Chapter 6

DISCUSSION
6. DISCUSSION

In spite of extensive research, treatment of diabetes without side effects remains a challenge. Hence, it is necessary to develop newer therapeutic agents with high efficacy and fewer side effects. Plant products are good source of drugs. Many of the currently used drugs were initially derived from plant sources and produced synthetically later on. Metformin, the most prescribed biguanide, used even today in the treatment of diabetes was synthesized from *Galega officinalis* (65). Therefore, phytochemicals are potential therapeutic substances that need to be scientifically validated and put to the best use for the betterment of humanity. In this context, naringin, a flavanone glycoside proved to have antidiabetic activity is taken in this present study to explore its mechanism of action on the pancreatic beta cells.

Adult beta cells are generally quiescent and less than 0.1 per cent of them undergo replication in a day (10). Various factors are being studied for their influence on adult beta cell replication. In this context, it has to be noted that as the age advances beta cells show decline in responsiveness to any mitogenic stimuli (137). Mitogenic stimuli may also pose a risk of turning the cells cancerous. Against this background, the present study tries to assess the effect of naringin, a well-established anticancer agent, on the beta cells of islets through two important transcription factors PDX-1 and FoxM1.

In the present study, STZ was used to induce diabetes as it selectively destroys beta cells and generates ROS, which also accelerates the beta cell destruction. STZ enters the beta cells selectively through GLUT-2 transporter and destroys beta cells by alkylation of DNA. In the process of repairing the DNA, beta cells deplete nicotinamide adenine dinucleotide (NAD⁺) that inhibits insulin synthesis and secretion by beta cells. At a later stage, mitochondrial genome and enzyme activity get damaged leading to inhibition of nutrient induced insulin secretion. Further, organs like kidney and liver expressing GLUT-2 are also susceptible to damage by STZ (138). These qualities of STZ make it a suitable substance for induction of diabetes, which helps to assess the central action of any therapeutic compound on beta cells. In the present study, the central action of naringin, a potent antioxidant is assessed using this model. This model additionally helps to assess the role of naringin on nutrient responsive insulin secretion and in diabetic complications like nephropathy.
6.1. Rationale for selecting naringin and the current dosage

Naringin’s therapeutic activity is based on its aglycone portion, naringenin. In permeability studies, naringenin was found to be absorbed better than naringin. Further, naringenin was proved to be a better antioxidant and shown to express better free radical scavenging activity than naringin in *in vitro* studies (139, 140). Despite naringenin having better attributes, glycoside naringin was resolved to be used in the present study for the following reasons: The sugar moiety attached to the aglycone facilitates the solubility of the aglycone in the aqueous cellular environment and protects the auto oxidation of reactive –OH groups. It also helps in the transport of aglycone from the endoplasmic reticulum to other components of the cells (60). In tissue cultures, the sugar moiety reduced the genotoxicity and cellular toxicity (141).

Works done by Punithavathi et al. (62) and Lim et al. (63) had demonstrated that higher dose of naringin administration yielded better results than lower doses. This point was indeed substantiated by the clinical trials that associate higher intake of flavonoids (≥550 mg/day) to reduction in the risk of non-insulin dependent diabetes (28). Therefore, in the present study, dosage of naringin was fixed as 100 mg/kg, the highest dosage reported in the literature to harness the best results.

6.2. Body weight, food and water intake

Normally rat’s food intake ranges between 5 g and 6 g per 100 g of bd.wt. and water consumption ranges between 10 ml and 12 ml per 100 g of bd.wt. (142). Subsequent to the induction of diabetes, the food consumption of diabetic animals doubled and water consumption tripled when compared to control animals. The diabetic animals also lost weight drastically. Naringin administration significantly regulated the food and water consumption and controlled the weight loss in diabetic animals (Figure 2 and Table 8). These changes were in accordance with the observations of Punithavathi et al. (62) and Xulu and Oroma Owira (108).

6.3. Blood glucose levels

Naringin administration to the diabetic rats brought down the fasting glucose gradually and at the end of the study, the levels were significantly lower than their
untreated counterparts (Table 9). The results were consistent with the previous works (61, 63, 110, 111, 115, 143). In studies done by previous authors, the dosage of naringin varied between 25 mg/kg and 100 mg/kg.

However, there exist two contrasting reports stating that naringin does not reduce hyperglycaemia. Study done by Xulu et al. (108) reported that treatment with naringin did not increase the insulin level and did not reduce hyperglycaemia in type 1 diabetes model. Whereas, Murunga et al. (124) documented that naringin treatment was unable to reduce the fasting blood sugar in spite of improved insulin levels. Xulu et al. has suggested that extra pancreatic mechanism might be the cause for reduction in hyperglycaemia. Both these studies used 50 mg/kg naringin in STZ-induced diabetic rats. The results of the present study contradicted the observations made in these two studies.

In addition, it is to be noted that in the present study, the glucose level of 8-week naringin treated animals were close to that of normal but never reached the normal range. Studies with duration of treatment greater than 8 weeks are to be conducted to ascertain whether prolonged use of naringin would bring the glucose within the normal range in diabetic animals.

6.4. Insulin secretion

Study done by Nzuza et al. (61) showed that naringin treatment to RIN5F cell lines increased insulin secretion proportional to the dosage of naringin in addition to reducing the oxidative stress. In the same lines, naringin administration showed improvement in the antioxidant and lipid peroxidation markers in the pancreatic tissue of diabetic animals (Table 12) and showed increase in beta cell insulin content and fasting serum insulin levels of the diabetic animals (Figures 4 & 9). This implies that naringin reduces the oxidative stress and not only helps in the synthesis and storage of insulin granules but also helps in the beta cell insulin secretion in response to hyperglycaemia. Increase in fasting insulin level seen in naringin treated diabetic rats were consistent with the results of previous studies (38, 63, 110, 112).
6.5. Light microscopic and ultrastructural changes

Single intraperitoneal injection of 50 mg/kg STZ was shown to produce necrosis and loss of beta cells and atrophy of islets (144). Likewise, in the present study, diabetic animals left untreated exhibited leukocytic infiltration of parenchyma, perivascular fibrosis and destruction of beta cells following the administration of STZ (Figures 5 & 6). The islet diameter was reduced by 50 per cent indicating marked atrophy of the islets (Figure 12A). These lymphocytes secrete cytotoxic cytokines that induces generation of ROS (81). Whereas, Group VI and VII animals showed minimal lymphocytic infiltration. Increase in the diameter and normal cellular architecture of the islets in these animals indicated recovery from the insult created by STZ and regeneration of the islets. These changes were in accordance with the observations of Lim et al. (63) affirming that naringin reduces the immunogenic destruction of islets.

Electron microscopic pancreatic images of different experimental groups substantiated that STZ affected the acinar portion of the pancreas in addition to beta cells and naringin ameliorated these deleterious changes. Beta cells of group VII displayed noticeable increase in the secretory granules and network of endoplasmic reticulum (Figure 8). These favourable transformations in the beta cells suggest that naringin had helped the cells to recover from the STZ-induced destruction and endowed them with potential to synthesize and store insulin granules. Similar outcome was documented by Sharma et al. (38) in HFD-STZ induced diabetic rats, where naringin treatment reduced the distension of endoplasmic reticulum and maintained adequate secretory granules.

6.6. PDX-1, FoxM1 and Insulin mRNA and protein expressions

6.6.1. Insulin

Impairment in the insulin production and secretion was noted in STZ induced diabetic rats, which was evident from insulin immunohistochemistry and fasting serum insulin levels (Figures 4 & 9). Following naringin administration, the above-mentioned disturbances appeared to resolve gradually. In group VII the Insulin gene was up-regulated and the volume of insulin-positive cells were three times higher than the diabetic animals left untreated (Figures 13A & 15A). Thus, increased
production and secretion of insulin in naringin treated animals resulted in reduction of hyperglycaemia (Figure 3). Immunohistochemistry exhibited the presence of insulin-positive cells only in the cytoplasm of the islets and not in the ductal cells or in the acini. Increase in the fractional volume of insulin-positive cells in naringin treated groups (VI and VII) indicated proliferation of functionally matured beta cells. These observations put together imply that the increase in functional beta cells were derived from pre-existing beta cells present in the islet and progenitors from duct-associated and exocrine lineages have no role in naringin mediated beta cell proliferation. Transdifferentiation of alpha cells to beta cells could not be ascertained, as the anti-insulin stained slides were not co-stained with anti-glucagon antibodies.

Splenic lobe partial pancreatectomy performed by Ackermann Misfeldt et al. (74) on pancreas wide FoxM1deleted adult mice revealed that beta cell did not proliferate in the duodenal lobe whereas neogenesis of beta cells within the regenerated splenic lobe remained unaffected. This suggested the requirement of FoxM1 for the multiplication of pre-existing beta cells. In the present study, FoxM1 expression were up-regulated in naringin treated groups, which endorsed the proposition of proliferation from pre-existing beta cells.

In the present study, up-regulation of PDX-1 and FoxM1 mRNA and protein expressions were reported in whole pancreatic tissues through PCR and immunoblot analysis. In order to prove that transcription factors expression was up-regulated within the beta cells, immunohistochemical staining was employed. The increased immunopositivity was observed in the nuclei of cells located in the central part of the islets. It is a well-established fact that in rodents, beta cells occupy the central portion of the pancreatic islets while the alpha and gamma cells are present in the periphery (69). Thus, a conclusion was drawn that the nuclei exhibiting immunopositivity belonged to beta cells of the islet.

6.6.2. PDX-1

Existing literature has established that PDX-1 is indispensable for activation of *Insulin* gene and for maintenance of adult beta cells (83). Further it is established that glucotoxicity alters the distribution of PDX-1 from nucleus to cytoplasm leading to
down-regulation of *Insulin* gene and reduction in insulin biosynthesis (145). In the present study, in contrast to the untreated animals, naringin treated animals exhibited up-regulation of *Pdx-1* gene and PDX-1 protein expressions (Figures 15B, 17A&B). Fractional volume of PDX-1-positive cells was in accordance with protein expression analyzed using Western blot (Figures 13B, 17A&B). Further, nuclear localization of PDX-1 observed in beta cells (Figure 10) confirmed the availability of the transcription factor to activate its downstream target *Insulin* gene. This was evident from the up-regulation of *Insulin* gene expression, increased insulin immunopositivity and increased serum insulin levels (Figures 4, 9 & 15A). These observations collectively implicate that naringin treatment up-regulates PDX-1 expression in diabetic animals that sequentially up-regulates the *Insulin* gene. *Insulin* gene in turn influences the synthesis of insulin hormone.

Glitazone receptor or PPARγ regulates the PDX-1 expression in beta cells and improves function of beta cells by regulating the downstream targets of PDX-1 (146). Earlier researchers have shown that PPARγ receptors in the liver and kidney of rats and in adipose tissue of db/db mice were up-regulated by administration of naringin (38, 147). Similarly, naringin mediated up-regulation of PPARγ expression in the beta cells has to be established to understand the mechanism resulting in the up-regulation of PDX-1.

Chronic hyperglycaemia induces oxidative stress and deteriorates the functions of beta cells through activation of JNK pathway. Cytoplasmic translocation of PDX-1 and its reduced binding to the promoter region of *Insulin* gene are related to activation of JNK. Further, oxidative stress down-regulates Akt expression resulting in phosphorylation of FoxO1, which is an important negative regulator of PDX-1. Phosphorylated FoxO1 causes nucleocytoplasmic translocation of PDX-1 and thereby results in down-regulation PDX-1 target genes (148). Naringin due to its antioxidant property may suppress the JNK kinase pathway, down-regulate FoxO1 expression and maintain the nuclear localization and increased expression of PDX-1 in beta cells.
6.6.3. FoxM1

Normal beta cell population predominantly consists of terminally differentiated cells with less than 0.1 per cent of cells undergoing proliferation (9, 10). Thus, FoxM1 expression in the control, group II and III animals were limited. Diabetic groups IV and V showed reduction in the expression of FoxM1 and exhibited hyperglycaemia. Group V animal expressed almost nil reactivity to the FoxM1 antibody. STZ specifically destroys beta cells. Of the surviving beta cells the cells that are proliferating would be still less. This could be the possible reason for the nil FoxM1 immunoreactivity seen in diabetic untreated animals of group V (Figure 11E).

Naringin treated diabetic animals (Group VI and VII) showed up-regulation of FoxM1 protein expression besides FoxM1 mRNA expression leading to beta cell proliferation. This was indicated by the increase in fractional volume of FoxM1-positive cells (Figures 11, 13C, 15C, 17C&D). The results were similar to the works done by Zhang et al. (98) and Golson et al. (99). Zhang et al. have shown that aged mice lacking FoxM1 had 60 per cent reduction in the beta cell mass and glucose intolerance. Whereas, activated FoxM1 expression in mice pancreas caused beta cell proliferation and enhanced secretion of insulin. Activation and repression of FoxM1 gene expression was carried out by Cre-Lox recombination technology. In the present study, naringin administration has brought about the up-regulation of FoxM1 gene and protein expressions in the pancreas.

Golson et al. (100) have shown that in mice with activated FoxM1, beta cell mass increased by virtue of increase in the size of the islet without much change in the size of the beta cells suggesting proliferation of beta cells. Similarly, in the present study, the islet diameter had increased significantly in group VI and VII animals, which synchronously exhibited higher expression of FoxM1.

Golson et al. also elucidated that lower level of FoxM1 expression was adequate to promote the function of beta cells, whereas higher level of expression was required for beta cell proliferation (100). This could partially explain the observations made in the present study. Group VII animals displayed two fold increase in the expression of FoxM1 protein than the control and 2.5 fold increase than the diabetic
group V (Figure 17D) which could substantiate the higher proliferation of beta cells seen in group VII. This subsequently resulted in improvement of insulin secretion and better control of hyperglycaemia. Whereas, in Group VI animals, in spite of modest increase in FoxM1 expression than group IV, noticeable improvement in the function of beta cells was not achieved. Beta cells could not secrete adequate insulin to bring hyperglycaemia under control.

Glucose is a mitogen for beta cells. Entry of glucose into beta cells increases the levels of intracellular calcium ions resulting in release of insulin granules. Rise in intracellular calcium also phosphorylates calmodulin and dephosphorylates NFAT. NFAT translocates to nucleus, binds to the response elements of FoxM1 gene and activates FoxM1 expression. This NFAT also represses cell cycle inhibitors namely p27 and p21. By this intracellular signalling pathway, therapeutic agents may control the cell cycle mechanism (149). In animals of groups VI and VII, increase in insulin levels was noted following naringin administration. This increase in insulin secretion requires increased intracellular calcium within beta cells. Based on these facts we hypothesize that naringin activates FoxM1 gene through the calcium-calmodulin-NFAT signalling pathway which brings about beta cell proliferation in addition to increasing the insulin secretion. Further investigation is needed to establish the effect of naringin on calcium-calmodulin-NFAT pathway converging on FoxM1 gene expression.

Shirakawa et al. (9) established that insulin signalling mediated through PI3K causes nuclear translocation of FoxM1 in beta cells. FoxM1 factor binds to promoter region of Cenp-A and Plk-1 genes and up-regulates them. Successive translation of these genes and deposition of CENP-A at the beta cell centromere causes segregation of chromosomes and mitosis of beta cells. In the present study, FoxM1 mRNA and protein expressions were up-regulated in diabetic rats treated with naringin. Further Insulin expression was up-regulated in these animals. Therefore, the signalling cascade mediated by insulin could possibly explain increased nuclear localization of FoxM1 in the beta cells. Further, nuclear localization of FoxM1 indicates the availability of transcription factor to up-regulate its downstream targets and result in beta cell proliferation.
PI3K, a signal transducer enzyme, associated with both glucose and insulin signalling pathways, helps in the nuclear translocation of both PDX-1 and FoxM1 factors (9, 83). Therefore, PI3K might be the target enzyme modulated by naringin in the activation of PDX-1 and FoxM1, which needs further research. The interaction between the PDX-1 and FoxM1 with the administration of naringin also needs to be explored further.

6.7. Protein and fat metabolism

Diabetes not only affects the glucose metabolism but also causes disturbances in protein and fat metabolism. Break down of tissue and plasma proteins causes increase in blood urea and creatinine levels (150). Increased urea and creatinine levels indicate impairment in the renal function of diabetic patients. Naringin administration caused reduction in the urea and creatinine levels signifying the protective role of naringin in diabetic nephropathy. Sharma et al. (38) has reported an identical result with naringin in type 2 diabetic model and has attributed the renoprotective effect of naringin to modulation of PPARγ, HSP-27, HSP-72 and NF-κB expressions.

In diabetic complications, dyslipidemia is an important risk factor. Reduced HDL and increased triglycerides are the common lipid disturbances seen in diabetic patients and these disturbances can be reversed by bringing the blood glucose under control (151). Naringin administration to diabetic rats significantly reduced the total cholesterol, triglycerides, LDL and VLDL levels and increased HDL level (Table 11). The results of the present study were matching with the results of Sharma et al. (38), Ahmed et al. (112), Pu et al. (53) and Xulu and Oroma Owira (108). According to Kim et al. (152), naringin supplementation to high fat high cholesterol fed mice reduced the HMG-CoA reductase and cholesterol acyltransferase in the liver. HMG-CoA reductase limits the rate of cholesterol biosynthesis, while cholesterol acyltransferase acylates cholesterol into cholesterol esters, which helps in the secretion of cholesterol by the liver. These activities of naringin were found to be mediated through up-regulation of hepatic PPARα gene and AMPK signalling pathway (53). This reinforces the protective effect of naringin against lipotoxicity and diabetic complications.
6.8. Oxidative stress

Chronic hyperglycaemia increases the oxidative stress. Oxidative stress increases lipid peroxidation, which in turn reduces cell membrane fluidity and causes cellular dysfunction. Beta cells have low intrinsic antioxidant defence mechanism, which make them more vulnerable to oxidative stress and this in turn reduces its insulin secretion (61, 153). Such susceptibility to ROS is probably due to low levels of key enzymes scavenging ROS, such as SOD, CAT, GSH (81). Additionally oxidative stress promotes the pathological changes related to hyperglycaemia resulting in diabetic complications (154).

In the present study, lower levels of enzymatic and non-enzymatic antioxidant markers observed in the diabetic rats improved with the administration of naringin. Naringin treatment also reversed the increase in lipid peroxidation marker in diabetic rats (Table 12). These changes can be ascribed to the established antioxidant activity of naringin. The results were comparable with the earlier studies by Sharma et al. (38), Parmar et al. (59) and Lim et al. (63). Thus, naringin offers protection to STZ induced diabetic animals from oxidative stress, which in turn improves insulin production, arrests the progression of disease and prevents complications.

Park et al. (155) reported that FoxM1 depleted cells were more susceptible to oxidative stress induced senescence and cell death. They further elucidated that cells expressing FoxM1 were more resistant to oxidative stress induced cell death. Naringin, a potent antioxidant by itself, up-regulates the expression of FoxM1 in beta cells. This expression of FoxM1 further augments the antioxidant defence mechanism of beta cells and protects the cells from apoptosis.

Moreover, the overall results of group VII animals treated with naringin for 8 weeks were much improved than the 4-week treated group VI animals. This suggests the need for prolonged use of naringin to achieve better therapeutic results.

Many molecules like GLP analogs, HGF, lactogen and IGF-1 have shown promising results in increasing the proliferation of rat and mouse beta cells. Nevertheless, their effects were limited in the human beta cells (156). Therefore, the changes observed in the rodent model should be replicated in the human beta cell
lines and reinforced by clinical trials enabling the development of naringin as a novel antidiabetic therapeutic agent. Further experimental investigations are needed to establish the up and down-regulatory mechanisms by which naringin modulates PDX-1 and FoxM1 expression to validate its clinical significance.

The present study establishes the role of naringin in up-regulation of molecular targets PDX-1 and FoxM1 involved in the maintenance and proliferation of beta cells. Further, it widens the scope of naringin usage from a mere antioxidant to pharmaceutical agent of proven mechanism, which can be developed into a novel drug for the treatment of diabetes.

6.9. Limitations

Even though the present study reports the role of naringin on beta cell transcription factors there are few limitations, which we are fully aware. The present study did not attempt to show immunohistochemically co-expression of insulin with PDX-1 or FoxM1 using double staining. Co-expression of insulin along with PDX-1 or FoxM1 would have proved beyond doubt that the cells expressing PDX-1 / FoxM1 are definitely beta cells. Further, the present study has not taken into account the fate of glucagon expressing cells in the islets. Therefore, it was not possible to determine the role of naringin on alpha cells and evaluate whether naringin facilitates transdifferentiation of alpha cells to insulin producing beta cells.

The main objective of the study was to ascertain the role of naringin on expression of PDX-1 and FoxM1 transcription factors. Therefore, we have not focussed on the up and down stream targets of these factors such as JNK, PPARγ, CENP-A and PLK-1 or the signalling pathways involving PI3K or calcium-calmodulin-NFAT. Having established the regulatory role of naringin on PDX-1 and FoxM1 expressions, we plan to include these up and down stream targets and signalling pathways in our future study to establish the molecular mechanism underlying naringin mediated beta cell maintenance and proliferation.