Immunohistochemical and Histochemical Studies of B-Cells Insulin Up-Regulation in Pancreatic Tissues of Streptozotocin-Induced Diabetic Albino Rats Treated with Melatonin and Magnesium

Elvis Tams Godam¹, Wilson Oliver Hamman², Enebeli S. Kelechi³, Sunday Oladele⁴, Modupeola Omotara Samaila⁵, Sunday Abraham Musa²

¹Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical Sciences, Rivers State University, Port Harcourt, Nigeria
²Department of Human Anatomy, Faculty of Basic Medical Sciences, Ahmadu Bello University Zaria Nigeria
³Department Of Pharmacology, Rivers State University Port Harcourt, Nigeria
⁴Department of Veterinary Pathology, Faculty of Veterinary Medicine Ahmadu Bello University, Zaria Nigeria
⁵Department Of Pathology, Faculty of Basic Clinical Sciences, Ahmadu Bello University Zaria Nigeria

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*Corresponding author: Elvis Tams Godam
Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical Sciences, Rivers State University, Port Harcourt, Nigeria

**Abstract**

**Background:** Improve insulin secretion and cellular availability to reduced blood glucose levels in diabetic subjects by bioactive compound especially antioxidants are the new focus to ameliorate the complication with diabetes mellitus. **Aims and objectives:** The aim of this study was to evaluate the effects of administration of melatonin and magnesium on the cytoarchitecture of the pancreatic tissue and to access immunohistochemically insulin release in streptozotocin-induced diabetic Albino rats. **Materials and methods:** To achieve this aim six normoglycaemic rats and forty eight Streptozotocin (STZ) induced diabetic rats was used in the study after two weeks acclimatization period. The animals were assigned into nine groups as follows, Normal control group (NC), Diabetic control (DC) group, Melatonin at 10mg/kgbw (MLD), magnesium dose group of 240 mg/kgbw (MgLD), melatonin and magnesium combined group of 10mg/kgbw+240mg/kgbw (MMgLD), melatonin group of 20mg/kgbw (MHD), melatonin and magnesium high dose combined group of 20mg/kgbw+480mg/kgbw (MMgHD) and insulin at 500mg/kgbw group (IN). Melatonin and insulin were administered through intraperitoneal injections (IP) while magnesium was by oral administration. The control groups were given placebo and all groups’ treatment was for twenty-one days. At the end of the study, the animals were aestheticized and euthanized to harvest pancreatic organ. The organs were fixed in neutral buffered formaldehyde (NBF). They were histologically prepared and stained using haematoxylin and eosin and immunohistochemically stained using insulin antibody to access insulin release. **Results:** Melatonin treatment at 10mg/kgbw and at 20 mg/kgbw showed histological improvement in histological tissues and insulin release while when combined with magnesium at dose of 10mg/kgbw and at 240 mg/kgbw showed better results. The administration of magnesium at 240 mg/kgbw, 480 mg/kgbw and when combined with melatonin at high doses does not show significant improvement in islet β-cell proliferation and insulin release. **Conclusion:** The administration of melatonin and magnesium at low doses regenerates pancreatic islet histoarchitecture and augments insulin release from treated diabetic albino rats.

**Keywords:** Pancreas, Diabetes, Magnesium, Melatonin, insulin, immunohistochemistry, insulin, beta cells.

**Introduction**

Diabetes is a global problem [1]. The latest data from the international diabetic federation shows that 463 million adults are living with diabetes [2] and that it will climb to 700 million (51%) people by 2045. Approximately 4.2 million adults aged 20–79 years are estimated to die as a result of diabetes and its complications in 2019 [2]. This is equivalent to one death in every eight seconds. Diabetes is estimated to be associated with 11.3% of global deaths from all causes among people in this age group and almost half (46.2%) of deaths associated with diabetes among the 20–79 years age group are in people under the age of 60.
years [2] and these figures will triple as a result of covid-19 pandemic. Myriads of pathological processes plays critical role in the development and progression of diabetes, from the autoimmune destruction of pancreatic beta cells and consequently insulin deficiency to processes that leads to insulin resistance. These processes are the basis of disorders in the metabolism of carbohydrates, lipids, and proteins. Disorders of insulin secretion and insufficiency of insulin function are commonly found in diabetic patients; it is often unclear which of these disorders the main cause of hyperglycemia is [2]. Diabetes is a chronic, progressive disease characterized by elevated levels of blood glucose. Diabetes of all types can lead to complications in many parts of the body and can increase the overall risk of dying prematurely [2, 3].

Type 1 diabetes mellitus results from autoimmune destruction of pancreatic β cells of the islets of Langerhans [5, 6]. Exogenous insulin is an important treatment for type 1 diabetes mellitus but it is not a physiological method to regulate blood glucose levels. Although various methods have been advanced and used in place of insulin like Beta cell replacement therapies using either the pancreas or pancreatic islet transplantation and many adjuvants have also been used as therapeutic alternative of exogenous insulin administration [7]. The risks associated with major invasive surgical procedures and consequents side effects of immunosuppressive therapies warrants the need for new alternative therapies [8], therefore there is need to regenerate endogenous pancreatic beta cells using known potent antioxidants and bioactive compounds like melatonin and magnesium to up regulate insulin secretion.

Melatonin is a hormone produced by the pineal gland and has shown enormous antioxidant properties. Studies have shown that melatonin reduces diabetic complications by attenuating oxidative damage [9, 10]. Pancreatic beta cells are prone to be associated with oxidative damage and have been shown to produce high endogenous levels of Reactive oxygen species (ROS) and are less expressive of the anti-oxidative enzymes. Melatonin serves as ROS scavenger and it has also shown potential to reduce the complications associated with diabetes mellitus such as diabetic syndrome, diabetic neuropathy, hyperglycemia and cardiovascular diseases [11]. Melatonin stimulates several anti-oxidative enzymes and acts on bone metabolism [12, 13]. The hormone exerts its effects both through activation of its receptors [14] and through the circulating levels of the hormone or in a more autocrine/paracrine fashion near target tissues [15, 16]. In addition, melatonin brings about vasoconstriction through the MT1 and vasodilation through the MT2 receptors [17]. Melatonin lowers cortisol secretion in the adrenal cortex, similar to the action shared with insulin [15, 16]. Moreover, human adipocytes, a major target tissue for insulin, express MT2 and have been shown to reduce expression of the insulin-dependent glucose transporter, Glut4, after melatonin stimulation [18]. Melatonin also stimulates glucose uptake in muscle cells by phosphorylation of insulin receptor substrate-1 (IRS-1) through MT2 signaling [19]. Melatonin can be able to bring anti-hyperglycaemic effects either by improving insulin sensitization and or by improvement of insulin secretion or both in pancreatic tissues [20, 21].

Magnesium (Mg) is an electrolyte of chief physiological importance in the body, being the most abundant divalent intracellular cation in the cells, the second most abundant cellular ion next to potassium and the fourth cation in general in the human body [22]. Type 2 diabetes mellitus (T2DM) is often accompanied by alteration of Mg status. An increased prevalence of Mg deficits has been identified in diabetes mellitus patients, especially in those with poorly controlled glycemic profiles, with longer duration of the disease and with the presence of micro and macrovascular chronic complication [23]. Magnesium deficiency may also be a factor implicated in diabetes mellitus complications. It was found that there is a relationship between ionic changes and echocardiographic indices alterations. Further observation is a significant association of reduced cellular Mg with cardiac hypertrophy in diabetes mellitus patients [24]. Magnesium is a cofactor in the glucose-transporting mechanism of the cell membrane and various enzymes in carbohydrate oxidation. It is also involved at multiple levels in insulin secretion, binding and activity. The almost universal involvement of magnesium in a wide variety of cellular processes critical to glucose metabolism, insulin action and cardiovascular functions has been well appreciated. The incidence of subclinical magnesium deficiency is common in diabetes and cardiovascular disorders [25].

Endocrine and metabolic disorders associated with magnesium deficiency in diabetes mellitus is the most common. Many studies have shown that plasma levels of magnesium are lower in patients with type 1 and type 2 diabetes mellitus, compared to non-diabetic control subjects. Inverse correlations between magnesium and fasting plasma glucose/ HbA1C/ HOMA-IR have been observed [26, 27]. Factors implicated in hypomagnesemia in diabetics include diets low in magnesium [28], osmotic diuresis causing high renal excretion of magnesium, insensitivity to insulin affecting intracellular magnesium transport, and thereby causing increased loss of the extracellular magnesium [9]. Rampant use of loop and thiazides diuretics promoting magnesium wasting [30, 31], diabetic autonomic neuropathies [25] and reduced tubular reabsorption due to insulin resistance [32]. Sometimes frequent use of antibiotics and antifungals such as aminoglycosides and amphotericin in patients with diabetes may also contribute to renal magnesium wasting [33]. Magnesium deficiency may result in disorders of tyrosine kinase activity of the insulin
receptor, event related to the development of post-receptorial insulin resistance and decreased cellular glucose utilization, that is, the lower the basal magnesium, the greater the amount of insulin required to metabolize the same glucose load, indicating a decreased insulin sensitivity [34]. The study aims to explore the combined effects of melatonin and magnesium on pancreatic tissue architecture and insulin release from regenerated beta cells in the islets of Langerhans of STZ induced diabetic albino rats.

MATERIALS AND METHODS

Materials

The following materials were used in the study, Plastic Cages, organ sample containers, Centrifuge, Temperature controlled refrigerator, Microwave oven, water bath, humidity chamber, Leica Auto processor, Leica Auto stainer, Leica DM750, Camera ICC50 E, AmScope D200 digital camera, MRC spectrophotometer, Microwave oven.

Bioactive compounds and drugs used in the study were

Melatonin M5250-1G (Sigma Aldrich USA), Magnesium (Randox, USA) Streptozotocin SP0130 (Sigma Aldrich, USA), Haematoxylin and Eosin Stain (H&E), insulin Antibody (Bioss, USA), antibody were used in the study.

Source of animals and management

Fifty-four Male Wistar rats weighing of 120–150 g, were purchased from the Faculty of Pharmaceutical Sciences Animal House of the Ahmadu Bello University, Zaria and selected for the study. The rats were maintained on a day and night cycle at room temperatures and have ad libitum access to food (Standard feeds, standard rat pellets) and water. All experiments were performed between 08:00 and 12 hours.

METHODOLOGY

Induction of Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) was induced after 2 weeks acclimatization period, a baseline blood glucose levels and behavioral and cognitive assessment were performed for all test animals. This was done to ensure that the animals were all normoglycaemic. Fifty-Eight male Wistar rats were randomly selected and given a single dose of intra peritoneal injection of streptozotocin, (STZ) (Sigma, Aldrich, USA), at 55mg/kg body weight in citrate buffer (0.1M, pH 4.5). The solution (STZ in citrate buffer) was used within 5 minutes to induce chemical diabetes in the Wistar rats after an overnight fast for twelve hours.

Hyper glycaemia screening and confirmation of T1DM

Four days after streptozotocin was used to induce diabetes mellitus, blood was collected from the tail vein following an overnight fast [35]. Fasting blood sugar (FBS) was measured with a standard glucometer (Optimum, Germany). The day that hyperglycaemia above 200mg/dl (11 mmol/l) was confirmed was considered to be diabetic day 1. Rats with fasting blood glucose levels lower than 200 mg/dL (11mmol/L) were excluded from the study.

Histochomical studies

The pancreatic tissue samples were harvested and fixed in 10% Neutral buffered formalin for 72 hours. The samples were grossed and labelled in tissues cassettes and processed histological by different concentration of alcohols (70, 80, 90 and 100%) for dehydration, cleared through three changes of Toluene, infiltrated, embedded in molten paraffin wax and blocked on cold ice packs. The tissues were sectioned using a Rotary Microtome (Leica, Germany) and the ribbons obtained were picked on clean grease free Leica charged slides in a float out water bath and drained for histological and immunohistochemical studies. The slides were drained and heat fixed on a hot plate at 2 degrees above wax melting point. The tissues were further taken to Leica Auto stainer machine and dewaxed in Toluene, cleared in decreasing reverse order of alcohol and taken to water before proceeding for Haematoxylin and Eosin staining procedure.

Immunohistochemical studies for insulin release

Insulin release in the pancreas of normal control, diabetic control, melatonin and magnesium treated animals was immunohistochemically studies using Bios antibodies (IHC) mouse polyclonal antibodies for insulin.

Methods

The pancreatic tissue was fixed in paraformaldehyde and paraffin wax embedded: antigen retrieval was by citrate buffer (0.1M, pH 6.0) and was placed in boiling bath for 90 minutes. Endogenous peroxidase was blocked by 3% hydrogen peroxide for 30 minutes and later blocked using buffer (normal goat serum, C-0005) AT 37 °C for 20 minutes.

It was further incubated with Rabbits/Mouse Insulin Polyclonal Antibody, Unconjugated with (bs-0862R) from Bios Antibodies Massachusetts USA, in 1: 500 dilutions for 20 minutes. Then conjugated to the secondary antibody (SP-0023) and DAB for 10 minutes (C-0010) and later stained with Heamatoxylin for nuclear contrast and mounted with DPX.

RESULTS

Histology of the Pancreas observed in the study using Haematoxylin and Eosin (H&E) Stains

Photomicrograph results of the histological examination of the pancreas in normal saline treated control group (Plate I) showed normal pancreatic cell mass. No observable pancreatic lesion in the tissues. Plate II (Diabetic control group) showed pancreatic nuclear pyknosis, vacuolation and areas of cellular degeneration. The pancreatic tissue samples were harvested and fixed in 10% Neutral buffered formalin for 72 hours. The samples were grossed and labelled in tissues cassettes and processed histological by different concentration of alcohols (70, 80, 90 and 100%) for dehydration, cleared through three changes of Toluene, infiltrated, embedded in molten paraffin wax and blocked on cold ice packs. The tissues were sectioned using a Rotary Microtome (Leica, Germany) and the ribbons obtained were picked on clean grease free Leica charged slides in a float out water bath and drained for histological and immunohistochemical studies. The slides were drained and heat fixed on a hot plate at 2 degrees above wax melting point. The tissues were further taken to Leica Auto stainer machine and dewaxed in Toluene, cleared in decreasing reverse order of alcohol and taken to water before proceeding for Haematoxylin and Eosin staining procedure.

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necrosis and reduced islet cell mass. Plate III (melatonin treated at 10 mg/kgbw) shows pancreatic islet of Langerhans cell mass recovery in STZ induced diabetic rats. Plate IV shows no islet cell mass recovery in Magnesium treated low dose group (240 mg/kgbw) STZ induced diabetic rats. There is pyknosis, vacuolation and reduced islet cell mass. Plate V shows pancreatic tissue section from melatonin and magnesium treated (10mg/kgbw+240 mg/kgbw) STZ induced diabetes Wistar rats with no cellular necrosis. Plate VI (melatonin treated 20 mg/kgbw group) shows mild pancreatic islet mass recovery in STZ induced diabetic rats. Plate 7 (magnesium treated at 480 mg/kgbw group) shows reduced pancreatic cell mass with pyknosis, vacuolation and cellular necrosis in STZ induced diabetic rats while plate VIII (melatonin 20 mg/kgbw +480 mg/kgbw Magnesium group) and plate IX (insulin treated at 2IU) shows similar results in plate VI.

Plate I: Photomicrograph of a section of pancreas tissue of Wistar rats from control group. The Pancreatic islet of Langerhans showed normal pancreatic islet cell mass (black arrows) (H&E, x250)

Plate II: Photomicrograph of a section of a pancreatic tissue from Wistar rats of STZ induced diabetes (diabetic control group) showing reduced islet of Langerhans mass, cellular degeneration, pyknosis and vacuolation. The reduction of the islet cell mass is due to the selective chemical destruction of β cells by streptozotocin, (black arrows), (H &E, x250)

Plate III: Photomicrograph of a section of pancreatic tissue from STZ induced diabetic rat treated with melatonin (10 mg/kgbw) with restored islet cells of Langerhans, (black arrows), (H&E, x250)

Plate IV: Photomicrograph of a section of pancreatic islet of Langerhans from magnesium treated Diabetic group at 240mg/kgbw. Islet cells mass are shrunken and degenerated (black arrows) and pyknotic cells, (red arrows), (H&E x100)

Plate V: Photomicrograph of a section of pancreas from STZ induced diabetes and treated with melatonin 10mg/kgbw and magnesium 240mg/kgbw, showing cellular restoration of islet cell, (black arrows), H&E x250

Plate VI: Photomicrograph of a section of pancreas of STZ induced diabetes with melatonin treated group at 20 mg/kgbw showing mild pancreatic islet mass recovery (black arrows) (H&E, x250)

Plate VII: Photomicrograph of a section of pancreas from STZ induced diabetes with magnesium treated group at 480 mg/kgbw showing reduced pancreatic cell mass with pyknosis, vacuolation and cellular necrosis (black arrows), (red arrows), (H&E x100)
Plate VI: Photomicrograph section of pancreas of STZ induced diabetic rats treated with Melatonin (20mg/kgbw) showing pancreatic islet of Langerhans cell mass restoration especially destroyed β cells (green arrows), (H&E x250)

Plate VII: Photomicrograph section of STZ induced diabetic rats treated with Magnesium at 480mg/kgbw (MgHD) group. Areas of islet cells necrosis are observed (blue arrow), Vacuolation (green arrow) and pyknosis (red arrow), H &E stain, x250

Plate VIII: Photomicrograph of a section of pancreas from STZ induced diabetic rats treated with melatonin (20 mg/kgbw) and magnesium (480 mg/kgbw) group (DMMgHD). Areas of Islet cells degeneration with necrosis (yellow arrow) and pyknosis (red arrow) observed, pancreatic Vein (black arrow), (H&E stain x250)

Plate IX: Photomicrograph of a section of pancreas from STZ induced diabetic rats treated with Insulin (2IU) group (IN), showing shrunken cell mass with vacuolation (red arrow). Areas of islet cells necrosis are observed, (H&E x250)

Insulin immunohistochemical Studies of the Pancreatic Islet of Langerhans

Plate X. Photomicrograph section of pancreatic tissue from Control rats (NC) administered normal saline shows pancreatic Islets of Langerhans with significant (p˂0.05) positive insulin expression (Brown stain-black arrows) from β cells mass. Similar results was obtained from Photomicrograph section of pancreas tissue from STZ induced diabetes rats treated with 10 mg/kg melatonin Plate XII (MLD group) showing Pancreatic Islets of Langerhans with significant (p≤0.05) positive insulin expression (Brown stain-yellow arrows) from regenerated β cells, Plate XIV Photomicrograph section of pancreas tissue from STZ induced Diabetic rats (MMgLD) group administered Melatonin (10 mg/kgbw) and Magnesium (240 mg/kgbw) showed pancreatic islets of Langerhans with significant (p≤ 0.05) positive insulin expression (Brown stain-black arrows) from regenerated β cells and Plate XV shows Photomicrograph of a section of pancreatic tissue from STZ induced diabetes treated rats administered melatonin 20 mg/kgbw (MHD group) showing pancreatic islets of Langerhans with significant (p≤0.05) positive insulin expression (Brown stain-black arrows) from regenerated β cells.

There was no significant insulin expression ((p>0.05) in diabetic control group (DC) as shown in Plate XI. The Low positive areas of insulin expression showed that STZ selectively destroyed β cells and are strongly diabetic as the results of the high blood glucose levels in the group confirmed it, while similar results were obtained in Plate XIII Photomicrograph section of pancreatic tissue from STZ induced diabetes rats treated with Magnesium at 240 mg/kgbw (MgLD) group showed pancreatic islets of Langerhans with significant (p≤ 0.05) negative insulin expression (brown stain-black arrows) from shrunken β cells mass, Plate XVI. Photomicrograph section of pancreatic tissue from STZ induced diabetes rats treated with Magnesium at 480 mg/kgbw (MgHD) group showing pancreatic islets of
Langerhans with significant (p<0.05) negative insulin expression (Brown stain-black arrows) from shrunken β cells. Plate XVII: Photomicrograph of a section of pancreatic tissue from STZ induced diabetes rats (MMgHD group) treated with melatonin (20 mg/kgbw) and Magnesium (480 mg/kgbw) showing pancreatic islets of Langerhans with significant (p<0.05) negative insulin expression (Brown stain-black arrows) from shrunken β cells while Plate XVIII is Photomicrograph section of pancreatic tissue from STZ induced diabetes rats (IN group) treated with insulin showing pancreatic islets of Langerhans with mild increase but not significant (p<0.05) insulin expression (Brown stain-black arrows) from shrunken β cells.

Plate-X: Photomicrograph of a section of pancreas tissue from Control rats (NC) administered normal saline showing pancreatic islets of Langerhans with significant (p<0.05) positive insulin expression (Brown stain-black arrows) from β cells mass, (Leica DM 750 ICC50 E, IHC; Insulin antibody, x400)

Plate-XI: Photomicrograph of a section of pancreas tissue from diabetic Control rats (DC) administered normal saline showing Pancreatic islets of Langerhans with significant (p<0.05) negative insulin expression (Brown stain areas) from shrunken β cells, (Leica DM 750 ICC50 E, IHC; Insulin antibody x400)

Plate-XII: Photomicrograph of a section of pancreas tissue from STZ induced diabetes administered melatonin 10 mg/kgbw (MLD group) showing pancreatic Islets of Langerhans with significant (p<0.05) positive insulin expression (Brown stain-yellow arrows) from regenerated β cells, (Leica DM 750 ICC50 E, IHC; Insulin antibody, x400)

Plate-XIII: Photomicrograph section of pancreas tissue from STZ induced diabetes rats (MgLD group) and treated with magnesium (240 mg/kgbw) showing pancreatic islets of Langerhans with significant (p< 0.05) negative insulin expression areas (Brown stain areas) from shrunken β cells, (Leica DM 750 ICC50 E, IHC; Insulin antibody, x400)

Plate-XIV: Photomicrograph of a section of pancreas tissue from STZ induced Diabetic rats (MHD group) administered melatonin (20 mg/kgbw) and magnesium (240 mg/kgbw) showing pancreatic islets of Langerhans with significant (p<0.05) positive insulin expression (Brown stain areas-black arrows) from regenerated β cells, (Leica DM 750 ICC50 E, IHC; Insulin antibody x400)
DISCUSSION

Histologic study of the Pancreas showed that melatonin and magnesium regenerated the pancreatic islet cell mass in STZ induced diabetic rats as observed in groups treated with melatonin at varying doses, and when combined with magnesium at low dose. There was shrunken pancreatic islet of Langerhans cell mass with vacuolation and necrosis in STZ induced diabetic group. Similar results of pancreatic islet cells damage and histoarchitechral cellular derangement and lesions was observed in magnesium treated STZ induced diabetic rats at low and high doses and when combined with melatonin at high dose. Results for insulin treated STZ induced diabetic rats did not show any restoration in pancreatic islet mass and cellular structure. Reports by [6] and [36] collaborates with our present study which corroborates that melatonin regenerates pancreatic islets cells by reducing hyperglycaemia, reduction of free radical and potentiating intracellular antioxidant SOD, CAT and GPx [37] showed that four weeks’ melatonin treatment of STZ induced diabetic rats restored pancreatic histoarchitectural damages such as restoration of cytoplasmic richness, augmented islet cell body, decrease vacuole size and regeneration of total pancreatic islets mass. This study is in agreement with our present study with the pancreas where melatonin and magnesium are combined at low doses resulting in regenerated and increase pancreatic islet of Langerhans cell mass cyto-architecture and insulin release. Hence these present results also collaborate with earlier report published by [37] stating that melatonin regulates glucose homeostasis and reports by [38] showed that melatonin also attenuates insulin resistance and down regulates amyloid beta accumulation

Results for the immunohistochemical studies of the pancreatic islet beta cells insulin expression showed that melatonin attenuates insulin release and positive expression from the pancreas in melatonin treated groups and when combined with magnesium at
low doses in this study. Islet insulin release in normal control group showed 100% insulin positive expression from the β cells of the pancreatic islet. There was 75%-85% insulin positive expression from regenerated β cells in the islets of Langerhans of melatonin treated group at low dose (10 mg/kgbw) and at high dose (20 mg/kgbw). Similar result of insulin positive expression was observed in the melatonin and magnesium combined group at low dose (about 85% insulin expression in beta cells). This showed that melatonin and magnesium synergistically combined to upregulates glucose uptake and cellular glucose metabolism by increasing insulin release and availability to the cells. The reduction of hyperglycaemia by melatonin and magnesium restored insulin secreting β-cells and augments insulin release. Reports by Arya et al. [39] and [40], showed that the volume size of pancreas islets, cellular nuclear intensity and cytoplasm richness can display the function of islet cells and that the predominant synthesis of insulin reflects islet regeneration and richer islet cells cytoplasm. These previous reports confirmed our findings that melatonin and magnesium antioxidant activities attenuate positive insulin expression and pancreatic islet of Langerhans regeneration. Reports by [41], also confirmed that the greater level of serum magnesium corresponds to greater degree of insulin sensitivity, therefore this improved the glycaemic index when magnesium is administered to STZ induced diabetic rats. These reports were able to established that magnesium co-administration with melatonin at 240 mg/kgbw and 10 mg/kgbw melatonin augments insulin release and glycaemic index with improved pancreatic islet histoarchitecture and immunohistochemical insulin up regulation of treated rats which diminished free radicals generated by streptozotocin induced diabetic hyperglycaemia.

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