

# Therapeutic Potential of Amniotic Fluid-Derived Stem Cells into Pancreatic Lineage in an Animal Model of Diabetes

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## Abstract

Diabetes is a major health concern and it is estimated that India became diabetic capital in the future. The therapeutic potential of various medications, cell therapy, and islet transplantation is explored in recent years. The therapeutic potential of Mesenchymal Stem cells (MSCs) from bone marrow and from other sources has been studied for the treatment of diabetes. MSCs from bone marrow ameliorate hyperglycemia but are unable to restore normoglycaemia in diabetic animals when injected with a single dose. The therapeutic potential of another more primitive source of MSCs from human amniotic fluid-derived stem cells (hAFSCs) has not been explored in diabetes.

In the present study, we have assessed the effect of hAFSCs in a rat model of diabetes. hAFSCs transplantation has been seen to control blood glucose levels, and promote weight gain and other physiological parameters. Immuno-histochemical results suggested an increase in islet mass and number after hAFSCs transplantation. Q-RT-PCR results showed transcription factors *Ins-1*, *Oct-4*, *MAF-A*, and *PDX-1* were markedly upregulated in the hAFSCs transplanted group in comparison with diabetes and control groups.

These data suggest that hAFSCs possess considerable therapeutic potential for diabetes through the restoration of insulin mass and up regulations of transcription factors of pancreatic lineage.

## Keywords

Amniotic fluid stem cells, diabetic mellitus, rat

## INTRODUCTION

Diabetes is caused by insufficient production of Insulin by either absolute lack (type 1 diabetes) or relative lack of (type 2 diabetes) beta cells in the pancreas, resulting in high circulating glucose levels.

Chronic hyperglycemia leads to secondary pathophysiological changes in the kidney, brain, eye, and skeletal muscles along with an increased risk of cardiovascular disease. Current therapy for diabetes focuses primarily on the administration of exogenous insulin to restore glucose homeostasis. Therapeutic uses of islet transplantation and bone marrow-derived Mesenchymal Stem Cells (MSCs) have been limited for diabetic treatment because it encounters problems with restricted growth capacity, and low levels of insulin secretions for a longer time [1-5]. Search for another source of stem cells for the treatment of diabetes leads to exploring amniotic fluid. The stem cells from amniotic fluid have a remarkable ability to expand and differentiate into various lineage types, and immunomodulatory properties without the risk of tumorigenesis. Amniotic fluid-derived stem cells (AFSCs) possess pluripotent stem cell markers along with MSCs

markers. The AFSCs are prime candidates for the development of cell-based therapy against congenital and acquired diseases as it lacks ethical concerns & legal limitations. The application of hAFSCs as repair and regenerative medicine is gaining attention [6-11]. A clinical report on the use of autologous amniotic membrane-derived stem cells for the treatment of T1DM with less requirement of insulin after 6 months of transplant has been published [12]. Here, we investigated whether human AFSCs (hAFSCs) could develop into glucose-responsive insulin-producing cells in diabetic rat models.

In the present study, the generation of pancreatic lineage cells ( $\alpha$  and  $\beta$  cells) by injecting hAFSCs into an experimental diabetic rat model has been explored. Assessing their ability to restore glucose levels and production of insulin through beta cell regeneration would have a profound impact on the treatment of a major health problem. Our study suggests that hAFSC can be used for  $\beta$ -cell regeneration in diabetic patients without genetic manipulation.

## MATERIALS AND METHODS

### Animals

Thirty female Wistar rats (160-250 g) were taken in the study & were housed (3-4 rats /plastic cage) at a temperature of  $25 \pm 2^\circ\text{C}$  with alternating 12-hour light and dark cycles and free access to standard food pellets and water. The rats were randomly distributed into three groups: Control rats (Normoglycemic,  $n= 6$ ), Diabetic rats (DM,  $n=12$ ), and Diabetic rats with stem cell infusion (DM+SCT,  $n =12$ ). Rats were housed for 24 hrs individually in metabolic cages for assessment of 24 hr water & food intake, and urine output. The rats from all groups were sacrificed after 8 weeks of stem cell therapy under anesthetic ether. All experimental procedures in rats in the study followed the guidelines of the Animal Ethics Committee of the Institute after approval of the committee.

### Induction of diabetes

Rats were fasted overnight and diabetes was induced by an intraperitoneal injection of Streptozotocin (STZ) at a dose of 50 mg/kg prepared fresh in 10 mmol/L of citrate buffer, pH 4.5 [13,14]. Soon after the STZ injection, 5% sucrose water is given for two days to prevent drug-induced hypoglycaemic shock in the rats. Glucose levels were estimated before, 24 h after the STZ injection, and weekly until 8 weeks of stem cell injection. Blood glucose levels equal / to or above 250 mg/dL after STZ injection and subsequent three similar readings were taken as diabetic rats.

### Amniotic fluid stem cells (AFSC) isolation, ex vivo expansion, and characterization

Amniotic fluid samples from 16-20 weeks of pregnancy were collected for prenatal diagnosis through amniocentesis. 0.5 ml of sample is diluted with PBS and centrifuged at 1000 rpm for 10 min. Cells in the pellet were suspended in  $\alpha$ -MEM media containing 16.5% FBS, 1% antibiotic solution, and 1% Glutamax and plated in a culture flask at the density of 2000 cells/cm<sup>2</sup> and kept in an incubator. After a few days, adherent cells were cultivated and replated for expansion [8,15,16,17].

### Phenotypic Characterization of AFSC

Five times passaged AFSCs are resuspended in Phosphate Buffered Saline containing 1% BSA for phenotypic characterization. Antigenic profiling of hAFSCs was determined by a flow cytometer. For detection of cell surface antigens of SSEA-4, Oct-4, CD34, CD29, CD166, CD 44, CD 45, and CD90,  $1 \times 10^6$  cultured cells were incubated with the Phycoerythrin (PE)-or Fluorescein Isothiocyanate (FITC)-conjugated antibodies against above surface antigens. Detection and quantitation of cell surface antigen were performed by Flow Cytometer (Becton Dickinson) and Cell Quest software respectively.

### AFSC transplantation

AFSCs were passaged five times,  $2 \times 10^6$  human AFSCs tagged with 2mM PKH 26 dye (Sigma USA) according to

manufacturer's instruction, & administered intra peritoneal to diabetic rats after 7 days of STZ injection [18].

### Physiological variables

The body weight, blood glucose, food and water intake, and urine output were monitored weekly throughout the experimental duration (8 weeks post AFSCs injection). Rats were kept in metabolic cages for 24 hr every week until eight weeks of stem cell injection to assess for water and food intake, and urine output.

### Measurement of albumin and creatinine in urine

To measure albumin and creatinine in the urine, rats were housed in an individual metabolic cage. Urine was collected for 24 hr, centrifuged and levels of albumin and creatinine were measured using kits following the manufacturer's instructions and expressed as mg albumin/ mg creatinine in 24 hr urine.

### Intraperitoneal glucose tolerance test

To monitor glucose tolerance, rats fasted overnight and D-glucose solution at a concentration of 2 g / kg body weight was injected into the intraperitoneal cavity. Blood glucose levels were measured at different times starting with 0 min up to 150 min by standard protocol.

### AFSC homing and histopathology analysis

hAFSCs were labeled 2 mmol/L PKH 26 (Sigma, USA) according to the manufacturer's instructions. After eight-week post-AFSC infusions in diabetic animals, animals were sacrificed and part of the pancreas was embedded in paraffin for preparation of tissue sections and part of the pancreas was stored at  $-80^\circ\text{C}$ . Paraffin sections of the pancreas were stained with hematoxylin-eosin and used for immunohistopathology. RNA was isolated from frozen pancreatic tissue for gene expression analysis.

### Quantification of islet number, & $\beta$ -cells

Histological samples of the pancreas from all groups of rats were prepared using a standard protocol. Pancreatic sections were treated with primary antibodies of insulin (Abcam) and glucagon (Cell Signalling Technology, USA) followed by incubation with secondary antibodies tagged with Alexa Flour 488 and Alexa Flour 594 for insulin and glucagon (Invitrogen) respectively. Pancreatic sections were counterstained with Hoechst dye at 0.1 mg/ml and observed under fluorescence microscopy. Slides were stained with hematoxylin and eosin for islet mass quantification and the number of islets was counted in serial sections of the pancreas in each group of rats.

### Determination of mRNA levels of functional $\beta$ cell genes, early and later stage developmental genes for pancreas by Real-time PCR

Total RNA from the pancreas of all group of rats were extracted using TRIzol reagent (Invitrogen) and the integrity of RNA was assessed before the synthesis of complementary DNA (cDNA). 5  $\mu\text{g}$  total RNA from each group was taken

for cDNA synthesis using a kit (cDNA super script kit, ABI, USA) with random primers. Real-time quantitative RT-PCR for various genes was performed with Brilliant SYBR Green Q PCR master mix (Roche) using human primers of specific genes and sequences listed in Table 1.

**Table 1.** Primer sequences of genes for Reverse transcription polymerase reaction

SNo	Gene	Primer sequence
1.	Oct-4	Forward: 5'-GGGTGGAGGAAGCTGACAAC-3' Reverse: 5'-GGTTGCCTCTCACTCGGTTTC-3'
2.	Isl-1	Forward: 5'-TGCAAGGACAAGAAGCGAAG-3' Reverse: 5'-GAGTTCCTGTCATCCCCTGG-3'
3.	PDX1	Forward: 5'-CCCTCCTACAGCACTCCACC-3' Reverse: 5'-CCGCTGTGTGTGTTAGGGAG-3'
4.	MAF A	Forward: 5'-TCATCCGGCTCAAGCAGAAAG-3' Reverse: 5'-GTTGGCACTTCTCGCTCTCC-3'
5.	Insulin	Forward: 5'-CAGATCACTGTCCTTCTGCC-3' Reverse: 5'-GTTGGTTCACAAAGGCTGCG-3'
6.	GAPDH	Forward: 5'-TGCACCACCAACTGCTTAGC-3' Reverse: 5'-GGCATGGACTGTGGTCATGAG-3'

The average threshold cycle (CT) of fluorescence units was used to analyze the genes. The mRNA levels of target genes

were normalized by levels of the housekeeping gene GAPDH. Quantification was calculated as  $\Delta CT$  of a particular gene (CT value of target gene- CT value of GAPDH gene).  $\Delta\Delta CT$  was calculated as  $\Delta CT_{Diabetes} - \Delta CT_{normal}$  and  $\Delta CT_{diabetic+SCT} - \Delta CT_{diabetic}$ .

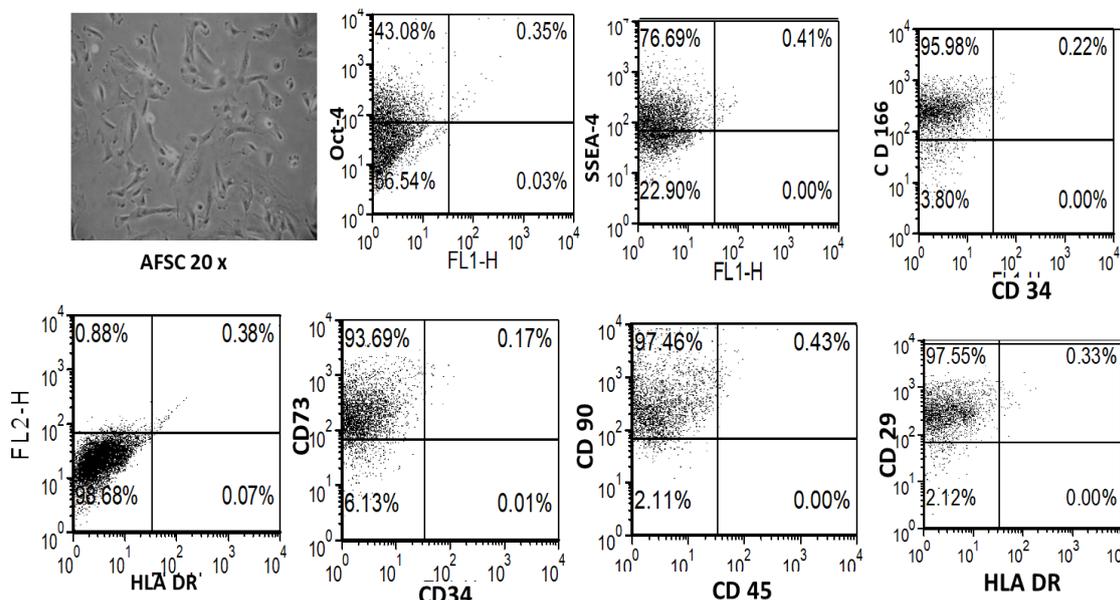
**Statistical analysis**

To find the significance, statistical analysis was performed with the use of SPSS software (SPSS Inc). All data are presented as mean  $\pm$  SEM. We used ANOVA followed by the Post Hoc test for the comparison of multiple groups. A value of  $p < 0.05$  was considered statistically significant.

**RESULTS**

**Morphology and Phenotypic Characterization of Human AFSCs**

Heterogeneous hAFSC populations are seen in culture (Figure 1) and cells are passage up to 20 populations. Human AFSC is cryopreserved after 4-5 passages, reseeded onto the flask, and doubling time is 36 hrs (same as unpreserved cells). Human AFSCs were stained for surface and nuclear markers and hAFSC stained positive for a number of markers typical of MSCs such as CD29, CD44, CD73, CD90, CD105, CD166 and negative for CD 45, CD 34, and HLA-DR. Human AFSCs are positive for embryonic stem cell markers Oct-4 (40%), and SSEA-4 (80%) (Figure. 1).



**Figure 1.** Morphological and phenotypic characterization of human amniotic fluid stem cells. Representative flow cytometry analysis of amniotic fluid stem cells for surface antigens SEA4, CD29, CD44, CD73, CD90, CD166, CD34, CD45, and HLA-DR Morphology shows the typical spindle shape cells. Representative flow cytometric dot-plots showing that AFSC is Oct-4 + , SSEA 4+ , CD166+/CD34 -, HLA DR -, CD73+/CD34 -, CD90+/CD45- , CD29+/HLADR

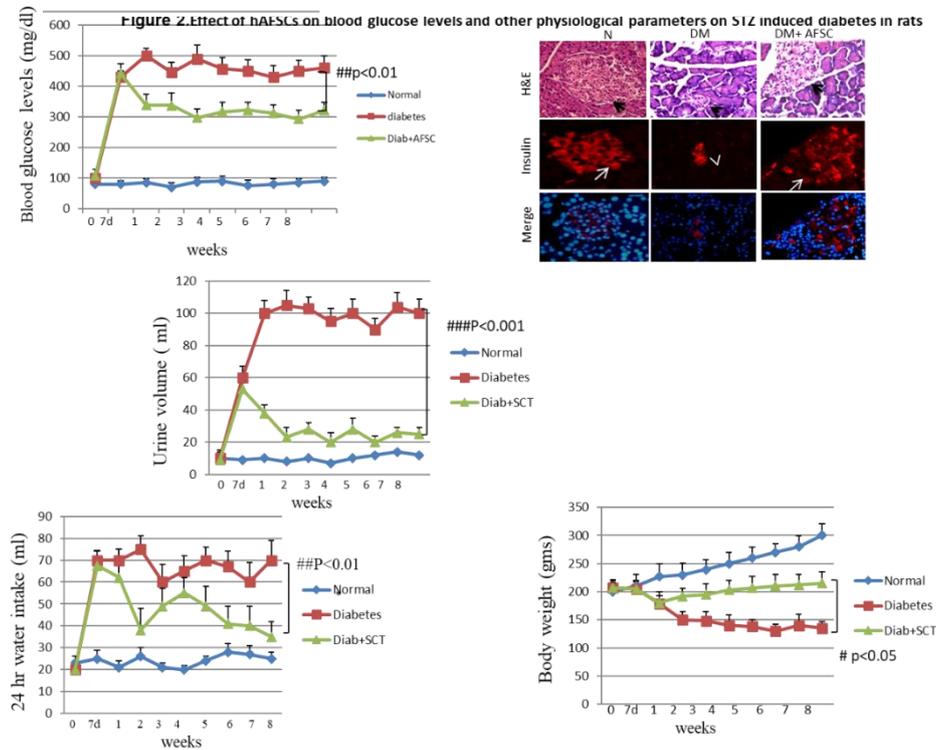
**Effect of Diabetes and stem cell therapy on physiological parameters**

All rats developed diabetes after STZ administration. Rats were assessed for basic parameters viz gain of body weight, food, and water intake, blood sugar, 24hr urine output every week for a period of 8 weeks post AFSC injection. Diabetic

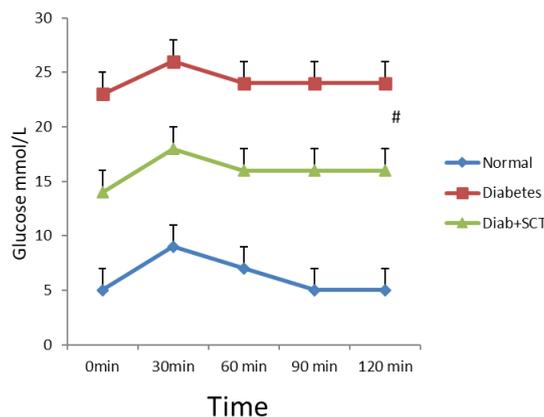
rats showed polyphagia, polydipsia, and polyuria with a significant increase in blood sugar levels (400mg/dl) compared to control rats (80mg/dl). The amount of diet consumed was more in diabetic rats but it did not result in weight gain compared to control rats (data not shown). AFSCs were injected after confirming diabetes post 7 days of

STZ injection, rats showed a significant decrease in blood glucose levels (300mg/dl), food and water intake started 1 week post-therapy (Figure 2). Significant weight gain was observed in the stem cell therapy group (Figure.2,  $p < 0.05$ ). Diabetes was established as a small size and number of Islet of Langerhans were observed by histochemical analysis

(Figure.2). Blood glucose levels were significantly high in the diabetic group that started with STZ injection and remained high after 8 weeks of study design, whereas glucose levels were decreased after AFSCs therapy in diabetic rats (Figure. 2).



**Figure 2.** Effect of hAFSCs on blood glucose levels and other physiological parameters on STZ-induced diabetes in rats. Reduced Islet mass was observed in diabetic rats vs control rats, as showed by hematoxylin and eosin staining and also by insulin immunofluorescence staining. Body weight, water intake, 24 hr urine output were measured in each group of rats weekly up to 8 weeks after AFSC injection. Data are mean  $\pm$  SEM ( $\#P < 0.05$ ,  $\#\#\#P < 0.01$  in Diabetes vs Diab+SCT group).



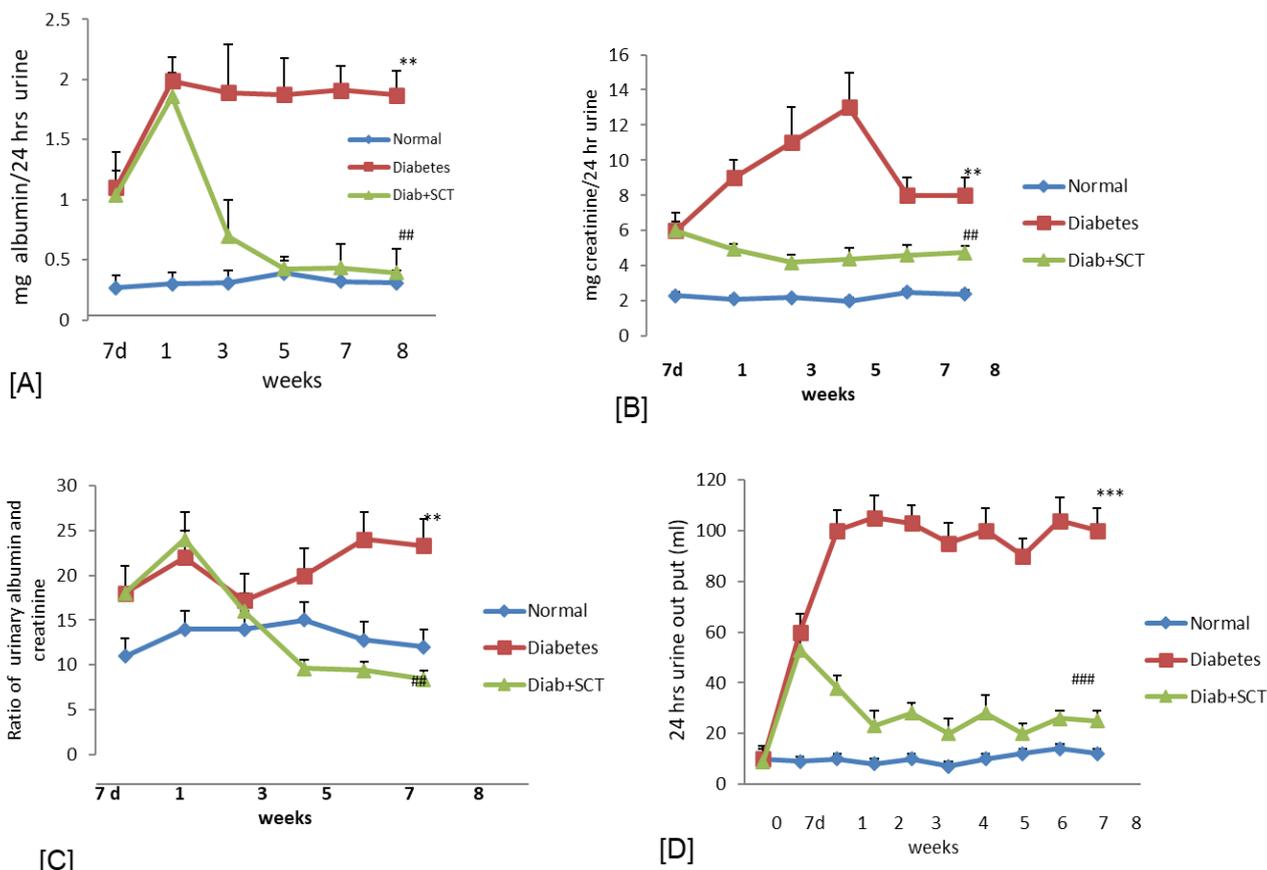
**Figure 3.** Effect of hAFSCs on glucose intolerance in STZ-induced diabetic rats.

Intraperitoneal glucose tolerance test was performed in control rats, diabetic rats, and diabetes with hAFSCs treated rats. Values are mean  $\pm$  SEM. \*  $P < 0.05$  compared with normal vs Diab,  $\#P < 0.05$  Diab vs Diab+SCT group.

**Effect of diabetes and stem cells therapy on severity of diabetes by estimating urine albumin/ creatinine ratio**

Urine creatinine and albumin were estimated weekly in 24-hour urine output in control, diabetes, and diabetes with stem cell therapy groups. There is a significant increase in albumin

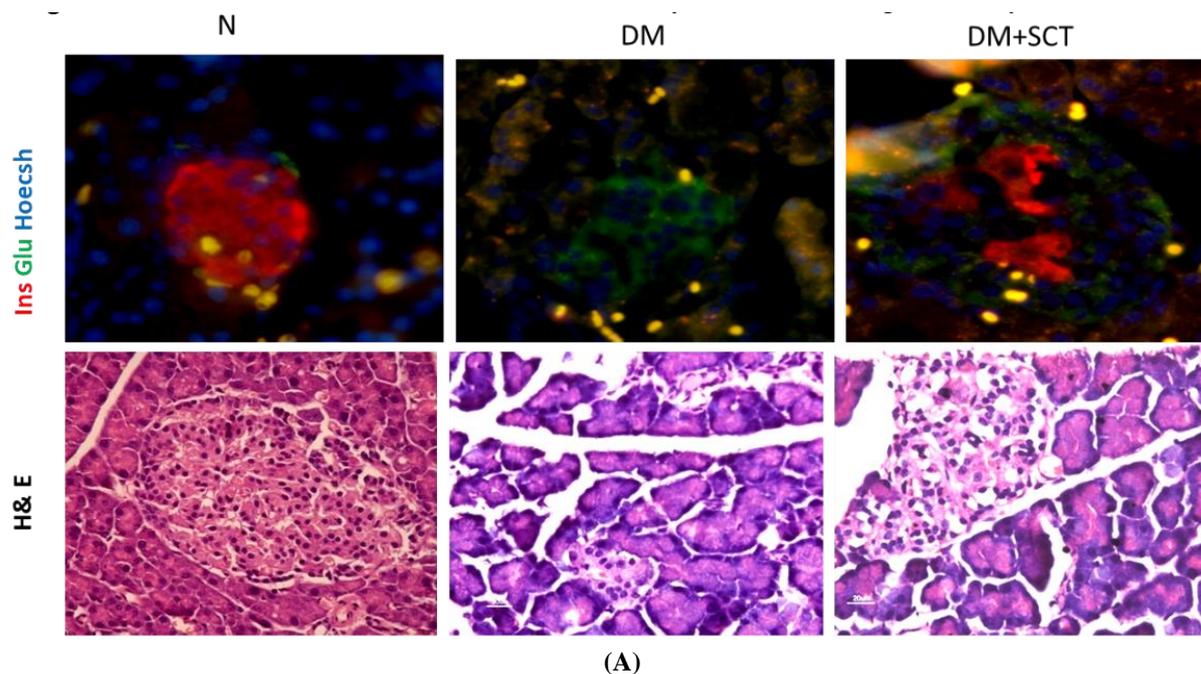
and creatinine ratio in the urine of diabetic rats when compared with control rats, whereas, in contrast, diabetes with stem cell therapy group showed a significant decrease in the ratio of albumin to creatinine when compared with diabetic rats (Figure. 4). Decrease in albumin to creatinine ratio showed the positive effect of stem cell therapy.

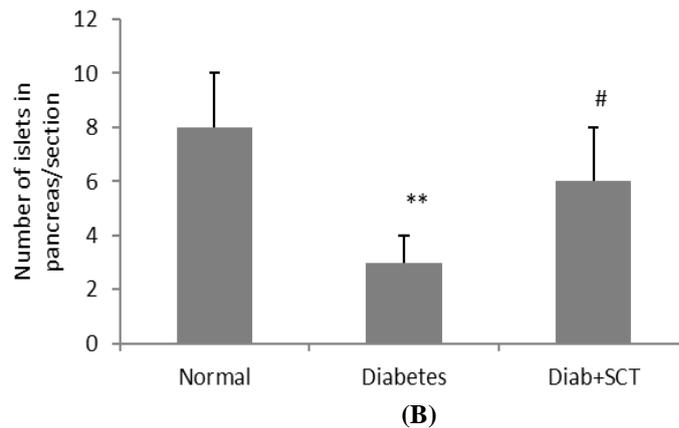


**Figure 4.** Physiological response to AFSC injection in STZ induced diabetic rats on, Micro albumin [A] and creatinine in 24 hr collected urine [B], Ratio of microalbumin to creatinine [C] 24 hr urine output (D), showed the positive effect of AFSC treatment. \*\*P < 0.01 normal vs Diab; ## P < 0.01 Diab vs Diab+SCT group.

**Effect of Diabetes and stem cell therapy on glucose tolerance test**

The normal pattern of glucose metabolism is seen in control rats on the glucose tolerance test (GTT). Impaired metabolism has been seen in diabetic rats and circulation levels of glucose were high. GTT results revealed better glucose metabolism following AFSCs injection (Figure. 3). Result suggests that hyperglycemia in diabetic rats could be ameliorated by hAFSCs.





**Figure 5.** (A) Histochemical analysis of islets of Langerhans of the pancreas with hematoxylin and eosin staining, 8 weeks post Injection in control (N), diabetic (DM) and diabetic + AFSC treatment (DM+SCT). Representative pancreatic islet from a normal control rat. Mass reduction and shrinkage of cell volume in islet from a diabetic rat treated with STZ as indicated by an arrow. STZ-treated rats receiving AFSC show, a normal islet morphology with no reduction of  $\beta$  cell mass. Detection of insulin expression by immunofluorescence, 8 weeks post-injection, in Normal control, STZ-treated, STZ-treated AFSC-injected rat. AFSC-injected rats show stronger expression of insulin compared to STZ-treated diabetic rats, where almost no production of insulin is detected. Detection of glucagon-expressing cells by immunofluorescence: normal control, STZ treated, and STZ-treated AFSC-injected rat. AFSC injected rat present a normal distribution of glucagon-expressing cells as it is in normal control animals. Distribution in diabetic rats is highly disorganized. Magnifications x 40. (B) Quantification of pancreatic islet numbers. Values are reported as the mean of three sections of the same samples taken from a 1mm distance. AFSC-injected rats show a greater number of islets compared to STZ-treated rats, though less abundant than in the control. \*\*  $p < 0.01$  control vs Diab; #  $P < 0.05$  when Diab vs Diab+SCT.

**AFSC engraftment and homing**

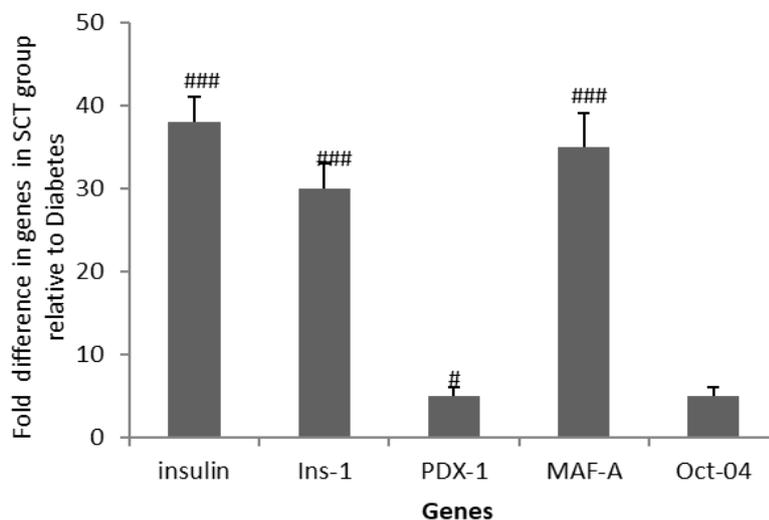
AFSC was labeled with the dye PKH 26 in order to be traced in the host tissue after injection. No AFSC was detectable in the exocrine/endocrine portion of the pancreas of the rats 8 weeks post-injection. (It is reported that engraftment of labeled stem cells was seen after 24 hrs of injections but in the later time of period viz 4, 8 weeks after, cells were diluted and not observed).

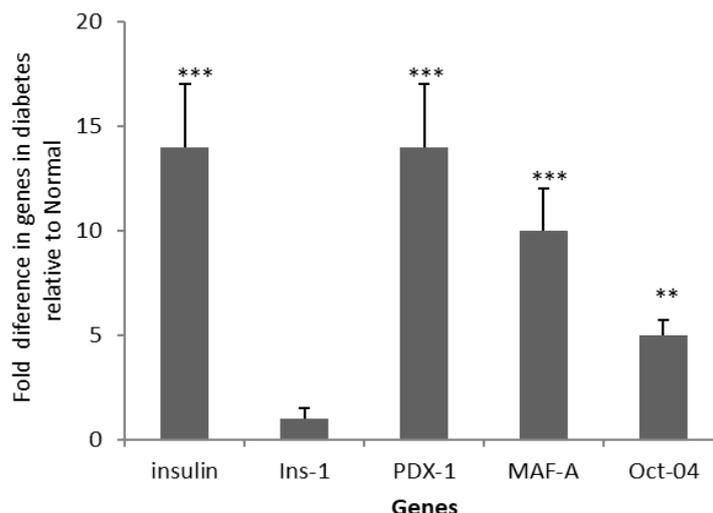
**Effect of AFSC on islets number and endocrine pancreatic markers (Insulin, Glucagon)**

Significant reduction in insulin expressing  $\beta$ -cells in STZ-treated rats compared when compared with control rats

(Figure. 5). Whereas AFSC treatment showed significantly higher  $\beta$ -cell numbers when compared with STZ-treated rats.

Histological examination showed significant alteration in islet structure as well a reduction in number was observed in the STZ-induced diabetic rats (Figure. 5) in comparison to AFSC-treated and control rats. The structure, number, mass, as well as insulin and glucagon-expressing cells of pancreatic islets of AFSC-treated rats, resembled similar to control rats (Figure. 5 A, B). However, a significant increase in Glucagon expressing  $\alpha$ -cells of pancreatic islets in the diabetic group was observed versus control rats (Figure. 5 B) in the present study.





**Figure 6.** Effect of AFSCs treatment on pancreatic hormone genes and transcription factors (Insulin, Pdx1, Isl-1, Maf A) in STZ-treated rats by QRT-PCR. Gene expression analysis was performed among normal control, STZ-treated rats (Diab), and Diab + AFSCs (SCT) group. Gene expression was evaluated for groups (normal control vs diabetic rats\*\*P < 0.01, \*\*\*P < 0.001, Diabetic and Diabetic +SCT group ## P<0.01, ###P<0.001)

**Alterations in expression of stem cell markers, early developmental markers, and pancreatic Lineage markers in hAFSCs-treated diabetic rats**

Significant several folds increased expression of stem cell marker as Oct-4, transcription factors for pancreas viz PDX-1, Ins-1, MAF-A, and Insulin were observed in hAFSC treated diabetic rats whereas decreased expression in transcription factors PDX-1, Ins-1 in diabetic rats was observed (Figure. 6).

**DISCUSSION**

Stem cell therapy is regarded as one of the alternative therapies for islet transplantation in diabetes. AFSCs are unique in origin, highly proliferative, and nontumorigenic and there are fewer ethical concerns about them. Here we present the therapeutic application of AFSCs obtained from human patients during amniocentesis against STZ-induced diabetes in rat models to preserve β cell function.

AFSC injection in diabetic rats was able to protect the rats as physiological parameters are improved and there is also the preservation of morphology, number, and mass of pancreatic islets as well as insulin and glucagon-secreting cells in islets up to 8 weeks of transplantation.

The study showed the isolation & expansion of stem cells from amniotic fluid and that these cells are suitable for therapeutic purposes and easier to culture. AFSCs positively express embryonic, mesenchymal, pluripotent stem cell markers and adhesion molecules.

However, AFSCs are negative for endothelial and hematopoietic markers and HLA-DR negative which makes them suitable for therapy [19]. The report suggests that AFSCs are non-tumorigenic and are free from ethical restrictions [16].

Improved physiological parameters (increase in body weight, fur coat, water, and food intake, and 24-hour urine

output) were observed in relation to hAFSC treated group which is in accordance with positive results of therapy and are well reported when beta cells are regenerated after injury [20,21]. A glucose tolerance test is not a gold standard nowadays to differentiate between type-1 and type-2 diabetes but is recommended to establish the diagnosis of gestational diabetes mellitus (GDM) [22]. We observed impaired glucose tolerance in diabetic rats whereas, in the stem cell therapy group, the anti-diabetic effect of hAFSC on OGTT showed lower blood glucose levels as compared to the diabetic control. It has been reported earlier that stem cell therapy has insulin-stimulative activity [23].

The presence of creatinine and microalbumin in urine (24 hr output) showed the effect of diabetes on kidneys which in later stages causes nephropathy. hAFSC treatment on diabetic rats showed decreased levels of creatinine and albumin in urine and the protective effect of stem cell therapy has been seen by a lower ratio of albumin to creatinine in urine [24].

Though the positive effect of stem cell therapy is seen by improved physiological parameters, we have not observed PKH-labeled stem cells in the pancreas after eight weeks of injection and it is reported by others also that at later stages labeled stem cells are not observed in tissue and seen by only in early stages that are after 3 days of injection [9,25]. Double labeling of insulin and glucagon in the pancreatic section in the stem cell-treated diabetic group reveals the augmentation in the number and mass of pancreatic beta cells in comparison to diabetic control, though size and number are less in comparison to normal control.

Further, to evaluate the potential of hAFSC to differentiate into β cells, real-time PCR was done from pancreatic RNA using human primers for different transcription factors. Expression of isl-1 & PDX1 (regulator of normal islet cell development) are increased several folds in the stem cell therapy group. MAF-A transcription factor insulin-producing cells are also induced several folds in the stem cell therapy

group which for better glucose response. It is reported that PDX1, MAF-A can induce mouse embryonic stem cells and induced pluripotent cells into insulin-producing cells when transplanted into diabetic mice and has the ability to restore normal glucose levels [26]. An increase in several-fold increased expression of OCT-4 (a marker for embryonic stem cells) in hAFSC-treated rats suggests that stem cells from the amniotic fluid are able to restore glucose-levels in diabetic rats by differentiating into insulin-producing cells which is in accordance with a study that showed human AFSCs differentiated into insulin-producing cells *in vitro* [27,28].

Though it is reported that genetic modification of amniotic fluid-derived stem cells produces pancreatic progenitors' *in vitro* condition [27,28]. Here in this study, we evaluated the potential of the non-genetically modified human amniotic fluid stem cells into pancreatic cell lineage *in vivo* (MAF A, PDX-1, Ins-1, insulin) and we observed the increased expression of genes and transcription factors which are necessary for the production of insulin, glucagon another pancreatic hormone.

The findings of this study suggest that AFSCs have therapeutic potential for diabetes mellitus by stimulating the regeneration of  $\beta$ -cell to restore normal glucose levels.

Competing Interests: The authors have declared that there are no competing interests

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