Evaluation the effect of autologous bone marrow – derived mesenchymal stem cells as a treatment in diabetic rabbits

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ABSTRACT

Background: Type 1 diabetes is the result of an autoimmune attack against the insulin-producing beta cells of the pancreas. Current treatment for patients with type 1 diabetes typically involves a rigorous and invasive regimen of testing blood glucose levels many times a day along with injections of recombinant insulin. Many recent researches have shown that stem cell therapy can be the best choice for treatment of this disease. The aims of this research were investigating regeneration of pancreatic beta cells of type 1 diabetic rabbits after stem cell transplantation.

Materials and Methods: 32 rabbits weighting an average of (2.5 - 3 kg) were used in this experimental study, and divided into 2 groups as follows; group A (contains 16 controlled diabetic rabbits received insulin as a treatment) and group B (contains 16 diabetic rabbits received autologous mesenchymal stem cells as a treatment). The induction of diabetes was achieved by a single dose of intravenous injection of the Alloxan, which was administered to the rabbits via the marginal ear vein, mesenchymal stem cells were differentiated into insulin - producing cells and reimplanted into the rabbits of group B with daily monitoring of blood glucose level and body weight.

Results: The insulin - producing cells regulated the hyperglycemia resulted from diabetic rabbits, 7 to 9 days after reimplantation the blood glucose level were decreased from about (400 mg/dl) into (180 mg/dl).

Conclusions: Islet-like functional cells can be differentiated from bone-marrow mesenchymal stem cells (MSCs), which may be a new procedure for clinical diabetes stem -cell therapy, these cells controlled blood glucose level in diabetic rabbits as the effect of insulin. MSCs play an important role in diabetes therapy by islet differentiation and transplantation.

Key words: Type 1 diabetes rabbits, bone marrow mesenchymal stem cell, stem cell therapy. (J Bagh Coll Dentistry 2012; 24(Sp. Issue 2):55-60).

INTRODUCTION

Diabetes mellitus is a chronic disease in which the body either does not produce enough, or does not properly respond to, insulin, a hormone produced in the pancreas. Hyperglycemia or high blood sugar, a common condition caused by the uncontrolled diabetes, damage the body system seriously, especially the nervous system and blood vessels. Current diabetes treatments just aim to lower the blood sugar through diet, exercise, medication with tablets and insulin. Therefore, researchers have been carrying out new diabetes treatments such as artificial CD3 antibody, pancreas graft and pancreatic islet cell graft in order to restore the insulin production of the body. However, these therapies are expensive, low compatibility, and easy to be rejected by the receiver's immune system (1,2). Diabetic mellitus (DM), one of the leading causes of morbidity and mortality in many countries, is caused by an absolute insulin deficiency due to the destruction of insulin secreting pancreatic cells (type 1 DM) or by a relative insulin deficiency due to decreased insulin sensitivity, usually observed in overweight individuals (type 2 DM).

In both types of the disease, an inadequate mass of functional islet cells is the major determinant for the onset of hyperglycemia and the development of overt diabetes. Islet transplantation has recently been shown to restore normoglycemia in type 1 DM (3).

Multipotent stem cells have been described within pancreatic islets and in nonendocrine compartments of the pancreas, and these cells have the capacity of differentiating into pancreatic islet-like structures. Furthermore, cells that do not reside within the pancreas, such as mesenchymal stem cells, hepatic oval cells and cells within spleen, have been differentiated into pancreatic endocrine hormone-producing cells in vitro and in vivo (4,12). Mesenchymal stem cells (MSC) have received widespread attention because of their potential use in tissue engineering applications (5).

MSC are defined as self-renewable, multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages. Also MSCs are defined as non-hematopoietic cells that are able to replicate for a long time while maintaining their multilineage differentiation potential. These cells were first recognized with the capacity to generate three osteoblastic, chondroblastic and adipocytic lineages (14,15). Many recent research studies have demonstrated that MSCs may possess more extensive differentiation potentials than expected. These cells have been shown that are able to...
differentiate into many other specialized phenotypes other than the skeletal lineages (osteocytes, chondrocytes and adipocytes) including neural cell, pancreatic cell, cardiomyocyte, renal epithelial cell, intestinal cell and keratinocyte. MSCs were first isolated using their plastic adherent properties and till now this property is utilized as current method for MSCs isolation from variety of species including human, mouse, rat, cat, rabbit, pig and baboon by many researchers.

**MATERIALS AND METHODS**

In this experimental study 32 adult rabbits weighting an average of (2.5 - 3 kg) were used and maintained under control conditions of temperature, drinking and food consumption. The experimental animals were divided into two groups as follows:

- **Group A**: contains 16 controlled diabetic rabbits received daily insulin as a treatment in a dose of 0.1 mg/ kg of body weight.
- **Group B**: contains 16 diabetic rabbits received autologous bone marrow-derived stem cells as a treatment.

**Induction of Diabetes Mellitus in Rabbits**

All animals were weighted to calculate the dose of anesthesia and alloxan which were given to them (Figure 1). The general anesthesia was induced by intramuscular injection of xylazine (0.4 mg/kg of body weight) plus ketamine hydrochloride (40 mg/kg of body weight). After 10 to 15 minutes the animals were anesthetized, the skin covering the ears of the rabbits was shaved carefully from the outer surface to expose the external auricular vein. The rabbits were injected by a single dose (120 mg/kg) intravenous injection of the pancreatic beta-cells toxin monohydrate (Alloxan), which was administered to the rabbits via the marginal ear vein (Figure 2). Severity of the induced diabetic state was assessed by daily monitoring of blood glucose levels with a calibrated glucose meter (few drops from the ear) and daily estimation of the body weight (Figure 3). For determination of blood glucose level, the animals whose blood glucose level was greater than 200mg / dl were indicated as hyperglycemic. Five to seven days after injection, Alloxan induced diabetes by destroying the beta cells of the pancreas; the blood glucose level was elevated above the 200mg/dl (Figure 4).

**Isolation of MSCs from the Bone marrow**

The surgery was performed under well sterilized condition and gentle surgical technique. The surgical towels were placed around the site of operation; the site chosen for operation was the proximal tibia metaphysis of the right limb (Figure 5). Skin incision was done by using a sharp blade to expose the muscle (Fig.6). Then the muscle was dissected to expose the tibia (Figure 7). By intermittent drilling with (1 mm surgical drill) and continuous, vigorous irrigation with sterile normal saline, a guide hole was made (Figure 8). By using sterile syringe (5ml) that contains few drops of heparin (to prevent blood clotting) the bone marrow was aspirated as soon as possible (Figure 9). After that the area was
washed very well with a sterile normal saline, the muscle was sutured with 3/0 absorbable (catgut) suture (Figure 10). The skin was sutured with interrupted 3/0 silk suture (Figure 11).

![Figure 5: The site of operation](image1)

![Figure 6: Skin incision](image2)

![Figure 7: Dissection of the muscle](image3)

![Figure 8: 1mm guide hole was made](image4)

![Figure 9: Aspiration of bone marrow](image5)

![Figure 10: Muscle sutured with cat gut suture](image6)

![Figure 11: Skin sutured with silk suture](image7)

Inside the hood (previously sterilized by UV Rays over night), the bone marrow was inserted into two test tubes (t.t.), equal volume of phosphate buffer saline(PBS) was added to (t.t.) and shake very well until the solutions became homogenous .Then the two t.t. was put inside the centrifuge (2000 RPM) for 10 minutes. Inside the hood the top two thirds of the solution were removed (that contains non adherent cells). RPMI-culture media was added to the precipitate 1/3 of the t.t. & shake very well Until the media was became homogenous, then the media was added into a well sterilized plastic falcons & covered very well by a parafilm, finally the media was incubated at (37 °C, 5% Co2 & 95% air).The cells were checked periodically under inverted microscope, the culture media was changed twice a week for two weeks. With the medium changes, almost all the non adherent cells were washed away.

**Differentiation of MSCs into Insulin producing cells**

1- Inside the hood about 2/3 of the medium in the falcons was removed and pre-inducing medium was added to the remaining 1/3 of the falcons, the pre-inducing medium containing low glucose–RPMI (L-RPMI) supplemented with 10 mM nicotinamide, plus 1 mM beta-
mercaptoethanol and 10% of fetal bovine serum (FBS), then covered by a parafilm and incubated at (37 °C, 5% CO2 & 95% air) for 24 hours.

2- The medium was changed with fresh inducing medium, containing serum free high glucose–RPMI (H-RPMI), supplemented with 10 mM nicotinamide, plus 1 mM beta-
mercaptoethanol, then covered by a parafilm and incubated at (37 °C, 5% CO2 & 95% air) (for 10-12 days).

**Detection of Insulin producing cells**

The insulin producing cells can be detected by dithiazine (DTZ) stain. DTZ is a zinc-chelating agent known to selectively stain pancreatic beta cells because of their high zinc content (18).

**Figure 12: Reimplantation of MSCs**

Inside the hood about 2/3 of the medium was removed from the falcon, then 2 ml of DTZ solution was added for the remaining 1/3 of the medium in the falcon that containing the MSCs, the cells were incubated at (37 °C, 5% CO2 & 95% air) for 30 minutes and examined under inverted microscope.

**Reimplantation of MSCs**

5 ml of the medium was reimplanted to the rabbits by subcutaneous injection (Figure 12).

**RESULTS**

About 4 to 5 days after incubation the stem cells adhered to the base of the sterilized falcons and started to be elongated and became spindle in shape just like a fibroblast cells, some of them became growing to have a neuron-like shaped (Figure 13). 14 to 16 days after incubation, the cells grew as a monolayer which were completely attached with each other by a network like connection and filled all the base of the sterilized plastic falcons (Figure 14). Ten to 12 days after adding the differentiated medium the undifferentiated MSCs were typical of adherent spindle and fibroblasts-like. However, under differentiation condition changed from spindle-like cells into round or oval types, these cells were morphologically similar to pancreatic islet cells (Figure 15).

**Figure 13: Colonies of MSCs 4 days after incubation, the cells became neurons-like shaped, inverted**

**Figure 14: The monolayer of MSCs, 2 weeks after incubation, inverted microscope 40X.**
Almost a lot of insulin-like cells were stained positive to DTZ stain and they appeared like crimson-red appearance under inverted microscope (Figure 16).

**DISCUSSION**

In pancreas, insulin is produced and secreted by specialized structures, islets of Langerhans. Diabetes, which affects thousands of million people in the world, results from abnormal function of pancreatic islets. The main obstacle to successful islet transplantation for diabetes is the limitation of available insulin-producing tissue (19). The present study introduced to generate cells expressing insulin from rabbit's bone marrow – derived mesenchymal stem cells. This approach may provide a potential new source of pancreatic islet cells for transplantation. Bone marrow is an important source of easily procurable adult stem cells, in addition to the ability of bone marrow-derived stem cells to reconstitute the hematopoietic system (20). In the present study MSCs was successfully isolated from the bone marrow, this finding was in agreement with (21), who found extra pancreatic proinsulin-producing cells present in the liver, BM, spleen, adipose tissue, and thymus in hyperglycemic animals and that the majority of these proinsulin producing cells were derived from the donor BM, as evidenced by BM transplantation experiments. The routine method for MSCs isolation from bone marrow samples is to plate the bone marrow cells in plastic dishes in the presence of appropriate medium and to incubate the cultures in an atmosphere of 5% CO2 and 37°C temperature. The next step is to discard the non adherent cells by medium replacement, keep and expand the adherent population which mainly possesses a fibroblastic morphology (22). The cells are cultured in glucose-rich medium. Glucose is a growth factor for beta-cell replication in vitro and in vivo. Glucose has been shown to increase the insulin content in cells derived from adult stem cells. In the proliferation phase, the high glucose content may support the extra energy needed for cell division. In the differentiation stage, it could modulate specific gene programs linked to glucose sensing and insulin secretion (23). Several in vitro studies have shown that bone-marrow–derived stem cells could be reprogrammed to become functionally insulin-producing cells under certain culture conditions (24,25). In this experimental study The MSCs were successfully differentiated into pancreatic islet β-like cells. These cells were morphologically similar to pancreatic islet cells and have the ability for regulating rabbit's blood glucose level. High glucose concentration was considered as a potent inducer for pancreatic islet differentiation. Nicotinamide was used to preserve islet viability and function (26), β-mercaptoethanol was commonly used as a neurocyte inducer. In the present experiment, high glucose alone could not effectively induce MSC to differentiate into islet-like cells. After adding nicotinamide, they effectively transformed MSCs into islet-like cells. This implies that nicotinamide has been an effective inducer, or it protected the differentiated cells from dying or transforming into other cell types. β-mercaptoethanol increased the potency of nicotinamide. DTZ, a zinc-chelating agent, is known to selectively stain pancreatic beta cells crimson red. As they contain a large amount of zinc. Using this characteristic of DTZ, insulin-producing cells derived from rabbit's bone marrow mesenchymal stem cells was identified as well as cellular clusters. DTZ is a zinc-binding substance, and pancreatic islets from such animal species as mouse, rabbit, rat, dog, pig, and human are known to be stained crimson red by its treatment, because of their higher zinc contents compared with other tissues (27,28).

**REFERENCES**

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