Chapter 3

In vitro effect of sitagliptin, melatonin, and combination treatment on β-cell proliferation in mouse pancreatic islets

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3.1 Introduction

Progressive deterioration of β-cell function and mass is a central parameter in the development of Type-1/2 Diabetes (T1/2D). Therefore, it is important to develop therapies that stop or even reverse such deterioration of β-cell function. Although insulin supplementation allows a reasonable control of blood sugar levels, T1/2D patients still suffer from long-term side effects of blood glucose variations, too often resulting in severe alterations of various organ functions. Most patients with T2D display a progressive worsening of glycemic control which is due to the gradual loss of β-cell function and mass, resulting in insulin deficiency (Cnop et al., 2007). Glucagon-like Peptide-1 (GLP-1) and Glucose-dependent Insulinotropic Polypeptide (GIP) are gut hormones/incretins that stimulate insulin secretion, prevent β-cell death, and promote β-cell regeneration (Marín-Peñalver et al., 2016). However, Dipeptidyl Peptidase-IV (DPP-IV) is a major enzyme responsible for degrading GLP-1 and GIP *in vivo* (Yabe and Seino, 2011). Therefore, maintaining β-cell function and/or halting further loss of β-cell mass may provide a promising strategy in managing or even reversing the course of the disease. To that end, over the last decade, many studies have been carried out exploring the potential benefits of incretin-based therapies on β-cell preservation and/or regeneration. Melatonin is endogenously produced in the pineal gland and in other organs. Melatonin is having a role in circadian rhythm regulation (Bixler, 2009), acts as an antioxidant (Ahmadi and Ashrafizadeh, 2020) and anti-inflammatory agent (Hacışevki and Baba, 2018), and is also functionally linked to glucose metabolism (Peschke et al., 2006). We hypothesize that melatonin would ameliorate oxidative stress and bring about insulin secretion via MT1 receptor activation whereas inhibition of DPP-IV would potentially improve the therapeutic efficacy of GLP-1 and GIP. Hence, we aimed to explore the therapeutic potential of a novel combination of melatonin and DPP-IV inhibitor on β-cell proliferation under glucotoxic and gluco-lipotoxic stresses *in vitro*.

3.2 Materials and Methods

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/BIOCHEMISTRY/IAEC/2019/9].

3.2.1 Islet Isolation and Dispersion

Pancreatic islets were isolated from C57BL/6J mice (8-9 weeks old) by Histopaque gradient separation (Ricordi and Rastellini, 2000). Briefly, mice were euthanized, and collagenase P (Roche, Basel, Switzerland) (1.7 mg/ml in Hanks' balanced salt solution) was injected into the common bile duct (3 ml/mouse) and the distended pancreata were excised out immediately in 50 ml falcon tube and was placed on ice until all pancreata were collected. Not more than five pancreata islet isolations were carried out at a given point of time. The distended pancreata were then incubated in a water bath at 37°C for 17 min. Collagenase was neutralized and washed off with Hanks' buffer + 10% Fetal Bovine Serum (FBS). Islets were filtered through 500µm wire mesh and separated by Histopaque (Sigma, St. Louis, MO, USA) gradient. Histopaque was washed off, and islets were incubated with 10 ml of Hanks's buffer. Islets were allowed to sediment by gravity for two minutes. 7 ml of Hanks's buffer was aspirated, and the remaining buffer containing islets was transferred to 10 ml of RPMI 1640 complete medium in a 35 mm plastic petri dish. Isolated mouse islets were cultured in RPMI 1640 complete medium (Life Technologies) containing 10% FBS, 5.5 mM glucose, and 1% penicillin-streptomycin for 24 hr.

Handpicked islets were dispersed by trypsin treatment. Briefly, islets were scattered by centrifugation at 1500 rpm for 10 min, washed twice in Phosphate Buffered Saline (PBS), re-suspended in 1 ml of 1 mg/ml trypsin, and incubated for 10 min at 37°C. During this digestion, the islets were dispersed by gentle pipetting up and down every 5 min for 10 sec. 500 µl of RPMI 1640 complete medium was then added to stop the digestion. The cells were then centrifuged for 5 min at 1500 rpm. The supernatant was removed, the pellet was re-suspended in RPMI 1640 complete medium, and cells were then plated on poly L-lysine treated coverslips with 50 µl cell suspension per coverslip.

3.2.2 Islet Culture

After dispersion, islets were incubated with RPMI 1640 complete medium containing either 5.5 mM (physiological), 25 mM glucose (high) alone; and 25 mM glucose + 250 µM palmitate for 48 hr. After 48 hr of incubation, the media were changed to RPMI 1640 complete media containing physiological glucose concentration, high glucose concentration + palmitate with varying sitagliptin concentrations (1, 10 and 100 µM for 24 and 48 hr) or varying melatonin concentrations (1, 10, 100 nM for 24 and 48 hr) for dose and time-dependent study (melatonin and sitagliptin were obtained from Sigma, St. Louis, MO, USA).

The standardized doses and time for sitagliptin, melatonin, and combination of sitagliptin and melatonin were selected for further experiments to assess β-cell proliferation.

3.2.3 Immunocytochemistry-Immunofluorescence (ICC-IF)

Islet cells on coverslips were fixed in 4% paraformaldehyde for 30 min at room temperature, washed with PBS and incubated in 100 µl blocking buffer [1.0% BSA, 0.5% Triton X 100, and 5% normal goat serum in PBS] for 1 hr at room temperature. Cells on coverslips were incubated with primary antibody (rabbit anti-Ki-67, Thermo Scientific; Cat# MA5-14520; 1:200 and guinea pig anti-insulin, DAKO; Cat# A0564; 1:1000) prepared in blocking buffer overnight at 4°C. Secondary antibody (goat anti-rabbit Alexa Fluor 594, Jackson ImmunoResearch, Cat# 111-585-144; 1:250 and goat anti-guinea pig Alexa Fluor 488, Jackson ImmunoResearch, Cat# 106-545-003; 1:250) which was prepared in blocking buffer was added and incubated for 1 hr in the dark at room temperature. The cells were washed with PBS and distilled water. Coverslip was mounted with a mounting medium with DAPI. Labelled cells were then visualized using laser confocal microscopy (Leica SP5 DM). The results shown are representative of three mouse islet preparations/group. 1000-2000 β-cells were counted in each section. β-cell proliferation was calculated as $Ki67^{+}$+$insulin^{+}$/total $insulin^{+}$ cells.

3.2.4 Statistical Analysis

All results were shown from at least three independent experiments. Statistical analysis of β-cell proliferation was performed by one-way analysis of variance (ANOVA) and multiple group comparisons by Tukey's post hoc test in Prism 5 (GraphPad Software, San Diego, CA, USA). The significance level was set as $p<0.05$. Results are expressed as mean±SEM.

3.3 Results

3.3.1 Assessment of β-cell Proliferation *In Vitro*

Dose and time-dependent studies on C57BL/6J mouse pancreatic islets suggest that the maximum effective dose (MaxED) was 100µM - 48 hr sitagliptin; 10nM melatonin - 24 hr and minimum effective dose (MinED) was 10µM sitagliptin - 48 hr; 1nM melatonin - 48 hr, to induce β-cell proliferation. MaxED showed no additive or synergistic effect in the combination (S+M) treated islets (Fig. 3.1 and 3.2A-C). However, MinED dose showed an additive effect of sitagliptin (S) and melatonin (M) on β-cell proliferation ($p<0.01$) as

compared to MaxED dose (Fig. 3.2D). Hence, MinED dose was selected for further experiments.

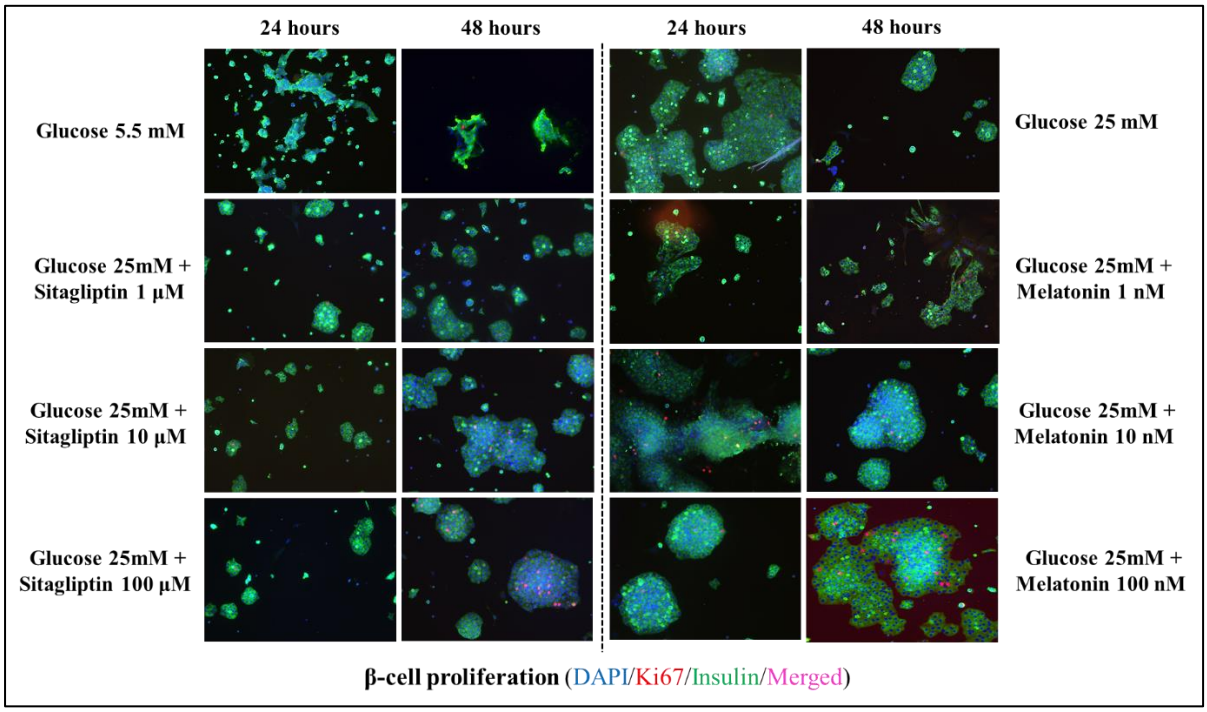

Figure 3.1 Dose and time-dependent effect of sitagliptin and melatonin on β-cell proliferation under glucotoxic stress - representative images. Scale: 50µm. Magnification: 20X.

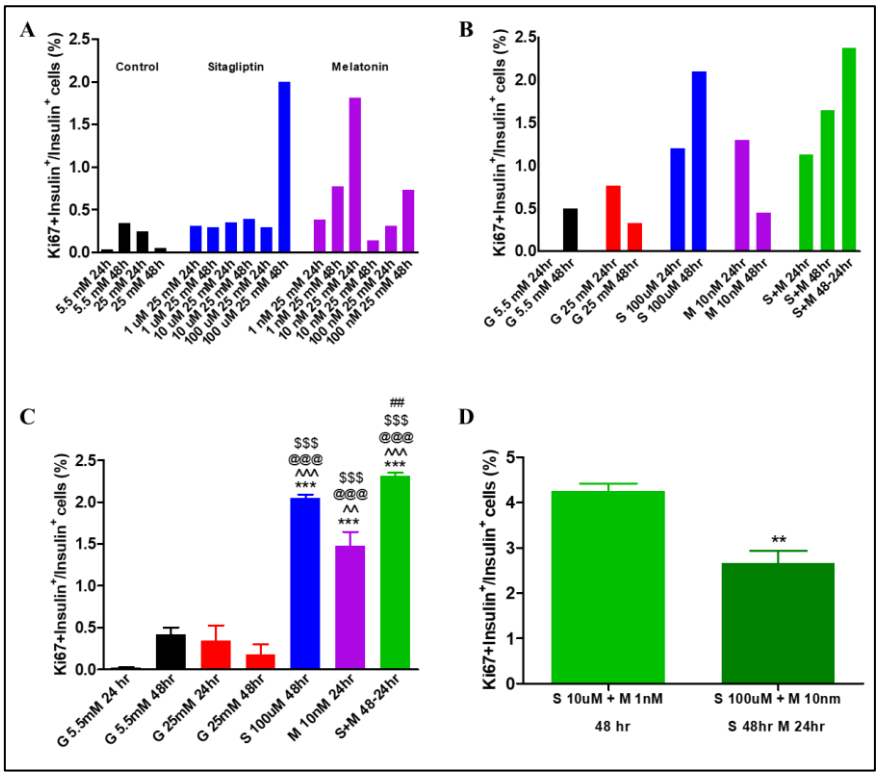

Figure 3.2 Dose and time-dependent effect of sitagliptin, melatonin, and combination treatment on β-cell proliferation under glucotoxic stress: A. The maximum effective doses (MaxED) of sitagliptin (S) and melatonin (M) observed on β-cell proliferation were 100µM for 48hr and 10nM for 24hr, respectively while the minimum effective doses (MinED) were 10µM and 1nm for 48hr, respectively. **B.** The MaxED was established but the percentage of β-cell proliferation (2 to 2.5%) was similar in the S, M, and S+M treated islets. **C.** β-cell proliferation was significantly increased in S, M, and S+M treated islet groups as compared to islets containing 5.5mM glucose and 25mM glucose (24hr and 48hr) but no significant difference was observed between S and S+M treated islets. **D.** A significant increase in β-cell proliferation was observed in S+M treated islets with MinED as compared to MaxED (4.2 to 4.5% vs. 2 to 2.5%). (**p<0.01, ***p<0.001 vs. G 5.5mM 24hr, ^^p<0.01, ^^^p<0.001 vs. G 5.5mM 48hr, $^{@@}p$<0.01, $^{@@@}p$<0.001 vs. G 25mM 24hr, $^{\$}p$<0.05, $^{\$\$}p$<0.01, $^{\$\$\$}p$<0.001 vs. G 25mM 48hr, $^{\&}p$<0.05 vs. S 10µM 48hr, $^{\#\#}p$<0.01 vs. M 1nM 48hr) (n=3 for 2.2C and 2.2D).

Under glucotoxic stress, the S, M and S+M treated islets showed a significant increase in the β-cell proliferation (p<0.001), as shown in Fig. 3.3.

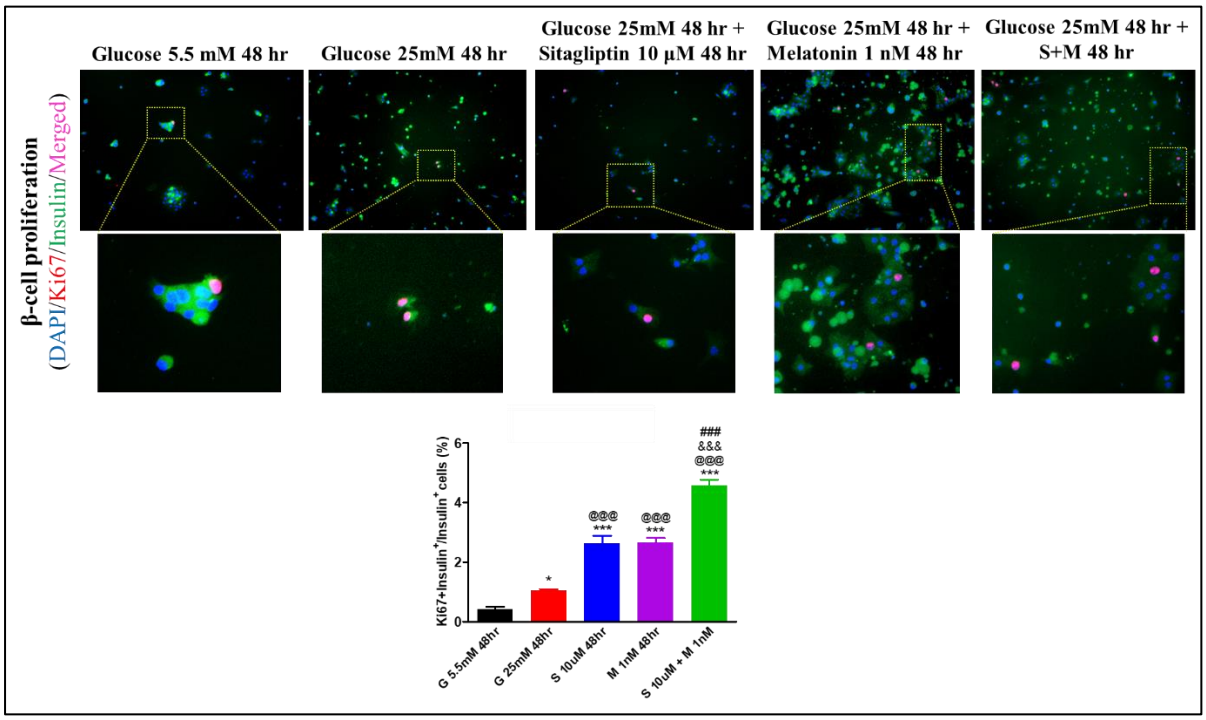

Figure 3.3 Effect of sitagliptin, melatonin and combination treatment on β-cell proliferation under glucotoxic stress: Combination of sitagliptin (S) and melatonin (M) showed additive effect (~5%) in β-cell proliferation as compared to monotherapies (~2.5%) under glucotoxicity, as shown by Ki67-insulin co-immunolabelling in dispersed mouse pancreatic islets (*p<0.05, ***p<0.001 vs. Glucose 5.5mM; $^{@@@}p$<0.001 vs. Glucose 25mM; $^{\&\&\&}p$<0.001 vs. Sitagliptin; $^{\#\#\#}p$<0.001 vs. Melatonin). Scale: 50µm, Magnification: 20X, (n=3).

Under gluco-lipotoxic stress, a significant increase in β-cell proliferation was observed in the S and M treated (p<0.05), and combination (S+M) (p<0.001) treated islets (Fig. 3.4).

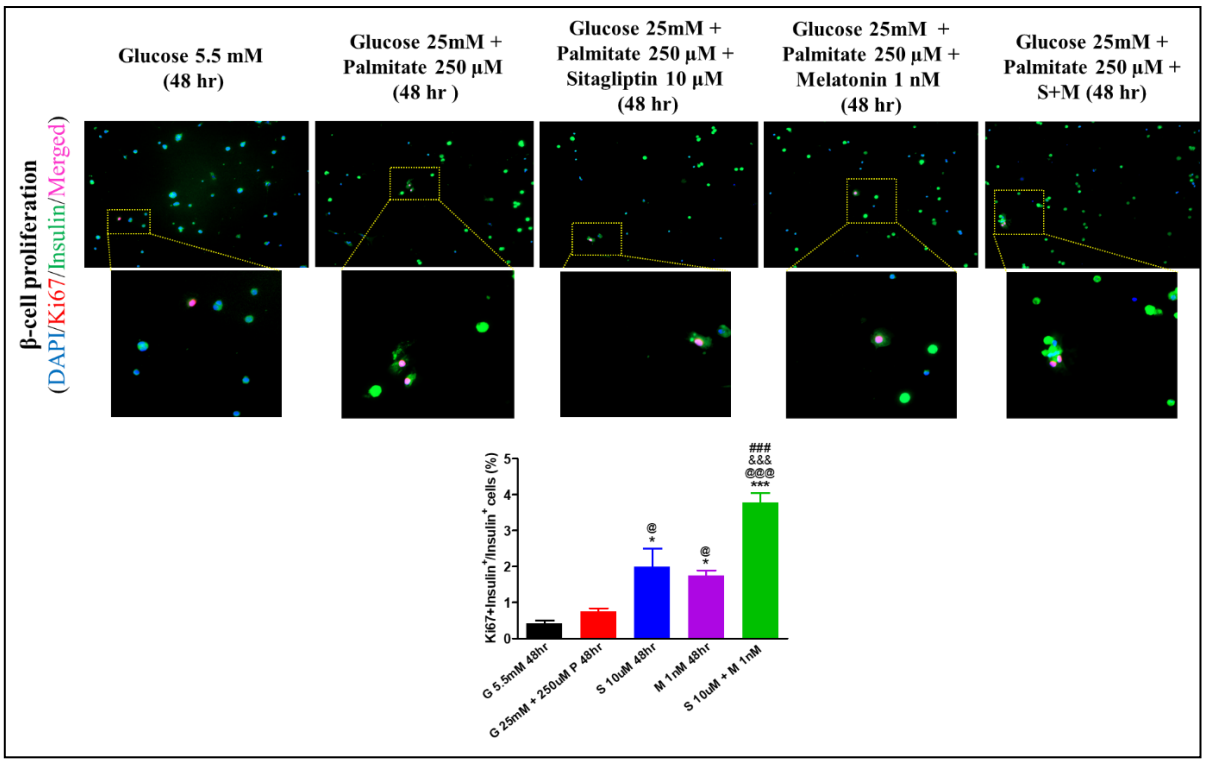

Figure 3.4 Effect of sitagliptin, melatonin and combination treatment on β-cell proliferation under gluco-lipotoxic stress. Combination of sitagliptin (S) and melatonin (M) showed additive effect (~4%) in β-cell proliferation as compared to monotherapies (~2%) under gluco-lipotoxicity, as shown by Ki67-insulin co-immunolabelling in dispersed mouse pancreatic islets. (*p<0.05, ***p<0.001 vs. Glucose 5.5mM; $^{@}p$<0.05, $^{@@@}p$<0.001 vs. Glucose 25mM; $^{\&\&\&}p$<0.001 vs. Sitagliptin; $^{\#\#\#}p$<0.001 vs. Melatonin). Scale: 50µm, Magnification: 20X, (n=4).

3.4 Discussion

T1D is a chronic metabolic disorder triggered by autoimmunity and oxidative stress among other factors leading to β-cell apoptosis. There is no potential drug available other than insulin replacement therapy at present to achieve glycemic control in T1D patients. Hence, we made an attempt to investigate the therapeutic potential of sitagliptin and melatonin on β-cell regeneration under gluco- and glucolipo-toxic stresses on mouse pancreatic islets *in vitro.* We found an additive effect of sitagliptin and melatonin in promoting β-cell proliferation.

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). On the other hand, 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). In human pancreatic islets, melatonin treatment increases insulin secretion and promotes β-cell survival via decreased JUN N- terminal kinase (JNK) activation (Chan et al., 2002).

Sitagliptin, a DPP-IV inhibitor, is an approved drug for T2D treatment. It is a highly selective inhibitor inhibiting more than 80% of DPP-IV enzyme activity thereby increasing the half-life of GLP-1 and GIP. 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). Sitagliptin reduces HbA1c and postprandial glucose levels alone, and in combination with other drugs. It also 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 reducing HbA1c levels, increasing β-cell mass, and ameliorating lipid profile. The effect of DPP-IV inhibitors on β-cells is mediated by GLP-1 (Matveyenko et al., 2009) which upon binding to its receptor increases β-cell proliferation and reduces β-cell apoptosis (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). There is currently no clear evidence that incretin-based therapies can increase β-cell mass in humans, other than a few reports that showed incretin mediated β-cell regeneration in rodents. This discrepancy might be due to several differences between human and rodent, including intrinsic species differences; differences in β-cell turnover, response to regenerative stimuli, amount of drug administered and the duration of treatment (Levine et al., 2008; Parnaud at el., 2008). 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 GLP-1 stabilization (Shah et al., 2013). DPP-IV mediates its action partly via GLP-1 and also has independent effects on β-cell. Gut is the primary source of GLP-1 which augments glucose-stimulated insulin secretion. Chambers et al., (2017) showed that GLP-1 is also produced in the α-cells of pancreatic islets, and is responsible for its incretin effect in mice. This raises the possibility that intra-islet GLP-1 also regulates β-cell function. Our studies

support this finding as we have observed β-cell proliferation *in vitro* in mouse islets. Interestingly, DPP-IV being an adipokine may also mediate its action directly via modulating chemokines and cytokines which are crucial for β-cell survival. For instance, saxagliptin, a DPP-IV inhibitor, induces β-cell proliferation *in vitro* and *in vivo* via stromal cell-derived factor-1α (SDF-1α)- a chemokine and its receptor (CXCR4) (Li et al., 2017). Thus, we assume that sitagliptin may be working in a similar manner, though its mode of action needs to be explored.

Thus, our results support previous studies on the effects of melatonin and DPP-IV inhibitor on β-cell proliferation. The effect of combination drug (S+M) treatment to islets is more effective to single drug (S and M) treatment, and S+M treatment additively promotes pancreatic β-cell proliferation *in vitro* under gluco- and gluco-lipotoxic stresses (Fig. 2.5).

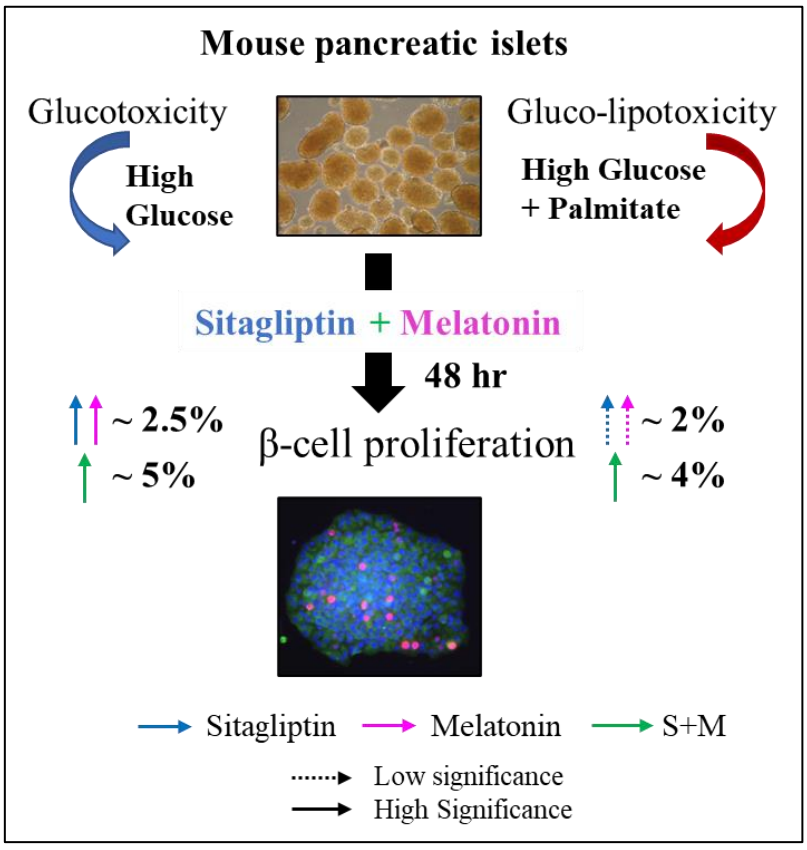

Figure 3.5 Additive effect of sitagliptin, melatonin and combination (S+M) on mouse β-cell proliferation under glucotoxicity and gluco-lipotoxicity *in vitro*.

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