

Protective effect of placental mesenchymal stem cells on histological changes of pancreas experimentally induced by alloxane in mice

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Abstract

In an attempt to find a treatment for diabetes by Placental mesenchymal stem cells, we induced diabetes in albino mice by injected them with the diabetic agent Alloxan intra peritoneal cavity dose 70 mg / kg of body weight for the group 1 and 2, then injection of the diabetic mice with Placental mesenchymal stem cells in the peritoneal cavity for the group 2 then the glucose levels in the blood of these mice were measured by using a GLUCOTREND 2, while group 3 was control injected with normal saline only. Pancreas tissue where tested by studying histopathological effect of Alloxan, the endocrine pancreas histologically showed decreased in the size and number of Langerhans islets with vacuolar degeneration and necrosis of almost all cells in the atrophied islets. Also, there was congestion of blood vessels in the interlobular space and there was distortion of exocrine pancreas. There was vacuolar degeneration and necrosis of the epithelial cells of the acini, also there was atrophy of acini which lead to edema and dilatation of interacini space. Moreover, there was thickening in the wall of blood vessels, and there was thrombus in some blood vessels. In addition to the infiltration of inflammatory cells in the interlobular septa. Compared with the section of pancreas of control group showed the normal structure of islets notice the normal cells shape surrounding by exocrine pancreas. Then the pancreas studied after injection of Placental mesenchymal stem cells, while there was regeneration of islets and acini which seem to be normal comparing with control group, there was improvement of normal histological appearance of mice treated with alloxan and then injected with placental mesenchymal stem cells. The results of the diabetic mice which treated with placental mesenchymal stem cells demonstrated present of significant differences ($P \leq 0.05$). Our conclusion of this study revealed that placental mesenchymal stem cells have a protective role against the histological changes of pancreas induced by alloxan.

Keywords: Placental mesenchymal stem cells, Diabetes mellitus, Pathological changes, Pancreas

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الدور الوقائي للخلايا الجذعية اللحمية للمشيمة على التغيرات النسيجية للبنكرياس المستحدثة تجريبياً باستخدام الالوكسان في الفئران

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الخلاصة

اهتمت الدراسة الحالية حول استخدام الخلايا الجذعية اللحمية للمشيمة في علاج داء السكري المستحدث تجريبياً في الفئران المهقاه بحقنها داخل التجويف الخلفي بمادة الالوكسان بجرعة ٧٠ ملغم/ كغم من وزن الجسم في المجموعة الأولى والثانية ثم حقنت الفئران المصابة بالسكري بالخلايا الجذعية اللحمية المعزولة من المشيمة في التجويف الخلفي في المجموعة الثانية وتم قياس نسبة الكلوكوز في دم هذه الفئران باستخدام Glucotrend2 بينما حقنت المجموعة الثالثة (مجموعة السيطرة) بالمحلول الملحي الفسلجي. تمت دراسة التغيرات المرضية لنسيج البنكرياس نتيجة تأثير الالوكسان، وتمثلت التغيرات النسيجية بصغر حجم وقلة في أعداد جزيرات لانكرهانز مع وجود التكتس الفجوي ونخر الخلايا مع ضمور في الجزيرات فضلاً عن وضوح الاحتقان في الأوعية الدموية في الحيز بين الفصيصات

واضطراب في نسيج البنكرياس الغدي. كما أظهرت النتائج وجود تورم حاد ونخر في الخلايا الظهارية للعنيدات Acini مع وجود ضمور للعنيدات والذي أدى إلى حدوث الوذمة، فضلاً عن وجود تنخر في جدران الأوعية الدموية ووجود الخثرات الدموية فيها مع وجود ارتشاح للخلايا الالتهابية في الحويصلات بين الفصيصات مقارنةً مع مجموعة السيطرة. تمت دراسة نسيج البنكرياس بعد حقن الفئران المصابة بداء السكر بالخلايا الجذعية اللحمية للمشيمة (المجموعة الثانية) حيث أظهرت النتائج حدوث تجديد للجزيرات والعنيدات التي بدت طبيعية مقارنةً مع مجموعة السيطرة حيث كان هنالك تحسن للمظهر النسيجي للبنكرياس لفئران المجموعة الثانية التي عولجت بحقنها بالخلايا الجذعية اللحمية للمشيمة وأظهرت نتائج التحليل الإحصائي لمستوى الكلوكون بالدم للفئران المصابة بالسكري والمعاملة بالخلايا الجذعية اللحمية المعزولة من المشيمة وجود ارتفاع معنوي عند المستوى ($P \leq 0.05$). نستنتج من الدراسة الحالية بأن للخلايا الجذعية اللحمية للمشيمة دور وقائي للحد من التغيرات التي أحدثتها حقن الالوكسان في البنكرياس.

Introduction

For the past few years, in an attempt to find new sources of cells that may be used in cell therapy numerous researchers have highlighted the particular properties of mesenchymal stem cells (1-4). Several studies have shown the superior role of stem cells in regenerative medicine and tissue engineering because their administration, following the isolation and *in vitro* culture leads to the cell's engraftment in certain tissues and the release of anti-inflammatory cytokines (5). This study focuses on placental mesenchymal stem cells (PMSCs) in an attempt to differentiate them *in vivo* into islets of Langerhans in order to treat the diabetes mellitus. The placenta is one of the most important sources of stem cells, and has been studied extensively over the past period. The placenta fulfills two main desiderata of cell therapy: obtaining high number of cells and use of non-invasive methods for their harvesting (6,7).

Material and methods

Animals used in the experiment

In this study we used albino mice of the balb /c strain, the mice were in good health, they were 2-3 months of age, weighing between 25-30 gm of both sex male and female. The animal was kept in a room at temperature between (20-25) °C, 12 hour light and 12 hour dark.

The drug used

In this study Alloxan used as a diabetogenic chemical, diabetes was induced by injecting the mice with 70 mg/kg of alloxan (intra peritoneal) after fasting the animals for 24 hours (8). While glucose solution in the drinking water of diabetic condition was confirmed 24 hours. After alloxan injection, not all the mice were diabetic, they were diabetic after 72 hours (9).

Measurement of glucose levels in animals

We regarded the mice which have more than 300 mg/ml glucose level, these mice appear tired and have polyuria. In this study we used the Glucotrend2

instrument (UK) to measure the blood taken from the tail vein of the mice then we measure the level of glucose in every 3 day in order to check the differences in the results.

Experimental design

The experiment recommended 30 mice, 20 of these mice were injected with alloxan so all of them were diabetic and 10 mice considered as a control, they were given normal saline. Group 1: Ten mice injected with alloxan. Group 2: Ten mice injected with alloxan and then injected with placental mesenchymal stem cell suspensions. Group 3: Ten mice which considered as control, injected with normal saline only.

The blood sugar was measured before inducing diabetes to record the value, then we measured the value after 3 days from injected the mice with alloxan were recorded and after injecting the mice with cells we measured the value were recorded every 3 days, and record the results. After 21 days the animals were scarified to examine in the pancreas grossly and to study the histopathological changes.

Preparation of alloxan solution

A dose of 70 mg/kg was recommended (9), then the dose was prepared, depending on the weight and number of mice in the experiment, where 35 mg of alloxan was freshly dissolved with 20 ml of normal saline injected to mice IP, since 1 ml of the solution can be given for each mouse, if diabetes doesn't occur, another dose is given.

Injected placental mesenchymal stem cell to the diabetic mice

After dissecting the animal chosen for extraction of mesenchymal stem cells of its placenta the extract PMSCs were injected directly into the peritoneal cavity at dose 1.0×10^6 cells/100 μ L phosphate-buffer saline (PBS) per mouse to diabetic animals (group 2), then the measure of blood sugar were every three days on a regular basis (10).

Histological technique of light microscope

Specimens of pancreas (of the diabetic mice and diabetic mice which injected with PMSCs) were kept in 10% neutral buffer formalin (11). The slides were prepared from pancreas according to (12). The specimens were dehydrated by series of ethanol alcohol, cleared in xylene then impregnated and embedded in paraffin wax (melting point 60°C in an oven for 4-6 hours. Then the specimens were blocked in paraffin wax serial transverse section of 4 microns thickness were cut by using rotary microtome. The section then floated in water bath at 37-40°C and mounted on a slide, then passed through hematoxylin and eosin staining method (12).

Placental mesenchymal stem cells isolation

Isolation of (PMSCs) is done according to methods used by (13,14).

Scanning of electron microscope examination

The substrate was prepared by dissolving 1 gm of gelatin in 100 mL of deionized distilled water and sterilized by autoclave, use this solution as the basic substrate grow on the placental stem cells, as it has been taking 0.5 ml of a sterile prepare solution of gelatin and doused in every hole of the tissue culture plate 4-well on pieces of a sterile cover glass slide therein and left to dry inside the hood. These prepared plates were used for the cultivation of cells that later used for scanning electron microscopic examination (15,16).

Results

Placental Mesenchymal Stem Cells (PMSCs)

Where the isolation of PMSCs was done at (14-16) days from pregnant mice and cultivated the cell suspension in the DMEM high –glucose media with the supplements and the suspension containing RBC, WBC and two types of the stem cells: Hematopoietic Stem Cells (HSCs) which decrease in numbers gradually and Mesenchymal Stem Cells (MSCs), in this stage we observed all types of cells took round shape as in (Figure 1). After (4 - 5) days we notice some cells began to adhere on the bottom of the tissue culture flask and beginning to take spindle shape which represent PMSCs while other stay plotting in the tissue culture flask represent (HSCs) (Figure 2). After two weeks of cultivation we observed confluence and forming monolayer as a result of attachment placental mesenchymal stem cells which have spindle shape-like fibroblastoid morphology (Figure 3).

We obtained pure culture of PMSCs after two weeks only of initial cultivation by changing the media for maintenance of the cells and discard the hematopoietic cells

which were not attachment while PMSCs staying because of ability to adherent to the plastic of tissue culture flask. The most important characteristic *in vitro* features of PMSCs are their ability to aggregate and form colonies of fibroblast-like cells appeared in culture because of division activity when initial culture began to form this colonies at 4-5 days which expanded and gradually grew to form large individual colonies and display fibroblast-like morphology with long and short processes, also the PMSCs showed the ability to stain with H&E stain (Figure 4).



Figure 1: The Placental derived cells suspension cultured in DMEM+10% FCS after one hour of culture under inverted microscope (200X).

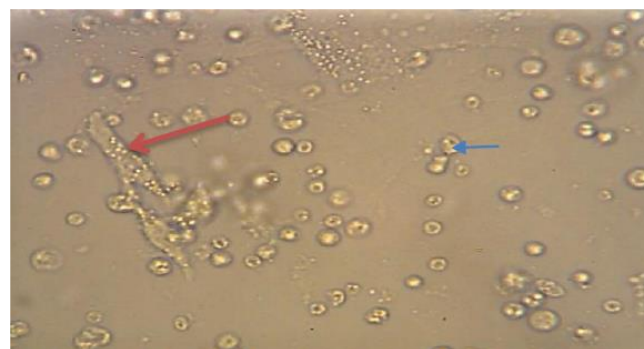


Figure 2: Placental derived cells after 4 days (—→) attachment of PMSCs and beginning to take spindle shape, were (—→) HSCs stay plotting (round shape) in the flask of culture under inverted microscope (200X).

Determination the cells viability

Testing and measuring the viability of the PMSc showed that the viability of the cells was 94.5% according to these equivalent: % viable cells = $[1.00 - (4 \div 73)] \times 100$. Viable cells = 94.5%

Harvesting PMSc and determination their number

PMSCs have high ability to proliferation and forming colonies, whereas the number of cells in harvesting cells from the initial culture after 21 days of cultivation was

14.6×10^4 and after culturing the cells in new tissue culture flasks, after 4-5 days we harvest the cells again which showing that the number increase to 23.9×10^4 .

Alkaline Phosphatase Activity (ALP)

The PMSCs in the 2nd and 4th passage was stained for ALP activity, and this surface marker served as biochemical marker for dedifferentiation stem cells, so the cells have taken dark red- brown color as in (Figure 5).

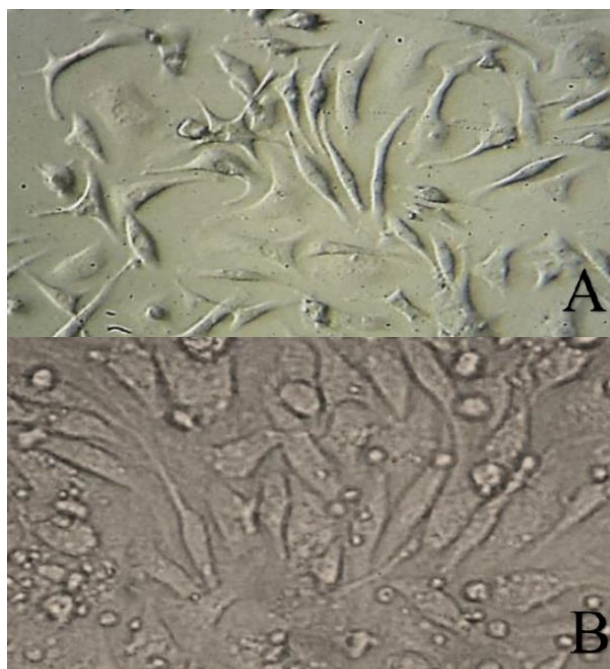


Figure 3: PMSCs forming monolayer after 2 weeks of cultivation under inverted microscope. (A) Under phase contrast (200X), (B) Under inverted microscope (400X).

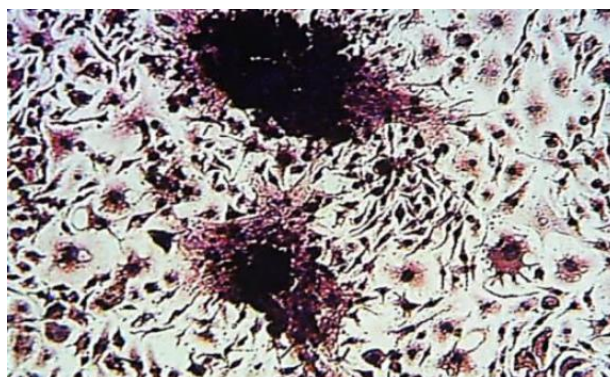


Figure 4: The ability of PMSCs to aggregate and form colonies of colony forming unit-fibroblast appeared under the inverted microscope (100x).

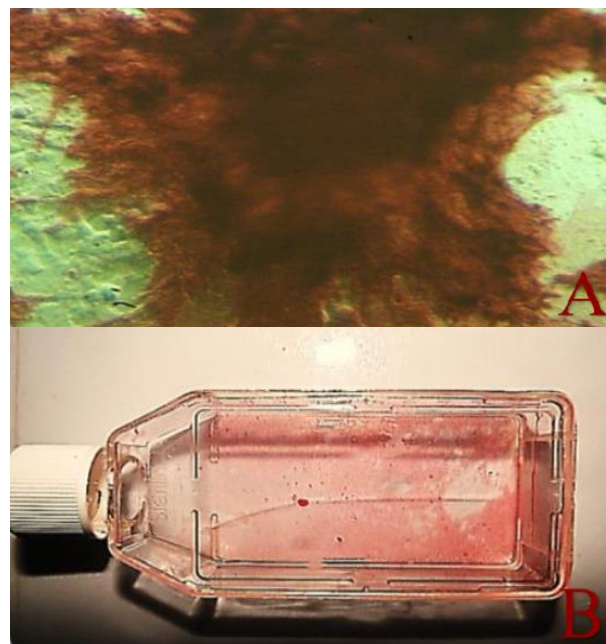


Figure 5: The alkaline phosphatase activity (dark red-brown) which represent the + ve result. (A) Under phase contrast microscope (200X). (B) The flask of alkaline phosphatase test.

Electron microscope study

In this study, we use the scanning electron microscope to observe the phenotypic characteristic of PMSc isolated from the placenta, where results showed the active and effective division of stem cell in its spherical shape as well as have been observed attachment of stem cells and the formation of protoplasmic extensions and observed filipodia which help the cells on the surface of the coverslips, also observed the lamilapoidia which represented of the surface receptors of cells and signals between the cells, we also observed the distinctive spindle shape of the mesenchymal stem cells as in (Figures 6-8).

Histopathological changes in pancreas occur by Alloxan

Alloxan induced diabetic mice, the endocrine pancreas histologically showed decreased in the size and number of Langerhans islets with vacuolar degeneration and necrosis of almost all cell in the atrophied islets as compared with control group (Figure 9). Also, there was congestion of blood vessels in the interlobular space (Figure 10), while other specimens showed that there was distortion of exocrine pancreas. There was degeneration (acute swelling) and necrosis of the epithelial cells of the acini. Also, there was atrophy of acini which lead to oedema and dilatation of inter acini space (Figure 11).

Moreover, there was thickening in the wall of blood vessels (Figure 12), and there was thrombus in other blood vessels, we saw that there was infiltration of inflammatory

cell in the inter lobular septa (Figure 13). Compared with the section of pancreas of control group showed the normal structure of islet notice the normal cells shape surrounding by exocrine pancreas.

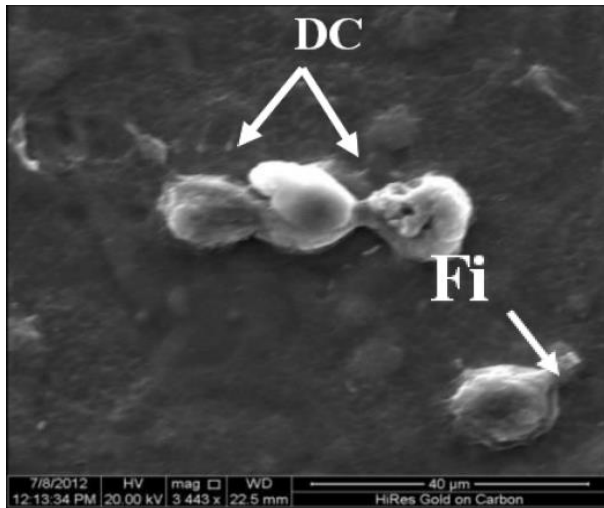


Figure 6: The scanning electron microscope shows the Filopodia (Fi) of the mesenchymal cells which is protoplasm prolong expanded from cell surface helping to connect the cells with each other's, and the division cells (DC) are clear here (3443 X).

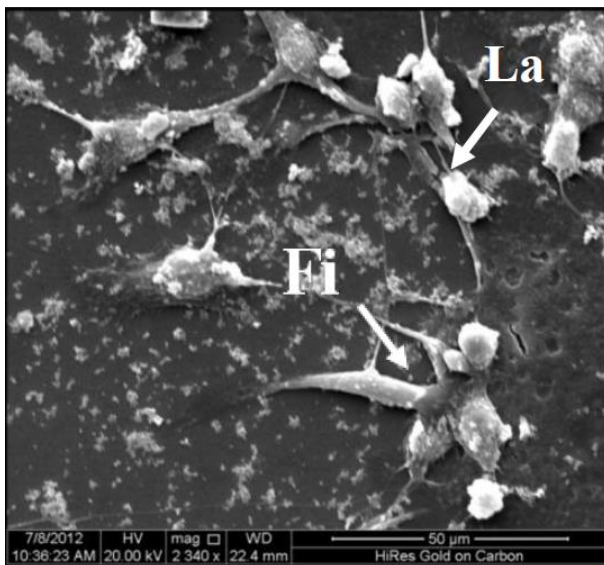


Figure 7: The scanning electron microscope shows the attachment of growing cells by the Filopodia. The Lamellipodia of the cells which represented the surface receptors (2340 X). (La) Lamellipodia, (Fi) Filopodia cell.

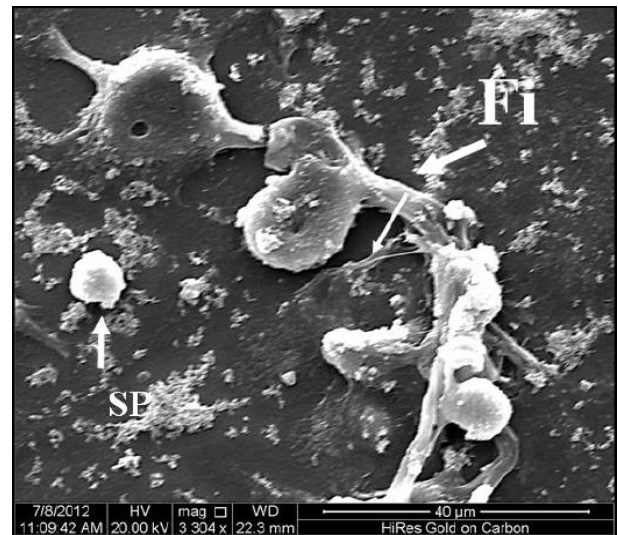


Figure 8: The scanning electron microscope shows the attachment of PMSCs by each other's by the Filopodia (3304 X). (Fi) Filopodia, (SP) Spherical cell.

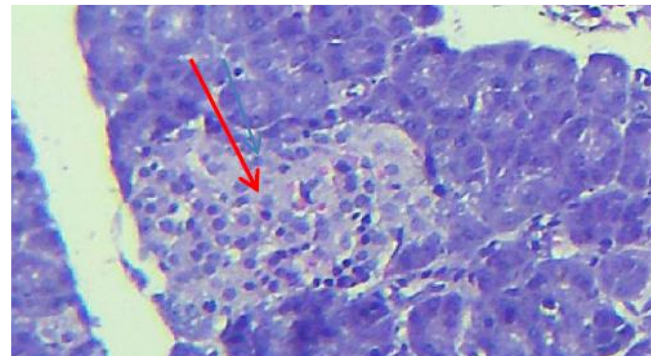


Figure 9: Section of pancreas of control group showed the normal structure of islet notice the normal cells shape surrounding by exocrine pancreas (→). H&E (420 X).

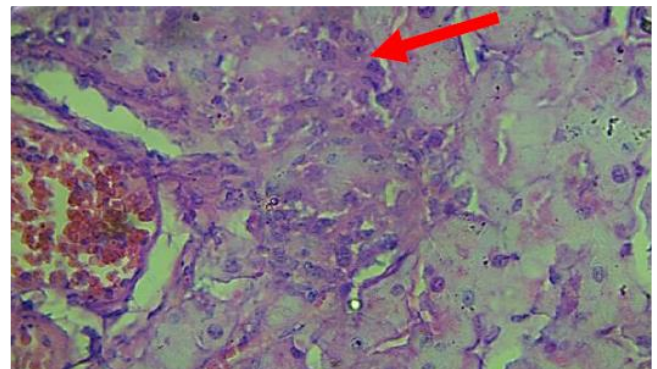


Figure 10: Section of mice pancreas of alloxan diabetic mice showed necrosis of islet cells (→). H&E (420X).

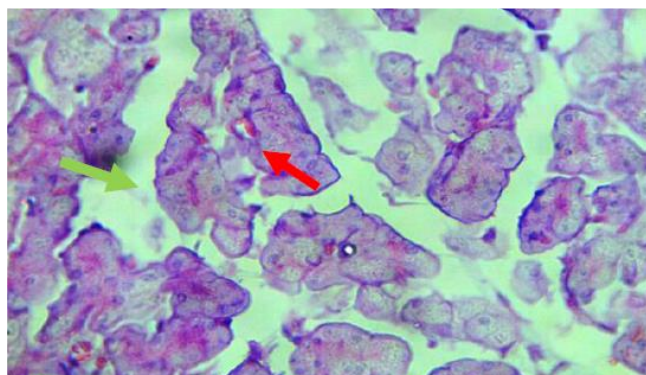


Figure 11: Section of pancreas showed distortion of exocrine pancreas, notice the degeneration and necrosis of acinar cells (→) and there is edema (→). H&E (420X).

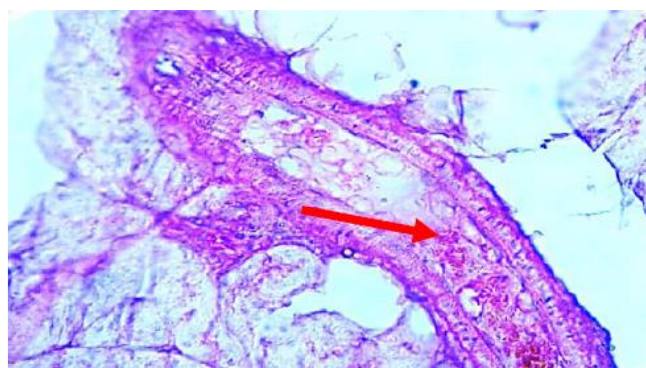


Figure 12: Section of pancreas showed the thrombus of the blood vessel. H&E (420X).

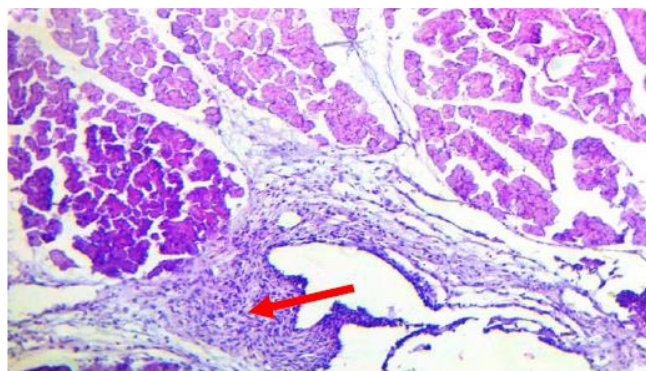


Figure 13: Section of pancreas showed inflammatory cells in the interlobular septa. H&E (420X).

Treatment of Diabetes Mellitus in Mice by mesenchymal stem cells

In this experiment the diabetic mice were grafted PMSCs via tail vein to treat the diabetic mice. There were

two groups of mice: diabetic mice were injected with PBS, and diabetic mice were injected with PMSCs via tail vein. While there was regeneration of islets and acini which seems to be normal compared with the control group (Figure 14). There was improvement of the histological appearance of pancreas of mice treated with alloxan and then injected with placenta mesenchymal stem cells, otherwise there was fibrous exudate with edema within the interlobular septa (Figure 15).

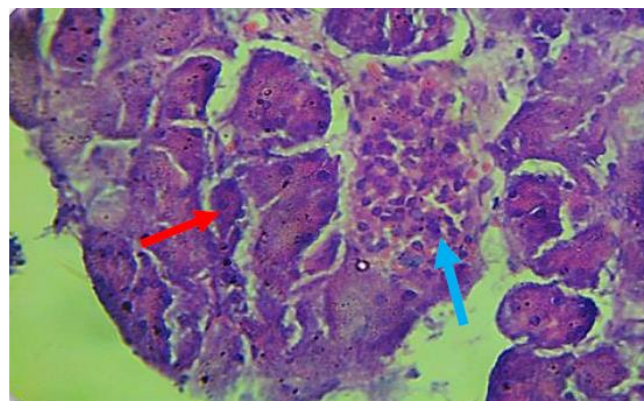


Figure 14: Section of pancreas shows stem cells treated mice pancreas showed the improvement of the exocrine (→) and endocrine of the pancreas (→). H&E (420X).

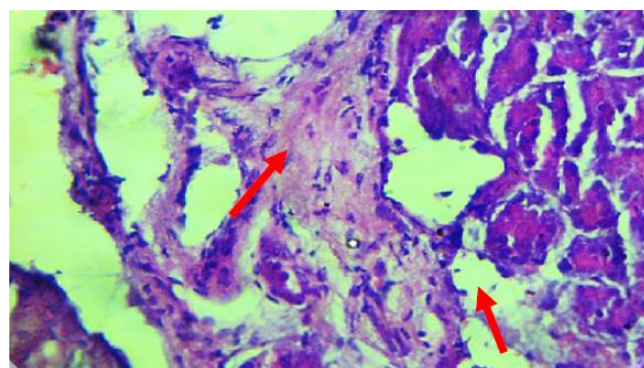


Figure 15: Section of pancreas shows mesenchymal stem cells treated mice pancreas showed fibrous exudate with edema in the interlobular septa, (→). H&E (420 X).

Measurement of glucose levels in animals

After inducing diabetes mellitus in mice we injected them with PMSCs as an attempt to treat them from diabetic mellitus so from recording the levels of sugar in the blood of mice which injected with the cells we observed a decrease in the blood sugar level in diabetic mice injected with the cells compared with control (diabetic mice which don't injected with cells) after that we dissected the mice

and took the pancreas and liver for histopathological study, to study the effects of Alloxan and the treatment with the PMSCs on these tissues.

Table 1: Explain change of the level blood sugar

Days	Blood sugar	
Before	111.89 ± 06.36	a
After	367.67 ± 14.30	b
3 D	273.00 ± 13.08	c
6 D	263.56 ± 10.56	c
9 D	195.78 ± 18.89	d
12 D	175.89 ± 15.22	d
15 D	166.89 ± 17.63	d
18 D	192.00 ± 26.67	d
21 D	188.89 ± 21.78	d

The same letters mean non –significant differences, the different letters mean significant differences, ANOVA $P < 0.05$.

Discussion

Induction of Diabetes mellitus

In this study we induced diabetes mellitus by alloxan an diabetic agent by injecting the mice with 70 mg/kg body weight and all the mice were found to be diabetic after 72 hours. this result is similar to that recorded by (9,17) who use the same diabetic agent other authors use another diabetic agent Streptozotocin and obtained the same result by Phuc *et al.* (18) and we can detect induce the diabetes in the mice by measuring the blood sugar level by using strips.

One of the aims of this study to improve the possibility of treatment diabetes mellitus by using new therapy PMSSc instead of insulin and others treatments and by taken the measurement of the blood sugar of these diabetic animals after injected with stem cells and when we observed a marked decreased in blood sugar level we dissect the animals, and the histological study improve the recovery of the pancreatic tissue.

These results are agreement with that obtained by Chen *et al* and Lumelsky *et al.* authors (19,20) when they recorded 3 methods to treatment of diabetic mice by injected Allogenic bone marrow and Xenograft of MSCs derived from umbilical cord blood, and when the treated diabetic by grafted insulin Producing cells IPCS and found that the insulin producing cells derived from MSCs had the best effect on recovering the insulin and blood sugar levels in diabetic mouse model. While Hori *et al.* (21) use Streptozotocin injected intravenously from caudal veins and glucose levels were tested one week later with Roche Accu-Chek glucose tester. After stable hyperglycaemia level was achieved 3 animals were subcutaneously injected with 5×10^6 differentiated cells the reaming 1 didn't receive any

cells. One week after cell injection, animal glucose level was recorded. He found that MSCs and successfully differentiated in to pancreatic islet β -like cells. These cells were morphologically similar to pancreatic islet cells. More importantly, they could also transcript, translate and excrete insulin. Cells were injected subcutaneously in to NOD rat model, although lack of statically data, these MSCs derived cells could regulate NOD blood glucose level. Nestin was regarded as a marker of precursors of pancreatic islet cells (22,23)

Treatment the diabetic Mice with PMSCs

After induced diabetes mellitus in mice we injected them with PMSCs as attempt to treat them from diabetic mellitus so from recording the levels of sugar in the blood of mice which injected with the cells we observed decrease the levels of sugar in diabetic mice which injected with the cells compared with control (diabetic mice which don't injected with cells) after that we dissected the mice and taken the pancreas for histopathological study, to study the effects of Alloxan and the treatment with the PMSCs on these tissues. To date (24) brought the evidence that umbilical cord blood cells, after culture in medium supplemented with fetal calf serum (in the absence of specific cytokines or growth factors ,show a panel of markers consistent with the characters of epithelial cells expressing genes considered essential in the differentiation steps towards pancreatic endocrine tissue (Isl-1, PDX-1 , Pax-4 and Ngn3).

The histopathological effect of alloxan in the animal's organs

Diabetes mellitus is the most common endocrine disorders in addition to the primary effects of diabetes, this disease is accompanied by increased risk factors such as hyperglycemia, hypertension, dyslipidemia, decreased fibrinolytic activity, seer atherosclerosis and increased platelet aggregation (25,26).

In agreement of our results, (27) found when he induce diabetes in rats and try to treat it with extract of plants found that Alloxan showing that necrotic changes of the islets of pancreatic, nuclear changes such as pyknosis, karyorrhexis, karyolysis, disappearance of nucleus and in some place residues of the destroyed cells were visible. Relative reduction in the size and number of the islets especially those around the central vessel together with severe reduction in the β - cells were demonstrated in these animals. Histopathological study of the treated diabetic rats that received garlic and Capparis spinosa extracts did not show a significant difference with the untreated diabetic rats. The cellular integrity and architecture of pancreases were intact in the control group. So, the histological change of pancreas in diabetic animals occur as a result of the pathogenesis of diabetes mellitus. Histopathological

changes of pancreas of diabetes mellitus, characterized by reduced of β cells mass result from either an impaired proliferative capability or an elevated rate of cell death (28).

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