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Mononuclear Derived from Human Umbilical Cord Normalize Glycemia in Alloxan-Induced Hyperglycemic Rat

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Key Words

Hyperglycemia • Human umbilical cord blood • Mononuclear cells • Diabetes • Alloxan • Insulin

Abstract

The present study explored whether mononuclear cells derived from human umbilical cord blood could resolve hyperglycemia. In order to test this hypothesis, mononuclear cells derived from Human umbilical cord blood (HUCB) were transplanted into alloxan-induced hyperglycemic rats. Mononuclear cells (MNCs) were isolated by a conventional centrifuge method through a Ficoll- paque. Hyperglycemia was induced in rats by a single injection of alloxan at 50 mg/kg body weigh intraperitonealy. Rats were divided into three groups of ten each. Group I, served as control; Group II received alloxan alone; Group III received both alloxan and MNCs. The serum glucose and insulin level were measured before the animals received the MNCs and at 1, 4, 7, 12 and 15 weeks following the treatment. Glucose levels were monitored by the glucose oxidase technique. The insulin level was measured following Elisa assay by the insulin kit specific for rats made by Mercodia Co., Sweden. The results indicated that glucose levels in alloxan-injected rats rose at week 1 and remained elevated 301.00 ± 6.43 mg/dl for 15 weeks. In contrast, in week 15, after treated with MNCs, the blood glucose levels were 108.26 ± 6.84, mg/dl. Within a week after MNCs administration, blood glucose levels significantly reduced (245.74 ± 2.37 mg/dl and reached a baseline almost close to the normal glycemic values 15 week later (108.26 ± 6.84 mg/dl). Treated with MNCs in alloxan diabetic rats caused a significant rise in serum insulin accompanied by a drop in the blood glucose level.

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Introduction

Evidence reveals that diabetes is the most common disease. Unfortunately the incidence of diabetes is increasing exponentially. In spite of drug therapy, progress of diabetes is out of control. Drug therapy is inadequate treatment and also, insulin replacement therapy can not treat diabetes. Therefore, alternative methods need to make sure of better glucose control. For these reasons, in the recent years cell based therapies have been proposed as an alternative treatment for diabetic patients.

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In this regard, embryonic and adult stem cells have been evaluated in animal models. On the basis of limitations of these types of cells ,much effort has been made to use the renewable source of stem cells. In this context, recent studies describe the potential use of umbilical cord blood. Umbilical cord blood (UCB) contains circulating stem/progenitor cells [1] which is an alternative source of haematopoietic progenitor cells for transplantation [2]. Cord blood is potentially considered as a valuable source of cells for tissue repairing in response to injury [3]. Ende et al. reported that transplantation of HUCB mononuclear cells in animal models of diabetes improved or reversed the hyperglycemia [4, 5]. Transplantation of HUCB cells in type 1 diabetic mice delay the onset of autoimmunity and insulitis and improve blood glucose level [5]. Previous studies recognized that following the intravenous transplantation of HUCB into NOD/SCID mice, insulin-producing cells of human origin were found in the mouse pancreatic islets [6]. Pessina and colleagues investigated the expression of endocrine pancreatic progenitor markers in HUCB cells [7]. Parekh et al. reported that mononuclear cells, derived from human umbilical cord blood, contained a subset of pancreas-committed cells with a potential to differentiate into insulin-producing β-cells [8]. Earlier studies suggested that HUCB may contain cells with a potential to develop into insulin-producing β-cells [9, 10] Henryk Zulewski reported that mesenchymal stem cells may have the potential to differentiate various tissues including insulin-producing cells *in vitro* [11]. UCB mononuclear cells contain a number of cells including β-cells, T-cells messenchymal and endothelial progenitor cells [12]. Compared to adult blood, cord blood contains an increased number of immature and atypical lymphocytes [13]. UCB cells can differentiate multiple cell types of non-haematopoietic origin [14, 15]. *In vitro* differentiation of UCB, stem cells to insulin and c-peptide–producing cells has been reported by Denner [16]. The study of umbilical cord blood stem cells to improve hyperglycemia still remains in early stages. In fact, the mechanism of umbilical cord blood stem cells still is not clearly defined. There are few reports in the literature related to this subject and the resolution of this mechanism requires further experiments. Based on the mentioned studies, following hypothesis is proposed that administration mononuclear cells derived from HUCB might contribute to improve of hyperglycemia animals. The aim of the present study was to investigate the potential role of mononuclear cells derived from HUCB in improving hyperglycemia in alloxan induced hyperglycemic rat.

Materials and Methods

Collection and preparation of human cord blood cells

Blood samples (n=10) from the umbilical cord were obtained from the Department of Gynecology and Obstetrics of Babol University, Babol, Iran. In accordance with the regulations set forth by the Human Investigations Committee of Babol University, informed consent was obtained from the mothers whose umbilical cord blood was collected. The approval of the Ethics Committee of Babol University was also obtained (#: 5393). Human UCB cells were obtained from full-term normal deliveries. Sample codes were:119651, 120133, 120922, 121219, 121931, 121825, 122176, 121790, 122470, 122821. Each cord blood sample was collected into a 50 ml sterile polypropylene test tube containing 5 ml of citrate phosphate dextrose (Sigma, USA) as an anticoagulant. The mean volume of cord blood varied from 41 to 52 mL. Immediately after collection of UCB samples, approximately 10 mL of collected cord blood was mixed with the same volume of phosphate-buffered saline (PBS). Units were placed in a 10 ml disposable centrifuge tube. An aliquot of 10 ml of this cell suspension was carefully layered over 10 ml of Ficoll-paque™ premium (Sweden 17-5442-02) and subjected to density–gradient centrifugation (Centrifuge, Behdad; Iran) at 1800 rpm for 30 min. After 30 mines of centrifugation in a swinging bucket rotor, the inter mediate layer was collected. Upon isolation, the cells of the mononuclear fraction were separated. The resulting pellet was suspended in 250 µl of PBS. Then the cells were washed three times with PBS and centrifuged for 15 min at 1800 rpm. An aliquot of 10 µl PBS was added to the pellet for counting (Hematology cell counter, Lc-10, Austria micros). The proportions of MNC cells were also different in each cord blood sample. At the beginning of the experiments, the nucleated cell count in each cord blood unit was different and ranged from 16- to 34.8*10^6 cells/250µl. Cell viability and counting were determined, and then mononuclear cells were centrifuged for 15 min at 1800 rpm. For the final dilution 250 µl of PBS solution was added and injected to the rats.

Cell preparation for injection

On the day of injection, cells were washed in PBS. Before infusion cell viability was determined by 0.4% trypan blue dye staining. Fresh MNCs were placed into a capped 5-ml-syringe prior to injection. Rats were deeply anesthetized and placed on supine position. Two days after injection of alloxan, direct cell injection into heart or tail vein was done. For cell injection, the tip of the 27-gauge-needle was placed with a 45- degree angle on the center of the surface anatomical region of heart and the needle is located in the right place. Then 250 µl PBS containing MNCs at dose of 23.56 *10^6 cells was gently infused. For controlling rats, only alloxan with equal volume of PBS was injected.

Characteristics of the MNCs isolated from umbilical cord blood

Over ten units of umbilical cord blood were isolated. The average volume was 45 ml (41-52 ml), including 5 ml anticoagulant, contained an average 23.56 *10^6 MNCs /250µl,

(16-34.8 *10^6 cells/250 µl). The number of viable cells was analyzed using 0.4 % trypan blue dye exclusion method (Hematology cell counter, Lc-10) before and following of transplantation. Cell viability ranged from 86 to 91 %. Transplant cell concentrations were adjusted to each animal (23.56 *10^6 cells /250 µl).

Animal

Thirty rats have been used with age from 8-12 weeks and weigh of 200 to 250 g. Rats of closed colony were prepared from the Animal Center of Babol University. Age-matched rats were used as control animals. The rats were housed in suspended bracket cages in a climate controlled room with temperature of 23±3 ºC. They housted in a cycle of 12 h light and 12 h dark with free access to food and water. All animals were carefully maintained under standard animal house conditions. All procedures were in accordance with the animal experiment guidelines of Babol University of Medical Sciences. Furthermore, all protocols involving animals were approved by Babol University Animal Care and Use Committee. Effort was made to minimize the number of animals.

Hyperglycemia was induced by a single dose injection (50 mg/kg, i.p) of freshly prepared solution of alloxan monohydrate (Aldrich, A7413-25G) dissolved in PBS intraperitonealy. By using a tuberculin syringe fitted with a 27-gauge needle alloxan solution was injected at a dose of 50 mg/kg days. The induction of hyperglycemia was confirmed after 48 hrs by blood glucose estimation and rats with fasting blood glucose levels above 250 mg/dl for 2 consecutive days were selected for the study. The animals were were randomly divided into three groups of ten as follows:

Group I: Control group, non treated animals received PBS.

Group II: The hyperglycemic animals received 50 mg/kg Alloxan intraperitoneally and did not receive any cells.

Group III: Hyperglycemic animals received 50 mg/kg Alloxan intraperitoneally and treated by MNCs with the total number up to 23.56 *10^6 /250 µl.

Blood sample for glucose estimation was collected from the tail vein or heart of the rats. Glucose levels were determined by the glucose oxidase method with single beam uv/visible spectrophotometer (Camspec, M 501) using the reagents supplied by Pars Azmoon, Tehran, Iran). The insulin level was measured following Elisa assay by the insulin kit specific for rats made by Mercodia Co., Sweden with EIA plate reader with 450 nm filter (Statfax 2100,USA). The serum glucose and insulin level were measured before the animals received the alloxan and MNCs and at 1, 4, 7, 12 and 15 weeks after treatment. The rats were followed for a mean of 15 weeks. Glucose levels were monitored by tapped tail vein blood under fasting condition every two days after transplantation for 15 weeks. Hyperglycemic rats were randomly divided into two groups. The treatment group received MNCs and the untreated group received Alloxan. Two days after alloxan injection, the blood glucose levels of all rats rose from normal to hyperglycemic status. Rats did not meet the blood glucose requirements for alloxan induced hyperglycemia were excluded. Control group just transfused 250 µl PBS. Body weight of rats were also measured at the time of blood glucose examination. The average amount of weight loss was compared between two groups after transfusion.

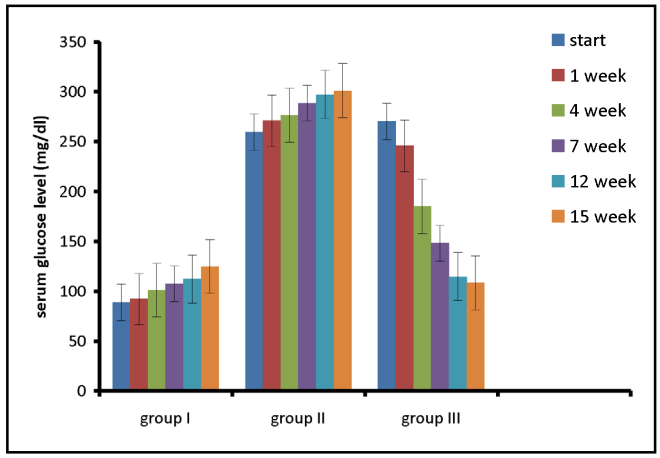

Fig. 1. Serum glucose level (mg/dl) in non hyperglycemic rats, non-treated animals received only PBS(group I). Hyperglycemic animals, received 50 mg/kg alloxan intraperitoneal did not receive any cells (group II). Hyperglycemic rats, received 50 mg/kg alloxan intraperitoneal treated with MNCs (group III). Each column represents the mean value ± SD of 6 separate experiments, $P < 0.05$. (column1 start; column 2, 1 week; column 3, 4 weeks; column 4 ,7 weeks; column 5, 12 weeks and column 6, 15 weeks after cell transplantation).

Statistics

All values are presented as mean ± standard error. Data were analyzed by unpaired student's t-test for comparison between two means. Probability values <0.05 were considered as statistically significant.

Results

Cell viability ranged between 78-96% averaged 92± 8%). Approximately 23.56*10^6 cells were transplanted into hyperglycemic rat. None of the rats receiving the MNCs dose died.

As demonstrated in Fig. 1, before injection of alloxan, the blood glucose levels were 88.94 ± 2.39 mg/dl in rats (group I) kept elevated 124.70 ± 3.54 mg/dl during 15 week. In contrast, after injection of alloxan, the blood glucose levels after 2 days were 259.54 ± 9.38 mg /dl in rats. Starting from the day of transplantation to week 15, the blood glucose levels of the alloxan-treated group remained hyperglycemic. Animals did not recover spontaneously, and 3 months later the onset of hyperglycemia was observed. The administration of

alloxan to the unfed rats (group II) markedly changed blood glucose levels (301.00 ± 6.43 mg/dl *vs.* 88.94 ± 2.39 mg/dl, P<0.05) during 15 week, as compared with control rats. Within 15 weeks after alloxan injection, the blood glucose concentration of hyperglycemic rats increased approximately 2.4 fold as compared with the control group. The blood glucose level of alloxan injected-rats did not drop compared to the PBS-treated group. Glucose levels in MNCs transplanted rats (group III) began to decrease at week 1 after transplantation, kept below 108.26 mg/dl during 15 week. Glucose levels in the MNCs transplanted rats remained low until the end of study. Administration of MNCs significantly reduced the hyperglycemia in alloxan-induced hyperglycemic rats (108.26 ± 6.84 vs. 270.30 ± 3.30 mg/dl, P<0.05). The maximum effect was observed with 23.56 *10^6 cells/100 µl at 15 week after treatment. As demonstrated in Fig. 1, glucose levels in MNCs implanted rats began to decrease at 1 week after transplantation 245.74 ± 2.37 mg/dl. On week 15 after transplantation, the blood glucose levels of treatment group (group III) average 108.26 ± 6.84 mg /dl, compare with 301.00 ± 6.43 mg/dl of the alloxan-injected group (group 2) (p<0.05).

In the hyperglycemic rat, UCB administered when hyperglycemic signs were evident, proved to revert hyperglycemia almost to the normal levels. In contrast, untreated UCB rat remained hyperglycemic until the end of study (Week 15; 301.00 ± 6.43 mg/dl).

Fig. 2 shows the body weight in the control, untreated, and HUCB–treated groups at 0 (start), 1, 4, 7, 12 and 15 weeks. As evident, the body weight was lower in the treated group than in the untreated group (241.4 ± 6.24 g *vs.* 299.6 ± 16.93 g) during 15 week. All hyperglycemic animals survived up to 15 weeks, maintaining their body weight constant and increasing food and water intake (Data not shown). The body weight of MNCs treated rats did not significantly decrease (241.4 ± 6.24 g compared to that of PBS–treated rats (264.3 ± 15.4 g) during 15 week after transplantation of MNCs. In short, we found drops in the blood glucose and body weight in hyperglycemic rats that caused by MNCs transplantation. The administration of alloxan to the unfed rats markedly changed body weight (299.6 ± 16.93 *vs.* 215.2 ± 6.69 g, P<0.05) compared to the control group during 15 week.

Animals in the control group (group I) survived up to 15 weeks, maintaining their body weight constant (264.3 *vs.* 215.2 g) and increasing food and water intake (Data not shown). Before injection of alloxan, the body weight of rats was 225.4 ± 20.6g. One week after injection of alloxan, the body weight was 234.7g. The administration of alloxan (50 mg/kg) to the unfed rats markedly changed body weight (225.4 ± 20.6 *vs.* 299.6 ± 16.93 g, P<0.05) during 15 week, in group 2. Administration of MNCs elevated the body weight in alloxan-induced

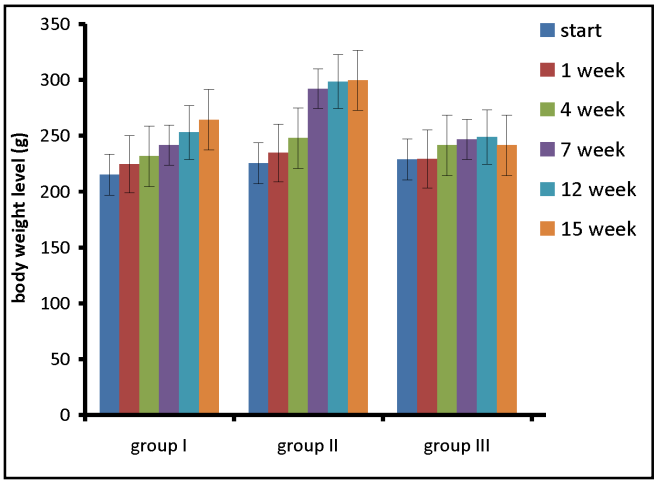

Fig. 2. Body weight level (g) in non hyperglycemic rats, non-treated animals received only PBS (group I). Hyperglycemic animals, received 50 mg/kg alloxan intraperitoneal did not receive any cells (group II). Hyperglycemic rats, received 50 mg/kg alloxan intraperitoneal treated with MNCs(group III). Each column represents the mean value ± SD of 6 separate experiments, $P < 0.05$. (column 1 start; column 2, 1 week; column 3, 4 weeks; column 4, 7 weeks; column 5, 12 weeks and column 6, 15 weeks after cell transplantation).

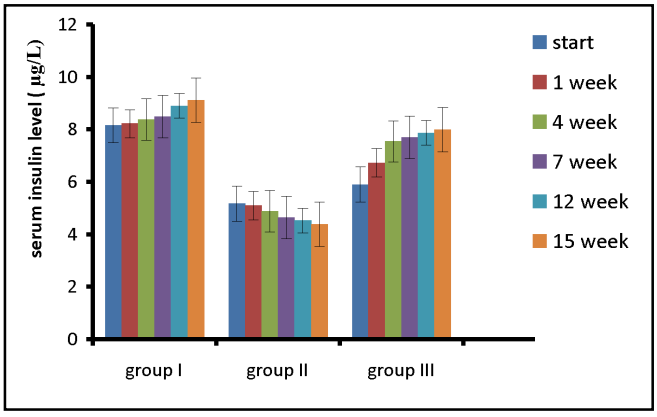

Fig. 3. Serum insulin level (µg/L) in non hyperglycemic rats, non-treated animals received only PBS (group I). Hyperglycemic animals, received 50 mg/kg alloxan intraperitoneal did not receive any cells (group II). Hyperglycemic rats, received 50 mg/kg alloxan intraperitoneal treated with MNCs (group III). Each column represents the mean value ± SD of 6 separate experiments, $P < 0.05$. (column 1 start; column 2, 1week; column 3, 4 weeks; column 4, 7 weeks; column 5, 12 weeks and column 6, 15 weeks after cell transplantation).

hyperglycemic rats (241.4 ± 6.2 *vs.* 228.8 ± 22.7 g ,P<0.05) in group III. The maximum effect was observed with 23.56 *10^6 cells /250µl at 15 week after treatment.

Fig. 3 shows the blood insulin levels in the control, untreated and HUCB –treated groups at start and after at 1,4,7, 12 and 15 week. As demonstrated in Fig. 3, before injection of alloxan, the blood isulin levels were 8.15 ± 1.15 µg/l in rats (group I). In contrast, after injection of alloxan, the blood insulin levels after 2 days were 5.16 ± 1.04 µg/l in rats. Insulin levels in MNCs transplanted rats (group III) began to increase at week 1 after transplantation, kept above 7.98 ± 1.12 µg/l during 15 week.

Discussion

Islet transplantation has been recently shown could be an efficient therapy for diabetic patients. However, the immune rejection of islet restricts its indication in clinical practice. Alternatively, much effort has been made to use the renewable source of stem cells. During the past few years, stem cell transplantation has become one of the major aspirations for the putative treatment of diabetes. Yoshida et al. reported that the UCB – derived stem cells could generate insulin-producing cells in mice [6]. As most of these secreted factors exert proliferation and differentiation effects, their release by HUCB cells might explain the potential effect observed upon trsnsplantations of these cells [17]. Cord blood cells contain many immature stem/progenitor cells with extensive proliferation capacity *in vitro* [18]. In addition to embryonic and adult stem cells, umbilical cord blood was shown to contain multipotent stem cells [19]. However, the mechanisms by which transplanted HUCBC induce functional benefit after hyperglycemia is not clear.

In recent years, the novel stem cell therapy method, particularly the mononuclear cells which appear to be the alternative therapies that can to improve the hyperglycemia and might be useful in diabetes. However, this ability must be proven by further investigation. Despite the importance of this subject, few related researches have been done on its role. Efficiency of these cells in treating hyperglycemic animals needs to be determined.

Therefore, the present study conducted to transplant MNCs into the body of alloxan- induced hyperglycemic rat to via heart and tail vein to determine the capacity of MNCs to correction of hyperglycemia in hyperglycemic rats. According to the present results, blood glucose levels increased in alloxan–injected rats during 15 week. But, the blood glucose levels were significantly low in the MNCs transplanted rats (Fig. 1). On the contrary, serum insulin levels were significantly increased after administration of MNCs when compared with control group (Fig. 3).This result confirms other results that showed transplantation of HUCB mononuclear cells into animal models of diabetes improved or reversed their hyperglycemia [4, 5]. As demonstrated in Fig. 1, glucose levels in MNCs implanted rats began to decrease in 1 week after transplantation, kept low during 15 weeks. Our results confirmed findings of other investigators [4, 5]. Long-term reduction of the blood glucose observed in the HUCB injected rats from day 2 up to 15 weeks after transplantation glucose levels of treated rats were continuously to be normal. All rats survived for at least 15 weeks post-transplantation and exhibited normal glucose levels during that time contrast to the un-transplanted rats that remained hyperglycemic. Un-transplanted alloxan-treated rats maintained hyperglycemia. These results were in good agreement with those reported previously [5]. The body weight of MNCs-treated rats were not significantly decreased to comparison of PBS–treated rats during the15 weeks after transplantation of MNC (Fig. 2). The interesting point was the animals that become hypoglycemic after transplantation of MNCs maintained normal body weights and glucose levels .These results strongly suggested that the cell therapy with MNCs could provide new possibilities for treatment of hyperglycemia. Our results reinforce the possibility of intravenous administered cell therapy as an effective approach to improve the hyperglycemia resulting diabetes.

For the first time, this study demonstrated a time–dependent decrease in blood glucose levels and increase insulin levels in rats that received HUCB mononuclear cells. To explain the hypoglycemic action of MNCs in alloxan-diabetic rat, we hypothesized that the reduction in blood glucose level following the administration of MNCs may be attributed to increase circulating insulin levels. It is possible that the many immature stem/progenitor cells with extensive proliferation capacity present in the MNCs are responsible for the islet regeneration and insulin release and/or they may have insulin-like properties. Also, the glucose- lowering activity observed in hyperglycemic animals may be due to the stimulation of β-cells of the pancreatic islets. In future, it will be important to investigate the putative mechanisms involved in these results. Based on our present findings, parallel with previous results, we suggest that

administrating MNCs may contribute to the regeneration of the pancreas in animals. In conclusion, investigation of potential treatment of MNCs to address diabetes is still in early stage. The results of this paper could encourage clinical studies to evaluate the potential benefit of MNCs administration. In the future, MNCs may be used as a therapeutic method in the treatment of diabetes. Studies are in their way in our laboratory to further elucidate the mechanism of the observed hypoglycemic effect.

Abbreviations

HUCB (Human umbilical cord blood); MNCs (Mononuclear cells); PBS (Phosphate-buffered saline); UCB (Umbilical cord blood).

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