Chapter 4

Effect of sitagliptin, melatonin and combination treatment on pancreatic β-cell regeneration in Streptozotocin (STZ)-induced T1D mouse model

4.1 Introduction

Type 1 Diabetes (T1D) is characterized by hyperglycemia due to pancreatic β-cell apoptosis and lack of β-cell regeneration. The most promising treatment for T1D is islet transplantation. However, its application remains restricted due to lack of donors and lifelong dependence on immunosuppressive drugs (Ricordi and Storm, 2004; Shapiro, 2012; Sharples et al., 2016). Thus, it is crucial to explore novel therapies that would aid in β-cell regeneration either by proliferation or neogenesis for T1D management. Monotherapies often fail in the long run due to other diabetes related complications and patient's lifestyle. Hence, combination therapy is recommended for long-term glycemic control.

Incretin-based drugs are currently categorized as novel therapies for Type 2 Diabetes (T2D) management. These include mainly two categories, GLP-1 receptor agonists (GLP-1RA) and dipeptidyl peptidase-IV (DPP-IV) inhibitors (Gilbert et al., 2020). Both drugs have a low risk of hypoglycemia and function differently to regulate glucose homeostasis. Sitagliptin, a DPP-IV inhibitor, prevents the degradation of incretins (GLP-1 and GIP), elevates their levels and thereby stimulate insulin secretion. Studies on T2D rodent model have shown that both the classes of drugs promote β-cell proliferation. However, studies demonstrated nominal beneficial effect of GLP-1RAs in the T1D model, possibly due to its limited immunomodulatory effects (Gilbert et al., 2020). DPP-IV inhibitors with immunomodulatory and glucoregulatory effects are considered as second line therapy for T2D management, but recently are being studied in T1D models to assess β-cell function and mass.

Melatonin, a pineal hormone known as the "hormone of darkness" has antioxidant (Reiter et al., 2016), anti-inflammatory and anti-apoptotic properties (Hardeland et al., 2019). It has also been associated with glucose homeostasis (Peschke et al., 2000; Owino et al., 2019). We have also shown that low levels of melatonin are implicated in the risk of T2D (Patel et al., 2018). Melatonin levels also reduce with ageing, and it has been previously reported that melatonin and insulin share antagonistic relationship (Peschke et al., 2011). In T1D, melatonin production increases as observed in streptozotocin (STZ)-treated Wistar rats (Peschke et al., 2008) and T1D patients (Kor et al., 2014), while β-cell loss decreases insulin production, resulting in hyperglycemia (Mok et al., 2019). Moreover, it has been reported that reduced antioxidant levels and simultaneous overproduction of free radicals were found in T1D patients (Marra et al., 2002). In this context, melatonin has been shown to have a protective role against STZ- induced T1D (Andersson and Sandler, 2001), including β-cell

proliferation (Kanter et al., 2006). Furthermore, β-cell proliferation and its regenerative capacity declines with age (Tschen et al., 2009) and so are the melatonin levels. Thus, it is important to assess β-cell regeneration in both young and old T1D mice upon melatonin administration.

Hence, we aimed to assess the therapeutic potential of melatonin and DPP-IV inhibitor on β-cell regeneration in STZ-induced young and old T1D mouse model.

4.2 Materials and Methods

4.2.1 Animals

Forty young (7-8 weeks old) and old (30 weeks old) BALB/c male mice, bred in our animal vivarium, were used for the experiment. The animals were maintained on a 12hr light-dark cycle starting at 7:00 AM. Food and water were provided *ad libitum*. All the experimental procedures were performed in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and were approved by Institutional Animal Ethical Committee (IAEC) (MSU/BC/13/2017).

4.2.2 T1D Mouse Model Development and Drug Treatment

T1D was induced in BALB/c mice by five consecutive intraperitoneal (i.p.) injections of streptozotocin (STZ) (50 mg/kg body weight). STZ (MP Biomedicals, Santa Ana, CA, USA) was freshly dissolved in 0.1 M cold sodium citrate buffer (pH 4.5). Control (n=8) and diabetic mice (n=32) were fed with a normal chow diet. Diabetes was confirmed two weeks later in mice having fasting blood glucose (FBG) >350mg/dL. FBG was measured in mice after 6 hours fasting by tail snipping method using a Glucometer (TRUEresult, NIPRO Diagnostics, Pune, MH, India). The diabetic mice were then randomly divided into four groups: i. Diabetic Control (DC), ii. Sitagliptin (S) treated, iii. Melatonin (M) treated, and iv) S+M treated. Melatonin (Sigma, St. Louis, MO, USA) was administered between 6 PM to 7 PM daily at a dose of 0.5 mg/kg BW i.p. in 0.9% saline. Sitagliptin (Januvia) (Merck & Co., Kenilworth, NJ, USA) was purchased in 100 mg tablets and was given at a dose of 5 g/kg diet. The treatment was given for six weeks along with bromodeoxyuridine (BrdU) (MP Biomedicals, Santa Ana, CA, USA) at a dose of 100 mg/kg BW (i.p.). FBG levels and BW were monitored twice a week.

4.2.3 Intraperitoneal Glucose Tolerance Test

Intraperitoneal glucose tolerance test (IPGTT) was performed post drug treatment (six weeks) in mice fasted for 6 hours. Mice were injected with glucose (2 g/kg BW i.p.). Blood glucose levels were measured immediately at 0, 15, 30, 90, and 120 minutes by tail snipping. The total area under the curve (AUC) was calculated.

4.2.4 Plasma Insulin Levels

Before sacrificing mice, 1 ml blood was collected from the orbital sinus into K_3 EDTA tubes and was centrifuged at 6000 g for 5 minutes at 4 °C. Plasma was separated and stored at -20°C for further analysis. Fasting plasma insulin levels were measured by a commercially available mouse insulin ELISA kit (RayBiotech, GA, USA).

4.2.5 Immunohistochemistry-Immunofluorescence (IHC-IF)

After six weeks of drug treatment, mice were sacrificed, and the pancreas was harvested. The pancreas was fixed in 10% neutral buffered formalin (NBF) for histological processing and paraffin embedding. 5µm sections were cut from the paraffin-embedded blocks. Immunofluorescence staining was carried out to study β-cell proliferation [Insulin/Glucagon/BrdU], neogenesis [Insulin/ Neurogenin 3 (NGN3)/ Pancreatic and duodenal homeobox 1 (PDX1)], α- to β-cell transdifferentiation [Insulin/ Aristaless related homeobox (ARX)/ Paired box gene 4 (PAX4)], and β-cell apoptosis [Insulin/apoptosis inducing factor (AIF) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Thermo Fisher Scientific, Cat# A23210)]. The sections were deparaffinized in xylene and rehydrated in a series of graded ethanol. Antigen retrieval was performed using 1N HCL for 45 minutes at 37 °C. Sections were blocked in 5% normal donkey serum (Jackson ImmunoResearch, Cat# 017-000-121) in PBST (PBS + 0.1% Tween 20) for 1 hour at room temperature and incubated with primary antibodies prepared in blocking buffer at 37 °C in a humidified chamber for 2 hours [guinea pig anti-insulin (DAKO, Cat# A0564; 1:200), rabbit anti-glucagon (Cell Signaling Technology, Cat# 2760; 1:200), rat anti-BrdU (Abcam, Cat# ab6325; 1:100), rabbit anti-NGN3 (Thermo Fisher Scientific, Cat# PA5-11893; 1:50), goat anti-PDX1 (Abcam, Cat# ab47383; 1:1000), rabbit anti-ARX (Sigma, Cat# AV36824; 1:500), goat anti-PAX4 (Sigma, Cat# SAB2501445; 1:500), and rabbit anti-AIF (Cayman chemicals, Cat# CAYM160773; 1:500)]. Sections were washed with PBS and were incubated with secondary antibodies prepared in blocking buffer at room temperature for 45 minutes in the dark [donkey anti-guinea pig Alexa Fluor 488 (Cat# 706-545-148; 1:500), donkey anti-rabbit Alexa Fluor 647 (Cat# 711-605-152; 1:500),

donkey anti-goat Rhodamine Red X (Cat# 705-295-003; 1:200), donkey anti-rat Rhodamine Red X (Cat# 712-295-150; 1:200), donkey anti-guinea pig Alexa Fluor 594 (Cat# 706-586-148; 1:500) (Jackson ImmunoResearch Laboratories, USA)]. Sections were washed with PBS and distilled water and were mounted with Slowfade® Gold Antifade mountant with DAPI (Thermo Fisher Scientific, USA), and the coverslip was sealed with nail varnish. Stained sections were observed under a confocal laser scanning microscope (Olympus FV10i, Tokyo, Japan). Image analysis was carried out in Image J software. Observations were made from three pancreatic sections per group from five different areas.

4.2.6 Statistical Analyses

Statistical comparisons of data were performed by one-way analysis of variance (ANOVA) and multiple group comparisons by Tukey's post hoc test in Prism 5 (GraphPad Sofware, San Diego, CA, USA). The significance level was set as $p<0.05$. Results are expressed as mean±SEM.

4.3 Results

4.3.1 Assessment of Body Weight, Fasting Blood Glucose, and Glucose Tolerance in Young T1D Mice

Metabolic phenotyping in young T1D mice suggests that DC group showed significant increase in FBG levels ($p<0.001$) with a reduction in plasma insulin levels ($p<0.01$) and glucose tolerance ($p<0.001$) as compared to control group. The six-week timeline showed reduction in BW in DC group as compared to control group. In addition, the monotherapies and the combination therapy significantly reduced FBG levels (S, $p<0.05$; M, $p<0.01$; S+M, $p<0.01$) by increasing insulin levels (M, $p<0.05$; S+M, $p<0.001$) with a concomitant increase in glucose tolerance (S, $p<0.05$; M; $p<0.05$; S+M, $p<0.01$) as compared to DC group. Furthermore, final FBG levels in the drug-treated groups were also significantly reduced as compared to their initial levels (S, $p<0.05$; M, $p<0.05$; S+M, $p<0.001$), however, sitagliptin group did not show significant increase in the plasma insulin levels ($p>0.05$) (Fig. 4.1A-E).

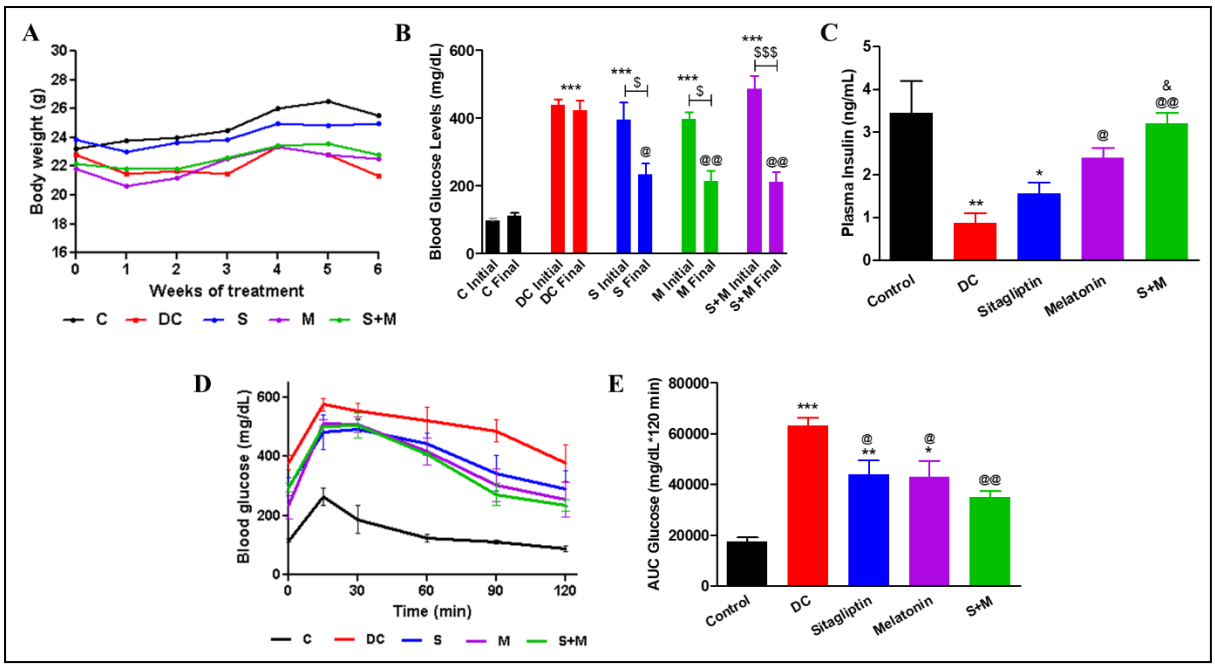

Figure 4.1 Assessment of body weight, blood glucose levels, and glucose tolerance in young mice: A. Body weight. Body weight was reduced in DC group as compared to control group over a period of six-weeks whereas no change in body weight was observed in M and S groups as compared to DC group while it remained constant in S group. **B. Fasting blood glucose levels.** A significant increase in FBG levels was observed in DC group as compared to control group while a significant decrease in FBG levels was observed in all the drug-treated groups as compared to their respective initial levels. Also, there was significant reduction in the FBG levels in the final drug-treated groups as compared to final DC group. **C. Plasma insulin levels.** A significant decrease in random plasma insulin levels was observed in DC group as compared to control group whereas the levels were significantly increased in M and S+M treated groups as compared to DC group. **D. Glucose tolerance test**. A significant decrease in glucose clearance was observed in DC group as compared to control group at all the time points. Increased glucose clearance was observed in all the drug-treated groups as compared to DC group at 60-, 90-, and 120-min. **E. Blood glucose AUC0–120.** A significant decrease in glucose tolerance was observed in DC group as compared to control group while all the drug-treated groups showed a significant increase in glucose tolerance as compared to DC. (*p<0.05, **p<0.01, ***p<0.001 vs. Control; $^{@}p$<0.05, $^{@@}p$<0.01 vs. Diabetic Control; $^{\&}p$<0.05 vs. Sitagliptin) (n=4-6/group).

4.3.2 Assessment of β-Cell Proliferation in Young T1D Mice

IHC analysis revealed that there was no change in β-cell proliferation in DC group as compared to control group as shown by $BrdU^{+}$ $insulin^{+}$ cells, however, S+M treated group showed significant increase in β-cell proliferation (S+M, p<0.001) as compared to DC group. Intriguingly, sitagliptin and melatonin groups did not show significant increase in β-cell proliferation as compared to DC group (p>0.05) (Fig. 4.2).

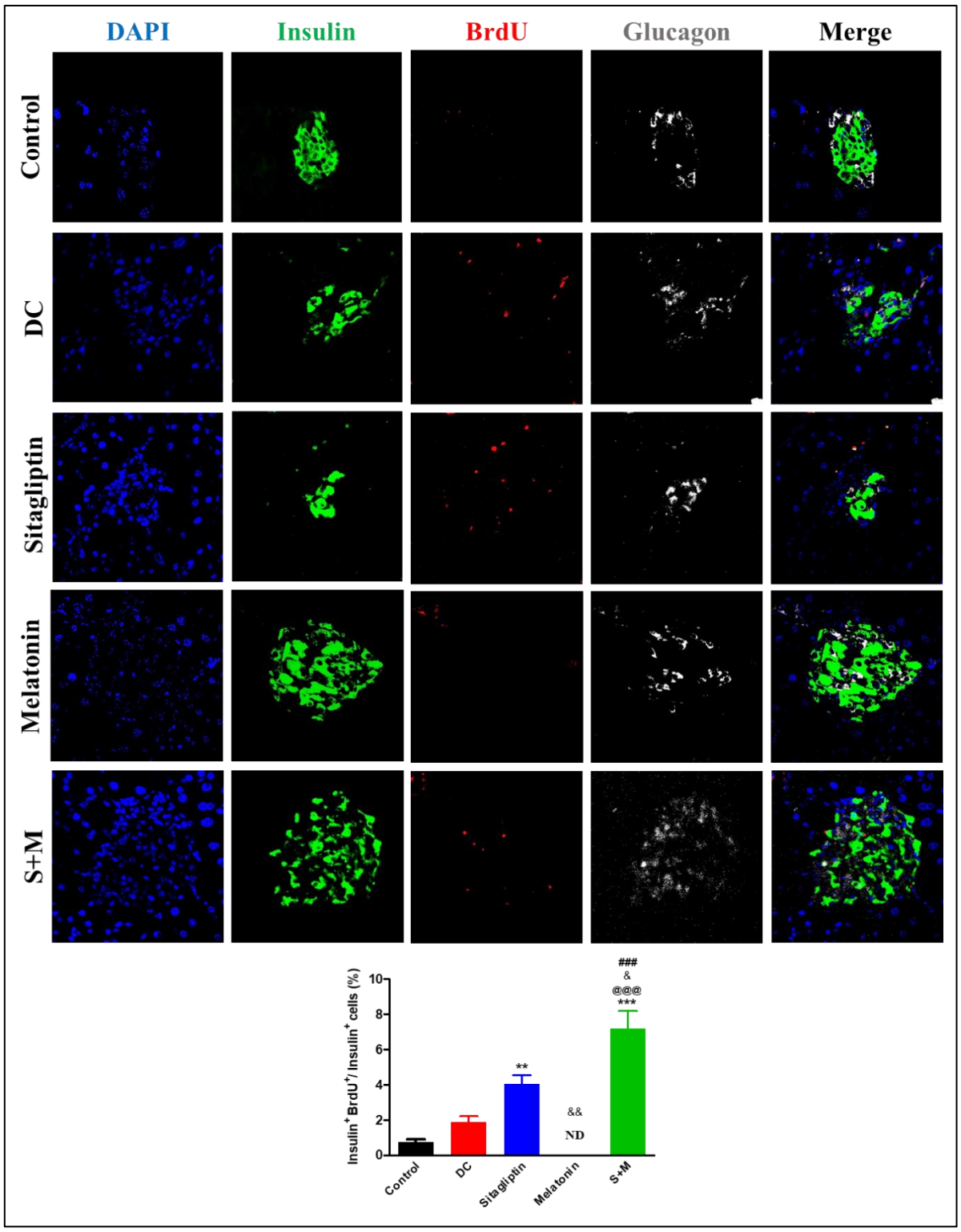

Figure 4.2 Immunohistochemical analysis for pancreatic β-cell proliferation in young T1D mice as shown by BrdU$^+$ insulin$^+$ cells. There was no significant change in β-cell proliferation in DC group as compared to control group. S+M treated group showed a significant increase in β-cell proliferation as compared to DC group. Sitagliptin and melatonin groups did not show significant increase in β-cell proliferation as compared to DC group. Scale: 50µm, Magnification: 60X. (**p<0.01, ***p<0.001 vs. Control; $^{@@@}p$<0.001 vs. Diabetic Control; $^{\&}p$<0.05, $^{\&\&}p$<0.01 vs. Sitagliptin; $^{\#\#\#}p$<0.001 vs. Melatonin) (n=3/group).

4.3.3 Assessment of β-Cell Neogenesis in Young T1D Mice

IHC analysis revealed that there was no β-cell neogenesis in young T1D mice as NGN3 was found negative. However, $PDX1^+$ $insulin^+$ cells were found in all the groups. Interestingly, nucleo-cytoplasmic translocation of PDX1 was observed in DC, sitagliptin and melatonin groups, while nuclear PDX1 was observed only in the control and S+M groups (Fig. 4.3).

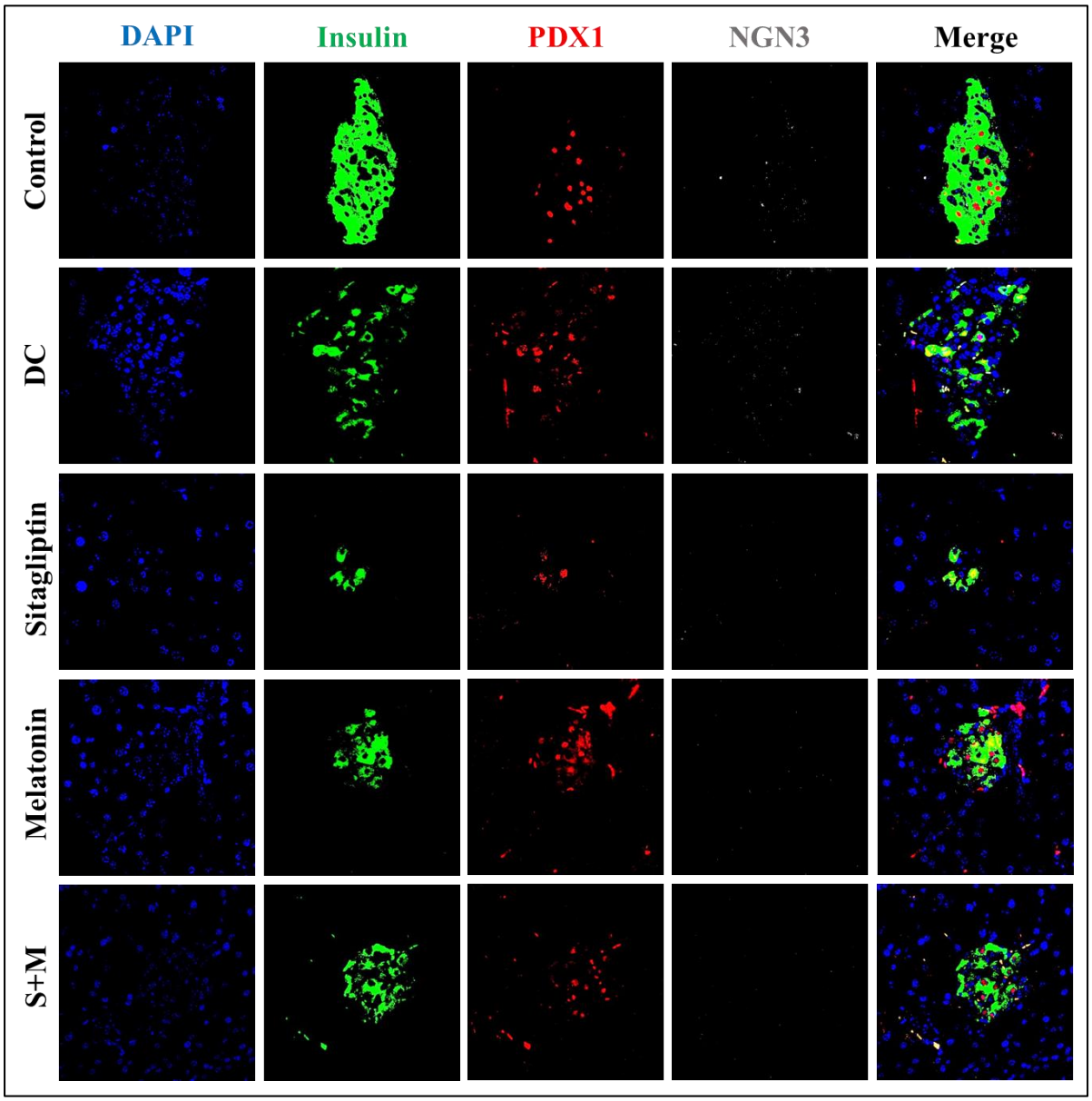

Figure 4.3 Immunohistochemical analysis for pancreatic β-cell neogenesis in young T1D mice as shown by $PDX1^+$ $NGN3^+$ cells. $NGN3^+$ cells were not detected in DC as well as in all the drug-treated groups. However, $PDX1^+$ cells were detected in all the groups while a nucleo-cytoplasmic translocation of PDX1 was observed in DC, sitagliptin and melatonin groups. Scale: 50µm, Magnification: 60X. (n=3/group).

4.3.4 Assessment of α- to β-Cell Transdifferentiation in Young T1D Mice

IHC analysis revealed that α- to β-cell transdifferentiation was detected in DC and all the drug-treated groups as shown by $PAX4^{+}$ ARX^{+} $insulin^{+}$ cells, while it was significantly increased in all the drug-treated groups as compared to DC group (S, $p<0.05$; M, $p<001$, S+M, $p<0.001$). Moreover, a nucleo-cytoplasmic translocation of PAX4 was also observed in control, and all the drug-treated groups (Fig. 4.4).

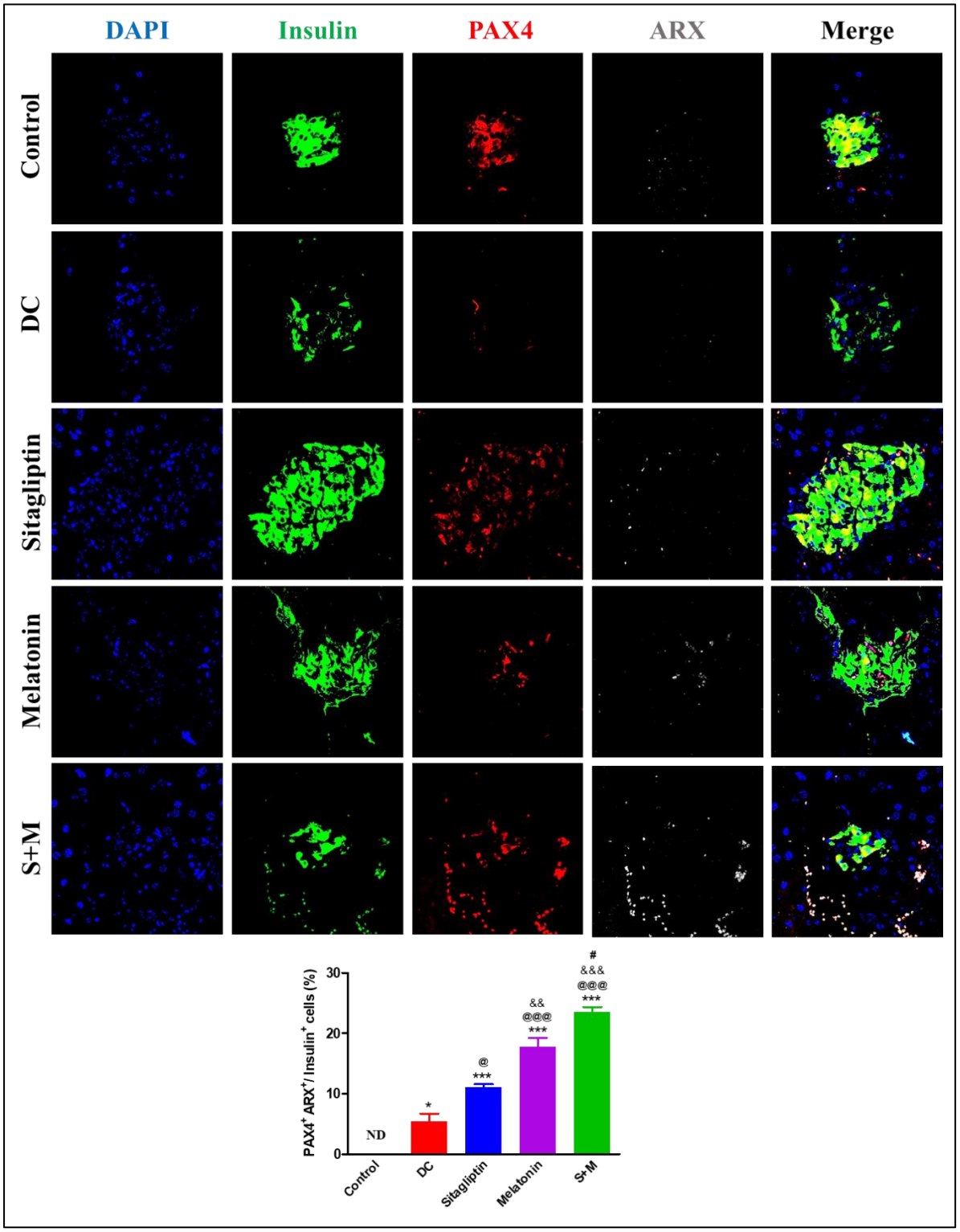

Figure 4.4 Immunohistochemical analysis for pancreatic α- to β-cell transdifferentiation in young T1D mice as shown by PAX^+ ARX^+ $insulin^+$ cells. α- to β-cell transdifferentiation was observed in DC and all the drug-treated groups, while it was significantly increased in all the drug-treated groups as compared to DC group. Moreover, a nucleo-cytoplasmic translocation of PAX4 was also observed in control, and all the drug-treated groups. Scale: 50μm, Magnification: 60X. (*p<0.05, ***p<0.001 vs. Control; $^{@}p$<0.05, $^{@@@}p$<0.001 vs. Diabetic Control; $^{\&\&}p$<0.01, $^{\&\&\&}p$<0.001 vs. Sitagliptin; $^{\#}p$<0.05 vs. Melatonin) (n=3/ group).

4.3.5 Assessment of β-Cell Apoptosis in Young T1D Mice

IHC analysis revealed that caspase independent β-cell apoptosis was observed in DC and S+M groups as shown by AIF^+ $insulin^+$ cells, whereas it was not detected in control, sitagliptin and melatonin groups. AIF translocation in the nucleus was significantly reduced in S+M group as compared to DC group (p<0.01) (Fig. 4.5).

Furthermore, β-cell apoptosis as shown by $TUNEL^+$ $insulin^+$ cells was significantly increased in DC group as compared to control group (p<0.001), while it was significantly reduced in S+M group as compared to DC group (p<0.001). However, no significant reduction in the monotherapy groups (p>0.05) was seen as compared to DC group (Fig. 4.6).

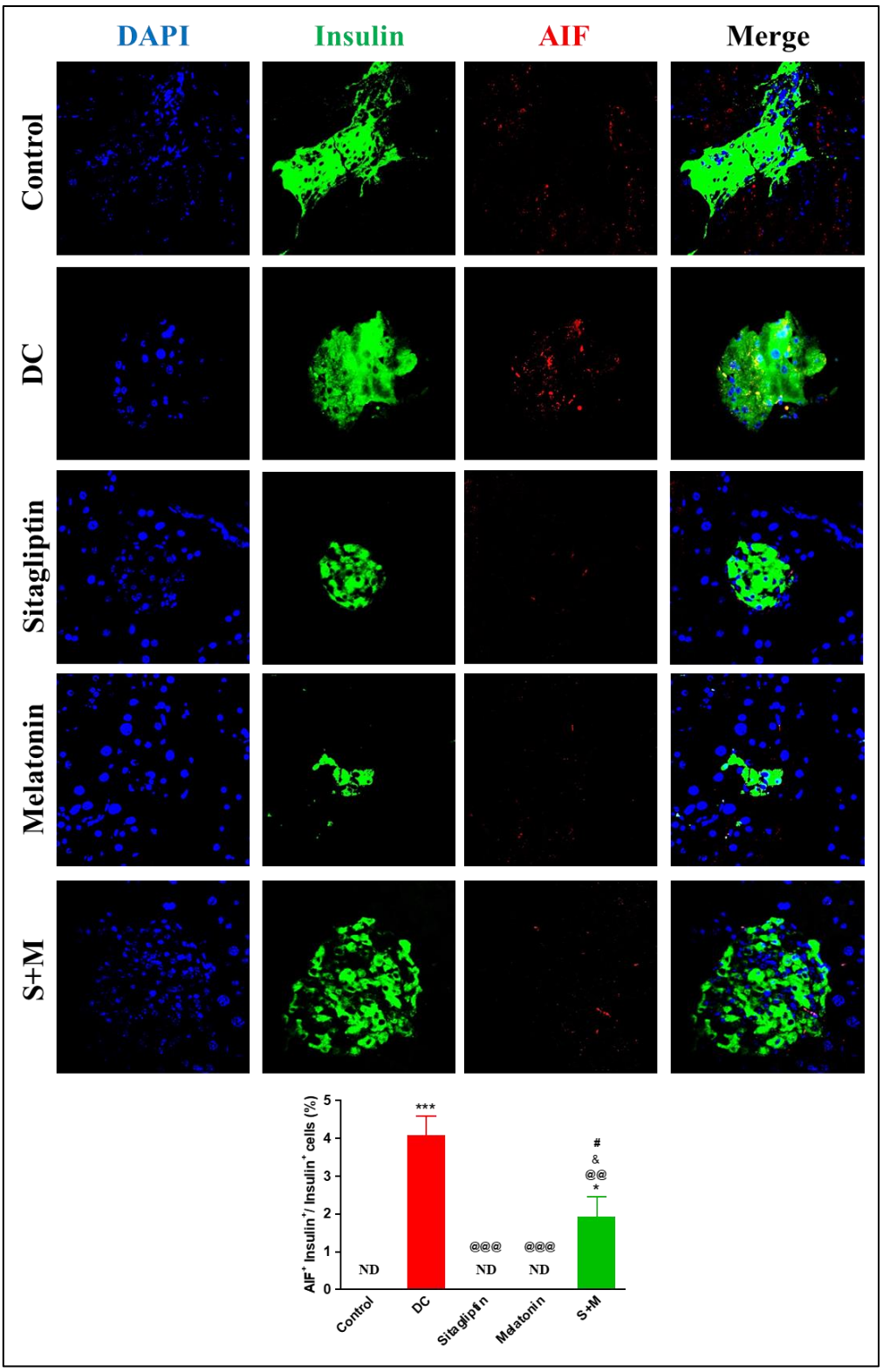

Figure 4.5 Immunohistochemical analysis for pancreatic caspase-independent β-cell apoptosis in young T1D mice as shown by AIF$^+$ insulin$^+$ cells. β-cell apoptosis was observed in DC and S+M groups as shown by AIF$^+$ insulin$^+$ cells, whereas it was not detected in control, sitagliptin and melatonin groups. AIF translocation in the nucleus was significantly reduced in S+M group as compared to DC group. Scale: 50µm, Magnification: 60X. (*p<0.05, ***p<0.001 vs. Control;

$^{@@}p<0.01$, $^{@@@}p<0.001$ vs. Diabetic Control; $^{\&}p<0.05$ vs. Sitagliptin; $^{\#}p<0.05$ vs. Melatonin) (n=3/group).

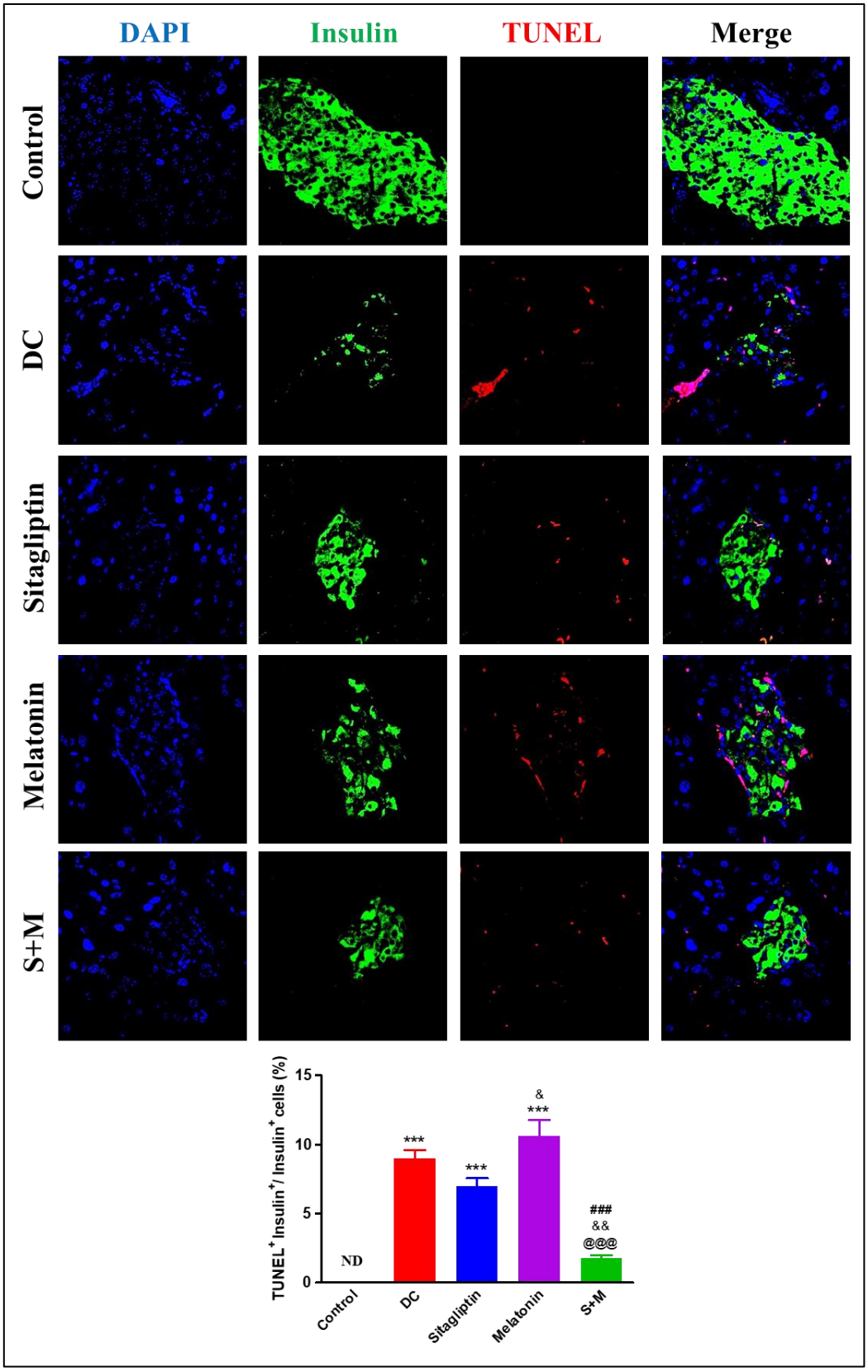

Figure 4.6 Immunohistochemical analysis for pancreatic β-cell apoptosis as shown by TUNEL^{+} insulin^{+} cells in young T1D mice. β-cell apoptosis was significantly increased in DC group as compared to control group while it was significantly reduced in S+M group as compared to DC group. No significant reduction in apoptosis was observed in monotherapy groups as compared to DC group. Scale: 50µm, Magnification: 60X. (***p<0.001 vs. Control; $^{@@@}p$<0.001 vs. Diabetic Control; $^{\&}p$<0.05, $^{\&\&}p$<0.01 vs. Sitagliptin; $^{\#\#\#}p$<0.001 vs. Melatonin) (n=3/group).

4.3.6 Assessment of Body Weight, Fasting Blood Glucose, and Glucose Tolerance in Old T1D Mice

Metabolic phenotyping in old T1D mice suggests that DC group showed significant increase in FBG levels (p<0.001) with a reduction in plasma insulin levels (p<0.01) and glucose tolerance (p<0.001) as compared to control group. The six-week timeline showed reduction in BW in DC group as compared to control group. In addition, the monotherapies and the combination therapy significantly reduced FBG levels (S, M, S+M, p<0.01) by increasing insulin levels (S+M, p<0.01) with a concomitant increase in glucose tolerance (S, p<0.01; S+M, p<0.01) as compared to DC group. Furthermore, final FBG levels in all the drug-treated groups were also significantly reduced as compared to their initial levels (S, p<0.01; S+M, p<0.01), however, sitagliptin and melatonin groups did not show significant increase in plasma insulin levels (p>0.05). Intriguingly, melatonin group neither showed reduction in FBG levels nor an increase in plasma insulin or glucose tolerance as compared to DC group (p>0.05). BW of S+M group was similar to control group while it was reduced in melatonin group similar to DC group in a period of six-weeks (Fig. 4.7A-E).

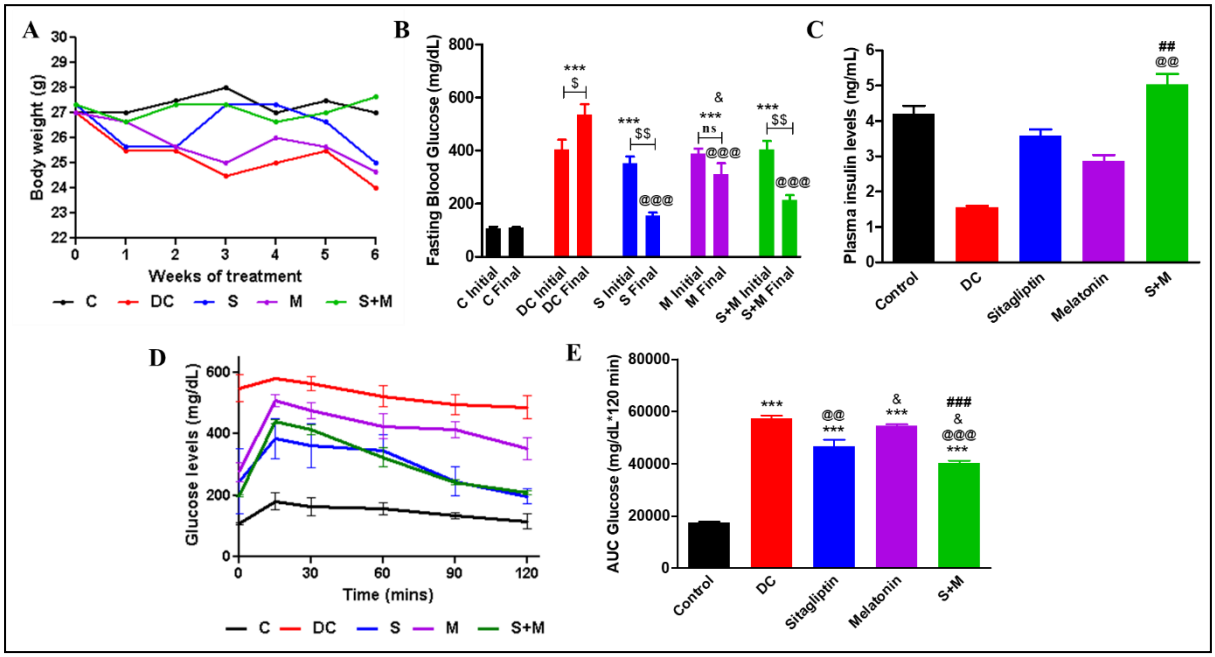

Figure 4.7 Assessment of body weight, blood glucose levels, and glucose tolerance in old mice: A. Body weight. A reduction in BW was observed in DC group as compared to control group while S+M group showed no reduction as compared to DC and monotherapy groups. **B. Fasting blood glucose levels.** FBG levels were significantly increased in DC as compared to control group while a significant decrease was observed in the final S and S+M groups as compared to their respective initial levels and DC. Melatonin group did not show a significant reduction in final FBG levels as compared to their initial levels, but it was significantly reduced as compared to DC group. **C. Plasma insulin levels.** Random plasma insulin levels were significantly reduced in DC group as compared to control group whereas it was significantly increased in S+M group as compared to DC and melatonin groups. **D. Glucose tolerance test**. Decreased glucose clearance was observed in DC group as compared to control group whereas increased glucose clearance was observed in the sitagliptin and S+M groups as compared to DC group at 30-, 60-, 90- and 120-min. **E. Blood glucose AUC0–120.** DC group showed significant reduction in glucose tolerance as compared to control group whereas S and S+M groups showed a significant increase in glucose tolerance as compared to DC. No significant difference was observed in glucose tolerance in melatonin group as compared to DC group (***p<0.001 vs. Control; $^{@@}p$<0.01, $^{@@@}p$<0.001 vs. Diabetic Control; $^{\&}p$<0.05 vs. Sitagliptin; $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. Melatonin) (n=4-6/group).

4.3.7 Assessment of β-Cell Proliferation in Old T1D Mice

IHC analysis revealed that there was no significant change in β-cell proliferation in DC group as compared to control group as shown by $BrdU^+$ $insulin^+$ cells, however, sitagliptin and S+M treated groups showed significant increase in β-cell proliferation (S, S+M, p<0.001) as compared to DC group. Intriguingly, melatonin group did not show a significant increase as compared to DC group (p>0.05) (Fig. 4.8).

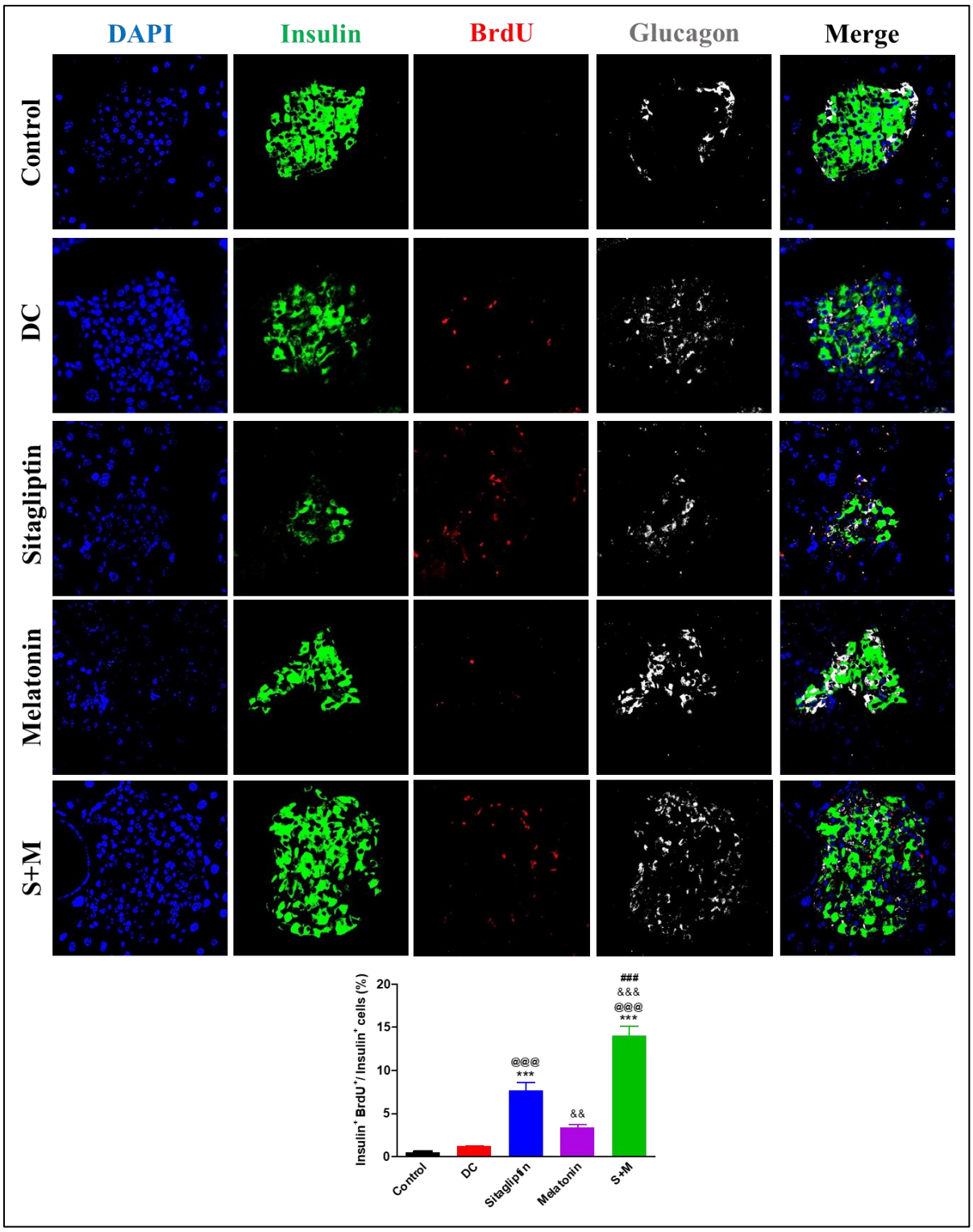

Figure 4.8 Immunohistochemical analysis for pancreatic β-cell proliferation in old T1D mice as shown by BrdU$^+$ insulin$^+$ cells. No significant change in β-cell proliferation was observed in DC group as compared to control group. Sitagliptin and S+M groups showed a significant increase in β-cell proliferation as compared to DC group while there was no significant change in melatonin group as compared to DC group. Scale: 50µm, Magnification: 60X. (***p<0.001 vs. Control; $^{@@@}p$<0.001 vs. Diabetic Control; $^{\&\&}p$<0.01, $^{\&\&\&}p$<0.001 vs. Sitagliptin; $^{\#\#\#}p$<0.001 vs. Melatonin) (n=3/group).

4.3.8 Assessment of β-Cell Neogenesis in Old T1D Mice

IHC analysis revealed that there was no β-cell neogenesis in old T1D mice as NGN3 was found negative. However, $PDX1^+$ $insulin^+$ cells were found in all the drug-treated groups. Interestingly, nucleo-cytoplasmic translocation of PDX1 was observed in sitagliptin and melatonin groups. Nuclear PDX1 was negative in the control and DC groups unlike in young T1D mice (Fig. 4.9).

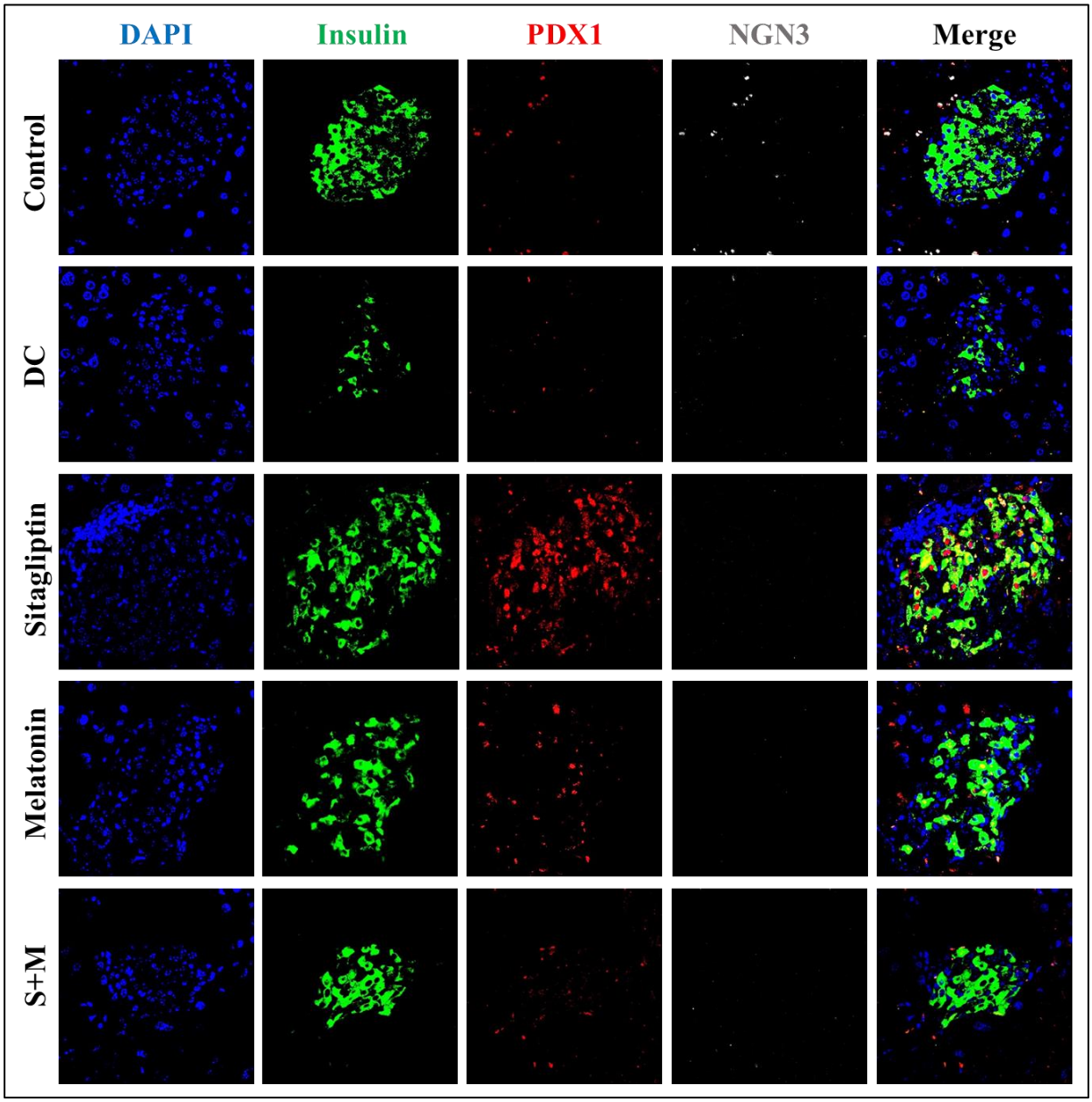

Figure 4.9 Immunohistochemical analysis for pancreatic β-cell neogenesis in old T1D mice as shown by $PDX1^+$ $NGN3^+$ cells. $NGN3^+$ cells were not detected in DC as well as in the drug-treated groups. However, $PDX1^+$ cells were detected in all the drug-treated groups while a nucleo-cytoplasmic translocation of PDX1 was observed in sitagliptin and melatonin groups. Scale: 50µm, Magnification: 60X. (n=3/group).

4.3.9 Assessment of α- to β-Cell Transdifferentiation in Old T1D Mice

IHC analysis revealed that α- to β-cell transdifferentiation was detected in DC, melatonin, and S+M groups as shown by $PAX4^+$ ARX^+ $insulin^+$ cells. It was significantly increased in melatonin and S+M groups as compared to DC group (M, p<001, S+M, p<0.01). (Fig. 4.10).

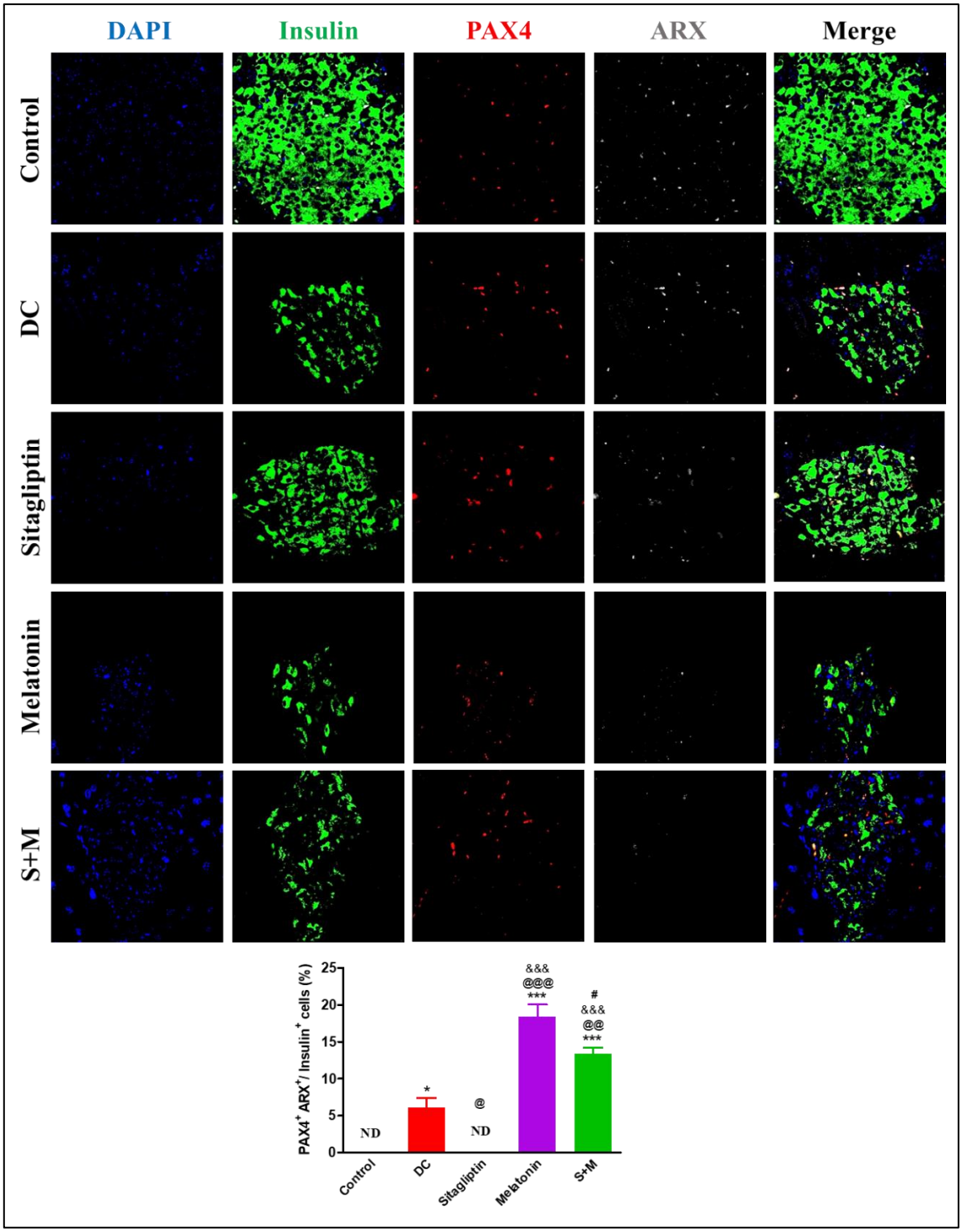

Figure 4.10 Immunohistochemical analysis for pancreatic α- to β-cell transdifferentiation in old T1D mice as shown by PAX^+ ARX^+ $insulin^+$ cells. α- to β-cell transdifferentiation was observed in

DC, melatonin, and S+M groups. It was significantly increased in melatonin and S+M groups as compared to DC group. Scale: 50μm, Magnification: 60X. (*p<0.05, ***p<0.001 vs. Control; $^{@}p$<0.05, $^{@@}p$<0.01, $^{@@@}p$<0.001 vs. Diabetic Control; $^{\&\&\&}p$<0.001 vs. Sitagliptin; $^{\#}p$<0.05 vs. Melatonin) (n=3/ group).

4.3.10 Assessment of β-Cell Apoptosis in Old T1D Mice

IHC analysis revealed that caspase independent β-cell apoptosis was not observed in any of the groups as shown by negative AIF translocation into the nucleus of insulin^{+} cells (Fig. 4.11).

Furthermore, β-cell apoptosis as shown by TUNEL^{+} insulin^{+} cells was detected in all the groups. However, a significant increase in TUNEL^{+} cells were observed in melatonin group as compared to DC group (p<0.001) while it was significantly reduced in S+M group as compared to melatonin group (p<0.001). (Fig. 4.12).

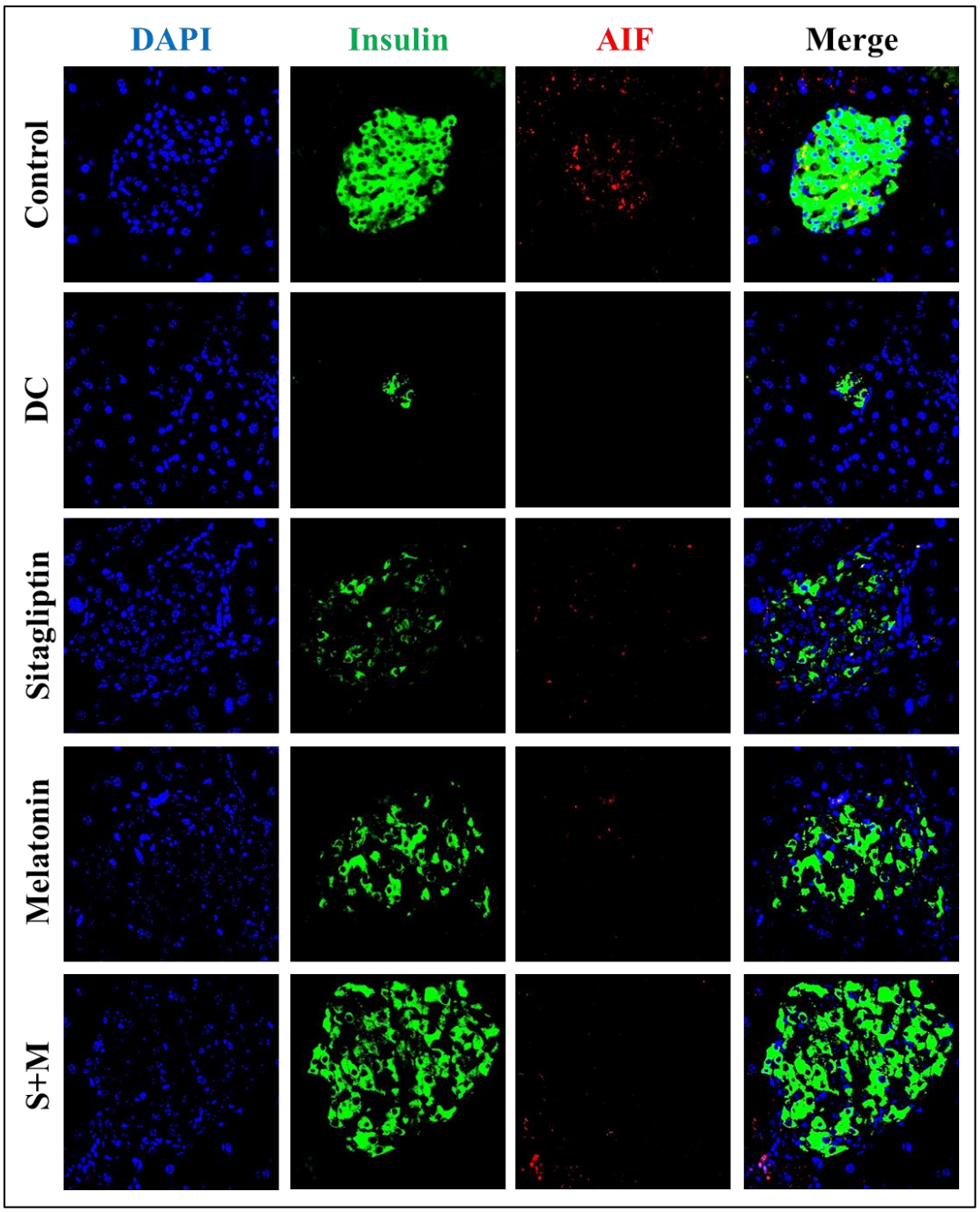

Figure 4.11 Immunohistochemical analysis for pancreatic caspase-independent β-cell apoptosis in old mice as shown by AIF$^+$ insulin$^+$ cells. None of the groups showed AIF translocation to the nucleus in insulin$^+$ cells. Scale: 50μm, Magnification: 60X. (n=3/group).

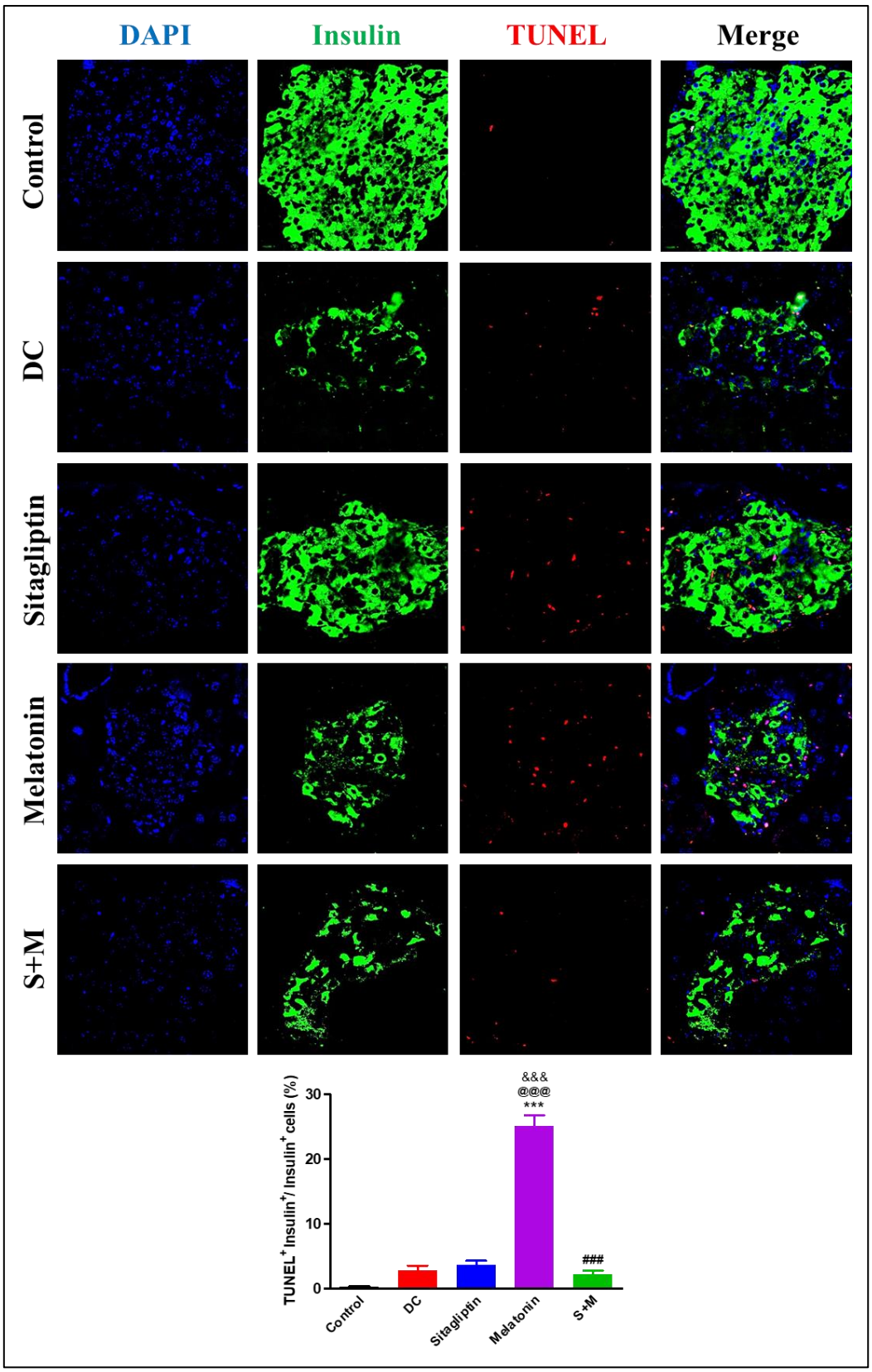

Figure 4.12 Immunohistochemical analysis for pancreatic β-cell apoptosis as shown by TUNEL$^+$ insulin$^+$ cells in old T1D mice. β-cell apoptosis was significantly increased in melatonin group as compared to DC group while it was significantly reduced in S+M group as compared to DC group.

Scale: 50μm, Magnification: 60X. (***p<0.001 vs. Control; $^{@@@}p$<0.001 vs. Diabetic Control; $^{\&\&\&}p$<0.001 vs. Sitagliptin; $^{\#\#\#}p$<0.001 vs. Melatonin) (n=3/group).

4.4 Discussion

T1D is a chronic metabolic disorder characterized by β-cell apoptosis mainly due to autoimmunity and oxidative stress. There is no potential drug therapy other than insulin replacement therapy to achieve glycemic control in T1D. Hence, the present study investigated the therapeutic potential of sitagliptin and melatonin on β-cell regeneration in STZ-induced T1D young and old mouse models. We found greater therapeutic effects of sitagliptin and melatonin combination in inducing β-cell regeneration and reducing β-cell apoptosis, increasing insulin levels, lowering FBG, and improving impaired glucose tolerance. Our findings show $BrdU^+$, $PDX1^+$, $PAX4^+$ β-cells, and reduced $TUNEL^+$ and AIF^+ β-cells in both monotherapy and combination therapy which revealed increased β-cell mass and reduced β-cell apoptosis.

Melatonin is a neurohormone mainly secreted from the pineal gland having anti-inflammatory, anti-apoptotic and anti-oxidative properties. Melatonin exerts its effects in the pancreas via two receptors, melatonin receptor 1A (MTNR1A) and melatonin receptor 1B (MTNR1B) (Nagorny et al., 2011). Melatonin has both inhibitory and stimulatory effects on insulin secretion. In a rodent cell line, it was established that melatonin reduces insulin secretion by activating Gi-coupled MTNRs in β-cells, further reducing PKA and cAMP levels (Peschke et al., 2000). It has also been reported to reduce intracellular Ca^{2+} levels in β-cells (Stumpf et al., 2009; Stumpf et al., 2008). However, by binding to Gq coupled MT2 receptors, melatonin stimulates the activity of PLC and IP3, leading to insulin secretion (Brydon et al., 1999). In addition, administration of melatonin *in vitro* protects β-cells from glucotoxicity, increases its survival and reduces oxidative stress in both human islets from T2D patients and rodent INS-1832/13 β-cells (Park et al., 2014; Costes et al., 2015). Melatonin showed protective role against STZ induced DNA damage and poly (ADP-ribose) polymerase (PARP) activation (Andersson and Sandler, 2001). Further, melatonin administration (10 mg/kg BW) to STZ induced rat T1D model partially restored β-cell mass (Kanter et al., 2006). Insulin also boosts melatonin secretion, insulin and melatonin are inversely related (Lynch et al., 1973). Studies on diabetic rodent models support the functional relationship between melatonin, insulin, and glycemic control (Peschke, 2008). Champney et al., (1983) reported that chemically induced diabetes abolishes melatonin secretion at nighttime apart from causing β-cell apoptosis. Exogenous treatment with

melatonin in pinealectomized rats improves glucose tolerance (Diaz and Blazquez, 1986). Moreover, melatonin administration (10 mg/100 ml) in drinking water normalized blood glucose levels in STZ induced rat T1D model (Hidayat et al., 2015). Yavuz et al., (2003) also reported that administration of melatonin (200 μg/kg i.p.) before diabetes induction could decrease oxidative stress and preserve β-cell integrity without affecting hyperglycemia. Later, it was reported that melatonin (10 mg/kg i.p.) administered for 6 and 8 weeks showed increased insulin secretion, β-cell proliferation (Kanter et al., 2006), reduced apoptosis and neogenesis from the ductal epithelium and centroacinar cells (Simsek et al., 2012). In human pancreatic islets, melatonin treatment increases insulin secretion and promotes β-cell survival via decreased c-JUN N-terminal kinase (JNK) activation (Chan et al., 2002). Lin et al., (2009) have reported that melatonin supresses autoimmune recurrence in NOD mice by inhibiting proliferation of Th1 cells, and melatonin signalling promotes β-cell survival function (Costes et al., 2015). It is known that with ageing, there is a decline in β-cell regenerative capacity and melatonin secretion. Our results on the old T1D mouse model suggest that exogenous treatment showed β-cell regeneration and reduced apoptosis without lowering hyperglycemia or improving glucose tolerance, despite increased plasma insulin levels. However, melatonin combined with sitagliptin showed similar results for β-cell regeneration in both young and old T1D mouse models. We assume that the dose administered, i.e., 0.5 mg/kg BW was insufficient to lower hyperglycemia, reduce apoptosis and induce proliferation in aged mice, unlike young mice. To our knowledge, there are no studies on the effect of melatonin in the T1D old mouse model. Thus, our findings support previous reports on the effect of melatonin on β-cell regeneration and insulin secretion.

Sitagliptin, a DPP-IV inhibitor, is an approved drug for T2D therapy. It inhibits more than 80% of its activity. However, it is a secondary line therapy and usually used in combination with metformin or other anti-diabetic drugs. Its action is to increase incretin hormones by 2 to 3- fold (Tian and Jin, 2016). It is shown to effectively reduce HbA1c and postprandial glucose levels in mono- and combination therapy. It stimulates insulin secretion from the pancreas (van Genugten et al., 2012). Several pre-clinical and clinical studies have been carried out in T1D and T2D models to assess its role in increasing HbA1c levels, β-cell mass, and lipid profile. The islets of sitagliptin treated animals showed increased insulin secretion than sulfonylureas-treated animals (Mu et al., 2006). The effect of DPP-IV inhibitors on β-cells is mediated by GLP-1 (Matveyenko et al., 2009). Further, GLP-1 treatment on INS-1 cells showed reduced STZ-induced apoptosis. *In vivo* studies suggest increased GLP-1

concentrations and improved β-cell mass and function, glucose-stimulated insulin secretion (GSIS), and islet neogenesis upon DPP-IV inhibitor administration in STZ induced rat T1D model (Pospisilik et al., 2003; Morita et al., 2016). Previous studies have shown that GLP-1 increases β-cell proliferation and reduces apoptosis by binding to its receptor (De Leon et al., 2003) via activation of P13K/Akt and CREB-IRS2 signalling pathways (Wang and Brubaker, 2002; Wang et al., 2004; Whalley et al., 2011). However, there is currently no clear evidence that incretin-based therapies can increase β-cell mass in humans. This discrepancy might be due to several differences between humans and rodents, including intrinsic species differences, differences in β-cell turnover, different responses to regenerative stimuli, and differences in the amount of drug administered and the duration of treatment (Levine et al., 2008; Parnaud at el., 2008). The effect of DPP-IV inhibitors on β-cell mass is yet to be evaluated in clinical studies. However, the effect of sitagliptin was evaluated in T1D patients who underwent HSCs transplantation, and in NOD mice with islet graft survival. Both these studies showed increased C-peptide levels, and the T1D patients were also not in need of insulin therapy for six months, confirming sitagliptins immunoregulatory role in T1D. However, there was no beneficial effect on hyperglycemic state (Couri et al., 2009; Kim et al., 2009). One of the DPP-IV inhibitors, Linagliptin, exerts a protective effect on β-cell turnover and function under diabetic conditions (gluco-, lipo-, and cytokine toxicity) via an anti-inflammatory/antioxidant pathway and the stabilization of GLP-1 (Shah et al., 2013).

β-cell development is dependent on a number of transcription factors (Gittes, 2009). The most important is PDX1 which is a pancreatic progenitor marker (Ohlsson et al., 1991). Following PDX1 expression, NGN3, an endocrine progenitor marker is expressed (Schwitzgebel et al, 2000). Amongst several transcription factors which differentiate endocrine precursor cells into β-cells and α-cells, PAX4 and ARX lie downstream of NGN3 differentiate endocrine precursor cells into β-cells and α-cells, respectively (Liew et al., 2008). Our results suggest that β-cell neogenesis is absent in all the drug-treated groups as the β-cells were $NGN3^-$ in both young and old T1D mice. However, PDX1 was observed in all groups in young mice whereas PDX1 was found only in drug-treated old mice. Moreover, a nucleo-cytoplasmic translocation of PDX1 was observed in DC and monotherapy groups in young T1D mice, and only in monotherapy groups in old mice but was absent in combination therapy group. Kawamori et al., (2003) reported the nucleo-cytoplasmic translocation of PDX1, which was induced by oxidative stress via activation of JNK. This implies that oxidative stress was significantly reduced in combination therapy group as compared to other

groups. Moreover, β-cell function and identity is also maintained by PDX1 (Gao et al., 2014). It was also reported that NGN3 did not co-localize with insulin as its expression was quenched upon differentiating into insulin expressed β-cell (Rukstalis and Habener, 2009). Further, $PAX4^+$ ARX^+ co-positive cells were observed in all drug-treated groups in young T1D mice, whereas only in melatonin and combination therapy groups in old T1D mice, indicating the presence of α- to β-cell transdifferentiation in these groups. Moreover, PAX4 also defines proliferating β-cell population (Brun et al., 2004; Lorenzo et al., 2015) which was clearly increased in all drug-treated groups. To our knowledge, there are no reports on α- to β-cell transdifferentiation upon exogenous treatment of sitagliptin or melatonin treatment in T1D mice. However, lineage tracing experiments are required to substantiate our results on β-cell transdifferentiation. Furthermore, our results suggest that β-cell proliferation was significantly increased in combination therapy group in young T1D mice, and in sitagliptin and combination therapy group in old T1D mice, whereas apoptosis was significantly reduced only in combination therapy group in young as well as old T1D mice.

Overall, our findings reinforce that sitagliptin and melatonin combination therapy has greater therapeutic benefits as compared to monotherapies in reducing hyperglycemia, improving glucose tolerance, and stimulating β-cell regeneration in the experimental T1D mouse model (Fig. 4.13). However, further studies are warranted to understand mechanisms underlying the action of sitagliptin and melatonin. Further, to explore the therapeutic potential of melatonin in combination with sitagliptin in clinical studies, it is essential to consider melatonin dosage (acute or chronic) and the relative timing of its intake with respect to glycemic challenge.

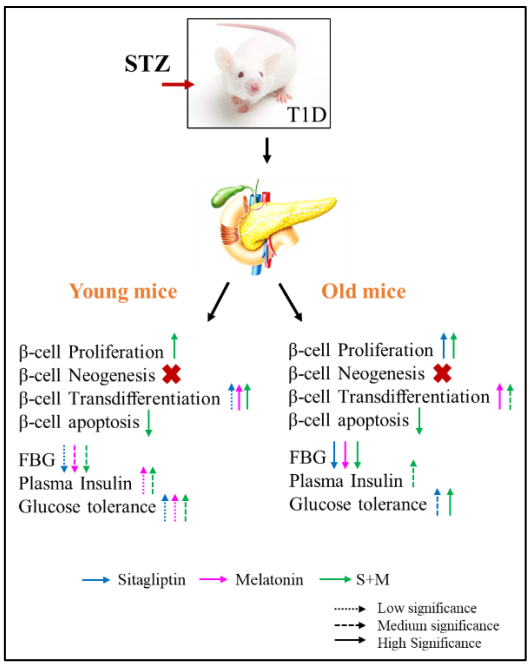

Figure 4.13 Effect of sitagliptin, melatonin and combination therapy on T1D manifestations in Streptozotocin (STZ)-induced T1D young and old mouse models. Combination therapy showed greater therapeutic benefits than monotherapies on β-cell regeneration and achieving glucose homeostasis in T1D young and old mouse models.

4.5 References

Andersson AK, Sandler S. Melatonin protects against streptozotocin, but not interleukin-1β-induced damage of rodent pancreatic β-cells. Journal of pineal research. 2001;30(3):157-65.

Brun T, Franklin I, St-Onge L, Biason-Lauber A, Schoenle EJ, Wollheim CB, Gauthier BR. The diabetes-linked transcription factor PAX4 promotes β-cell proliferation and survival in rat and human islets. The Journal of cell biology. 2004;167(6):1123-35.

Brydon L, Roka F, Petit L, de Coppet P, Tissot M, Barrett P, Morgan PJ, Nanoff C, Strosberg AD, Jockers R. Dual signaling of human Mel1a melatonin receptors via Gi2, Gi3, and Gq/11 proteins. Molecular Endocrinology. 1999;13(12):2025-38.

Champney TH, Brainard GC, Richardson BA, Reiter RJ. Experimentally-induced diabetes reduces nocturnal pineal melatonin content in the Syrian hamster. Comparative biochemistry and physiology. A, Comparative physiology. 1983;76(1):199-201.

Chan AS, Lai FP, Lo RK, Voyno-Yasenetskaya TA, Stanbridge EJ, Wong YH. Melatonin mt1 and MT2 receptors stimulate c-Jun N-terminal kinase via pertussis toxin-sensitive and-insensitive G proteins. Cellular signalling. 2002;14(3):249-57.

Costes S, Boss M, Thomas AP, Matveyenko AV. Activation of melatonin signaling promotes β-cell survival and function. Molecular endocrinology. 2015;29(5):682-92.

Couri CE, Oliveira MC, Stracieri AB, Moraes DA, Pieroni F, Barros GM, Madeira MI, Malmegrim KC, Foss-Freitas MC, Simoes BP, Martinez EZ. C-peptide levels and insulin independence following autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. Jama. 2009;301(15):1573-9.

De Leon DD, Deng S, Madani R, Ahima RS, Drucker DJ, Stoffers DA. Role of endogenous glucagon-like peptide-1 in islet regeneration after partial pancreatectomy. Diabetes. 2003;52(2):365-71.

Diaz B, Blazquez E. Effect of pinealectomy on plasma glucose, insulin and glucagon levels in the rat. Hormone and metabolic research. 1986;18(04):225-9.

Gao T, McKenna B, Li C, Reichert M, Nguyen J, Singh T, Yang C, Pannikar A, Doliba N, Zhang T, Stoffers DA. Pdx1 maintains β cell identity and function by repressing an α cell program. Cell metabolism. 2014;19(2):259-71.

Gilbert MP, Pratley RE. GLP-1 analogs and DPP-4 inhibitors in type 2 diabetes therapy: review of head-to-head clinical trials. Frontiers in endocrinology. 2020;11:178.

Gittes GK. Developmental biology of the pancreas: a comprehensive review. Developmental biology. 2009;326(1):4-35.

Hardeland R. Aging, melatonin, and the pro-and anti-inflammatory networks. International journal of molecular sciences. 2019;20(5):1223.

Hidayat M, Mahar Y, Wasim B. Effect of melatonin on serum glucose and body weights in streptozotocin induced diabetes in albino rats. Journal of Ayub Medical College Abbottabad. 2015;27(2):274-6.

Kanter M, Uysal H, Karaca T, Sagmanligil HO. Depression of glucose levels and partial restoration of pancreatic β-cell damage by melatonin in streptozotocin-induced diabetic rats. Archives of toxicology. 2006;80(6):362-9.

Kawamori D, Kajimoto Y, Kaneto H, Umayahara Y, Fujitani Y, Miyatsuka T, Watada H, Leibiger IB, Yamasaki Y, Hori M. Oxidative stress induces nucleo-cytoplasmic translocation of pancreatic transcription factor PDX-1 through activation of c-Jun NH2-terminal kinase. Diabetes. 2003;52(12):2896-904.

Kim SJ, Nian C, Doudet DJ, McIntosh CH. Dipeptidyl peptidase IV inhibition with MK0431 improves islet graft survival in diabetic NOD mice partially via T-cell modulation. Diabetes. 2009;58(3):641-51.

Kor Y, Geyikli I, Keskin M, Akan M. Preliminary study: Evaluation of melatonin secretion in children and adolescents with type 1 diabetes mellitus. Indian journal of endocrinology and metabolism. 2014;18(4):565.

Levine F, Itkin-Ansari P. β-cell regeneration: Neogenesis, replication or both?. Journal of Molecular Medicine. 2008;86(3):247-58.
Liew CG, Shah NN, Briston SJ, Shepherd RM, Khoo CP, Dunne MJ, Moore HD, Cosgrove KE, Andrews PW. PAX4 enhances beta-cell differentiation of human embryonic stem cells. PLoS One. 2008;3(3):e1783.

Lin GJ, Huang SH, Chen YW, Hueng DY, Chien MW, Chia WT, Chang DM, Sytwu HK. Melatonin prolongs islet graft survival in diabetic NOD mice. Journal of pineal research. 2009;47(3):284-92.

Lorenzo PI, Fuente-Martín E, Brun T, Cobo-Vuilleumier N, Jimenez-Moreno CM, Gomez IG, Noriega LL, Mellado-Gil JM, Martin-Montalvo A, Soria B, Gauthier BR. PAX4 defines

an expandable β-cell subpopulation in the adult pancreatic islet. Scientific reports. 2015;5(1):1-4.

Lynch HJ, Eng JP, Wurtman RJ. Control of pineal indole biosynthesis by changes in sympathetic tone caused by factors other than environmental lighting. Proceedings of the National Academy of Sciences. 1973;70(6):1704-7.

Marra G, Cotroneo P, Pitocco D, Manto A, Di Leo MA, Ruotolo V, et al. Early increase of oxidative stress and reduced antioxidant defenses in patients with uncomplicated type 1 diabetes: a case for gender difference. Diabetes Care. 2002;25(2):370-375.

Matveyenko AV, Dry S, Cox HI, Moshtaghian A, Gurlo T, Galasso R, Butler AE, Butler PC. Beneficial endocrine but adverse exocrine effects of sitagliptin in the human islet amyloid polypeptide transgenic rat model of type 2 diabetes: interactions with metformin. Diabetes. 2009;58(7):1604-15.

Mok JX, Ooi JH, Ng KY, Koh RY, Chye SM. A new prospective on the role of melatonin in diabetes and its complications. Hormone molecular biology and clinical investigation. 2019;40(1).

Morita A, Mukai E, Hiratsuka A, Takatani T, Iwanaga T, Lee EY, Miki T. Distinct effects of dipeptidyl peptidase-4 inhibitor and glucagon-like peptide-1 receptor agonist on islet morphology and function. Endocrine. 2016;51(3):429-39.

Mu J, Woods J, Zhou YP, Roy RS, Li Z, Zycband E, Feng Y, Zhu L, Li C, Howard AD, Moller DE. Chronic inhibition of dipeptidyl peptidase-4 with a sitagliptin analog preserves pancreatic β-cell mass and function in a rodent model of type 2 diabetes. Diabetes. 2006;55(6):1695-704.

Nagorny CL, Sathanoori R, Voss U, Mulder H, Wierup N. Distribution of melatonin receptors in murine pancreatic islets. Journal of pineal research. 2011;50(4):412-7.

Ohlsson H, Thor S, Edlund T. Novel insulin promoter-and enhancer-binding proteins that discriminate between pancreatic α-and β-cells. Molecular Endocrinology. 1991;5(7):897-904.

Owino S, Buonfiglio DD, Tchio C, Tosini G. Melatonin signaling a key regulator of glucose homeostasis and energy metabolism. Frontiers in endocrinology. 2019;10:488.

Park JH, Shim HM, Na AY, Bae KC, Bae JH, Im SS, Cho HC, Song DK. Melatonin prevents pancreatic β-cell loss due to glucotoxicity: the relationship between oxidative stress and endoplasmic reticulum stress. Journal of pineal research. 2014;56(2):143-53.

Parnaud G, Bosco D, Berney T, Pattou F, Kerr-Conte J, Donath MY, Bruun C, Mandrup-Poulsen T, Billestrup N, Halban PA. Proliferation of sorted human and rat beta cells. Diabetologia. 2008;51(1):91-100.

Patel R, Rathwa N, Palit SP, Ramachandran AV, Begum R. Association of melatonin & MTNR1B variants with type 2 diabetes in Gujarat population. Biomedicine & Pharmacotherapy. 2018;103:429-34.

Peschke E, Fauteck JD, Mußhoff U, Schmidt F, Beckmann A, Peschke D. Evidence for a melatonin receptor within pancreatic islets of neonate rats: functional, autoradiographic, and molecular investigations. Journal of pineal research. 2000;28(3):156-64.

Peschke E, Hofmann K, Bähr I, Streck S, Albrecht E, Wedekind D, et al. The insulin-melatonin antagonism: studies in the LEW.1AR1-iddm rat (an animal model of human type 1 diabetes mellitus). Diabetologia. 2011;54:1831–40.

Peschke E. Melatonin, endocrine pancreas and diabetes. Journal of pineal research. 2008;44(1):26-40.

Peschke E, Wolgast S, Bazwinsky I, Pönicke K, Muhlbauer E. Increased melatonin synthesis in pineal glands of rats in streptozotocin induced type 1 diabetes. Journal of pineal research. 2008;45(4):439-48.

Pospisilik JA, Martin J, Doty T, Ehses JA, Pamir N, Lynn FC, Piteau S, Demuth HU, McIntosh CH, Pederson RA. Dipeptidyl peptidase IV inhibitor treatment stimulates β-cell survival and islet neogenesis in streptozotocin-induced diabetic rats. Diabetes. 2003;52(3):741-50.

Reiter RJ, Mayo JC, Tan DX, Sainz RM, Alatorre-Jimenez M, Qin L. Melatonin as an antioxidant: under promises but over delivers. Journal of pineal research. 2016;61(3):253-78.

Ricordi C, Rastellini C. Methods in pancreatic islet separation. Methods in Cell Transplantation. 2000:433-8.

Ricordi C, Strom TB. Clinical islet transplantation: advances and immunological challenges. Nature Reviews Immunology. 2004;4(4):259-68.

Rukstalis JM, Habener JF. Neurogenin3: a master regulator of pancreatic islet differentiation and regeneration. Islets. 2009;1(3):177-84.

Schwitzgebel VM, Scheel DW, Conners JR, Kalamaras J, Lee JE, Anderson DJ, Sussel L, Johnson JD, German MS. Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. Development. 2000;127(16):3533-42.

Shah P, Ardestani A, Dharmadhikari G, Laue S, Schumann DM, Kerr-Conte J, Pattou F, Klein T, Maedler K. The DPP-4 inhibitor linagliptin restores β-cell function and survival in human isolated islets through GLP-1 stabilization. The Journal of Clinical Endocrinology & Metabolism. 2013;98(7):E1163-72.

Shapiro AJ. Islet transplantation in type 1 diabetes: ongoing challenges, refined procedures, and long-term outcome. The review of diabetic studies: RDS. 2012;9(4):385.

Sharples EJ, Mittal SM, Friend PJ. Challenges in pancreas transplantation. Acta diabetologica. 2016;53(6):871-8.

Simsek N, Kaya M, Kara A, Can I, Karadeniz A, Kalkan YI. Effects of melatonin on islet neogenesis and beta cell apoptosis in streptozotocin-induced diabetic rats: an immunohistochemical study. Domestic animal endocrinology. 2012;43(1):47-57.

Stumpf I, Bazwinsky I, Peschke E. Modulation of the cGMP signaling pathway by melatonin in pancreatic β-cells. Journal of pineal research. 2009;46(2):140-7.

Stumpf I, Mühlbauer E, Peschke E. Involvement of the cGMP pathway in mediating the insulin-inhibitory effect of melatonin in pancreatic β-cells. Journal of pineal research. 2008;45(3):318-27.

Tian L, Jin T. The incretin hormone GLP-1 and mechanisms underlying its secretion. Journal of diabetes. 2016;8:753–765.

Tschen SI, Dhawan S, Gurlo T, Bhushan A. Age-dependent decline in β-cell proliferation restricts the capacity of β-cell regeneration in mice. Diabetes 2009;58(6):1312-20.

Van Genugten RE, Van Raalte DH, Diamant M. Dipeptidyl peptidase-4 inhibitors and preservation of pancreatic islet-cell function: a critical appraisal of the evidence. Diabetes, Obesity and Metabolism. 2012;14(2):101-11.

Wang Q, Brubaker P. Glucagon-like peptide-1 treatment delays the onset of diabetes in 8 week-old db/db mice. Diabetologia. 2002;45(9):1263-73.

Wang Q, Li L, Xu E, Wong V, Rhodes C, Brubaker PL. Glucagon-like peptide-1 regulates proliferation and apoptosis via activation of protein kinase B in pancreatic INS-1 beta cells. Diabetologia. 2004;47(3):478-87.
Whalley NM, Pritchard LE, Smith DM, White A. Processing of proglucagon to GLP-1 in pancreatic a-cells: is this a paracrine mechanism enabling GLP-1 to act on b-cells. Journal of Endocrinology. 2011;211:99-106.

Yavuz O, Cam M, Bukan N, Guven A, Silan F. Protective effect of melatonin on β-cell damage in streptozotocin-induced diabetes in rats. Acta Histochemica. 2003;105(3):261-6.