the body with a special immuno-isolated position, the risk of destruction of the transplanted beta cells by the autoimmune process in the transplantation receptor was completely eliminated (1). The technique of transplantation of the Langerhans islets cells inside a capsule in the absence of immunological inhibitors to support the transplanted tissue against the host immunity system is a new way of success in this path (16). In this process, the islets can be encircled in a semi-permeable membrane that allows food and oxygen to reach the Langerhans islets and the insulin to be released into the blood flow while, at the same time, it creates a mechanical barrier separating the potentially destructive immunity cells and the antibodies from the islets cells and thus preventing the rejection of the transplantation (17). Statistical data relating to F, df, values of P, food, water, urine, body weight and SEM in the entire test groups, compared to the findings of Lanza et al, Gray et al, and Pipellers et al, (1, 3) show the higher success of the transplantation of the Langerhans islets in rats as achieved in their work.

The Langerhans islets constitute 1-2% of the weight of the pancreas. Their diameter is 0.2-0.5 mm. The number of the separable Langerhans islets is 360,000 islets. Each islet has thousands of cells. In the infusion method of the islets through the portal vein into the liver and reproduction of islets there and returning them back-up, approx. 9,000 Langerhans islets per kilogram of body weight of the rat are needed. In other words, for a rat weighting 250 gr., approx. 2,500 islets are needed. Secondly, in this method, other than the further need to islets, there is the major problem of the HLA compatibility

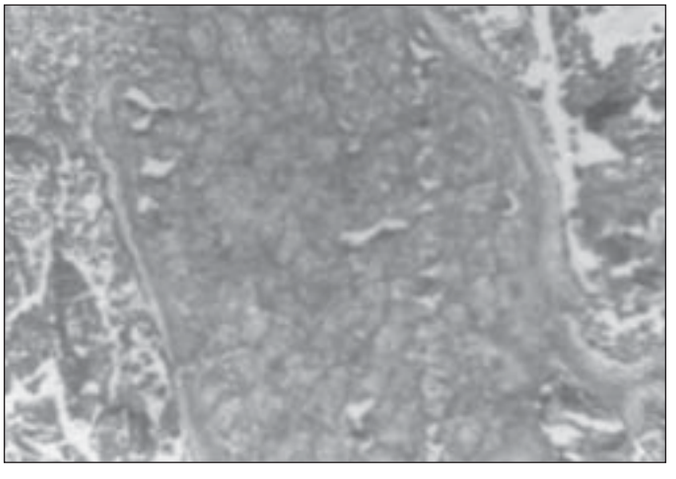


Fig 2 : The cells of the tissue under the testis subcutaneous of the normal rat While no changes is observed in it

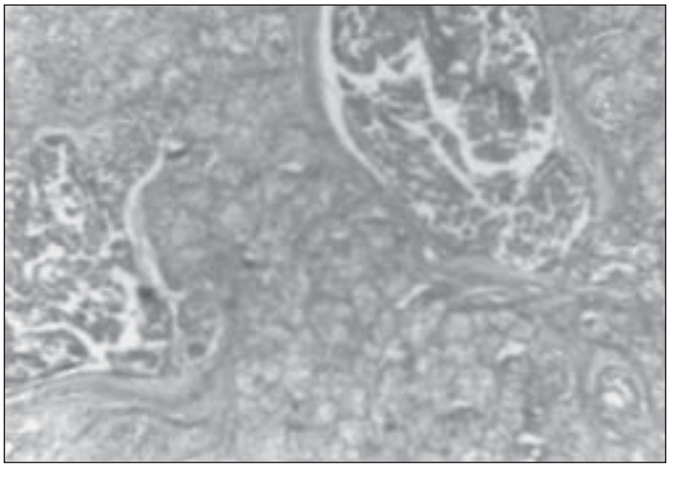


Fig 3 : The cells of the tissue under the testis subcutaneous of the diabetic rat While no changes is observed in it

complex of the main tissue in the receptor and donor of the islets, which we are studying further. Thirdly, this method is inexpensive and available to everyone. However, the method of transplantation of the Langerhans islets inside the testis subcutaneous of diabetic rats (Fig. 1) successfully shows the transplanted Langerhans islets, i.e. in an immunity-quarantined space, it prevents access of the immunity cells to the external transplanted cells and prevents rejection of the transplantation. The rat is cured 100% as a result of secretion of the transplanted Langerhans islets cells under the testes. In this method, approx. 5,000 islets are needed for each kg of rat body weight. This method is important because of its simplicity and accessibility. Actually, by purifying the pancreas of an adult normal rat a successful transplantation to a diabetic rat can be done, while no change is observed in the tissue inside the testis subcutaneous of the normal and diabetic rats (Fig. 2 and 3). We are still studying intra-peritoneal injection method.

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