Chapter 2

To assess the efficacy of melatonin, GABA and combination therapy in amelioration of diabetic manifestations in streptozotocin induced T1D mouse model

2.1 Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by progressive loss of functional β-cell mass resulting from insulitis, leading to insulin deficiency. Therefore, it is essential to develop therapies that prevent or even reverse the deterioration of β-cell function. Islet transplantation has been an ultimate treatment for subjects with severe T1D, but its application is limited due to the lack of donors and the need for intense immunosuppression (Sharples et al., 2016). Thus, it is pertinent to develop novel therapeutic approaches that can increase the survival rate and proliferation of endogenous pancreatic β-cells and immunosuppressive effects. However, none of the current therapies effectively achieve both the goals (Atkinson et al., 2014). To achieve long-term glycemic control, combination therapy is recommended due to the limitations of monotherapies and complications related to diabetes and the patient's lifestyle (Jeon et al., 2018).

Melatonin, a pineal hormone, has a role in circadian rhythm regulation, and it acts as an antioxidant (Galano et al., 2011), anti-inflammatory and anti-apoptotic agent (Chahbouni et al., 2010); additionally it is functionally linked to the glucose metabolism (Mulder et al., 2009). It has been reported that the levels of melatonin decrease with age and that melatonin and insulin have an antagonistic relationship (Peschke et al., 2011). The production of melatonin increases in individuals with T1D, as observed in both STZ-treated Wistar rats and T1D patients (Peschke et al., 2008; Kor et al., 2014). The loss of β-cells reduces insulin production, leading to hyperglycemia in T1D patients. Additionally, T1D patients exhibit lower antioxidant levels and overproduction production of free radicals (Mok et al., 2019; Marra et al., 2002). Nevertheless, studies have shown that melatonin can protect against STZ-induced T1D and promote β-cell growth (Andersson and Sandler, 2001; Kanter et al., 2006). Moreover, our recent study suggest that melatonin in combination with sitagliptin (a DPP-IV inhibitor) can regenerate pancreatic β-cells in rodent and human islets (Patel et al., 2022).

Gamma (γ) - aminobutyric acid (GABA), initially identified as an inhibitory neurotransmitter and produced by pancreatic β-cells in large quantities, has emerged as a new anti-diabetic dietary supplement (Adeghate and Ponery, 2002; Ramos-Ruiz et al., 2018). GABA has astonishing physiological effects in diabetes mellitus (DM) due to its anti-diabetic, antioxidant, anti-inflammatory, and immunomodulatory properties (Ramos-Ruiz et al., 2018; Rashmi et al., 2018). In an islet, GABA acts via $GABA_AR$ on the β-cells, enhancing insulin secretion through

membrane depolarization (Rorsman et al., 1989). Therefore, GABA is vital in regulating islet cell function and glucose homeostasis. GABA stimulates β-cell replication, protects β-cells against apoptosis, attenuates insulitis, regulates islet-cell function and glucose homeostasis, and suppresses detrimental immune reactions (Liu et al., 2017; Tian et al., 2004; Tian et al., 2011; Purwana et al., 2014). Moreover, our recent study has suggested that GABA in combination with CR (Calorie restriction) diet can regenerate pancreatic β-cells in diabetic mice (Rathwa et al., 2022).

Thus, we aimed to investigate the therapeutic potential of melatonin in combination with GABA on diabetic manifestations in the STZ-induced T1D mouse model.

2.2 Materials and methods

2.2.1 Animals:

Forty male BALB/c mice (7-8 weeks old), 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. These animals had free access to a standard chow diet (Keval Sales Corporation, Vadodara, India) and water. All the experimental procedures were conducted as per the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and were approved by Institutional Animal Ethical Committee (IAEC) (MSU/BC/02/2019).

2.2.2 Induction of T1D and treatment:

For T1D induction, 32 BALB/c mice were given five consecutive intraperitoneal (i.p.) injections of 50 mg/kg body weight (BW) STZ (MP Biomedicals, Santa Ana, CA, USA), freshly dissolved in 0.1 M cold sodium citrate buffer (pH 4.5). Eight mice were kept separately as a non-diabetic control group. Diabetes was confirmed two weeks later in mice having fasting blood glucose (FBG) >350mg/dL. FBG was measured in mice after 6 hours of fasting by tail snipping method using a Glucometer (TRUEresult, NIPRO Diagnostics, Pune, MH, India). The diabetic mice were then randomly assigned to four groups: i. Diabetic Control (DC), ii. Melatonin (M) treated, iii. GABA (G) treated, and iv. M+G 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 (Patel et al., 2022). GABA (Sigma–Aldrich, United States) was given at a dose of 18 mg/day (Liu et al., 2017) by oral gavage. DC group received 0.9% saline i.p. and water by oral gavage as a vehicle. The treatment was given for six weeks along with

bromodeoxyuridine (BrdU) (MP Biomedicals, Santa Ana, CA, USA) on alternative days at 100 mg/kg BW, i.p. dose. FBG levels and BW were measured twice a week.

2.2.3 Intraperitoneal glucose tolerance test (IPGTT):

Glucose tolerance was evaluated by IPGTT at the end of treatment. For IPGTT, all mice were fasted for 6 hours and injected with glucose (2 g/kg BW i.p.). Blood glucose levels were measured immediately at 0, 15, 30, 60, 90, and 120 minutes by tail snipping method using a Glucometer. The total area under the curve (AUC) was evaluated.

2.2.4 Plasma Insulin Levels:

1 ml blood was collected into K_3EDTA tubes by the orbital sinus method after 6 hours of fasting before sacrificing the mice. Blood was centrifuged at 6000 g for 5 minutes, and plasma was separated and stored at -20°C for further analysis. Plasma insulin levels were measured by commercially available mouse insulin (RayBiotech, GA, USA) ELISA kits as per the manufacturer's protocols.

2.2.5 Pancreatic tissue preparation and immunohistochemistry-immunofluorescence (IHC-IF) analysis:

The pancreas were harvested and fixed in 10% neutral buffered formalin for histological processing and paraffin embedding. 5µm sections were prepared from the paraffin-embedded blocks. Immunofluorescence staining was carried out to study β-cell proliferation (Insulin/Glucagon/BrdU), β-cell neogenesis (Insulin/NGN3/PDX1), α- to β-cell transdifferentiation (Insulin/ARX/PAX4), β-cell apoptosis by TUNEL assay (Thermo Fisher Scientific, MA, USA) and insulin/apoptosis-inducing factor (AIF) staining. The sections were deparaffinized in xylene and rehydrated in a series of graded ethanol (100%, 95%, 80%, and 70%). Antigen retrieval was performed using 1N HCL for 45 minutes at 37 °C. Sections were blocked in 5% donkey serum in PBST (PBS + 0.1% Tween 20) for 1 hour at room temperature, and antibodies were diluted as indicated in table 2.1. The sections were incubated with primary antibody at 37°C in a humidified chamber for 2 hours. Tissue sections were washed with PBS and incubated with secondary antibodies at room temperature for 45 minutes in the dark. Tissue 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 (Zeiss LSM 780, Oberkochen, Germany) at 63X for β-cell regeneration and apoptosis. Image analysis was carried out in Zeiss Zen software. Results were expressed as the percentage of specified markers for β-cell regeneration and apoptosis (Patel et al., 2022). Observations were made from three pancreatic sections per group from five different areas.

Table 2.1. List of antibodies used for the IHC studies.

Primary Antibody	**Secondary Antibody**	**Excitation (nm)**	**Emission (nm)**
Anti-Insulin (1:200, Guinea Pig) [DAKO Agilent, USA]	Alexa 488 (1:500, Donkey) [Jackson ImmunoResearch Laboratories, Inc. USA]	493	519
Anti-Glucagon (1:200,rabbit) [Cell Signaling Technology, USA]	Alexa 647 (1:500, Donkey) [Jackson ImmunoResearch Laboratories, Inc. USA]	651	667
BrdU (1:100, Rat) [Abcam, USA]	Rhodamine Red (1:200, Donkey) [Jackson Immuno Research Laboratories, Inc. USA]	570	590
NGN3 (1:50, rabbit) [Thermo Fisher Scientific, USA]	Rhodamine Red (1:200, Rat) [Jackson Immuno Research Laboratories, Inc. USA]	570	590
PDX-1 (1:1000, Goat) [Abcam, USA]			
ARX (1:500, Rabbit) [Sigma-Aldrich, Germany]			
PAX-4 (1:500, Goat) [Sigma-Aldrich, Germany]			
AIF (1:100, Rabbit) [Abcam, USA]			

2.2.6 Statistical Analyses:

Statistical data comparisons were performed by one-way analysis of variance (ANOVA) and multiple group comparisons by Tukey's post hoc test in Graphpad Prism 6 (GraphPad Sofware, San Diego, CA, USA). The significance level was set at $p<0.05$. Results were expressed as mean±SEM.

2.3 Results

2.3.1 Assessment of body weight, fasting blood glucose, and glucose tolerance:

Metabolic profiling in mice suggests that the DC group showed a significant increase in FBG levels ($p<0.001$) with a reduction in plasma insulin levels ($p<0.001$) and glucose tolerance ($p<0.001$) as compared to the control group. The monotherapies and the combination therapy

significantly reduced the FBG levels (M, p<0.001; G, p<0.001; M+G, p<0.001) by increasing plasma insulin levels (M, p<0.01; G, p<0.001; M+G, p<0.001) with a consequent increase in glucose tolerance (M, p<0.001; G, p<0.001; M+G, p<0.001) in the drug-treated groups as compared to the DC group. Furthermore, final FBG levels in the drug-treated groups were also significantly reduced as compared to their initial levels (M, p<0.01; G, p<0.001; M+G, p<0.001). However, none of the drug-treated groups showed a significant change in the body weight after the treatment (Figure 2.1A-E).

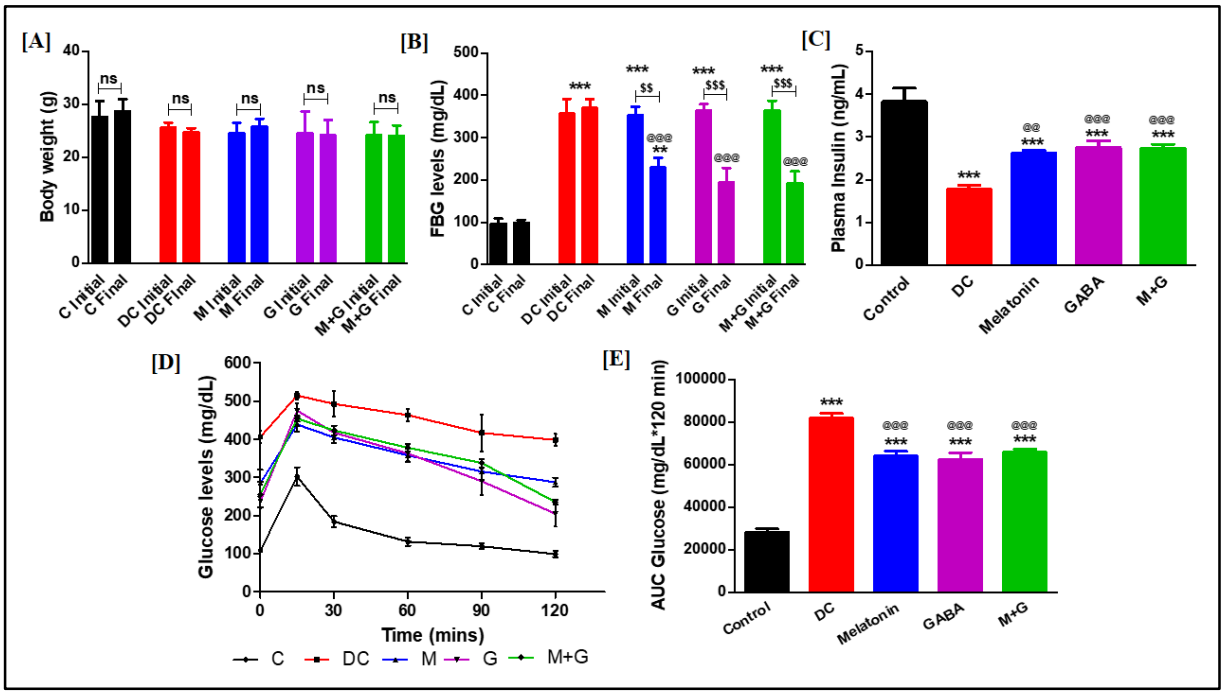

Figure 2.1. Assessment of Body Weight, Fasting Blood Glucose levels, and Glucose Tolerance. [A] Body weight: No significant change in the body weight was observed before and after treatment in all the treated groups. **[B] Fasting blood glucose levels:** A significant increase in FBG levels was observed in the DC group compared to the control group. In contrast, a significant decrease in FBG levels was observed in all the drug-treated groups compared to their initial levels. Also, there was a significant reduction in the FBG levels in the final drug-treated groups compared to the final DC group. **[C] Plasma insulin levels:** A significant decrease in random plasma insulin levels was observed in the DC group compared to the control group. In contrast, the levels were significantly increased in all the drug-treated groups compared to the DC group. **[D] Glucose tolerance test:** A significant decrease in glucose clearance was observed in the DC group compared to the control group at all times. Increased glucose clearance was observed in all the drug-treated groups compared to the DC group at all times. **[E] Blood glucose AUC0–120:** A significant decrease in glucose tolerance was observed in the DC group as compared to the control group. In contrast, all the drug-treated groups showed a significant increase in glucose tolerance compared to DC. (***p<0.001 vs. NCD; $^{@@}p$<0.01, $^{@@@}p$<0.001 vs. HFD; ns, p>0.05) (n=6/8 per group).

2.3.2 Assessment of Pancreatic β-cell regeneration and Apoptosis:

IHC analysis showed no β-cell proliferation or transdifferentiation in DC group as compared to the control group, as shown by $BrdU^+/insulin^+$ cells and $PAX4^+/ARX^+$ $insulin^+$ cells, respectively. In comparison, both proliferation and transdifferentiation were significantly increased in all the drug-treated groups (proliferation: M, $p<0.01$; G, $p<0.01$, M+G, $p<0.001$; transdifferentiation: M, $p<0.01$; G, $p<0.001$, M+G, $p<0.001$). Moreover, a nucleo-cytoplasmic translocation of PAX4 was observed in DC and in all the drug-treated groups. Intriguingly, the analysis revealed no β-cell neogenesis as NGN3 was found to be negative. However, we observed $PDX1^+/insulin^+$ cells in all the groups indicated by the nucleo-cytoplasmic translocation of PDX1.

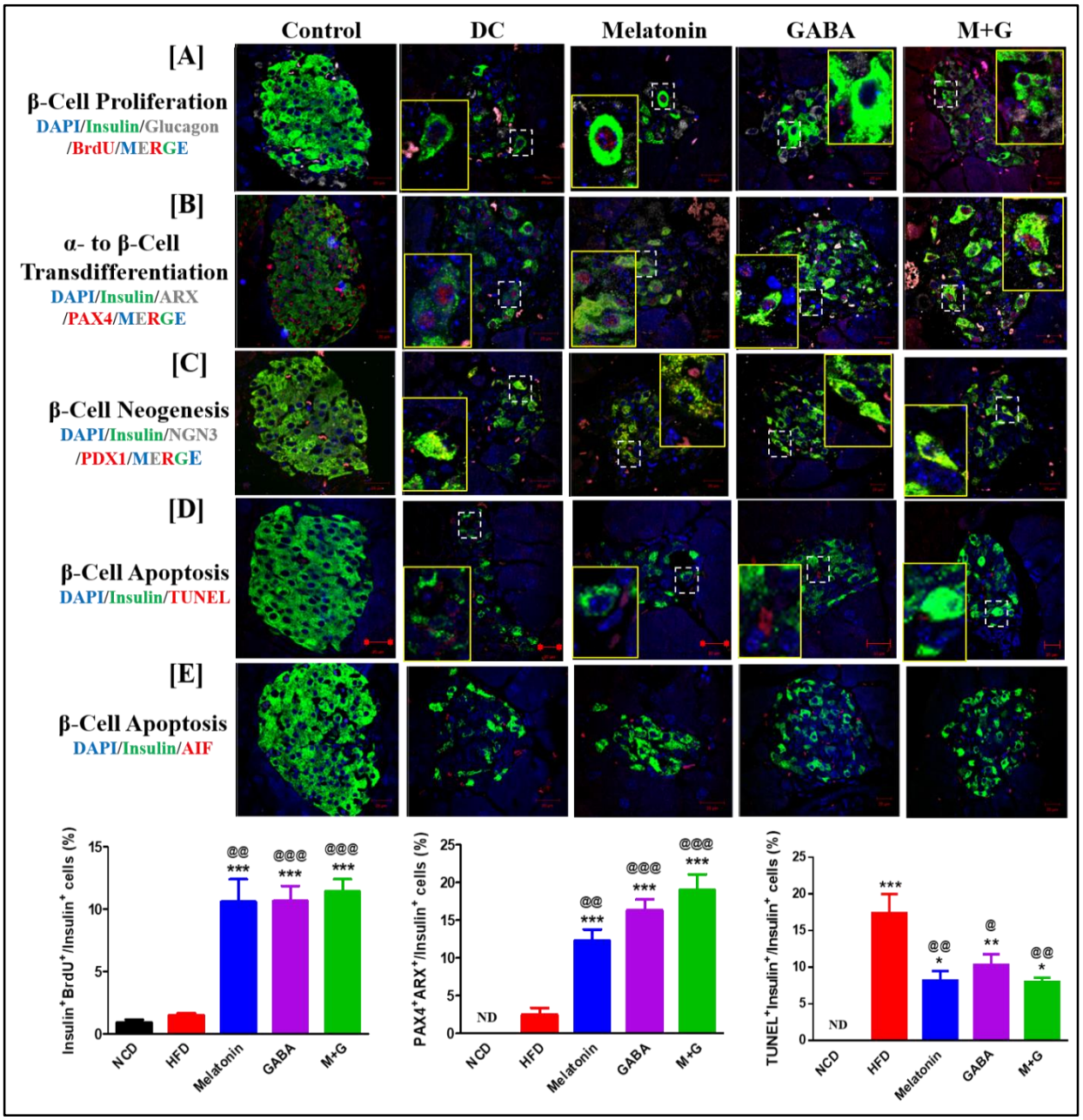

Figure 2.2. Assessment of pancreatic β-cell regeneration and apoptosis. [A] β-cell proliferation: No significant change in β-cell proliferation was observed in the DC group compared to the control group. All treated groups showed a significant increase in β-cell proliferation compared to the DC group. **[B] α- to β-cell transdifferentiation:** 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 compared to the DC group. Moreover, a nucleo-cytoplasmic translocation of PAX4 was also observed in DC and all the drug-treated groups. **[C] β-cell neogenesis:** $NGN3^+$ cells were not detected in DC or the drug-treated groups. However, $PDX1^+$ cells were detected in all the groups with their nucleo-cytoplasmic translocation. **[D] β-cell apoptosis by AIF:** None of the groups showed AIF translocation to the nucleus in $insulin^+$ cells. **[E] β-cell apoptosis by TUNEL:** β-cell apoptosis was significantly increased in the DC group compared to the control group and significantly reduced in all the drug-treated groups compared to the DC group. Scale-20 µm. Magnification-63X. (*p<0.05, **p<0.01, ***p<0.001 vs. NCD; $^{@}p$<0.05, $^{@@}p$<0.01, $^{@@@}p$<0.001 vs. HFD) (n=3/group).

Additionally, 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. However, β-cell apoptosis, as shown by $TUNEL^+$/$insulin^+$ cells was significantly increased in the DC group (p<0.001) as compared to the control group (p<0.001), whereas it was reduced significantly in all the drug-treated groups (M, p<0.01; G, p<0.05, M+G, p<0.01) (Figure 2.2A-E).

2.4 Discussion:

T1D is characterized by β-cell destruction caused by an autoimmune process and oxidative stress, leading to absolute insulin deficiency and chronic hyperglycemia. The management of DM involves two main approaches: i) increasing the survival rate and proliferation of endogenous pancreatic β-cells with immunosuppressive effects, and ii) increasing insulin-mediated glucose uptake by the peripheral tissues. However, none of the current therapies effectively achieve both the goals (Atkinson et al., 2014). Recent studies suggest that melatonin and GABA both individually can stimulate β-cell replication, protect β-cells against apoptosis, attenuate insulitis, regulate the islet-cell function and glucose homeostasis, improve peripheral insulin sensitivity and suppress the detrimental immune reactions (Patel et al., 2022; Rathwa et al., 2022; Purwana et al., 2014; Liu et al., 2017; Tian et al., 2004; 2011a). Hence, the present study investigated the therapeutic effects of melatonin in combination with GABA in the STZ-induced mouse T1D model. Our results showed that monotherapies are as effective as the combination therapy in inducing β-cell regeneration, as suggested by proliferation and transdifferentiation markers in the β-cells, reduction of β-cell apoptosis ($TUNEL^+$), increased

insulin levels, decreased FBG levels, and improvement in impaired glucose tolerance.

Melatonin mediates its action via melatonin receptor 1A (MTNR1A) and melatonin receptor 1B (MTNR1B) to regulate metabolic functions in insulin sensitive tissues (Cecon et al., 2018). Melatonin has both inhibitory and stimulatory effects on insulin secretion. Melatonin reduces insulin secretion by activating Gi-coupled MTNRs in β-cells, and it has been shown to reduce PKA and cAMP levels (Peschke et al., 2000) in a rodent cell line. Melatonin has also been reported to reduce the intracellular Ca^{2+} level in the β-cells (Stumpf et al., 2009; Stumpf et al., 2008). Melatonin triggers insulin secretion by activating PLC and IP3 through binding with Gq-coupled MT2 receptors (Brydon et al., 1999). Chan et al. (2002) showed that the melatonin treatment increases insulin secretion and promotes β-cell survival via decreased c-JUN N-terminal kinase (JNK) activation in human pancreatic islets. In addition, melatonin administration protects β-cells from glucotoxicity and oxidative stress and increases their survival in both human islets from T2D patients and rodent INS-1832/13 β-cells (Park et al., 2014; Costes et al., 2015). Yavuz et al. (2003) also reported that administering melatonin (200 μg/kg i.p.) prior to the onset of diabetes could reduce oxidative stress and preserve β-cell integrity without influencing hyperglycemia. Moreover, melatonin has a protective effect against DNA damage induced by STZ and also prevents the activation of poly (ADP-ribose) polymerase (PARP) (Andersson and Sandler, 2001). Further, melatonin administration (10 mg/kg BW) partially restored the β-cell mass in the STZ-induced rat T1D model (Kanter et al., 2006). Melatonin (10 mg/kg i.p.) administration for 6 and 8 weeks showed an 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). Studies carried out on diabetic rodent models have demonstrated that there is a functional correlation between melatonin, insulin, and the regulation of blood sugar levels. (Peschke, 2008). Champney et al. (1983) reported that chemically induced diabetes causes β-cell apoptosis and abolishes melatonin secretion at nighttime. Moreover, Lin et al. (2009) have reported that melatonin suppresses autoimmune recurrence in NOD mice by inhibiting the proliferation of Th1 cells and decreasing the production of pro-inflammatory cytokines. Thus, our findings support the previous reports on the effect of melatonin on β-cell regeneration and insulin secretion.

GABA has a proven role in islet-cell hormone homeostasis, preserving the β-cell mass, suppressing immune reactions, and consequent apoptosis (Wang et al., 2019). GABA exerts its

biological effects by activating GABA receptors ($GABA_AR$ and $GABA_BR$) that are expressed in a variety of peripheral tissues, including pancreatic islet cells and immune cells, such as T and B lymphocytes (Tian et al., 2004). Within an islet, GABA acts via $GABA_AR$ on the α-cells and suppresses glucagon secretion due to membrane hyperpolarization. In contrast, the β-cells enhance the insulin secretion through membrane depolarization. The activation of GAB_ARs via exogenous GABA treatment or specific agonists significantly inhibits β-cell apoptosis and protects pancreatic β-cells in STZ-induced hyperglycemic mice, following the islet transplantation into NOD/SCID mice (Tian et al., 2013). Ligon et al. (2007) demonstrated that GABA treatment increases β-cell survival in isolated rat islets. Also, it decreased apoptosis and increased the proliferation of β-cells. Similarly, GABA and GAB_AR agonists promoted β-cell replication in hyperglycemic mice and human islets (Bansal et al., 2011; Wang et al., 2014). GABA-induced β-cell membrane depolarization and Ca^{2+} influx can activate the PI3K/Akt signalling cascade (Soltani et al., 2011; Purwana et al., 2014). This pathway has been shown to play a pivotal role in β-cell protection and survival (Wang and Brubaker, 2002; Wang and Jin, 2009; Wang et al., 2004). GABA promotes the phosphorylation of CREB (cAMP response element-binding protein) (Purwana et al., 2014), a key transcription factor responsible for the maintenance of insulin gene transcription and β-cell survival in rodent and human islets (Shao et al., 2013). In human islets, GABA-induced CREB activation could be inhibited by either $GABA_AR$ or $GABA_BR$ antagonists (Purwana et al., 2014), suggesting the role of both receptors. Studies with pathway inhibitors in human islets and a rodent clonal β-cell line (INS-1) indicate that the effects of GABA are independently governed by the Akt and CREB pathways, and the activation of both pathways is required for an optimal response (Purwana et al., 2014). Among targets of CREB, the insulin receptor substrate-2 (IRS-2) may be a crucial gene in regulating β-cell mass and function. Studies in rodent islets or clonal β-cells suggest that glucose-induced upregulation of IRS-2, in a Ca^{2+} and CREB-signalling dependent fashion is vital for β-cell plasticity in response to an increased demand for insulin. GABA regulates cytokine secretion from human PBMCs, suppresses β-cell-reactive $CD8^+$ cytotoxic T lymphocytes (CTLs), and increases regulatory T-cell in T1D models (Soltani et al., 2011). GABA's anti-inflammatory and immunomodulatory actions are governed by SIRT1-mediated inhibition of the NF-κB pathway, which further protects the pancreatic β-cells against apoptosis (Prud'homme et al., 2014).

The plasticity and transition of α- to β-cells are possible during pancreatic islet development as

both arise from the same multipotent progenitors (Shih et al., 2013). The β-cell development depends on several transcription factors (Gittes, 2009). The most important is PDX1, a pancreatic progenitor marker (Ohlsson et al., 1991). Following PDX1 expression, NGN3, an endocrine progenitor marker, is expressed (Schwitzgebel et al., 2000). ARX is the critical transcription factor for α-cell, while PAX4 and other transcription factors determine the β cell's identity (Rutter 2017). Due to the lack of NGN3 expression in all the drug-treated groups, we suggested that β-cell neogenesis was absent in all the groups. However, we observed $PDX1^{+}$/ $insulin^{+}$ cells in all the groups as indicated by the nucleo-cytoplasmic translocation of PDX1. Moreover, β-cell function and identity are 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^{+} and $insulin^{+}$ co-positive cells were observed in all drug-treated groups, indicating the presence of α- to β-cell transdifferentiation in these groups. Moreover, PAX4 also defines the proliferating β-cell population (Brun et al., 2004; Lorenzo et al., 2015), which was increased in all the drug-treated groups. However, lineage-tracing experiments are required to substantiate our results on β-cell transdifferentiation. Furthermore, our results suggest that β-cell proliferation (INS^{+} $BrdU^{+}$ co-positive cells) was significantly increased, and apoptosis (INS^{+} $TUNEL^{+}$ co-positive cells) was significantly reduced in all the drug-treated groups.

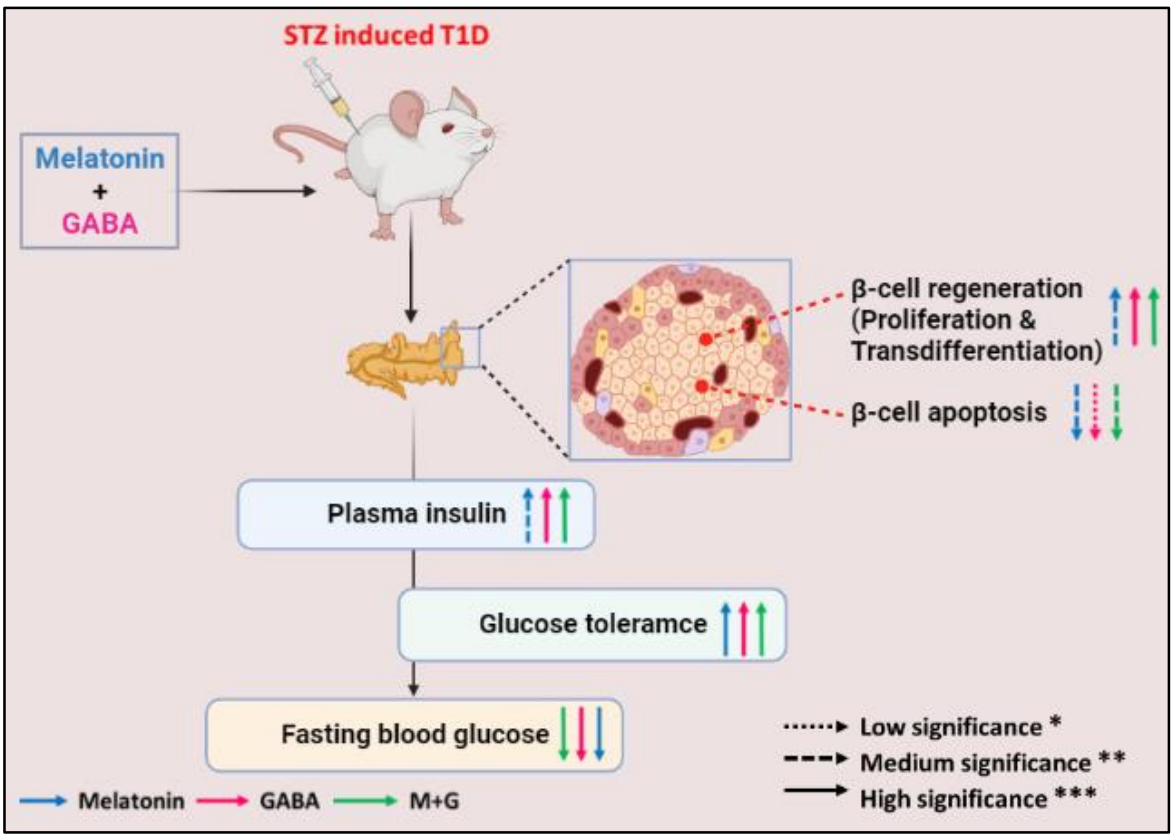

Figure 2.3. Effect of melatonin, GABA, and combination therapy in amelioration of STZ-induced T1D manifestations in a mouse model. Monotherapies are as effective as combination therapy on β-cell regeneration and achieving glucose homeostasis in T1D mouse model.

Thus the present study suggests that monotherapies are as effective as the combination therapy in reducing hyperglycemia, improving glucose tolerance, and stimulating β-cell regeneration in the STZ-induced T1D mouse model (Fig. 2.3). However, further studies are warranted to understand the mode of action of melatonin and GABA. Furthermore, to explore the therapeutic potential of melatonin in combination with GABA in clinical studies, it is essential to consider the duration and dosage of melatonin besides the relative timing of its intake concerning the glycemic challenge.

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