Life Sciences 239 (2019) 116908

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Investigating curcumin potential for diabetes cell therapy, in vitro and in vivo study

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ARTICLE INFO

Keywords:
Regenerative medicine
Nanocurcumin
MSCs
IPCs
Diabetes type I model rats

ABSTRACT

Aims: An important obstacle on the way of cell-based therapy is the risk of tumorigenicity in the patients benefit from these transplanted cells due to undifferentiated cells which participate in transplantation. Curcumin, the main compound of spice turmeric -as one of the natural products-was demonstrated to possess effective anti-cancer properties, with no significant effect on normal cells in dose and/or time-dependent manner. Furthermore many studies have been accomplished using curcumin for diabetes treatment. Therefore in this study we examined the efficacy of IPCs treated with curcumin in vivo.

Main methods: Differentiation efficiency investigated by flowcytometry. RNA extraction and real-time PCR performed for important genes in IPC differentiation and tumorigenesis including *Insulin*, *Nestin*, *Ngn3*, *Pdx1*, *P21*, and *P53*. Finally we investigated the efficiency of these differentiated and treated cells in diabetic rats.

Key findings: Our data indicates that nanocurcumin -in a specific dose-reduces the expression of *Nestin* with no significant effect on insulin expression in mRNA and protein level. Besides blood glucose level of diabetic rats which treated with DNC + cells, decreased from average 350 (mg/dI) to 100 (mg/dI). Checking out the pancreases of these rats, demonstrated that their endocrine segment was rebuilt. Moreover hematoxylin & eosin staining and IF results revealed that the Langerhans Islands were reformed.

Significance: IPCs' which treated with DNC were able to efficiently control the blood glucose level in diabetic rats which these cells were transplanted to them. Hence Curcumin has the potential to be employed in this kind of cell therapy.

1. Introduction

According to WHO recent statistics, 422 million adults have diabetes; this means 1 out of 11 persons is affected [1]. Generally we can divide various types of diabetes into three categories: type I, type II, and temporary which may be seen during pregnancy. Type 1 diabetes mellitus (T1D) is a chronic, multifactorial autoimmune disease that involves the progressive destruction of pancreatic β-cells, ultimately resulting in the loss of insulin production and secretion. The goal of clinical intervention is to prevent or arrest the onset and progression of autoimmunity, reverse β-cell destruction, and restore glycometabolic and immune homeostasis. Despite promising outcomes observed with islet transplantation and advancements in immunomodulatory therapies, the need for an effective cell replacement strategy for curing T1D still persists. Cell therapy approach seems to be a promising alternative for existing approaches (including organ/islet transplantation and etc.) [2,3].

In cell therapy we benefit from the pluripotency ability of stem cells which makes them powerful applicable sources for differentiation towards desired cell lineages. We can get stem cells from different sources including: Embryonic stem cells (ESCs) [4–6], induced pluripotent stem cells (iPSCs) [7], and adult stem cells (e.g. human bone marrow mesenchymal stem cells (hBM-MSCs)). It is clear that if hBM-MSCs from patients themselves are utilized; the risk of immunological responses can be omitted. The main challenge we are facing with in cell therapy, is the risk of tumor formation in recipients. These tumors are originated from undifferentiated stem cells which participate in transplanted cells. This devastating phenomenon has been proved to arise when we use either ESCs and iPSCs or adult MSCs [8–14].

Recently three strategies have been developed to decrease the risk of tumor formation in cell therapy: Terminal differentiation or complete elimination of residual pluripotent stem cells from culture, interfering

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https://doi.org/10.1016/j.lfs.2019.116908
Received 25 August 2019; Received in revised form 17 September 2019; Accepted 23 September 2019
Available online 11 October 2019
0024-3205/

with tumor-progression genes to prevent tumor formation from the residual pluripotent cells, and finally tumor detection and elimination after formation in the patient's body. It is obvious that the first strategy is the safest approach. Benefiting from herbal products [15–17] in cell therapy for complete differentiation and/or eradication of undifferentiated stem cells is newly emerging field which can be set in first of these three strategies [18–26].

In this study we employed Curcumin (the main compound of *curcuma longa*) to eliminate undifferentiated cells after differentiation of hBM-MSCs into insulin producing cells (IPCs). Curcumin is a natural product which can induce apoptosis in cancerous cells in specific doses and times through different pathways; interestingly it does not have significant effects on normal cells in these doses/times. Curcumin is water insoluble, so in this project we utilized Curcumin in complex with a dendrosomal carrier (this complex is abbreviated as DNC). After differentiation of hBM-MSCs into IPCs, the expression level of differentiation markers including pancreatic and duodenal homeobox 1 (*Pdx1*), neurogenin 3 (*Ngn3*) and insulin increased. After treatment of these cells with 20 μM of DNC, undifferentiated cells eliminated and hence the expression of *Nestin* (gene which is expressed only in undifferentiated cells) decreased significantly, furthermore this treatment did not affect expression level of the mentioned differentiation markers. Cells from different groups obtained from in vitro studies were further investigated for their in vivo functional ability. For this aim we benefited from diabetic rats models which obtained from streptozotocin (STZ) treatment. Intraperitoneal glucose tolerance (IPGT) test revealed that diabetic rats that received differentiated/DNC treated cells were able to response to glucose increasing as well as normal rats. Immunohistochemistry and hematoxylin/eosin staining revealed that the endocrine part of pancreas has been rebuilt in these groups.

2. Materials & methods

2.1. Materials and reagents

To culture hBM-MSCs, DMEM (Dullbecco's modified Eagle's medium)-low/high glucose, supplemented with 10% FBS (fetal bovine serum), 100 mg/ml streptomycin and 100 U/ml penicillin (all from GibcoBioCult, Scotland, UK) were utilized. PBS (Phosphate-buffered saline) and trypsin-0.25% EDTA were also from GibcoBioCult. Nicotinamide, DTZ and DMSO were provided from Merck (Germany). 2,5-diphenyltetrazolium bromide (MTT), agarose, β-mercaptoethanol, STZ and other reagents were from Sigma-Aldrich (USA).

2.2. hBM-MSCs isolation

In this study adults' human bone marrow mesenchymal stem cells (hBM-MSCs) were utilized. These cells were isolated exactly with the same protocol which described in our previous study [15,27].

2.3. Cell viability assay

To investigate either different doses of DNC has the ability to cause cellular death, MTT assay (2,5diphenyltetrazolium bromide) performed. In this assay living cells are able to convert MTT into formazan crystals, this reaction accomplishes with their mitochondrial reductase enzyme activity and hence this test is actually monitoring cells which are metabolically active. 24 h prior to treatment with DNC (nanocurcumin prepared exactly with the same protocol of our previous study [28]), 8×10^3 cells were seeded in each well of 96-well plates. After 24 h media were replaced with fresh media plus a concentration range of nanocurcumin (0–40 μM) (3 replicates were used for each concentration). After 24 h from DNC treatment, the media replaced by 180 μl fresh media plus 20 μl MTT solution (5 mg/ml in PBS). Plates incubated at 37 °C, 5% CO_2 (plate was kept in darkness) for 3 h. After this time purple formazan crystals sediments appeared at the wells bed, media containing MTT was removed; then sediments were dissolved in 200 μl DMSO [29–31]. Eventually, the absorbance of each well was recorded in 570 nm by Biotek ELX800 microplate reader (BioTek Instruments, Inc.). To further investigate the kind of cellular death in these cells Annexin V/Propodium iodide (Annexin V/PI) flowcytometery test performed by Annexin V/PI Roche kit (Switzerland) exactly based on manufacture protocol.

2.4. Differentiation of hBM-MSCs into IPCs and DTZ staining

To differentiate hBM-MSCs into IPCs we exactly utilized the same protocol discussed in our previous study [15]. Briefly, we utilized passage 3 hBM-MSCs and cultured them till they obtained 80% confluency. These cells were then cultured in differentiation media. Control group was the cells which were cultured in serum free hDMEM.

To investigate whether hBM-MSCs were differentiated into IPCs, we should test them in different levels including: *morphological changes*, *DTZ staining* which shows ability of differentiated cells to store insulin in hexamer form in complex with Zn^{2+}, *transcriptome level*, and ability of them to *secrete insulin* to the media after differentiation. DTZ (from Merck) staining performed to examine the ability of differentiated cells to store insulin [15]. After staining, clusters which have insulin hexamer in complex with Zn^{2+} become crimson red.

2.5. RNA extraction and real-time PCR

Total RNA was extracted from different groups by using TRIzol reagent (Invitrogen) based on manual's instruction. After extraction, quality and concentration of extracted RNAs determined utilizing 1% agarose gel electrophoresis and spectrophometery respectively. Same amount of RNAs (1 μg) used for cDNA synthesis (cDNA synthesis kit, Takara, Japan). Primers designed to specifically amplify mRNAs of important genes in differentiation of hBM-MSCs into IPCs including: *Nestin*, *Insulin*, *Pdx1*, *Ngn3*, *P53* and *P21* (*Gapdh* utilized as internal control) (Table 1) (some primers sequences achieved from previous studies on the corresponding gene, in this case all criteria including primers Tm, amplicon size, and etc. Checked out to be commensurate [32,33]. Real-time PCR performed by Applied Biosystems 7500 Fast real-time PCR device and SYBR® Premix Ex Taq™ kit (Takara, Japan) (reaction program was the program which recommended by Takara kit's manual).

2.6. Induction of diabetes in rats

For in vivo studies, we induced diabetes in wistar rats by STZ (purchased from Sigma). All rats were 8 weeks males. 50 (mg/Kg) of STZ were injected intraperitoneally. Blood glucose level of treated rats increased and reached into stable level after 14 days and these rats

Table 1
Primers sequences utilized for real-time PCR.

Gene	Primers sequences	Amplicon (bp)
Gapdh	**F:** 5′ GTGAACCATGAGAAGTATGACAAC 3′ **R:** 5′ CATGAGTCCTTCCACGATACC 3′	109
Nestin	**F:** 5′ AACGAGGCTTCTTCTACACACC 3′ **R:** 5′ CCACAATGCCACGCTTCTG 3′	130
Insulin	**F:** 5′ TGCGGGCTACTGAAAAGTTC 3′ **R:** 5′ AGGCTGAGGGACATCTTGAG 3′	144
P53	**F:** 5′ TCCTCAGCATCTTATCCGAGTG 3′ **R:** 5′ AGGACAGGCACAAACACGCACC 3′	265
P21	**F:** 5′ ACTCTCAGGGTCGAAAACGG 3′ **R:** 5′ GATGTAGAGCGGGCCTTTGA 3′	150
Ngn3	**F:** 5′ TTTGCGCCGGTAGAAAGGAT3′ **R:** 5′ AGTGCCAACTCGCTCTTAGG3′	248
Pdx1	**F:** 5′ CAAAGCTCACGCGTGGAAAG 3′ **R:** 5′ TTTTTCCACTTCATGCGGCG 3′	234

Table 2
Different groups employed in this study.

In vivo groups	
DNC + rats	Diabetic rats which received in vitro differentiated/ nanocurcumin treated cells
DNC- rats	Diabetic rats which received in vitro differentiated cells
BMSCs rats	Diabetic rats which received undifferentiated BMSCs
- rats	Normal rats
+ rats	Diabetic rats which didn't receive any other treatment
Ins rats	Diabetic rats which received insulin every day
curcumin rats	Diabetic rats which received different dose of curcumin instead of insulin

became hyperglycemia [34–36]. All animal experiments performed in accordance with animal ethics committee of Tarbiat Modares University. Rats were anesthetized by IP injecting mixture of ketamine (40 mg/kg)/xylazine (5 mg/kg).

2.7. Cells transplantation to rats

10^6 cells were injected locally into the tip of spleen location of each diabetic rat after 14 days. Each group contained at least 7 rats. Different rats groups included: *DNC + rats*, diabetic rats which received in vitro differentiated cells which treated with nanocurcumin; *DNC- rats*, diabetic rats which received in vitro differentiated cells which didn't treat with nanocurcumin; *BMSCs rats*, diabetic rats which received undifferentiated BMSCs; *- rats*, negative rats which did not receive STZ and hence didn't have diabetes; *+ rats*, diabetic rats which didn't receive any more treatment of rather cells or other compounds; *Ins rats*, diabetic rats which received insulin every day, *curcumin rats*; diabetic rats which received different dose of curcumin instead of insulin (different in vivo groups are summarized in Table 2).

2.8. Intraperitoneal glucose tolerance (IPGT) test

In vivo functional improvement analysis performed by IPGT test. After 2 months of different treatment on rats from different groups, to investigate in vivo functionality of their tolerance to blood glucose level, IPGT test performed. These rats underwent 16 h fasting treatment, after that they received 2 (g/Kg) glucose intraperitoneally and changes in their blood glucose level determined in specific times after injection (included: just after injection (time = 0), 1 h and 2 h after injection) [37–40].

2.9. Investigating langerhans islands formation in pancreases

To study whether treatment of diabetic rats induced reformation of islets in these rats, their pancreases isolated, prepared and finally stained by hematoxilin and eosin. Briefly, tissues were fixed in paraformaldehyde for 48 h and paraffinized. These paraffinized tissues were then sectioned by microtome instrument. Sections were deparaffinized and hydrated, stained with hematoxilin for 5 min and eosin for 30 s [40–43]. To further investigate Langerhans islands existence in different samples, Immunofluorescence (IF) performed using antibody against insulin protein (purchased from Abcam, ab181547). Tissues were fixed with paraformaldehyde and blocked with 10% of serum for 45 min at 25 °C. These samples were then incubated with primary antibody (1/1000 in PBS) for 1 h at 37 °C. Goat anti-Rabbit IgG (ab150077) used as the secondary antibody.

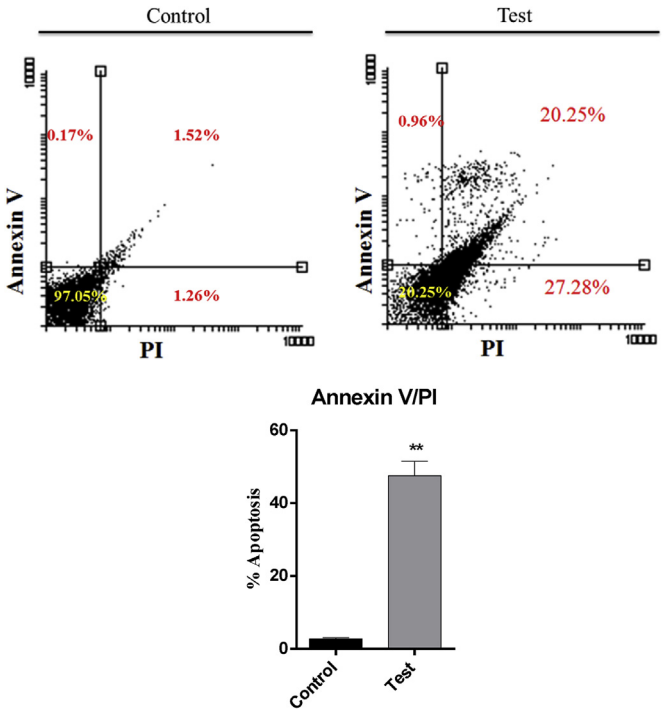

Fig. 2. Investigating the kind of cellular death in hBM-MSCs after treatment with 24 h IC50 of DNC. Annexin V/PI results demonstrated that treatment of hBM-MSCs with IC50 dose of DNC for 24 h [15] induces about 50% apoptosis in these cells (right up and down quadrant in right diagram) compared with untreated cells (left diagram). We can conclude that treatment of hBM-MSCs with 20 μM of DNC induces cellular apoptosis in these cells post 24 h.

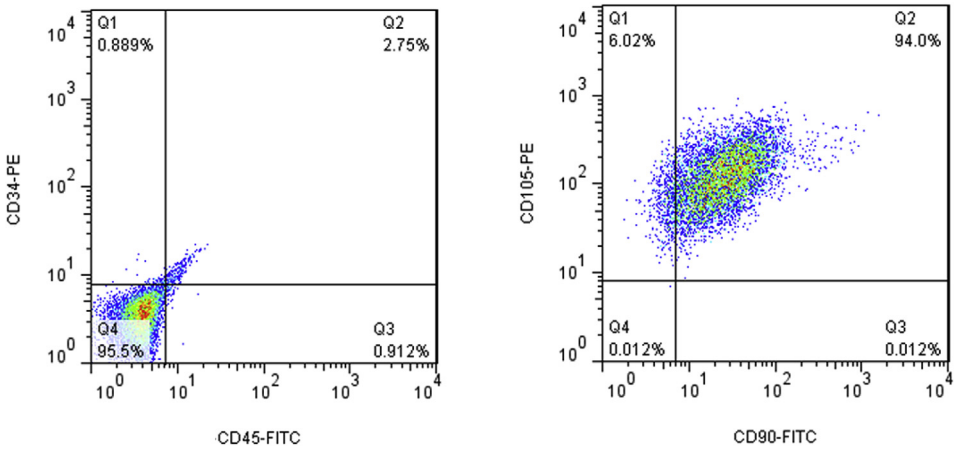

Fig. 1. Studying negative surface markers (CD34 and CD45) and positive markers (CD105 and CD90) in isolated hBM-MSCs by flowcytometery. Average of 98.2% of cells expressed neither CD45 nor CD34 (down left quadrant in left diagram) and average of 91% of these cells were positive for CD105 and CD90 cell surface markers (up right quadrant in right diagram). Isolated cells express positive markers and not negative markers for bone marrow MSCs.

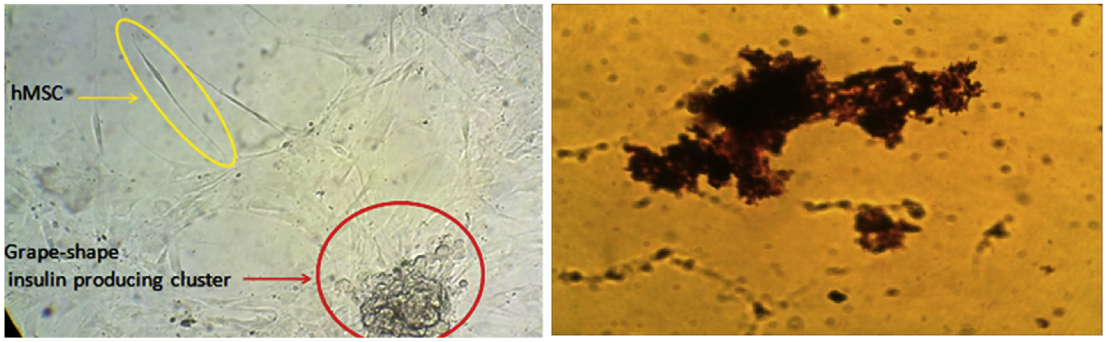

Fig. 3. Morphological changes post differentiation of hBM-MSCs into IPCs. After differentiation of hBM-MSCs into IPCs grape shape like clusters appears. Heterogeneous cells population appears after differentiation (left picture, yellow arrow: undifferentiated hBM-MSCs, red arrow; differentiated grape shape like clusters). After staining with DTZ, these clusters become crimson red (right picture) (magnification 200X). Morphological changes and the crimson red stained clusters confirm at least at cellular level, that differentiation towards IPCs occurred. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

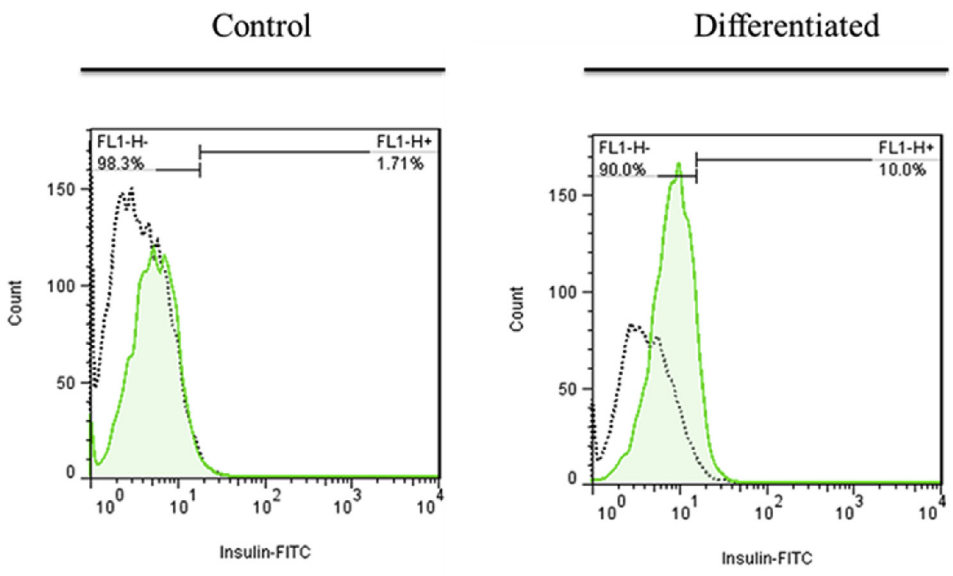

Fig. 4. After differentiation of hBM-MSCs into IPCs, about 10% of cells were insulin positive (labeled as differentiated) (undifferentiated hBM-MSCs were used as control).

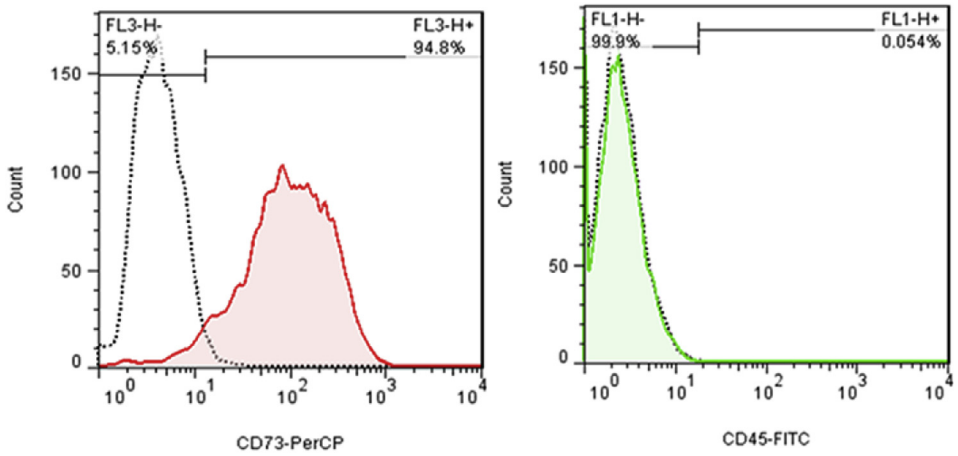

Fig. 5. Investigating BM-MSCs positive CD marker CD73 and negative CD marker CD45 in cells detached after treatment of differentiated samples with DNC. 94.8% of these cells were positive for CD73 and less than 1% were CD45 positive; which confirms that these cells which affected by DNC were undifferentiated.

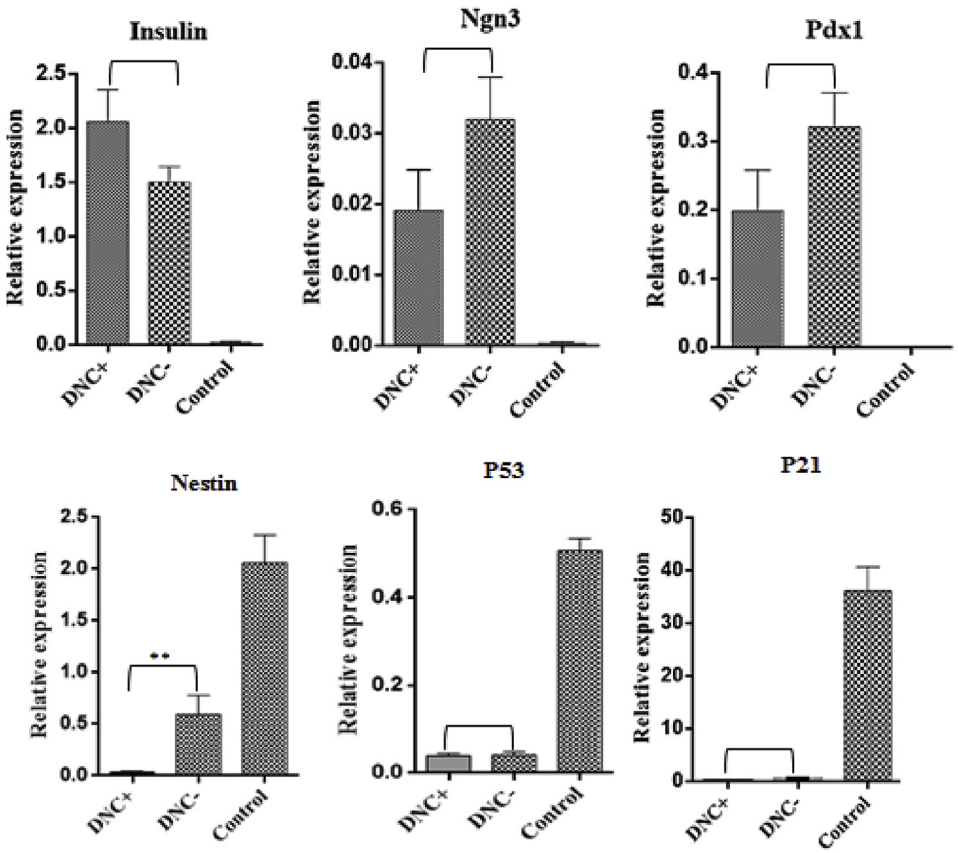

Fig. 6. Real-time PCR results for IPCs positive markers (*Insulin*, *Ngn3*, and *Pdx1*), negative markers (*Nestin*), and the important gene involve in cell cycle (*P21*) and *P53* which has tumor suppressor activity, performed on different samples in vitro. The expression levels of *Insulin*, *Ngn3*, and *Pdx1* increase after differentiation; *Nestin* is down-regulated after differentiation and DNC + samples seems that do not express this gene. Both DNC+ and DNC- samples showed endogenous overexpression of IPCs positive markers and negative markers down-regulated in these samples, which further confirms the IPCs differentiation. Furthermore DNC treatment does not have significant impact on the expression of positive markers. *P53* and *P21* expression level decreases in DNC+ and DNC- samples.

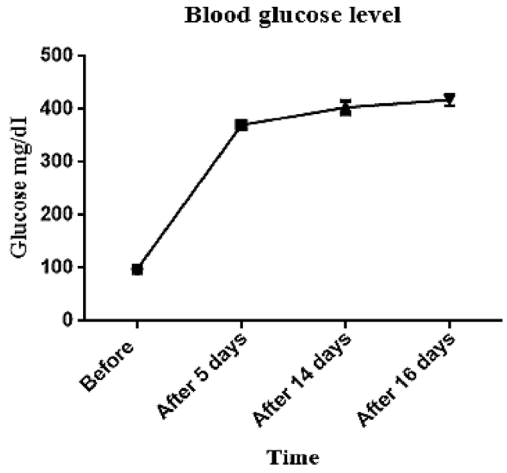

Fig. 7. Changes in blood glucose level of rats after injection of STZ. 50 (mg/Kg) of STZ intraperitoneally induced blood glucose level increasing. Left, columnar diagram and right, the same linear diagram, shows that after 14 days blood glucose level of these rats reached to a stable level of 400 (mg/dl). (Normal untreated rats' blood glucose levels were 81–88 (mg/dI), which are not shown in this picture.). Before: just before STZ injection, after: after STZ injection.

2.10. Statistical analysis

All statistical analysis in this study performed by GraphPad Prism software version 6.01 (*p value* less than 0.05 was considered as significant). To compare different groups with the corresponding control student t-test performed, in cases we wanted to compare different groups one-way ANOVA was employed.

3. Results

3.1. Confirmation of isolated hBM-MSCs

Flow cytometery results demonstrated that about 91% of isolated cells were positive for both CD90/CD105 markers and average of 98.2% of these cells were negative for both CD45/CD34 markers (Fig. 1).

3.2. Cell viability assay

The MTT assay results revealed that 20 μM of DNC is able to induce 50% cellular death (IC50) in hBM-MSCs for 24 h (data not shown here, reported in our previous paper [15]). Annexin V/PI flowcytometery confirmed that the kind of cellular death induced in these cells was apoptosis. Treatment hBM-MSCs with the IC50 dose of nanocurcumin induced about 48% apoptosis (late and early apoptosis) in these cells (Fig. 2).

3.3. Differentiation of hBM-MSCs into IPCs

After differentiation, morphological changes happen, and fibroblast like hBM-MSCs produce grape shape like clusters. If these clusters stain with DTZ they become crimson red which shows the ability of these clusters to store insulin in complex with Zn^{2+} (Fig. 3). As it is shown in Fig. 3, after differentiation we will have heterogeneous population of cells including undifferentiated MSCs and differentiated clusters. Differentiation efficiency was investigated by flowcytometry. With this regard insulin positive cells' frequency studied by flowcytometry (Fig. 4). About 10% of cells were insulin positive post differentiation.

According to the literature during differentiation process some cells remain undifferentiated; however these cells are not necessarily normal and may undergo alterations with may have the potential to form tumors in recipients [23]. To further confirm that DNC treatment affects

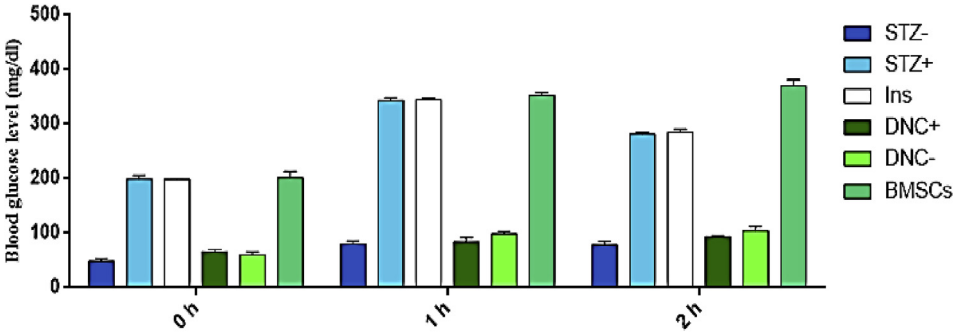

Fig. 8. Investigating functionality of injected cells in vivo by IPGT test. After injection of glucose, blood glucose level of *STZ-* (= *-rats* refer to materials and methods) (dark blue columns in this diagram), *DNC ± rats* (dark green and lawn green columns respectively) did not increased and it seems that they tolerated glucose increasing. Other groups including *STZ* + (= + *rats*), *Ins/BMSCs rats* (respectively, sky blue, white and sea green columns) could not tolerated glucose increasing, their blood glucose level elevated to about 400 (mg/dI) after 2 h of glucose injection. These results demonstrate that *DNC ± rats* are able to resist in front of increasing blood glucose level likewise to the *STZ*-samples. (*DNC+*, diabetic rats which received in vitro differentiated cells which treated with nanocurcumin; *DNC-*, diabetic rats which received in vitro differentiated cells which didn't treat with nanocurcumin; *BMSCs*, diabetic rats which received undifferentiated BMSCs; *STZ-* (= - *rats*), negative rats which did not receive STZ and hence didn't have diabetes; STZ+ (= + *rats*), diabetic rats which didn't receive any more treatment of rather cells or other compounds; *Ins*, diabetic rats which received insulin every day (one-way ANOVA performed for statistically analyze the mean values, p value = 0.0129). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

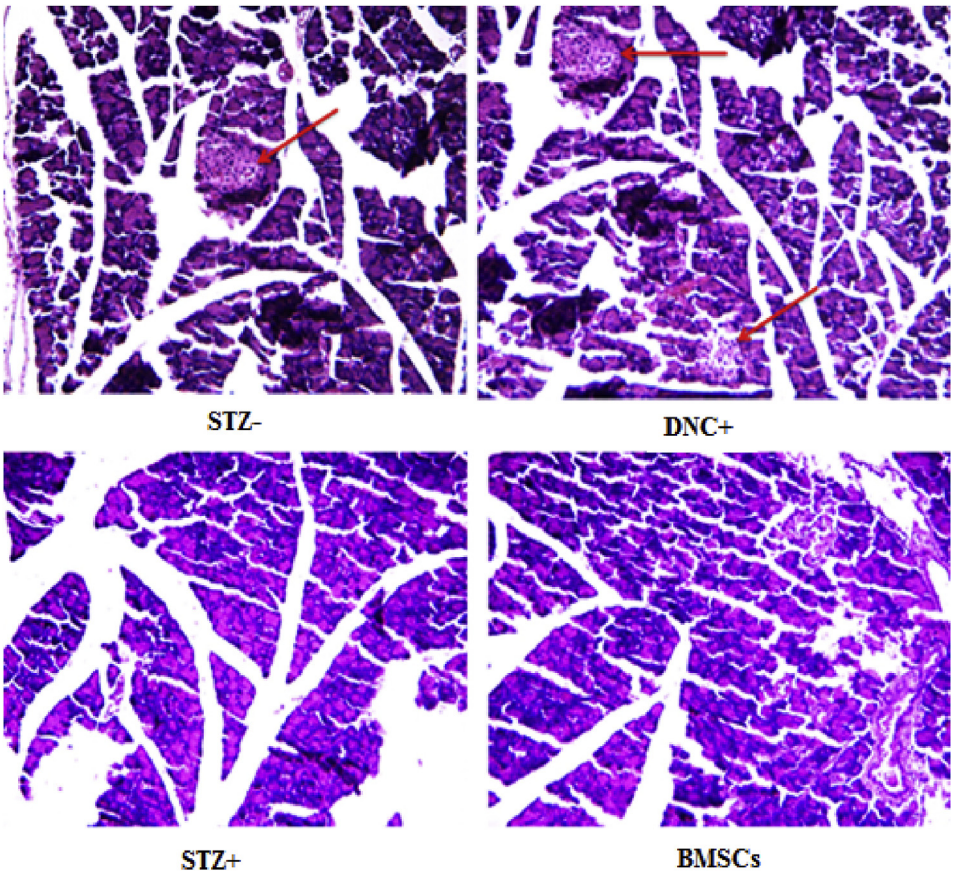

Fig. 9. H & E staining results to study the existence of Langerhans islands in pancreas isolated from *DNC* + *rats*. Tissues sections prepared from STZ – and DNC + rats' pancreases had Langerhans Islands (up left and right in this picture), tissues sections prepared from STZ + and BMSCs rats' pancreases however did not have any of these Islands (down left and right in this picture) (magnification 200X).

undifferentiated cells, after differentiation, DNC treatment performed and as it is shown in Fig. 3, cells surrounding the clusters detached. We harvested these detached cells and performed flowcytometery for BMSCs surface positive CD marker CD73 and negative CD marker CD45 [44–46]. As it is clear in Fig. 5, 94.8% of these cells were positive for CD73; which confirms that these cells which affected by DNC were undifferentiated.

3.4. Real-time PCR

To further investigate whether differentiated cells express important molecular markers after differentiation, expression level of these genes at mRNA level investigated by real-time PCR in different groups. After differentiation the expression level of *Insulin*, *Pdx1*, and *Ngn3* increased (DNC+ and/or DNC- samples in Fig. 6); the expression level of *Nestin* decreased after differentiation and vanished after treatment of these cells with DNC (DNC + sample). Differentiated cells express the gate keeper gene *P53* and its downstream gene *P21* less than undifferentiated sample (Fig. 6).

3.5. Induction of diabetes in rats

After injection of 50 (mg/Kg) of STZ intraperitoneally, blood glucose level of these increased. 14 days post injection their blood glucose level became stable and about 400 (mg/dI) (Fig. 7).

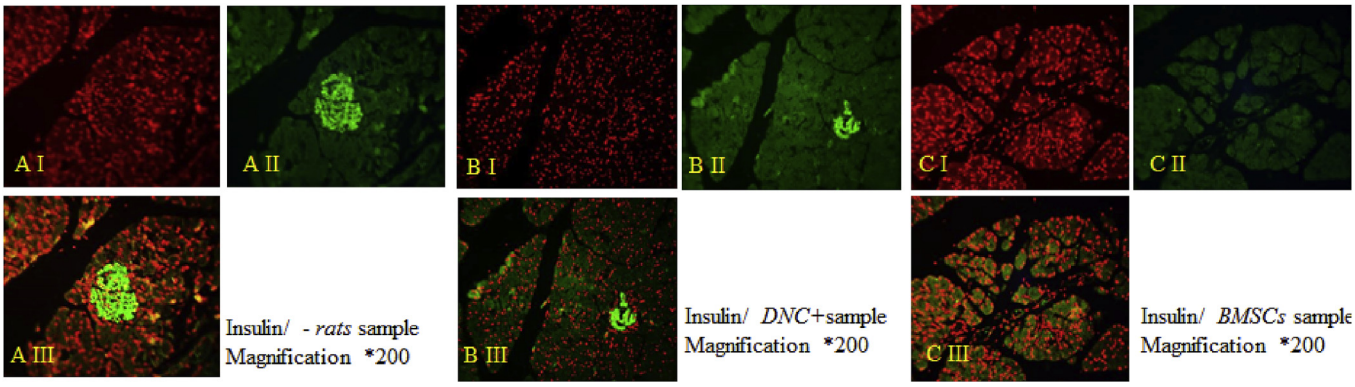

Fig. 10. IF test for -, *DNC+, and BMSCs rats*' pancreases to further confirm the existence of Langerhans Islands in *DNC + rats*. AI, BI, and CI are cells nuclei stained with propidium iodide (PI); AII, BII, and CII are the same section treated with antibody against insulin; AIII, BIII, and CIII are the merge pictures of AI/AII, BI/BII, and CI/CII respectively. As we can see in AII and AIII and/or BII and BIII in this picture, Langerhans Islands do exist in the sections prepared from – *rats* (AI – AIII) and *DNC + rats* (BI – BIII). The Islands in DNC + rats' samples were immature and hence smaller than that of – *rats*' samples (magnification 200X).

3.6. IPGT test

IPGT test revealed that rats in DNC+ and/or DNC- groups, reacted like STZ- (*-rats*, which did not received STZ, refer to materials and methods part). After injection of glucose, blood glucose level of rats in these three groups did not elevate to be more than 100 (mg/dl). On the other hand, *BMSCs/Ins/ + rats* blood glucose level increased after glucose injection and elevated into about 400 (mg/dl) (Fig. 8).

3.7. Hematoxylin & eosin and IF

Pancreases of rats from *STZ+/-, DNC+, BMSCs rats* isolated after IPGT test for H & E staining. As it is shown in Fig. 7, the Langerhans Islands were detectable in pancreases of *DNC+/-, - rats*. We did not detect any of these Islands in sections prepared from *BMSCs rats*' pancreases (Fig. 9). These pancreases from *-/DNC + and BMSCs rats* were further investigated with IF test for the existence of Islands. As it is demonstrated in Fig. 8, sections from – and/or *DNC + rats* did have Islands, with this different that the Islands of – *rats* were completely mature but that of *DNC + rats* were immature and hence were smaller in size (maybe because of reformation of these Islands). Sections obtained from *BMSCs rats'* pancreases did not have any signs of these Islands (Fig. 10).

4. Discussion

It seems that there is a long way which should be paved before cell therapy and/or regenerative medicine is translated into translational-clinical medicine for public benefit [47]. One of the main problems we are facing in cell therapy is the risk of tumorigenicity in recipient patients due to the involvement of undifferentiated cells in transplantation. MSCs are known to exert both anti-tumor and tumor progression activity [48–53]; it is of importance to mention that these cells have been genetically engineered and utilized to deliver anti-cancer agents to the tumor sites, due to their tumor homing ability [54,55]. However, in addition to tumor-associated fibroblasts, inflammatory cells, and etc. MSCs are attracted to tumor sites as well, and in this microenvironment, it is reported that these cells can secrete cytokines which are beneficial for tumor growth and progression [56,57]. As an example supporting tumor progression role of MSCs, Rodini et al. revealed that secretome profiling of MSC-GBM (glioblastoma multiform) co-cultures contains 126 differentially expressed proteins; and MSCs are able to induce GBM growth at least partially by secreting TGF-β1 [58]. In this study our main goal was to struggle to eradicate undifferentiated cells post differentiation of hBM-MSCS into IPCs, as much as possible, in order to escape from tumorigenicity risk of these cells in recipients; and at that time functionality of these cells remains.

Newly emerging joint between herbal medicine and regenerative medicine seems to be promising in solving some obstacles in this so called translational medicine [59]. Amounting of studies performed trying to reveal the anticancer and/or anti diabetic targeted effects of herbal remedies [50]. This is of great importance to notice that these targeted effects of herbs which are able to induce apoptosis in cancerous cells and not in normal cells, are time/dose dependent.

Based on WHO statistics, diabetes prevalence is increasing worldwide. Many studies performed with this aim, to find out real curing strategy rather than treatment. As providing resources for Islet/organ transplantation are limited, these struggles including new differentiation methods to find applicable source of cells for differentiation [60,61]. In our previous study we showed that curcumin has the potential to eliminate undifferentiated cells in vitro [15]; as it has been proved, stem cells treated with differentiation media may undergo changes including genomic instability [23,62,63], and after differentiation we would have heterogeneous population of cells including differentiated and undifferentiated hazardous cells. Hence it seems that nanocurcumin in dose/time dependent manner has the ability to specifically eliminate these undifferentiated cells with no significant effect on differentiated cells. In this study we tried to further confirm the functionality of these cells in vivo. First we had to demonstrate that DNC treated differentiated cells had the ability to produce and secrete insulin. We tried to investigate these two characteristics in 4 steps including: A) *morphological changes after differentiation*: hBM-MSCs are fibroblast like and after differentiation they form grape shape like clusters. B) *DTZ staining*: differentiated grape shape like clusters become crimson red in this staining if they store insulin in complex with Zn^{2+}, the characteristic which is seen in normal beta cells [64–66]. C) *MRNA expression level for important genes*: the expression level of Ngn3, Pdx1, and Insulin genes is higher in DNC+ and DNC- cells compared with undifferentiated cells. D) *ELISA*: determines the insulin secretion into the medium (data not shown, refer to our previous study [15]). After these in vitro studies, we transplanted cells from different in vitro groups into STZ diabetic rats. After transplantation of these cells, the IPGT test results revealed that diabetic rats which received DNC + or DNC- cells, reacted just like the control group (- or *STZ-rats*); but BMSCs rats which received undifferentiated cells reacted like *STZ + rats* (diabetic rats which didn't receive cells). H & E staining demonstrated that Islands of Langerhans do exist in *DNC + rats* but not in *BMSCs rats*. IF data confirmed the reformation of immature Islands in *DNC + rats*.

Amounting studies conducted, shedding light on the therapeutic potential of MSCs for diabetes cell therapy (Bohacova and Holan, [67–71]; in a case report study performed by Pradeep V. Mahajan et al., they transplanted undifferentiated MSCs in to a 17 years old female

suffering from T1D in 2 sessions. They demonstrated that these transplanted cells enabled the recipient to discontinue insulin consumption [72]. However we did not see the same result in our in vivo study, diabetic rats which received merely undifferentiated BM-MSCs showed similar manner to the + rats in IPGT test (Fig. 8), and we didn't see any trace of β like cells in H&E or IF experiments in samples obtained from this group (Figs. 9 and 10). Besides their marvelous characteristics including immunomodulatory and pro-angiogenic properties, that can help in arresting β-cell destruction; based on these in vivo results, it seems that undifferentiated BM-MSCs are not capable to be utilized merely and we expect them to regenerate destructed β-cells. Furthermore we propose that effect of DNC investigated in the current study is worthy to be investigated in more differentiation protocols to further confirm the ability of DNC in the area of cell based therapies.

5. Conclusion

Together our data demonstrates the curcumin not only has the potential to eliminate undifferentiated cells in vitro, but also these differentiated/treated cells are functional in vivo.

Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116908.

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