

# Effect of Platelet Rich Plasma (PRP) Injection on the Endocrine Pancreas of the Experimentally Induced Diabetes in Male Albino Rats: A Histological and Immunohistochemical Study

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## Abstract

**Introduction:** Diabetes mellitus is a global problem and several restoration approaches have been developed to induce beta ( $\beta$ ) cells regeneration. Platelet rich plasma (PRP) is an autogenous and economical source of growth factors which nowadays used in the tissue repair.

**Aim:** To test the hypothesis that PRP could play a role in the improvement of the structural changes occurred in the endocrine pancreas of experimentally- induced diabetic rats and the possible mechanisms through which PRP induced its effects to shed a light on the possible use of such application in the clinical field.

**Material and methods:** Sixty male albino rats were used; 20 for obtaining the PRP and 40 were divided into 4 equal groups (10 rats each): control, PRP-group, diabetic group, PRP/diabetic group. Diabetes was induced by single intra-peritoneal injection of streptozotocin (50-60 mg/kg). The PRP was administered by SC injections in a dose of 0.5 mg/kg twice weekly for 3 weeks.

**Results:** The diabetic group showed a significant increase in blood glucose levels compared to the control. Treatment with PRP significantly reduced the blood glucose levels compared to the diabetic group. The diabetic group showed variable marked morphological changes which diminished by the PRP administration. PRP/diabetic group had a significant increase in the mean number of pancreatic islets and  $\beta$ -cells/islet compared to the diabetic group. The islet cells appeared normal with scarcely seen vacuolations. The duct system showed several changes; stratifications, invagination of the surface epithelium to the underlying connective tissue, and sprouting of the ductal epithelial cells in between the lobules. Numerous small islets were noticed in a close association with the intra-lobular ducts. Small newly lobules with abundant connective tissue were organized. There were significant increases in the insulin immunopositive  $\beta$ -cells and PCNA positive cells in PRP/diabetic group compared to diabetic group.

**Conclusion:** This study provides an evidence of the diabetic pancreatic islet regeneration in response to PRP treatment. The PRP stimulated islet cell regeneration and stimulated the induction of other sources of  $\beta$ -cells generation as the exocrine portion of the pancreas; ductal and acinar cells. In addition, PRP might put the pancreas into an environment similar to the postnatal developmental one where new lobules were formed. These will pave the future for a novel treatment for diabetes.

**Keywords:** Diabetes mellitus; Streptozotocin; Platelet rich plasma; Rats

## Introduction

Diabetes mellitus (DM) is a global problem. It was the direct reason of 1.5 million deaths in 2012 [1]. It is predicated that the number of diabetic person in the world could reach up to 366 million by the year 2030 [2]. Type 1 diabetes results from the autoimmune destruction of pancreatic beta ( $\beta$ ) cells of the islets of Langerhans [3]. Exogenous insulin is an important treatment for type1 diabetes but it is not a physiological method to regulate the blood glucose levels, as it was not administered in relation to glucose concentration. Beta cell replacement therapies using either the pancreas or the islet transplantation were used as a therapeutic alternative to the administration of exogenous insulin [4]. However, these procedures associated with many problems such as risks of major invasive surgery along with side effects of immunosuppressive therapy [5]. Special alternative ways to generate  $\beta$ -cells from endogenous sources should be found as a way for the development of treatment. This is to avoid the complication of tissue matching and surgical procedures [6]. To date several restoration approaches have been developed to induce  $\beta$ -cells regeneration through the induction of the proliferation of remaining  $\beta$ -cells, neo-genesis;

de novo islet formation from pancreatic progenitor cells, and trans-differentiation; converting non- $\beta$ -cells within the pancreas to  $\beta$ -cells. These models of induction are the most simple, direct, and least invasive ways to increase  $\beta$ -cell mass [7].

Streptozotocin (STZ) is a naturally occurring compound. It has also been used as an antibiotic and cancer treatment. The STZ has been widely used for inducing experimental diabetes mellitus [8]. Growth factors (GFs) are natural biological mediators that control

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growth, differentiation, and have role in the process of tissue repair and regeneration [9]. Recently, long-term administration of a low dose of epidermal growth factor (EGF) induces  $\beta$ -cell neo-genesis in diabetic mice and induced ductal cell differentiation into  $\beta$ - cells [10]. Platelets convey many growth factors (GFs) [11]. Platelet-Rich Plasma (PRP) is a low-cost procedure to deliver high concentrations of autologous GFs [12]. PRP has been defined as an autologous concentration of platelets that is 3 to 5 times greater than physiologic concentration of thrombocytes in whole blood [13]. PRP therapy represents a relatively new approach in regenerative medicine and accumulated considerable attention over the two last decades [14]. It was used in many medical and surgical fields [15] such as dentistry, orthopedics, neurosurgery, ophthalmology, maxillofacial surgery, and cosmetic surgery [16].

PRP has been the subject in different studies in medicine, but there was a lack of studies that handling the effect of PRP injection on the diabetic pancreas. The aim of this was to test the hypothesis that PRP could play a role in the improvement of the structural changes occurred in the endocrine pancreas of experimentally-induced diabetic rats and the possible mechanisms through which PRP induced its effects to shed a light on the possible use of such application in the clinical field.

## Materials and Methods

### Animals

This study was conducted in the Histology Department, Faculty of Medicine, Minia University and carried on 60 adults male albino Wistar rats weighing approximately 150-250 gm, of 8-10 weeks which were pathogenically free. Animals were obtained from the animal house of faculty of agriculture, Minia University. Rats were housed in clean plastic cages and fed a standard laboratory diet with free access to water and diet at room temperature with normal light/dark cycles. All aspects of animal care and treatment were carried out according to the local guidelines of the ethical committee of the Faculty of Medicine, Minia University.

### Reagents

- Streptozotocin (STZ): A vial containing 1.5 g powder of STZ and 220 mg citric acid (Sigma Aldrich, Egypt) which was freshly dissolved in 0.1 M sodium citrate buffer (pH 6) according to the manufacture instructions and was used within 5 minutes of preparation.
- Anti-insulin antibody: A monoclonal mouse antibody, ultra-vision one detection system, HRP Polymer & DAB plus Chromogen (Thermo Fisher Scientific, USA).
- Anti-proliferating cell nuclear antigen (anti-PCNA): A monoclonal mouse antibody, horseradish peroxidase coupled to an inert polymer backbone & DAB Plus Chromogen (Dako-EPOS, USA).

The platelet rich plasma (PRP) was freshly prepared.

### Experimental design

Twenty male rats were used for obtaining PRP and the other 40 rats were randomly divided into 4 equal groups (10 rats each):

- The control group: Rats received i.p injections of citrate buffer saline in a dose of 0.5 ml /kg twice weekly for 3 weeks.
- The PRP-group: Rats received PRP in a dose of 0.5 mL/kg by subcutaneous injection (SC) injection twice weekly for 3 weeks.

- The diabetic group: Rats received a single i.p injection of STZ in a dose of 50-60 mg/kg body weight for induction of diabetes. After 48 hours (considered day 0) of STZ injection, animals were fasted overnight and blood samples were collected from the tail vein and glucose levels were measured. Individual glucose levels reached above 250 mg/dl was considered diabetic [16].
- The PRP/diabetic group: Rats received a single i.p injection of STZ in the same dose as the diabetic group. At day 0; after the confirmation of the induction of the diabetes, the PRP was given in the same dose as PRP-group, twice weekly for 3 weeks.

### Procedures

**Preparation of PRP:** The PRP preparation was performed at the pharmacology department of faculty of medicine, Minia University. The PRP preparation was carried out by adapting the protocol of the double centrifugation tube method [17]. In brief, rats were anesthetized with ether, 2 ml of blood was collected under aseptic technique from the retro-orbital plexus using capillary tube initially dipped in 3.2% sodium citrate, then collected into tubes containing 0.3 mL of the anticoagulant. The blood was subjected to double centrifugation method, in the first centrifugation the tubes were centrifuged at 1600 revolutions per minute (rpm) for 10 minutes. This resulted in 3 different density compartments; the inferior layer contained red blood cells, the intermediate layer contained buffy coat of white blood cells, and the superior layer contained plasma. The plasma was pipetted and the portion just above buffy coat was obtained without disturbance of the buffy coat. The plasma was centrifuged again at 2000 rpm for 10 minutes. This resulted in 2 parts: the top consisted of platelet-poor plasma (PPP) and the bottom consisted of the platelet button. Part of the PPP was discarded and part was remained in the tube along with platelet button which then gently agitated to promote platelets resuspension. This procedure resulted in the production of platelet-rich plasma (PRP). For conformation of the platelet's concentration, 80  $\mu$ L of the PRP sample was counted in an automatic apparatus to verify that the platelet count was greater than 1,000,000/ $\mu$ L.

**The PRP administration:** 0.5 ml PRP was dissolved in phosphate buffer saline (PBS) (PRP 1:1 PBS), then immediately aspirated with micropipette, placed in a sterile insulin syringe, and injected subcutaneously [18]. Rats were sacrificed at the end of 3rd week for all groups by decapitation under light halothane anesthesia. Pancreas was rapidly removed and fixed.

**Biochemical study:** Blood glucose levels were detected by the use of one touch Accu-check glucometer<sup>®</sup> and compatible blood glucose test strips [19]. At the beginning of the study, the blood glucose levels were measured for all animals before grouping them to ensure that the animals were all normoglycaemic. Then blood glucose levels were measured for the different study groups and measures were statistically analyzed.

**Histological study:** Pancreatic specimens were fixed in 10% buffered formalin for 24 hours, dehydrated in a graded alcohol series, cleared in xylene, and embedded in paraffin. 5  $\mu$ -sections were mounted on glass slides for further staining with haematoxylin and eosin (H&E) and other sections with Masson trichrome according to Bancroft et al. [20].

**Immunohistochemical study:** Other 5  $\mu$ -sections were used for immunestaining. Briefly [21] sections were deparaffinized in xylene, rehydrated in descending grades of alcohol and immersed in 0.1% hydrogen peroxide for 15 minutes to block the endogenous peroxidase activity. Then sections washed by phosphate buffer, followed by

incubation in the ultravision block for 5 minutes at room temperature to block the non-specific background staining. The primary antibody (anti-insulin antibody) was diluted at 1:200 in antibody diluent while the anti-PCNA antibody was ready to use. Sections were incubated in the primary anti-insulin antibody for 30 minutes while incubated for 60 minutes with anti-PCNA antibody at room temperature. The reaction was visualized using; Ultravision one detection System, HRP Polymer & diaminobenzide (DAB) Plus Chromogen. After completion of the reaction, counter staining was done using hematoxylin. Sections were dehydrated by ascending alcohol concentrations, cleared by xylene, and mounted. Positive cells for the anti-insulin antibody showed brown cytoplasmic reaction and positive cells for the anti-PCNA antibody showed brown nuclear reaction.

The positive control for anti-insulin was the normal pancreatic tissue of the control animals while positive control for the anti-PCNA antibody was the sections from colon of the control animals. For negative control slides, the same steps were applied but the 1ry antibody was not added to the pancreatic sections from the control group (figures not included).

**D-Morphometrical analysis:** Three sections were examined from each animal in the different groups. The morphometrical studies were made using Leica Qwin 500 Image Analyzer computer system (Leica Microsystem Imaging Solution Ltd., Cambridge, UK).

#### Quantitative data were collected for 3 parameters

- The number of islets per square millimeter of each section
- The islets of the pancreas were counted in H&E slides randomly under 10 power fields. The number of islets was assessed by counting all islets per one square millimeter.
- The number of  $\beta$ -cells in the islet
- The  $\beta$ -cells of the pancreas were counted in the anti-insulin immunostained sections under 40 power fields. The number of  $\beta$ -cells was assessed by counting the nuclei of all positive cells inside one islet in the field. A total number of 30 islets for each group were counted.
- The number of PCNA positive cells in the islet
- The PCNA positive cells in the islet of pancreas were counted in the anti-PCNA immunostained sections under 40 power fields. The brown nuclei inside one islet in the field were counted. A total number of 30 islets were counted for each group.

**Statistical analysis:** Quantitative data was analyzed by SPSS (IBM Corp. Released 2010. Windows, Version 19.0). The mean and standard deviation (sd) was calculated for the parameters of each group. Values were expressed as means  $\pm$  sd. One-way analysis of variance (ANOVA) test was used for the detection of significant differences between groups, followed by the use of Tukey-Kramer as a post hoc test. The results were considered statistically significant when the p-values were  $<0.05$ .

## Results

### Biochemical results

The PRP-group showed normal blood glucose levels with no significant difference compared to the control group ( $p=0.900$ ). There was a significant increase in blood glucose levels after STZ injection in the diabetic group compared to the control group ( $p=0.0001$ ). There was a significant decrease in the blood glucose levels in the PRP/diabetic group compared to the diabetic group ( $p=0.0001$ ), but it showed a significant increase if compared to the control group ( $p=0.046$ ).

### Histological results

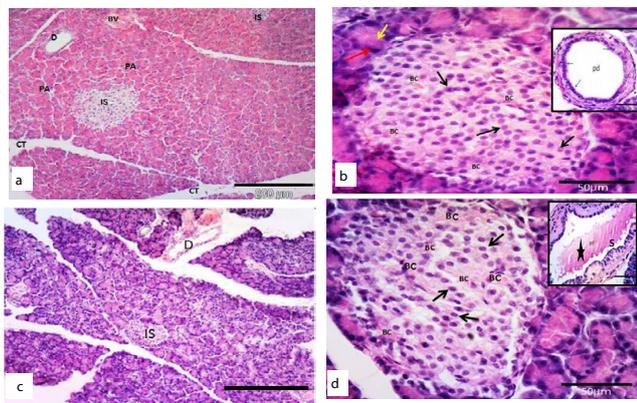
**H&E and Masson trichrome stains results:** H&E stained sections from the control group (Figure 1a and 1b), showed normal histological structure of the pancreatic tissue that was formed of lobules packed with acini and separated from each other by a delicate connective tissue. The islets of Langerhans appeared as pale stained rounded or oval areas surrounded by the acini. The islets were formed of cords of cells separated by blood capillaries. The main pancreatic duct was lined by simple columnar epithelium resting on basement membrane and surrounded by CT.

The PRP-group (Figure 1c and 1d), showed histological features that nearly similar to the control group except that some blood vessels appeared congested and the main pancreatic duct was dilated with wide lumen and stagnant secretion. Some areas of stratification were noticed in the lining epithelium.

In the diabetic group (Figure 2), STZ caused variable marked morphological changes in the pancreatic tissue structure. There was widening of the inter-lobular spaces and the islets were less numerous. Some islets became massively degenerated with a reduction of the cell mass while others were completely devoid of cells. Dilatation of the intra-lobular duct and numerous dilated blood vessels loaded with RBCs and inflammatory cells were seen in between the lobules. The degenerated islets showed ballooned cells with vacuolated cytoplasm and were separated with congested blood capillaries. Other islets showed shrunken cells with acidophilic cytoplasm and pyknotic nuclei. Fibroblast like cells were seen infiltrating the islet. The main pancreatic duct was dilated and had areas of stratification in its epithelial lining.

PRP administration to diabetic rats of the PRP/diabetic group (Figure 3), resulted in marked improvement in the morphological changes observed in the diabetic animals. Numerous islets of variable sizes were seen. Some islets were observed connected to the nearby one by stream of cells and appeared in close associations with the ducts. The islet cells appeared normal with scarcely seen vacuolated cells. The duct system showed several changes. Regarding the main pancreatic duct, the lining epithelium showed increased stratifications, invagination of the surface epithelium to the underlying connective tissue, and sprouting of the ductal epithelial cells in between the lobules. Numerous intra-lobular ducts were dilated and had invaginations in their lining epithelium. Some acini and islets enclosed within large amount of connective tissue were noticed in close association with the wall of the intra-lobular duct. Interestingly, scattered areas of the parenchyma had begun to be organized into small lobules with abundant connective tissue. This lobule was formed of aggregates of small acini, small ducts, and numerous islets.

Sections of the pancreas stained with Masson trichrome from the control group (Figure 4a and 4b) and PRP-group (Figure 4c and 4d) revealed delicate collagen fibers in the septa between lobules and in-between acini. They were also observed surrounding the intra-lobular ducts, the blood vessels and in the wall of the main pancreatic duct. There were traces of delicate collagen fibers surrounding the margins of the islets and in between their cells. The diabetic group (Figure 4e and 4f) showed increased collagen deposition around the mentioned sites. There was extensive intra-islet collagen deposition forming strands of fibrous tissue dividing the islets into nests of endocrine cells. The PRP/diabetic group (Figure 5) showed a relative increase in collagen deposition of the inter-lobular connective tissue, the peri-ductal collagen fibers, the intra-lobular ducts, and the blood vessel. In contrast there was a decrease in the amount of collagen deposition within the



Figures 1: Photomicrographs of rat pancreatic tissue:

- Control group showing normal lobular architecture; islets of Langerhans (IS) and the pancreatic acini (PA). Notice the interlobular connective tissue (CT), the intra-lobular duct (D) and blood vessel (BV).
- The islet's cells of the control group forming cords (black arrows) separated by a network of blood capillaries (BC). The inset showing the main pancreatic duct (pd) lined by simple columnar epithelium (arrows) and surrounded by connective tissue (C). Notice the basal basophilic (red arrows) and apical acidophilic cytoplasm (yellow arrows) of pancreatic acinar cells.
- The PRP-group showing preserved lobular architecture. Notice the congested blood vessels (BV).

The PRP-group showing numerous blood capillaries (BC) in between the cords of cells (arrows). Inset showing dilated main pancreatic duct (pd) with areas of stratification (s) and stagnant secretion (star).  
H&E, scale bar: a,c X200  $\mu$ m; b,d, insets X50  $\mu$ m.

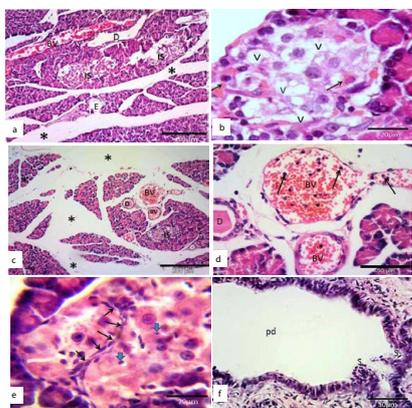


Figure 2: Photomicrographs of rat pancreatic tissue of the diabetic group showing:

- Degenerated islets (IS), dilated intra-lobular duct (D) and congested blood vessel (BV). Notice empty area of massively damaged islet (E) and wide inter-lobular spaces (\*).
- A degenerated islet showing numerous cells with vacuolated cytoplasm (V) and some cells with densely acidophilic cytoplasm (arrows).
- Dilated inter-lobular spaces (\*), degenerated islet (IS), inter-lobular duct (D) and congested dilated blood vessel (B.V).
- Congested and dilated blood vessel (BV) filled with RBCs (\*) and inflammatory cell (arrows). Notice the stagnant secretion in the inter-lobular duct (D).
- Another islet showing deeply stained cells with pyknotic nuclei (blue arrows). Notice the fibroblast like cells (black arrows) infiltrating between islet's cells.
- Dilated main pancreatic duct (pd) with areas of stratification (s) in its epithelial lining.

H&E, scale bar: a,c X200  $\mu$ m; b,e X20 $\mu$ m; d,f X 0 $\mu$ m.

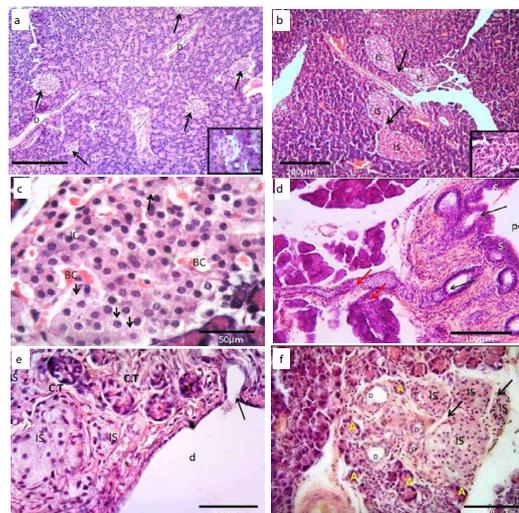


Figure 3: Photomicrographs of rat pancreatic tissue of the PRP/diabetic group showing:

- Restored lobular architecture with numerous islets (arrows) associated with intralobular ducts (D). Inset showing higher magnification of a small islet.
- Some islets are inter-connected with each other (arrows). Notice the small duct (D). The inset shows higher magnification of a connecting area between two islets.
- An islet with numerous apparently normal cells (IC) and rich vasculature (BC). Notice the scarcely vacuolated cells (arrow).
- Increased stratifications (S) in the epithelium lining of the main pancreatic duct (pd), and the invagination of the surface epithelium to the underlying connective tissue (black arrows). Notice the ductal epithelial cells in between the lobules (red arrows).
- A dilated intra-lobular duct (d) with invagination of its epithelial lining (arrow). Notice the acini (A) and islets (IS) in a relatively large amount of connective tissue (CT).
- An apparently newly formed small lobule containing small ducts (D), some acini (A) and islets (IS) embedded in connective tissue (arrows).

H&E, scale bar: a,b X200  $\mu$ m; c,e, inset X50  $\mu$ m; d,f X100  $\mu$ m

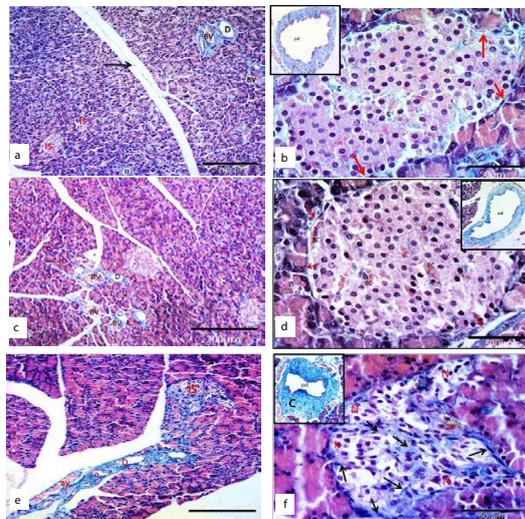
islet. Some areas had small clusters of endocrine cells closely associated with the duct and each cluster was encapsulated by connective tissue. The wall of the main pancreatic duct was invested with a large amount of collagen fibers compared to the control group.

### Immunohistochemical study

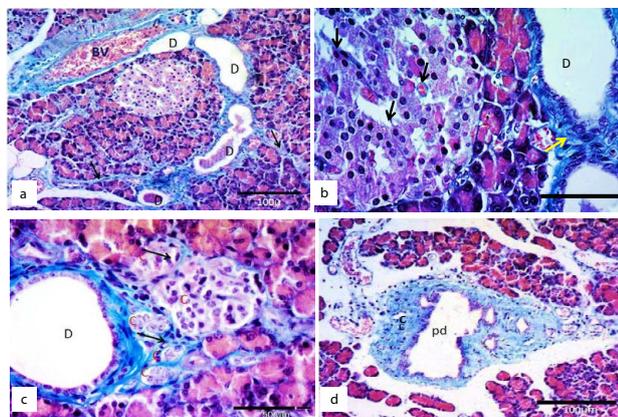
**Immunohistochemical analysis of sections immunostained for insulin:** The insulin secreting cells;  $\beta$ -cells, of the control group (Figure 6a) and the PRP-group (Figure 6b) represented the major cell population of the islets. The ductal epithelium showed negative reaction. In the diabetic group (Figure 6c), some islets showed reduced immune reactivity while others showed negative expression. The ductal epithelium had also a negative reaction.

The PRP/diabetic group (Figure 7) showed increased immunostained cells in the islet compared to the diabetic group. Scattered newly formed small islets composed of positive cells were observed in the lobules. The connection of cells that were observed connecting two islets had also positive expression. There was a positive expression in the epithelial lining of the nearby intra-lobular ducts. Some immunostained cells were observed forming a stream between the islets and the nearby ducts. Surprisingly, few exocrine acinar cells showed positive expression.

**Immunohistochemical analysis of sections immunostained for PCNA:** The control group (Figure 8a and 8b), showed few positive



**Figure 4:** Photomicrographs of rat pancreatic tissue showing collagen fibers in the septa between lobules, between acini (arrows), surrounding the intralobular ducts (D) and blood vessels (BV) in : a ) Control group, c) The PRP treated group, e) Diabetic group. Higher magnifications showing the islets and the inset showing the main pancreatic ducts of b) Control group, d) The PRP-group. The diabetic group: e) Dense collagen deposition around the congested dilated blood vessel (BV) and the inter-lobular ducts (D). f) The extensive intra-islet collagen deposition (black arrows) forming strands dividing the islet into nests of endocrine cells (N). The inset shows the main pancreatic duct (pd) with extensive collagen (C) fibers in its wall. Masson trichrome, scale bar: a,c X200 µm; b,d,f X50 µm; e, insets X100 µm



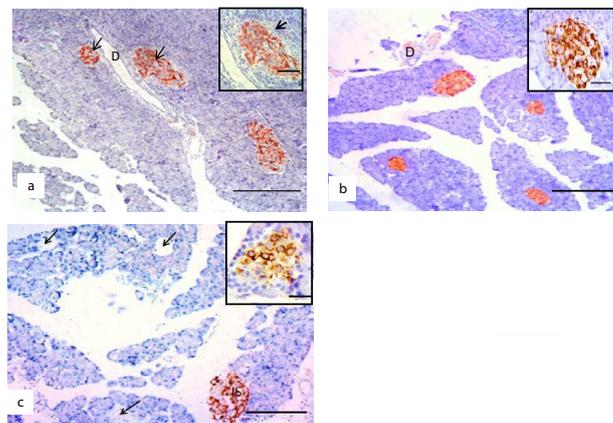
**Figure 5:** Photomicrographs of rat pancreatic tissue of the PRP/diabetic group showing:

- A relative increase in collagen deposition surrounding lobules (arrows), intra-lobular ducts (D) and blood vessel (BV).
  - An islet with little amount of collagen fibers (black arrows) in between islet cells. Notice prominent collagen fibers (yellow arrow) around the duct (D).
  - Small clusters of endocrine cells (C) encapsulated by connective tissue (arrows) and closely associated with the intra-lobular duct wall (D).
  - The main pancreatic duct (pd) with extensive connective tissue rich in collagen surrounding its wall (C).
- Masson trichrome, scale bar: a,d X100 µm; b,c X50 µm

nuclear expression for PCNA in the cells of the islets and in the epithelial lining of the intra-lobular ducts. In the PRP-group (Figure 8c and 8d), more immunoreactive nuclei were noticed in the islets of Langerhans and in the epithelial lining of the intra-lobular ducts. Unfortunately,

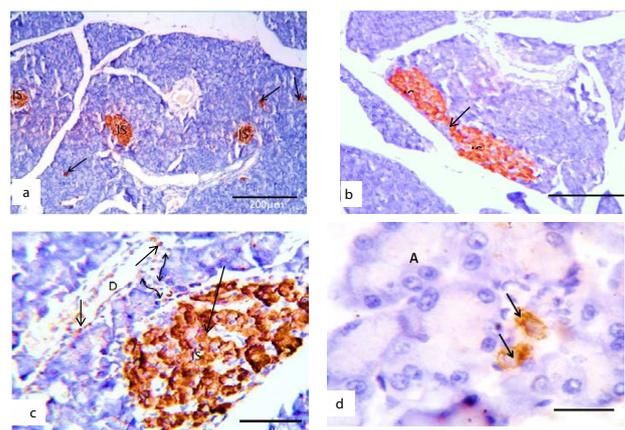
these positive nuclei had atypical appearance with abnormal shape and sizes.

In the diabetic group (Figure 8e and 8f), scarce immunopositive cells were noticed in the islets, while the lining epithelium of intra-lobular duct and the acinar cells showed numerous immunopositive nuclei.



**Figure 6:** Photomicrographs of rat pancreatic tissue immunostained for insulin of:

- The control group, and b) The PRP-group; showing positive immunostained β-cells (brown color) occupying most of the islet's tissue (black arrows) with negative reaction in the duct (D) and acini (A). Inset is higher magnification of the positive immunostained β-cells .
- The diabetic group showing reduction in the immunohistochemical expression in one islet (IS) and completely negative expression in other islets (arrows). Inset showing marked reduction in the expression in β-cells (arrows). Immunohistochemistry, counterstained with H, scale bar: a,b,c X200 µm; insets, d X50 µm



**Figure 7:** Photomicrographs of rat pancreatic tissue of the PRP/diabetic group immunostained for insulin showing:

- strong reaction in the islet's cells (IS). Notice numerous small islets scattered in the lobules (arrows).
- two islets (IS) communicated to each other by immunopositive cells (arrow).
- immunopositive cells (arrows) in the islet (IS) and in the nearby epithelium lining the intra-lobular duct (D) . Notice stream of immunopositive cells in between the duct and the islet (arrows).
- acini (A) and positive expression in two of acinar cells (arrows). Immunohistochemistry, counterstained with H, scale bar: a,b X200µm; c X50 µm; d X20 µm.

In the PRP/diabetic group, there was an apparent increase in positive immunoreactive nuclei among the cells of the islets and in the intra-lobular ductal lining epithelium. Some immunostained cells appeared as a stream of positive nuclei that were crawling from the duct towards the islet. Interestingly the connective tissue surrounding the inter-lobular duct and cells of acini showed immunopositive reaction (Figure 8g).

### Morphometrical results

**The number of islets per square millimeter (islets/mm<sup>2</sup>):** There was no significant difference in the mean number of pancreatic islets/mm<sup>2</sup> of the PRP-group compared to the control group (p=1.068) but there was a significant decrease in the diabetic group compared to the control group (p=0.001). On the other hand, there was a significant increase in the mean number of pancreatic islets in PRP/diabetic group compared to both the control group (p=0.001) and diabetic group (p=0.0001) (Tables 1 and 2).

**The number of insulin positive β-cells per islet:** There was no significant difference in the mean number of anti-insulin positive β-cells of the PRP-group compared to the control group (p=0.200), but a significant decrease in the mean number of immunopositive β-cells in the diabetic group compared to the control group (p=0.0001)

	Blood glucose levels (mg/dl)	
	Mean ± sd	p-value
Control group	93.4 ± 12.6	
PRP-group	91.3 ± 12.5	0.900c
Diabetic group	393.3 ± 14.9	0.0001c*
PRP/diabetic group	167 ± 54.4	0.046 c* 0.0001d*

\*p<0.05 is significant, c versus the control group, and d versus the diabetic group.

**Table 1:** The blood glucose levels (mg/dl) in the studied groups (n=10).

was observed. On the other hand, there was a significant increase in the mean number of immunoreactive β-cells of PRP/diabetic group compared to both the control group (p=0.046) and the diabetic group (p=0.0001).

**The number of PCNA positive cells per islet:** There was a significant increase in the mean number of PCNA positive cells in the islets of PRP-group compared to control group (p=0.040). There was a significant decrease in the diabetic group compared to the control group (p=0.001). On the other hand there was a significant increase in the mean number of PCNA positive cells in the islets of PRP/diabetic group compared to both the control and the diabetic group (all p=0.000).

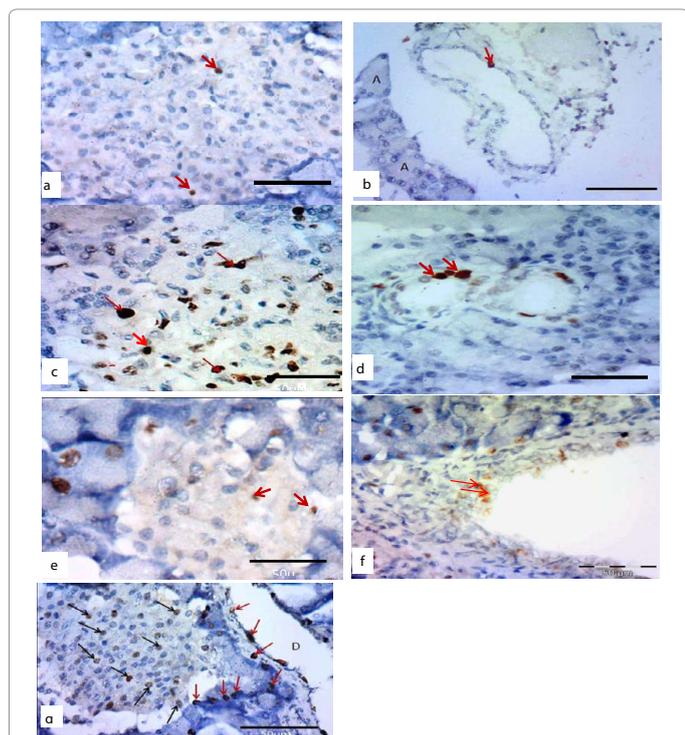
### Discussion

Platelet rich plasma (PRP) is an autogenous and economical source of growth factors which nowadays used in the tissue repair [12]. In this study, the PRP preparation depended on double centrifugation method that resulted in a platelet concentration 3 times higher than the initial blood sample [17]. It was also depended on the endogenous activation of platelets to allow the platelets to make their action spontaneously. The repeated dose regimen provided a constant elevated level rather than a sudden flood of growth factors [22].

In pancreatic disorders, there were pancreatic microcirculatory disturbances and oxidative stress production which resulted in congestion, extravasation of RBCs, and escape of tissue fluids. These changes led to widening of the interlobular spaces with inflammatory cell infiltration [23]. This was observed in the diabetic group beside the extensive islet destruction. The STZ induced hyperglycemia by β-cells destruction [24].

In terms of β-cell regeneration, the present results demonstrated the effect of PRP injection on the regeneration and restoration of pancreatic islet cell mass and clarified the different mechanisms through which PRP influence the improvement of the diabetic damage effects.

New islet cells could develop from progenitors through a process called neo-genesis in postnatal life in rodent studies (Bonner-Weir et al. [25]). The GFs were used to stimulate pancreatic β-cell proliferation in vivo. These molecules such as vascular endothelial growth factor (VEGF) and connective tissue growth factor, had been investigated as potential therapies for diabetes. It can stimulate β-cell proliferation and insulin production [7]. Administration of PRP to the diabetic rats in our study showed improvement of the pancreas and the islet morphology. Numerous islets approached the corresponding healthy pancreatic sections with an increase in β-cell number. Sections showed increased positive PCNA cells; increased proliferation. Numerous cells were functioning cells as the anti-insulin positive cells; β-cells, represented the major cell population of the islets. Hyperplastic and proliferative changes occurred in pancreas under the effect of certain stressors or stimuli led to formation of recent β-cells (Jurczyk et al.



**Figure 8:** Photomicrographs of rat pancreatic tissue immunostained for PCNA of: **a)** The control group showing few immunoreactive nuclei in the islet's cells (arrows), and **b)** few immunoreactive nuclei in the epithelial lining of the inter-lobular duct (arrow) and negative expression in the acini (A). **c)** The PRP-group showing numerous islet cells (IS) with positive nuclear expression. Notice atypical abnormal shape and size of nuclei (arrows), and **d)** some immunopositive nuclei in the epithelium lining of the intralobular duct. **e)** The diabetic group showing immunoreactive nuclei of islet cells (arrows) and in the acinar cells (\*), and **f)** immunoreactive nuclei in the lining epithelium of intra-lobular duct (arrows). **g)** The PRP/diabetic group showing many immunoreactive nuclei in the lining epithelium of the intralobular duct (D) crawling towards the islet (red arrows) and among the islet's cells (black arrows). Immunohistochemistry, counterstained with H, scale bar: a,c,d,e,g X50 μm; b,f X100 μm.

	islets/m <sup>2</sup>		β-cells/islet		PCNA +ve cells/islet	
	Mean ± SD	p-value	Mean ± SD	p-value	Mean ± SD	p-value
Control group	2.5 ± 0.97		88.7 ± 8.7		5.9 ± 3.1	
PRP-group	2.5 ± 0.85	1.068c	94 ± 7.5	0.200c	8.5 ± 6.1	0.040c*
Diabetic group	1 ± 0.85	0.001c*	23.6 ± 22.8	0.0001c*	1.2 ± 1.8	0.001c*
PRP/diabetic group	4.4 ± 0.96	0.001c* 0.0001d*	104.50 ± 21.9	0.046 c* 0.0001d*	24.1 ± 6.6	0.0001c* 0.0001d*

\*p<0.05 is significant. <sup>c</sup>versus the control group. <sup>d</sup>versus the diabetic group.

**Table 2:** The number of islets per square millimeter (islets/m<sup>2</sup>), insulin positive β-cells per islet (β-cells/islet), and PCNA positive cells per islet (PCNA +ve cells/islet) in the studied groups (n=10).

[26]). Therefore, this study suggested that GFs of the PRP might act as stimuli for proliferation of these cells that led to an increase in β-cell number.

The pancreatic duct cells may serve as a source of regeneration in adult rats by undergoing a reproducible trans-differentiation into β-cells [27]. β-cell progenitors located in the ductal lining epithelium could be activated in the injured adult mouse pancreas (Xu et al. [28]). In this study, the ductal epithelial lining showed areas of stratifications of the main pancreatic duct in the diabetic group which might be a trial to increase the cell numbers as a way for regeneration of β-cells. However, the capacity of the pancreas to regenerate was limited and the exhaustion of cells led to diabetes because of the imbalance between β cell destruction and β cell formation [29].

In the PRP/diabetic group, several areas of stratification and invagination of the epithelial lining of the ducts were observed and most of the islets and small clusters of endocrine cells were seen closely associated with ducts. PCNA immunostained sections showed increased positive cells in the lining epithelium of the intra-lobular duct. Interestingly, a stream of PCNA-positive cells appeared as if it was crawling from the duct towards the nearby islet through the connective tissue between them. Furthermore, numerous cells in the connective tissue showed insulin immunoreactivity. These findings could be clarified by lineage-tracing data described by Xu et al. [28] who mentioned that in adult pancreas some cells originate from hormone progenitors present near ducts and become hormone-positive islet cells in adult mice. This enforced our suggestion that platelet GFs released in injured pancreas might act as stimuli for ductal stem cell activation and its further differentiation. This was in agreement with Song et al. [30] who reported that the TGF-α; one of PRP GFs, induced the expansion of ductal cells leading to an increase of areas of islet in a process of neogenesis.

In addition, some acini in our results had PCNA positive cells and some insulin positive cells in their lining. Acinar cells could be converted to mature β-cells after injury as a compensatory mechanism [31]. Acinar to endocrine conversion was demonstrated using in vitro cultured primary acinar cells treated with GFs such as EGF. Accordingly, endocrine cells can regularly be detected in exocrine cells in each rodents and humans [32].

The findings of this study were in line with Jurczyk et al. [26] who found that the histological studies on human tissue in adult who exposed to any stress factors showed insulin-expressing cells in ducts, isolated β-cells in the pancreatic parenchyma, and small islet clusters. PRP had consistently shown to potentiate stem cell proliferation, migration, and differentiation (Masoudi et al. [33]). The PRP peptide GFs such as EGF were the principal external indicators that activate the mitogen-activated protein kinase and had a role in trans-differentiation of pancreatic acinar and ductal cells into endocrine islet cells (Abban-Mete et al. [34]). Another one of PRP GFs; insulin growth factor,

localized to the focal areas of regeneration and may play an important role in pancreatic regeneration by autocrine mechanisms through stimulation of DNA synthesis [35]. This could take place by using replication of already differentiated β-cells (β-cell plasticity) or neo-genesis from putative islet stem cells in the ductal or acinar epithelium [26].

Therefore, PRP could restore β-cells by stimulating the different sources of β-cells progenitors in adult rat through proliferation and trans-differentiation process. This was in contrast to others [36,37] who suggested that adult pancreatic β-cells are formed by self-duplication rather than stem-cell differentiation. Also, it was in opposing to Kopp et al. [37] who reported that the derivation of endocrine cells from the ducts occurred only in early postnatal life, but no endocrine or acinar cell neo-genesis occurred in adult mice either physiologically or after pancreatic duct ligation.

For assessment of the role of the PRP injection in stimulation of extracellular matrix formation in pancreatic tissues, Masson trichrome stain was used. After STZ injection in the diabetic group, extensive collagen depositions were observed. In diabetes, there was inter-acinar and inter-lobular fibrosis which suggesting that insulinopenia might cause these injurious effects on exocrine pancreas [38]. Concerning this issue, previous reports localized the pancreatic stellate cell (PSCs) in the islets. Upon activation, PSCs started to proliferate, change their morphology into myofibroblast-like cells, and start to secrete extra cellular matrix components [39].

In this study, fibroblast-like cells were seen invading some islets. This observation could explain the extensive intra-islet collagen deposition forming strands of fibrous tissue which divided the islets into nests of endocrine cells. Kim et al. [40] in their study owed the fibrotic changes to activation and proliferation of the PSCs. Invasions of the pancreatic islets by these cells resulted in fibrotic islet destruction that led to the limited capacity of β-cell proliferation and accelerated apoptosis in diabetic patients. This was responsible of delaying a complete tissue restoration in diabetes [41].

Activated PSCs subsequently develop functional alterations including: increased proliferation and migration, synthesis of excessive ECM proteins (collagen, fibronectin, laminin), secretion of GFs and cytokines which exert both paracrine and autocrine effects to enhance the cell growth and migration [42]. In our study, PRP/diabetic group revealed an obvious decrease in collagen deposition in the islet and a relative increase in the peri-ductal collagen and within the inter-lobular connective tissue.

The increased vasculature observed in this group could be explained by Bir et al. [43] results who mentioned that injection of PRP resulted in increasing the neo-vascularization due to release of VEGF.

One of the most characteristic findings of this work was the appearance of aggregates of small acini, small ducts, and numerous small

islets abundant connective tissue arranged into small lobules. These results could provide an evidence of new lobule formation through PRP activation of ECM formation together with the neovascularization in order to build a scaffold for the proliferated progenitors in the ducts. Then the ductal epithelium gives rise to all pancreatic epithelial lineages, i.e. duct, acinar and endocrine cells [44]. These were similar to Hardikar, who described the same consequence for endocrine pancreas development during the postnatal period. Therefore, this could support our suggestion that the PRP administration might put the pancreas in an environment similar to the postnatal period of development.

Regarding the morphometric results of this study, two important observations must be taken in consideration: in the PRP/diabetic group there was significant increase in the number of PCNA positive cells compared to controls. Therefore, its recommended to perform several studies for adequate adjustment of the dose and duration of PRP therapy before its clinical application. The PRP-group exhibited increased PCNA positive cells which had atypical shape and size of nuclei. Therefore, our study recommends not to inject the PRP in normal conditions to avoid overstimulation of cell proliferation, which could lead to hyperplasia or tumor formation.

## Conclusion

This study provides an evidence of the diabetic pancreatic islet regeneration in response to PRP treatment. The PRP stimulated islet cell regeneration and stimulated the induction of other sources of  $\beta$ -cells generation as the exocrine portion of the pancreas; ductal and acinar cells. In addition, PRP might put the pancreas into an environment similar to the postnatal developmental one where new lobules were formed. These will pave the future for a novel treatment for diabetes.

## Recommendations

Some limitations to this study were acknowledged. Further studies are required to identify the exact mechanisms responsible for these results and to confirm the process of neo-genesis and trans-differentiation among pancreatic cells.

## Conflicts of Interest

There is no conflict of interest to declare.

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