Research Paper

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Uses of Stem Cells in Treatment of Experimentally Induced Diabetes Mellitus in Dogs

Medical Science

KEYWORDS :

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ABSTRACT

The current study aimed to highlight the ability of using differentiated stem cells in treatment of experimentally induced diabetes mellitus in dogs. Twenty native local male dogs aged 4-6 months were divided equally into four groups: Group I was control (left without any treatment), groups II, III, and IV were injected a single dose of alloxan (65 mg / kg wt) for inducing diabetes mellitus. Group II left as diabetic control (not treated), group III was given intravenous single dose of (1 ml/Kg wt) of cell free culture media at 10 days post alloxan administration, simultaneously group IV was administered by the same route autologous differentiated mesenchymal stem cells into insulin producing cells as a single dose of 2.5×106 cells/ml/Kg BW. The result showed typical clinical signs of diabetes mellitus, observed three days post alloxan injection and at the same time significant (P≤ 0.05) increase of blood glucose levels were recorded, these levels continued in elevation for 73 days in both groups II and III post alloxan injection, whereas significant (P≤ 0.05) lowering of blood glucose levels noted 4 days post cellular therapy, the gradual decline of glucose levels continued to 63 days. Conclusively the use of differentiated mesenchymal stem cells is an encouraging step in the treatment of diabetes mellitus and gives a glimmer hope to diabetic patients.

Introduction

Diabetes mellitus is a world-wide spread disease in human and to a lesser extent in dogs, besides the current global increase in the incidence of diabetes type – I, (reach 3% per year), it was predicted that the incidence will be 40% higher in 2010 than 1998 (Onkamoet.al. 1999). Stem cells are a subject of intense and increasing interest because of their biological properties and potential medical importance in treating and repairing the injured and damaged tissues, they are regarded as undifferentiated cells having the ability for proliferation, self-renewal and regeneration of tissues (Morrisonet.al.1997,Kochar 2004). Mesenchymal stem cells (MSCs) can be differentiated into many specific other cells such as osteoblast, neuron, chondrocytes… etc. (Dimoset.al. 2008). The surface markers of dogs MSCs are different according to their origin: those derived from umbilical cord blood (UCB-MSCs) are expressed surface markers such as CD29, CD44 and CD105 , while MSCs derived from bone marrow are expressed surface markers such as CD44 and CD90, (Csakiet.al. 2007, Kisiel et.al. 2012). Few researches had been done on the implications of cellular therapy in health practice in Iraq, therefore this is a preliminary manuscript in the veterinary field, aimed into studying the morphological characters of MSCs derived from bone marrow and their ability to differentiated into insulin producing cells (IPCs), used in treating diabetic dogs.

Materials and methods

Experimental animals:

Twenty male dogs, native local breed aged 4-6 months were used in the experiment, they were housed in special canneries belonging to the department of Surgery and Obstetric / College of Veterinary Medicine, University of Baghdad, Iraq .They were kept on ordinary pellet diet of commercial source ad-lib, water consumption was measured by using special containers.

Glucosedetermination:

It was determined in fastened dogs (for 12 hours) of all groups (prior to induction of diabetes mellitus) according to Barham and Trinder (1972), for baselinedata andthen through the periods of experiment .

Bone marrowaspiration: It was carried out from the trochanteric fossa of the femur bone of each dog in group (IV) only, according to Wellman and Radin (1999), special needle for aspiration of bone marrow with disposable syringe were heparinized and used for this purpose.

Isolation and Culturing of-derived MSCs: Isolation and purification of MSCs was carried out according toSun Yu et. al. (2007) with modification . Five ml of bone marrow samples diluted with 5 ml of mesencult ® (Invitrogen-USA), were centrifuged(8C°) at 2000 rpm for 10 minutes , the fat and serum layers were discarded and the cells pellet re-suspended with 5 ml of the same mesenchymal culture media supplemented with 10% fetal calf serum (FCS). The cell suspension was loaded on 5ml of (60%) percoll to be layered on top of a density gradient solution in sterile conical tube, and centrifuged (8C°) at 2000 rpm for 20 minutes to obtain the mononuclear cells (MNCs) from buffy coat layer, then washed three times with phosphate buffered saline (PBS) to get ride of the percoll by cool centrifugation (8C°) as done previously. The cells pellet was resuspended again in 10 ml ofmesencult® (with 10% FCS) supplemented with penicillin(100 U/ml, streptomycin 5 mg/ml) andincubated at 37 C°, in 5% $CO_2$for 24 hours in humidified atmosphere, sub-culturing from the first culture was done and incubated three days at the same previous condition, resubculturing repeated towtime in three flasks, using new culture media at a density of 1×10^6 , to obtain more expansion of cells in culture, after that the non-adherent cells discarded and the adherent cells on wall of cultured flask were detached by trypsin solution (0.25 mMol trypsin +1 mMol EDTA) in order to get ride of dead cells by trypsin digestion, then cells were cultured in low (5.56 mMol/L) glucose dulbeccos modified eagales media (L-DMEM)®, supplemented with 10% FCS, penicillin 100 U/ml, streptomycine 5mg/ml,and incubated for 3 days in the same conditions, trypsinzation was done and the culture was examined for morphological characters, cells viability and counted too, and re -cultured for 24 hours in humidified atmosphere at 37 C° and 5% CO_2 in the new media (L-DMEM).

Induction of bone marrow derived MSCs to insulin producing cells:

These cells were transferred into new culture media: H-DMEM

(glucose 25 mMol/L) at adensity of 1×10^5 cell/ml , supplemented with β-mercaptoethanol (0.5 mMol/L) and incubated for 48 hours at 37 C° , 5% CO_2 in humidified atmosphere, and one cultured flask left without addition of β-mercaptoethanol as control flask, after that the cultures of cells examined for morphological characters and compared with control, then cells collected and cultured again in H-DMEM containing: 1% non-essential amino acid, 20 ng/ml β-fibroblast growth factor,2% B27, 2 mMol/L L-glutamin in three flasks and incubated for 8 days(the media changed every 3 days) then cells transferred into new H-DMEM supplemented with 10 mg/ml β-celluline, 10 ng/ml activin A , 2% B27, 10 mMolnicotinamide and incubated again for 7 days (media changed every 3 days). Cells examined for their morphological characters and compared with control cultured flask(cells in culture media without addition of differentiated compounds),then cells collected and cultured at a density of 1×10^6 cells/ml in new L-DMEM for 24 hours then cells examined for morphological characters, viability and presence of insulin granules by dithiozon®(diphenylthiocarbazoneInvitrogen -USA) stain.

Counting of viablecells:
Equal volumes (0.5 ml) of cells suspension and the trypan blue stain were incubated at 37 C° for 30 minutes. 0.02 ml of the mixture was put in counting chamber slide (hemocytometer) and examined under light microscope (40x).The stained cells were counted, if they were over 30% , the sample was discarded and resub-culturing of cells was repeated by using new mesenchymal media (Pollard and Walker 1997) .

Dithiozone stain.:
10 mg of dithiozone stain dissolved in 10 ml of dimethyl sulfoxide(DMSO) and stored at 20 C° as stock solution, the working solution was immediately prepared 'before using', by diluting the stock solution at a ratio of 1:10 in PBS. According to Li-BoChen, et al. (2004) with slight modification. 1 ml of cell suspension mixed with 0.5 ml of diluted stain stock solution in a conical tube, and incubated at 37 C° for 30 minutes, a drop of mixture was spread on slide, stained and non-stained cells were counted under microscope (40x), when the stained cells in tissue culture suspension 80% or more ,the cultured cells were used for therapy.

Induction of diabetes mellitus:
This performed by intravenous administration of fresh alloxan monohydrate solution (Lenzenet.al.1988) in a dose of 65mg/ Kg wt as a single dose , alloxan solution was sterilized by using 0.22 µm Millipore filter unit.

Experimental design:
Twenty dogs were left in animal house 2 weeks for adaptation, they were equally allocated into four groups as below:

Group I (control) left without any treatment.

Group II was administered alloxan solution to induce diabetes mellitus and kept as control diabetic group and left without cellular treatment.

Group III was administered alloxan solution and after that injected intravenously a suspension of pure (cell free) culture media (L-DMEM)* 1 ml/Kg wt, ten days post alloxan injection.

Group IV: Mesencymalcells (MSCs) were obtained from their individuals only, after that they were administered alloxan solution and then treated with autologous differentiated stem cells (IPCs) suspension in a dose of 2.5x106 cells / ml / Kg wt intravenously ten days post alloxaninjection, the therapeutic dose was determined by hemocytometer (Aremanet.al.,1992).

Statistical analysis:
Statistical analysiswas made according to system (SAS Institute 2001), t-student's test was used for comparison. Data were analyzed statistically by using Complete Randomize Design (C.R.D) in factorial experimental (Two-way ANOVAs) by using following model:

$$Y_{ijk} = \mu + G_i + D_j + (G\,D)_{ij} + e_{wk}$$

Y_{ijk} = Observed variable

μ = Commom mean

G_i = Effect of groups

D_j = Effect of Days

(G D)ij = Effect of Interaction between groups and days.

Data were analyzed by using SPSS, 2008 Program.

Results
Dogs in groups: II,III and IV showed typical clinical signs of diabetes mellitus 3 days post alloxan administration: polyuria , low activity and increase appetite but there was neither ocular cataract nor dull hair-coat observed, insignificant variation among clinical parameters were recorded in all diabetic groups , whereas group I (control) remained healthy (no clinical sign observed) and active.

The water consumption in groups II, III and IV were increased 3 days after induction of diabetes mellitus (2.4± 0.24, 2.0± 0.21, 2.5±0.24 L/day, respectively) as compared with their consumption before administration of alloxan(1.7±0.12, 106±0.14, 1.6±0.5 respectively) and with control group-I (1.8±0.1 L/ day),such increases were continued along the period of experiment in groups II and III (Fig – 1), whereas the increase in water consumption was halted in group IV, 7 days post cellular therapy (3.2±0.23 L/day), and the gradual decline continued to minimum level at the 56th day post cellular therapy (1.5 ± 0.14 L/day).

Similarly the serum glucose level was significantly elevated 3 days post alloxan administration in groups II, III and IV (149.2 ± 2.30 , 149.8 ± 1.70, 150±2.3 mg/dl respectively) as compared with control group-1(113.2±0.54) Fig -2 , the elevation of glucose levels in groups II, III also continued for 73th post alloxan injection, whereas the increase of blood glucose level in group IV was halted also 4 days post intravenous administration of cellular (IPCs) suspension (2.5x106 cells /ml /kg body weight), moreover a noticeable gradual decline in glucose level in the group IV start at 4 days post cellular therapy and reached the minimum level (116±0.7 mg/dl) 63th day post cellular therapy (Fig -2). Simultaneously slight disappearance of clinical signs were observed, besides that no significant variation observed in the blood glucose levels between this group(IV) and (control) group-1 (113.6±0.52 mg/dl at this period.

The percoll gradient density procedure was of high efficient and successful technique for separation of MNCs from variety of cells in bone marrow samples. Also the uses of mesencult® culture media with fetal calf serum was helpful to obtain the predominant MSCs, the optimum period was 7 days to obtain large quantity and good expansion of MSCs, as the mesencult is a specific media, although some cells were floating but the huge number adhered to the flask wall progressively. The MSCs proliferated in culture medium within three days, they formed colonies of adherent cells, and sparse too, they appeared as fibroblast and spindle-like, the adhered MSCs proliferated increasingly 3 days after sub-culturing in new mesencult media®. The confluencespindle – like cells were surrounded by the same cells, which gave appearance of more dense cellular masses (Figure - 3), these characters were highly indicated for presence of the MSCs. Also the effect of changing different medias on the cells was alleviated beside preparing them for exposure to differentiating compounds, were achieved by transfer MSCs into L-DMEM for 18- 24 hours this, might aid in perpetuating the viability and improve the differentiation of MSCs. The differentiation was induced by three stages: in the first 48 hours MSCs started in differentiation as cells loss their confluence and became swollen in shape as compared with contol flask, in the second stage (culturing in L-DMEM+non-essential amino acid+fibroblast growth factor+B27+L-glutamine, for 8 days),

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the cells appeared as slated to oval shape with marked border, as the transformation progressed, cells showed spherical appearance, contained small rounded nucleus (under microscopical examination of stained slide), in the thrid stage cells tend to aggregate and take form of cluster shape then appeared as islet grape-shaped cells within 7 days of incubation, these events had not been seen in the cells of control flask(without addition of β-mercaptoethanol and nicotinamide), the cells formed clusters, gave appearance of islet grape- shaped cells, and contain intra-cytoplasmic granules, undistinguished in culture media by inverted microscope, which represented insulin granules, they were checked by dithiozone stain,these cellstransferred to new L-DMEM (without addition of any compound) just before administration to diabetic dogs, in order to avoid the influences of chemicals on animals.

Discussion

Diabetes mellitus was easily induced by intravenousinjection of alloxan monohydrate in a dose of 65 mg/kg wt in dogs without shock, and no deathsoccurred, although variant doses were mentioned in different studies (Cooperstein and Watkins 1981, Rastogiet.al.1992,Kaneko et.al.1997, Nelson 2000, Belcheret. al.2005).

The primitive, undifferentiated as well as theregenerative ability of MSCs were exploit to replace damaged cells or tissue (Morrison et al.1997). Furthermore the autologous graft procedure was used in this study to reduce the limitation of cell viability and undesirable immune reaction. The blood glucose levels in diabetic group(IV) treated with IPCs, were decreased significantly (P≤0.05) at 4 days post cellular administration (Fig-2), this possibly pointed to successful differentiation of MSCs into functional IPCs, beside that the cellular transformation was monitored periodically by examination of the cultured media under inverted microscope, the morphological changes and special characteristics were observed and compared with control flask(undifferentiated MSCS cells) , beside the intra-cytoplasmic insulin granules were detected by dithiozone stain, thus these should be considered for confirming of MSCs transformation, also this was supported by successful work of others(Li-Bo Chen et. al.2004, Jong et.al.2008), the marked improvement in the condition of diabetic dogs as: gradual disappearance of clinical signs as well as decrease in water consumption (Fig-1), complies with lowering blood glucose, however some researchers looked for more proofs: as using surface marker antigens of MSCs as CD44, CD99 (Kisielet. al. 2012), and/or for the expression of specific genes of IPCs as nestin, PDX-1(Sun Yu et. al. 2007), the current procedure was more applicable, saved time and reduced the cost, moreover their results were feasible. Scientists are aware that time of recurrence of clinical signs of diabetes mellitus in animals treated with MSCs are imprecise, however in this study the low blood glucose levels and slight disappearance of clinical signs were continued to 63 days post cellular therapy (treated diabetic dogs not followed after this time). These results were encouragable and might be attributed to uses of MSCs from healthy individual (MSCs aspirated before inducing of diabetes mellitus), leading to high activity and successful transformation of these cells. The high dose of IPCs might be required particularly when administered via intravenous route, however less risk might be expected to be happened due to administration of differentiated than those undifferentiated MSCs, also the differentiated cells (IPCs), might be distributed to different organs or tissues in the body, it was reported that IPCs were reside in renal sub-capsular region (Lim et. al.2007) and hepatic sub-capsular and parenchymal region (Lijurret .al.2002), our suggestion that intra-arterial administration may be a favorable route used in the future but it is associated with technical difficulties. In conclusion there is a light in the end of tunnel substantiating a hope for diabetic patients, although extensive efforts are required to solvemany problems and challenges that might face workers in this field, on other hand preparation IPCs can be made easy.

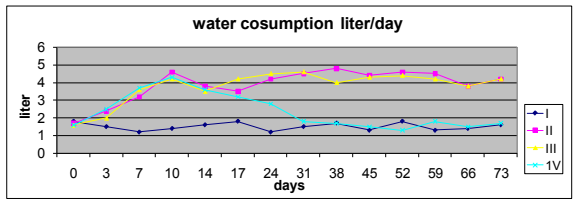

Figure - 1

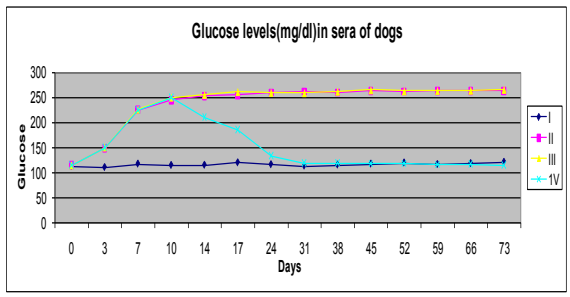

Figure - 2

Photo -1: Bone marrow - MSCs in culture (Mesencult®) after seven days of culturing (X100).

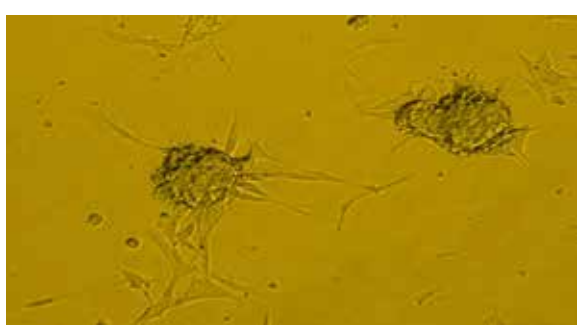

Photo-2: Differentiated MSCs in LDMEM aftersevendays of culturing.

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