CHAPTER 2
MATERIALS AND METHODS

Spirogyra neglecta extract preparation

Dried SN was weighed and blended thoroughly followed by boiling at 100°C for 1 hr. The extract was then filtered through filter paper (Whatman, Kent, UK) with negative pressure pump (Hicovac, Köln, Germany). The filtrate was subsequently evaporated using lyophilizer (GEA process engineering inc., SC, USA). Lyophilized SN extract was stored at 4°C prior subsequent experiments.

Due to the previous study found that 1 g of SN extract contained antioxidant compounds, polyphenol, of 77.7 ± 3.6 mg GAE/g extract (Peerapornpisal et al., 2009). Therefore, to maintain the quality of SN extract, the purity was quantified to reach the minimum of 77.7 ± 3.6 mg GAE/g of SN extract before use in this study. In addition, it has been shown that there was no acute toxic effect in rats at the dose as high as 2000 mg/kg BW (Amornlerdpison et al., 2011) while the dose of 1000 mg/kg BW had anti-hyperglycemic and anti-hyperlipidemia in T2DM experimental rats (Lailerd et al., 2010). Therefore, SN extract at the dose of 1000 mg/kg BW daily was applied in this study.

Animal

Male Wistar rats with 120-150 g were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Thailand. The animal facilities and protocols were approved by the Laboratory Animal Care and Use Committee at Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. All experimental rats were housed in a room maintained at 25±1 °C on a 12:12 h dark–light cycle and allowed to acclimatize at least 1 week before the beginning of the experiments.

Induction of type 2 diabetic rats

Thirty rats were randomized and divided into two groups: normal and high-fat diet groups. The normal diet fed rats were consumed commercial available normal

chow diet (C.P. Mice Feed Food no. 082, Bangkok, Thailand), containing 20% of fat of total energy in the diet (%E) while the high-fat diet fed rats were received high-fat diet containing 60% energy of fat, ad libitum. The composition of diet is show in the Table 2.

Table 2 Compositions of normal and high-fat diet (high-fat diet ingredients were modified from Wikes, Bonen and Bell, 1998)

Ingredients	Normal diets		High-fat diet (HF) (g)	
	g	%E	g	%E
Carbohydrates	49.53	51.99	19.08	14.27
Fat	8.37	19.77	35.22	59.28
Protein	26.90	28.24	35.36	26.45
Vitamins and minerals	6.54	-	9.92	-
Fiber	3.43	-	4.32	-
Total	94.77	100	103.9	100
Kcal/g	4.02 kcal/g		5.15 kcal/g	

Energy per gram (kcal/g): carbohydrate = 4; fat = 9; protein = 4

After 2 weeks of dietary manipulation, the rats were induced type 2 diabetic model using the modified method as previously described (Srinivasan et al., 2005). The high-fat diet rats were intra-peritoneally injected with a single dose of 40 mg/kg BW of STZ (Sigma Aldrich, MO, USA) dissolved in 0.1 M citrate buffer while normal diet fed rats were given vehicle citrate buffer. Ten days after injection, the overnight fasting blood glucose was measured from whole blood collected from tail vein by a portable Glucometer (Roche Diagnostics Limited, IN, USA). The rats with fasting blood glucose level exceeded 250 mg/dl were considered diabetes. If any rat was not diabetes, it was excluded from this study.

Experimental design

A total of 30 rats (12 normal rats, 18 diabetic surviving rats) were divided into 6 groups (Figure 7):

Group 1: Normal control (NC)

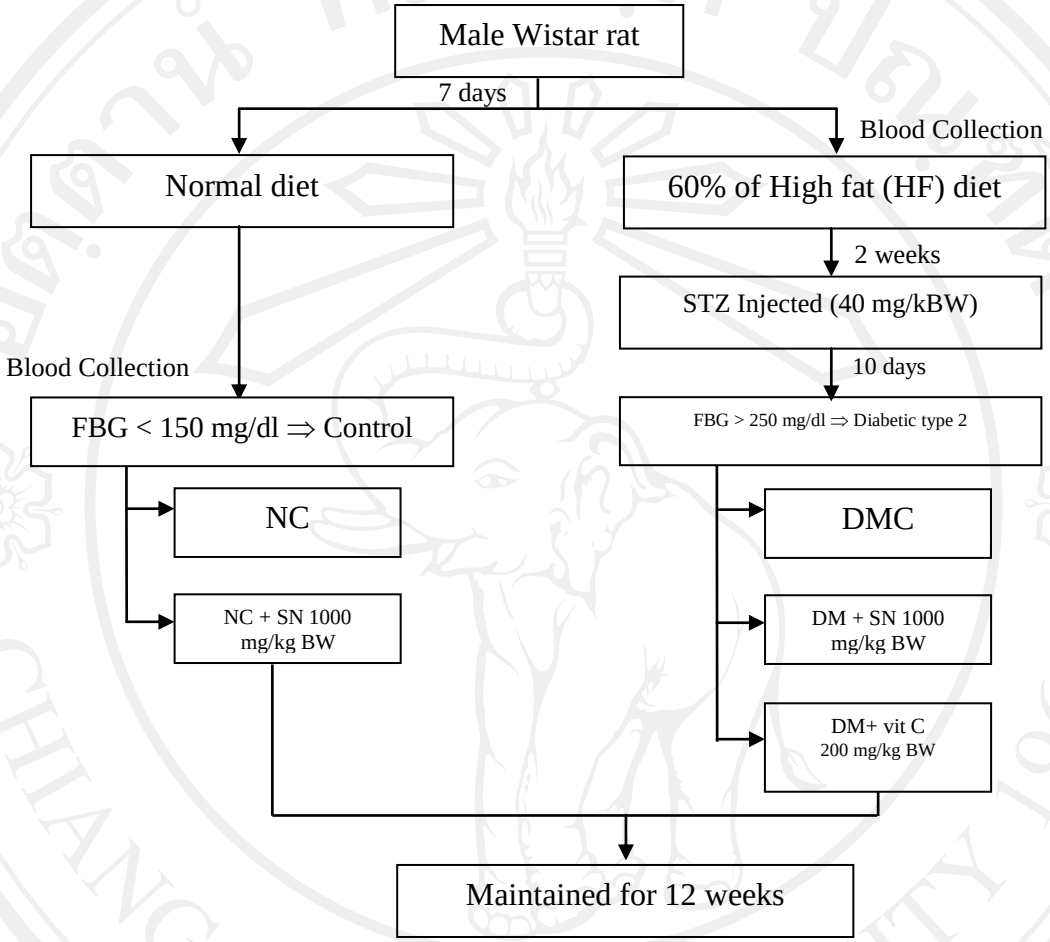
Group 2: Normal and SN extract supplement at the dose of 1000 mg/kg BW (NC+SN1000)

Group 3: Diabetic control (DMC)

Group 4: Diabetic and SN supplement at the dose of 1000 mg/kg BW (DM+SN1000)

Group 5: Diabetic and ascorbic acid (vit.C) (Merck, NJ, USA) supplement at the dose of 200 mg/kg BW (DM+vit.C)

SN extract and vit.C were dissolved in deionized water. Dose of SN was referred from the preliminary study of lipid peroxidation in renal tissues, showing that diabetic treated with SN extract at the dose of 1000 mg/kg BW improved lipid peroxidation. The dose of vit.C was modified from previous in-dependent studies, indicating that daily vit. C administration at the dose of 200 mg/kg BW significantly reduced plasma glucose and triglyceride ($p<0.01$) (Owu et al., 2006). The animals in group 2 and 4 were gavage-fed daily with SN extract at the dose of 1000 mg/kg BW until the 12th week after diabetic rats were considered. Similarly, the animals from group 5 were administered with vit.C at the dose of 200 mg/kg BW. The body weight of each rat was recorded weekly until the end of this study. The rats were allowed to continue feeding on their respective diet until the end of experiment.



Note: FBG = Fasting blood glucose, vit C = vitamin C

Figure 7 Diagram demonstrates the experimental design of this study.

Animal sacrifice

At the end of the experiments, the overnight fasted rats were euthanized using pentobarbital (Sanofi, Brussels, Belgium) at the dose of 40-50 mg/kg BW. The blood samples were obtained from the right atrium into 1.5-ml microcentrifuge tubes, containing either NaF or EDTA for determination of plasma parameters. The plasma was separated by centrifugation and stored at -20^{o}C until being assayed for triglyceride, insulin and glucose levels.

Subsequently, the kidneys were removed and weighed, and the renal cortex was then cut and used to determine the level of lipid peroxidation, rOat1 and rOat3 transport function and regulation.

Biochemical assays

To determine hyperglycemia, hyperlipidemia and insulin concentration, the quantitative total plasma glucose and triglyceride were determined by enzymatic colorimetric assays using commercial available kits obtained from Biotechnical Nology (Bangkok, Thailand). Furthermore, the plasma insulin concentrations were obtained using a Sandwich ELISA assay kit from LINCO research (Millipore, MA, USA). Insulin resistant index was estimated using the homeostasis assessment of insulin resistant (HOMA index) that was calculated by the following formula:

$$\frac{\text{Fasting plasma insulin (}\mu\text{U/ml)} \times \text{Fasting plasma glucose (mmol/L)}}{22.5}$$

Determination of renal oxidative stress condition

The measurement of renal cortical malondialdehyde (MDA) levels is a well-established method for determination of oxidative stress condition as previous study (Ramsammy et al., 1985). Renal tissues were prepared and modified according to the published method (Ohkawa et al., 1979). Briefly, renal cortical tissues were cut and suspended in lytic buffer, containing protease inhibitors according to the manufacturer's protocol. The tissues were then homogenized and centrifuged at 2,500 g for 10 min at 4°C and supernatant were collected for thiobarbiturate-malondialhyde (TBAR-MDA) assays (Cayman chemical, MI, USA).

Measurement of rOat1 and rOat3 transport functions and regulations using rat renal cortical slice experiments

Rat renal cortex was sliced to 0.5-mm thickness using a Stadie-Riggs microtome as previously described (Pritchard, 1990). Renal cortical slices were then maintained in freshly oxygenated ice-cold modified Cross and Taggart saline buffer, designated as an incubation buffer. To determine rOat1 and rOat3 functions and their regulatory functions, slices were incubated at room temperature (RT) for 30 min in the absence or presence of insulin at the concentration of 30 μg/ml in the incubation buffer and subsequently incubated with 20 μM of glurate, to generate an outwardly directed glutarate gradient, containing either 5 μM of [^{3}H]para-amino-hippurate (PAH), typical substrate for Oat1 and Oat3, or 50 nM [^{3}H]estrone sulfate (ES), specific substrate for Oat3, for 30 min. At the end of the experiment, the reaction was stopped using 0.1 M $MgCl_2$ and the slices were then blotted on filter paper, weighed and dissolved in 0.5 ml of 1 N NaOH and neutralized with 0.5 ml of 1 N HCl. The radioactivity was quantified by liquid scintillation spectroscopy (Perkin elmer, MA, USA). Transport activity was calculated as tissue to medium ratios (dpm/mg tissue ÷ dpm/ul medium).

Determination of rOat3 and the regulatory gene expressions of rOat1 and 3 using western blotting analysis

Subcellular Fractionation

Subcellular fractions were modified and obtained according to the previous study (Lockwich et al., 2000). Briefly, renal cortical tissues were cut, suspended in lytic buffer, containing protease inhibitors and subsequently centrifuged at 5,000 g for 10 min at 4°C. The supernatant was designated as *whole cell lysate* and the pellet was re-suspended in the same solution and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was then designated as *nuclei fraction*. In addition, whole cell lysate fraction was subsequently centrifuged at 100,000 g for 2 hrs at 4°C. The supernatant fraction from the spin was designated as the *cytosolic fraction*. The crude membrane pellets were re-suspended in the same buffer and designated as *membrane fraction*.

Protein concentration of the samples was measured using Bradford's assays (Bio-Rad, PA, USA) and stored at –80°C prior to use in subsequent experiments.

Determination of protein concentration

The quantitative total protein concentration was determined by colorimetric Bradford protein assay using commercially available kits (Bio-Rad, PA, USA). The principle of this method is based on the fact that an absorbance shifts in the Coomassie dye. The red form of Coomassie dye first donates its free electron to the ionizable group on the protein of interest, which causes a disruption of the native state of the protein, consequently exposing its hydrophobic pockets. The pockets of tertiary protein structure bind non-covalently to the non-polar region of the dye via van der Waals forces, positioning the positive amine groups in proximity with the negative charge of the dye. The bond is further strengthened by the ionic interaction binding of the protein, which stabilizes the blue form of Coomassie dye, and thus the amount of complex present in solution is a measurement for the protein concentration using absorbance spectrometry. Protein samples were stored at –80°C prior to use in subsequent experiments.

Electrophoresis and Western Blotting

Protein samples were resolved in 2X Laemmli solution and electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare, WI, USA). Non-specific bindings were then eliminated by blocking with 5% (w/v) non-fat dry milk in 0.05% Tween 20 in Tris-buffered saline (T-TBS) for 1 hr and subsequently incubated overnight with 1:500-1:1000 dilution of a desire specific primary antibody (rOat3, PKCα, p-PKCα, p65NFκB, PKCζ, p-PKCζ, β-actin, Na^+-K^+-ATPase). The membranes were then washed with T-TBS buffer and incubated with a corresponding 1:10,000 dilution of horseradish peroxidase-conjugated ImmunoPure secondary goat anti-rabbit IgG or anti-mouse IgG (Millipore, MA, USA) for 1 hr. The proteins were detected using Super Signal West Pico Chemiluminescent Substrate (GE Healthcare, WI, USA) and quantitatively analyzed by a Image J

program from Research Services Branch (RSB) of the National Institute of Mental Health (NIMH), (MD, USA).

Determination of oxidative gene expressions using polymerase chain reaction (PCR) technique

Total ribonucleic acid (RNA) extraction

Total RNA was purified from freshly isolated rat renal cortical tissues using total RNA extraction kit (Amresco, OH, USA). Briefly, 0.1 mg of rat cortical tissue was homogenized by Polymix PX-SR 90 D in 1 ml of the extraction reagent. Subsequently, chloroform was added and mixed to separate the three solution phases. RNA was then recovered in upper aqueous phase, which was mixed with 70% ethanol for extraction RNA out of other molecules and applied to the spin column. Subsequently, RNA was then bound to the column while DNA and nucleotides were removed in the washing steps using the washing buffer. The purified total RNA was extracted by elution buffer. The purity and integrity of the total RNA were determined by Synergy H4 spectrophotometry (Biotek, VA, USA). RNA concentration and the 260/280 optical density ratio were calculated. The purity ratio in the range of 1.8 to 2.0 was accepted and subsequently used in the further experiments.

First strand complementary DNA (cDNA) synthesis

First strand cDNA was obtained using iScript™ cDNA synthesis kit (Bio-rad, CA, USA). In brief, 1 μg of each total RNA template was added in the reaction mixture, containing cDNA master mix and reverse transcriptase. After spinning down, the reaction was placed in the PCR MJ Mini™ Gradient Thermal Cycler machine (Bio-Rad, CA, USA) in the condition of amplification as follow: 25 ° C for 5min, 42 °C for 30 min, 85 °C for 5 min.

Polymerase chain reaction (PCR)

The PCR reaction was obtained using commercial available kit (Vivantis, Selangor Darul Ehsan, Malaysia) by adding 0.5 μg of cDNA template in PCR master mix, containing PCR buffer, tag polymerase and specific primer set as shown in Table

3. The condition of DNA amplification was set as the following steps: 94 ° C for 2 min, 92 °C for 2 s, 58 °C for 30 s, 72 °C for 30 s and 72 °C for 7 min for 25-35 cycles of amplification depends on gene of interest. PCR products were subsequently separated in 2% agarose gel electrophoresis and band density was quantitatively determined using a Image J program from RSB of the NIMH/NIH, (MD, USA). The housekeeping gene, actin, was used as an internal control

Table 3 Details giving primer sequences and expected product sizes for the genes amplification

cDNA	Genbank Accession No.	Forward primer	Reverse primer	RT-PCR product size
Cu-Zn SOD	X05634	5'-GCA GAA GGC AAG CGG TGA AC	5'-TAG CAG GAC AGC AGA TGA GT	387 bp
GPx	NM030826	5'-CTC TCC GCG GTG GCA CAG T	5'-CCA CCA CCG GGT CGG ACA TAC	297 bp
CAT	AH004967	5'-GCG AAT GGA GAG GCA GTG TAC	5'-GAG TGA CGT TGT CTT CAT TAG CAC TG	670 bp
GADPH	NM002046	5'-AGC CTT CTC CAT GGT GGT GAA GAC	5'-CGG AGT CAA CGG ATT TGG TCG	308 bp
Actin	NM031144	5'-ATG GTG GGT ATG GGT CAG AA	5'-GGG GTG TTG AAG GTC TCA AA	241 bp

Cu-Zn SOD-copper zinc superoxide dismutase; GPx-glutathione peroxidase; CAT-catalase ; GAPDH- Glyceraldehyde 3-phosphate dehydrogenase.

Data analysis

Results obtained from the experimental groups were compared and analyzed. The information obtained from this objectives allowed us to investigate the physiological effect of SN extract and identify the mechanisms involved with the functions and regulations of rat renal transporters among normal control, type 2 diabetes and type 2 diabetes treated with SN extract.

Statistical analysis

Data was expressed as mean ± standard error (SE). Statistical differences were assessed using one-way ANOVA, followed by tukey post-hoc test using Statistical package for social sciences (SPSS version 17) (IL, USA). The differences were considered to be significant when $p < 0.05$.