Chapter-6

Discussion

The major finding of our study is that PO-TRF as well as RBO-TRF has a potential to act as a nephroprotective agent against DN through a multifactorial approach. Supplementation of PO-TRF and RBO-TRF ameliorated the severity of renal dysfunction in type 1 and type 2 DM by attenuating hyperglycemia, hyperlipidemia and oxidative stress induced expression of TGF-β which in turn prevented the increased expression of fibronectin and collagen type IV in kidney. Another important finding is that amongst the two major sources of tocotrienols, PO-TRF can be considered as a better hypoglycemic and nephroprotective agent than RBO-TRF.

Animal models allow performing detailed and mechanistic testing, including direct examination of tissue to assess pathology that is difficult to perform in clinical studies. Also, the genetic background in inbred animal models is homogeneous and environmental factors can be controlled, therefore, they are valuable in genetic dissection of multifactorial diseases which is difficult in humans due to complex interaction among multiple susceptibility genes and between genetic and environmental factors. Moreover, diabetes research in humans is impeded by obvious ethical considerations, because provocation of disease is strictly impermissible in man. Hence we preferred to use an animal model for this study. At present, most of the available models are based on rodents because of their small size, short generation interval, easy availability and economic considerations (Srinivasan and Ramarao, 2007). Rat strains with genetic defects causing type 1 and 2 DM are expensive and are not easily available for the investigative purpose as well as regular screening experiments. The observations derived from these highly inbred genetic strains may not always be satisfactorily extended to the human population as a whole because of the large heterogeneity in the latter (Srinivasan et al., 2005). In addition, the genetic defect has not been well characterized yet in most of the models. Considering all these facts, many efforts were made to develop non genetic rat models for type 1 DM and type 2 DM that would on the one hand closely mimic the pathophysiology of human DM and on the other hand would be cheaper, easily available and useful for the investigation as well as preclinical testing of various compounds.

The non genetic rat model of type 1 DM used in our study was developed by injecting a single intraperitoneal injection of high dose of STZ (55mg/kg bw) as described in several studies (Budin et al., 2009; Kuhad and Chopra, 2009a). STZ has a selective cytotoxic effect on pancreatic β cells. It enters β cells via a glucose transporter GLUT2 (Schnedl et al. 1994), causes alkylation of DNA (Elsner et al. 2000) and induces generation of free radicals. Destruction of pancreatic β cells leads to reduced insulin secretion, thereby increasing plasma glucose levels. In the absence of proper glucose metabolism, the body starves. To compensate, more food is required in an attempt to provide energy to the body cells, hence there is almost an unavoidable intake of more food (*Polyphagia*). Also, the release of triglycerides from adipose tissue and catabolism of amino acids in muscle tissue is triggered to give energy. This leads to loss of body fat as well as lean mass resulting in loss of body weight as well increased serum levels of triglycerides. A build up of glucose within the bloodstream signals the body's natural defence to expel it. Consequently, the kidneys that tend to deal with many waste products excrete it in the form of frequent urination (*Polyuria*). As more fluid gets excreted, the body becomes dehydrated and a thirst signal is generated leading to increased consumption of fluids (*Polydipsia.*). In line with these facts, we found that the high dose STZ induced type 1 diabetic rat model used in our study developed hyperglycemia with insulin deficiency, dyslipidemia (hypertriglyceridemia), polyphagia, polydipsia and polyuria along with loss in body weight. These metabolic abnormalities are very similar to those seen in human type 1 DM.

The non genetic HFD/STZ rat model of type 2 DM used in our study was developed by feeding a high fat diet to the rats and then administration of low dose of STZ as described by Srinivasan et al. (2005) and Danda et al. (2005). Excessive fat intake causes an increased influx of triglycerides into the blood leading to an excess of plasma levels of free fatty acids, which induces insulin resistance (Le Marchand-Brustel et al., 2003). On the other hand, a single low dose of STZ causes a partial destruction of β cell mass to produce a mild insulin deficient state. The combined effect of HFD and low dose of STZ results in hyperglycemia. The increased levels of glucose stimulates pancreatic β

cells to secrete more and more insulin, generating hyperinsulinemia, which further triggers the elevation of triglycerides and closes the vicious circle (Kraegen et al., 2001). In concordance with these reports, we found that the HFD/STZ rat model of type 2 DM in our study developed obesity due to HFD, frank hyperglycemia, insulin resistance and hypertriglyceridemia. These metabolic abnormalities are very similar to those seen in human type 2 DM. Thus, we were able to successfully develop the non genetic rat models of both type 1 and type 2 DM. Both of these models also developed progressive proteinuria, mesangial expansion, diffuse and nodular glomerulosclerosis, tubular atrophy, elevation in BUN, increased serum creatinine and decreased GFR similar to human DN (Alebiosu et al., 2002; Loon, 2003). Thus, we believe that these are appropriate models to investigate therapeutic strategies against DN.

To our knowledge, the present study is the first to compare the hypoglycemic and nephroprotective effects of TRF obtained from PO and RBO against DN in experimental diabetic rat models. We demonstrated that when type 1 and type 2 diabetic rats were administered with PO-TRF and RBO-TRF, then PO-TRF lowered the blood glucose and improved the renal function more effectively than RBO-TRF at similar low doses and also the optimal dose of PO-TRF that showed maximal effect was achieved at a dose lower than optimal dose of RBO-TRF. One possible reason for the observed response could be the comparatively higher concentration of α-TOC in RBO-TRF. Although both PO and RBO are rich sources of T3, PO-TRF has a higher percentage of T3 than RBO-TRF. It is the T3 that is biologically more potent than TOC in terms of its hypoglycemic, hypolipidemic and antioxidant activities (Fang et al., 2010; Ajuluchukwu et al., 2007; Serbinova et al., 1991; Gu et al., 1999). It is also reported that T3 are less bioavailable after oral ingestion compared with α-TOC because of the presence of an alpha tocopherol transfer protein (α-TTP) that preferentially selects α-TOC than other forms of vitamin E (Arita et al.1995; Hosomi et al., 1997). Hence it is plausible to speculate that higher concentration of TOC in RBO-TRF might have reduced the bioavailability of T3 and diminished the threshold concentration of T3 in the kidney tissues that is required to afford effective protection against hyperglycemia, oxidative stress and lipid induced

kidney damage. On the other hand, the higher concentration of T3 in PO-TRF as well as the least interference by TOC might be responsible for its greater efficacy.

The role of hyperglycemia in the pathogenesis of DN has been previously established by a number of studies conducted in experimental animal models and human studies (Coimbra et al., 2000; Nangaku et al., 2005). It is reported that the elevation of glycosylated haemoglobin beyond 7% generally leads to DN in type 1 DM and type 2 DM (Loon, 2003). In our study, we found that the level of blood glucose was significantly high in both type 1 diabetic control rats (due to insulin deficiency) and type 2 diabetic control rats (due to insulin resistance). The level was much higher in type 1 than type 2 diabetic rats, indicating a relatively mild condition in type 2 DM. Thereafter, exposure of hemoglobin (Hb) to high levels of plasma glucose caused non-enzymatic glycation of Hb resulting in higher percentage of glycosylated Hb (8.2% in type 1 diabetic control rats and 7.8% in type 2 diabetic control rats). Inability of body to utilize the available blood glucose triggered the release of triglycerides from adipose tissue to give energy. Accordingly, we found that the serum triglycerides level was also significantly elevated in both type 1 and type 2 diabetic control rats consistent with previous reports (Danda et al., 2005; Mohammadi et al., 2009). The glycemic status and the serum triglyceride level of TRF supplemented diabetic rats improved significantly at the end of study period in both type 1 and type 2 DM. Our results are in concordance with earlier findings (Baliarsingh et al., 2005; Qureshi et al., 2002; Budin et al., 2009). This could be attributed to the finding of Fang et al. (2010) who established that T3 (and not TOC) within the TRF, act as modulators of PPAR (peroxisome proliferator-activated receptors). PPAR are transcriptional factors that regulate the expression of genes involved in carbohydrate and lipid metabolism. Binding of tocotrienol to PPAR enhance the expressions of glucose transporter 4(Glut 4), carnitine palmitoyl transferase 2 (CPT2) and uncoupling protein 3 (UCP3) which in turn promote insulin mediated glucose uptake and β-oxidation of fatty acids. Thus, besides triggering fatty acid utilization, TRF improves whole body glucose utilization by enhancement of insulin sensitivity.

Dyslipidemia is common in patients with DM and is considered as a risk factor for the progression of DN (Ravid et al., 1998). Diabetic patients often have multiple lipoprotein abnormalities such as, increased plasma levels of very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and triglycerides (Shoji et al., 2001). Several studies have found correlations between baseline lipid measures and rate of decline in kidney function (Manttari et al., 1995; Muntner et al., 2000; Schaeffner et al., 2003). Dominguez et al. (2000) found a direct linkage between renal injuries of rats with type 2 DM and elevated levels of blood LDL cholesterol. In the present study, we found that the level of TC, LDL-C and VLDL-C were significantly elevated whereas the level of HDL-C was significantly reduced in diabetic control rats compared to control rats in both type 1 and type 2 DM studies. The lipid levels improved significantly in TRF supplemented groups compared to diabetic control rats. Our results are in line with earlier reports (Budin et al., 2009; Chou et al., 2009). The mechanistic action behind the ability of T3 within TRF to lower serum cholesterol level had been attributed to its suppressive action on Hydroxy methyl glutaryl CoA reductase (HMGCR), a key enzyme in the cholesterol synthesis pathway (Parker et al., 1993). In vitro studies by Zaiden et al. (2010) have also demonstrated that T3 (but not TOC) suppress the upstream regulators of lipid homeostasis genes viz. Diacyl glycerol O-acyltransferase 2 (DGAT2), apolipoprotein B (APOB2), Sterol regulatory binding proteins-1/2 (SREBP1/2) and HMGCR leading to suppression of triglycerides, cholesterol and VLDL biosyntheses. Further, he also demonstrated that T3 enhances LDL efflux through induction of LDL receptor expression. Recent studies have suggested that metabolites of the mevalonate pathway play a critical role in cell proliferation (Corsini et al., 1993). Mevalonate is synthesized intracellularly from HMGCoA by the action of HMG-CoA reductase. Thus, it is plausible to speculate that inhibition of HMG-CoA reductase by T3 leads to loss or significant reduction of mevalonate, subsequently inhibiting DNA synthesis as well as cell proliferation in several types of cells, including mesangial cells. In our study, PO-TRF was found to be a better hypolipidemic agent than RBO-TRF. However, there are some conflicting reports, wherein Qureshi et al. (1997) suggested that the cholesterol-

lowering effect of TRF from RBO were even more encouraging than the effects seen in their previous studies with PO-TRF (Qureshi et al., 1991). We support our finding with the fact reported by Qureshi et al. (1996) who demonstrated that in chickens, the cholesterol-lowering action of the T3 might be attenuated by α-TOC. Hence it is plausible to speculate that higher concentration of α-TOC in RBO-TRF might have interfered with cholesterol-lowering action of T3 within the RBO-TRF, unlike PO-TRF where the percentage of T3 is much greater than TOC.

Reactive oxygen species (ROS) play an important role in DN. Superoxide anion has been implicated in the oxidation of LDL (Steinbrecher, 1988). Ox-LDL may be taken up by scavenger receptors on mesangial cells and monocyte-macrophages, resulting in foam cell formation (Schlondorff, 1993), alteration in renal hemodynamics via arachidonic acid metabolites (Kaplan et al., 1990), and induction of macrophage infiltration (Hattori et al., 1994). These events may contribute to renal damage. Increased oxidative stress in the kidney may also induce apoptosis that further contribute to the development of DN (Zhang et al., 1997; Murata et al., 2002). Generation of ROS and increased oxidative stress has been linked to hyperlipidemia and hyperglycemia. Hyperlipidemia increases vascular oxidative stress and generates an excess of superoxide anion within hypercholesterolemic vessels that can damage the membrane lipids of the vessels (Weiss et al., 1978; Cai and Harrison, 2000). Hyperlipidemia also leads to enhanced formation of peroxynitrite (Onody et al., 2003). Peroxynitrite can induce DNA damage, increase lipid peroxidation, and cause post-translational modification of proteins (e.g. nitration, oxidation of thiol groups), thereby activating or inhibiting certain enzymes like aconitase, superoxide dismutase etc. (Pacher et al., 2005). Synergistically, hyperglycemia also induces oxidative stress in the rat kidney (Sharma et al., 2006a; Sharma et al., 2006b) by not only generating more ROS via auto-oxidation of glucose but also by attenuating antioxidative mechanisms through glycation of the scavenging enzymes (Ha and Kim, 1999). In agreement with these findings, we found that the level of lipid peroxidation was significantly increased, whereas the levels of antioxidant enzymes like SOD, catalase, GPx and GR were significantly reduced in kidneys of

diabetic control rats compared to control rats in both type 1 and type 2 DM studies. We also found that TRF supplementation significantly restored the levels of lipid peroxidation and antioxidant enzymes in the kidneys of both type 1 and type 2 DM studies as demonstrated previously by Budin et al. (2009) and Kuhad and Chopra (2009a) , indicating protective role of TRF against oxidative stress. The antioxidant activities of T3 and TOC found in the TRF are rooted in their ability to donate phenolic hydrogens (electrons) to lipid radicals, acting as a peroxyl radical scavenger that terminates chain reactions of oxidation of polyunsaturated fatty acids (Kamal-Eldin and Appelqvist, 1996; Margherita et al., 2007). Additionally, their hypoglycemic and hypolipidemic activities prevented modification of antioxidant enzymes by high levels of glucose and lipids. Thus, it is prudent to conclude that oral supplementation with TRF might have protected nephrons in DN by antioxidant dependent mechanisms like suppressing oxidative stress induced apoptosis. Again PO-TRF displayed a stronger antioxidant activity than RBO-TRF in our study. This is explained by the presence of greater percentage of T3 than TOC in PO-TRF in contrast to RBO-TRF. Several studies have established that tocotrienols have higher antioxidant activity than tocopherol (Serbinova et al., 1991; Gu et al., 1999). Factors responsible for the higher antioxidant activity of T3 compared with TOC has been attributed to more uniform distribution of T3 in membrane bilayer than TOC that results in stronger disordering of membrane lipids, more effective collision with radicals and greater recycling activity of chromanoxyl radical than TOC All these factors positively correlate with inhibition of lipid peroxidation (Packer et al., 2001).

Accumulation of extracellular matrix (ECM) components in mesangial matrix, glomerular basement membrane and tubular basement membrane is the central abnormality in DN (Kim et al., 1991; Steffes et al., 1992). A growing body of evidences suggests that transforming growth factor-β (TGF-β), a fibrogenic cytokine is the final common mediator of the principal renal lesions such as glomerular hypertrophy and extracellular matrix expansion (Ziyadeh and Han, 1997,Goldfarb and Ziyadeh, 2001; Ziyadeh, 2004). In line with these findings, we observed that the protein expression levels of TGF β was significantly elevated in whole kidney lysates of diabetic control rats in

both type 1 and type 2 DM and correspondingly, there was substantial mesangial expansion accompanied with thickening of tubular basement membrane in histopathological slide of diabetic control rat kidney. Our results are in agreement with Kiyomoto et al. (1997) who noticed an increased expression of TGF β in the glomeruli of diabetic rats. Studies also report that TGF-β in particular, stimulates the expression of fibronectin, laminin, collagen IV, and other ECM proteins (Park et al., 1997; Mason and Wahab, 2003) in glomerular epithelial cells, glomerular mesangial cells and tubular epithelial cells (Creely et al., 1992; Yamamoto et al., 1996). In concordance, we found that the protein levels of ECM proteins like fibronectin and collagen type IV were significantly elevated in whole kidney lysates of diabetic control rats in both type 1 and type 2 DM. Our finding is in complete agreement with Danda et al. (2005) who demonstrated an increased expression of TGF-β, fibronectin and collagen type IV in whole kidney lysates of type 1and type 2 diabetic rats. However, the levels of TGF-β, fibronectin and collagen type IV were comparatively higher in kidney of type 2 diabetic rats as compared to type 1 diabetic rats in our study. The probable explanation could be that the immunublot analysis was carried out after a long period of 16 weeks in type 2 DM during which a greater damage occurred to the kidney as compared to damage that took place after 8 weeks study period in type 1 DM. The protein expression level of fibronectin was greater as compared to collagen type IV in all groups, indicating that fibronectin was mainly deposited in glomerulus and tubules of diabetic kidney and there was only a small deposition of collagen type IV.

The expression levels of TGF-β, fibronectin and collagen IV were significantly down regulated in TRF supplemented groups compared to diabetic control groups and there was only mild mesangial expansion and occasional thickening of tubular basement membrane in histopathological slide of P-200 and R-400 rat kidney in both type 1 and type 2 DM related study. Several studies have shown that high glucose concentrations stimulate the expression of TGF-β (Gilbert et al., 1998; Hill et al., 2000) in diabetic rat kidney. ROS like hydrogen peroxide is also reported to increase TGF-β and fibronectin production in mesangial cells (Ha and Kim, 1997). Further, Nishida et al. (1997) has

demonstrated that at certain concentrations, LDL, IDL and VLDL enhance the secretion of fibrogenic cytokines, such as TGF-β and/or other growth factors in human mesangial cells. All these findings confirm that hyperglycemia, hyperlipidemia and ROS act together to stimulate the expression of TGF-β in kidney. Hence it is plausible to speculate that TRF down regulated the expression of TGF-β owing to its hypoglycemic, hypolipidemic and antioxidant activities. Down regulation of TGF-β, in turn, prevented accumulation of fibronectin and collagen type IV in mesangial matrix and tubular basement membrane, thus preserving the glomeruli and tubules in TRF supplemented diabetic rats.

Mesangial expansion could also be attributed to increase in interglomerular pressure due to increased blood flow induced by greater NO production. Available data suggest that increased NO production leads to preferential afferent arteriolar dilation, glomerular enlargement and glomerular hyperfiltration (Bank and Aynedjian, 1993; Komers et al., 1994; Sugimoto et al., 1998). We found that the serum and urinary NO levels were higher in diabetic control rats compared to control in both type 1 and type 2 DM related studies. Our finding is consistent with the previous studies in animals and humans (Maree et al., 1996; Choi et al., 1999; Apakkan Aksun et al., 2003). We also found that supplementation of TRF significantly reduced the level of NO in both serum and urine. This protective effect of TRF can be linked to the finding of Wu et al (2008) who demonstrated that TRF exhibits anti-inflammatory property by suppressing lipopolysachride mediated expression of inducible NO synthase (iNOS).

When mesangium expands, it restricts and distorts glomerular capillaries and diminishes capillary filtration leading to decreased GFR. Increased serum creatinine and BUN in diabetic rats is taken as an index of altered GFR in diabetic nephropathy (Sugimoto et al., 1999). In agreement with Sugimoto et al, our results show that the levels of BUN and serum creatinine were significantly elevated whereas creatinine clearance was significantly reduced in diabetic control rats of both type 1 and type 2 DM. The observed alteration in GFR could be related to TGF-β stimulated expression of fibronectin and collagen type IV in glomerular mesangial cells and also to increased

interglomerular pressure resulting from increased NO production that ultimately caused substantial mesangial expansion in diabetic control rats. We also demonstrated that administration of TRF for 8 and 16 weeks significantly improved the GFR in type 1 and type 2 diabetic rats respectively, by lowering the levels of BUN and serum creatinine and increasing the creatinine clearance, implicating its nephroprotective action. We suggest that TRF improved GFR by downregulaing TGF-β induced expression of fibronectin and collagen type IV in the glomerular matrix and also by suppressing lipopolysachride mediated expression of inducible NO synthase (iNOS) to prevent increased production of NO that may lead to mesangial expansion.

The proteinuria that develops after induction of diabetes is mainly due to an increased excretion of an array of low molecular weight proteins where albumin comprises a relatively low proportion (5 to 20%) of total urinary protein (Koliakos et al., 2001). The mechanism for increased urinary excretion of low molecular weight proteins has been considered generally to be decreased tubular reabsorption of filtered plasma proteins (Woo and Lau, 1997). Recent reports provide evidence that high ambient glucose can induce DNA fragmentation (Ishii et al., 1996) and stimulate expression of apoptosis-regulatory genes (Ortiz et al., 1997) in renal proximal tubular epithelial cells; this may contribute to atrophy of the tubular epithelium in DN. TGF-β is also known to activate NADPH oxidase in tubular epithelial cells resulting in an increased ROS production in these cells (Jiang et al., 2003). In addition to their ability to directly inflict macromolecular damage, ROS can function as signaling molecules to activate a number of cellular stress-sensitive pathways that cause cellular damage (Ha and Lee, 2000). Consistent with these findings, we found that the tubules were extensively damaged in kidneys of diabetic control rats in both type 1 and type 2 DM rats and the amount of total protein excreted in urine was also significantly elicited in these groups. Several studies have also demonstrated that glomerular hyperfiltration leads to the development of proteinuria by increasing the blood flow and vascular permeability that provides the driving force for protein infusion into bowman's space (Mogensen, 1971; Chiarelli et al., 1995; Vedel et al., 1996) We found that the degree of proteinuria was reduced to a

significant level in TRF supplemented diabetic rats compared to diabetic control rats and the degree of proteinuria positively correlated to tubular damage in our study. The level of proteinuria was close to control values in PO-TRF supplemented rats and correspondingly the tubules were almost undamaged in P-200 group as seen in the histopathological analysis, whereas the level of proteinuria was higher in RBO-TRF supplemented group as compared to control and the tubules showed limited damage in R-400 group as evident from the histopathological slide. We suggest that TRF improved proteinuria by preventing hyperglycemia and TGF-β induced tubular injury and also by inhibiting NO synthesis that can induce glomerular hyperfiltration leading to protein infusion into bowman's space.

All these functional findings are in close agreement with the histological data. Significant structural abnormalities were seen in the glomerulus and tubules of diabetic control rats of both type 1 and type 2 DM. The observed glomerular and tubular injury could be attributed to TGF-β stimulated expression of fibronectin and collagen IV in glomerular mesangial cells, glomerular epithelial cells and tubular epithelial cells and also hyperglycemia and TGF-β induced oxidative stress in proximal tubular cells. The severity of the above mentioned abnormalities decreased considerably by treatment with TRF, indicating an effective protection offered by TRF in DN.

Experimental and clinical studies have shown that the optimal therapeutic approach in the treatment of DN should be intensive combined therapy targeting hyperglycemia, hypertension, proteinuria, and dyslipidemia (Graede et al., 1999). Our results suggest that both PO-TRF and RBO-TRF significantly ameliorated the development of abnormalities in renal structure and function of diabetic rats and the amelioration of DN by TRF was not restricted to only lowering of the blood glucose and proteinuria, but also involved improvement of the serum lipid profile, NO level and antioxidant status, thus providing a multifactorial approach for prevention of DN. Moreover, we did not observe any untoward effect of TRF in healthy rats in our study. Therefore, TRF may be a good source of nutrition and may provide beneficial health effects in diabetes related complications.