CHAPTER 2

MATERIALS AND METHODS

2.1 Animal preparation and study design

Male Wistar rats (180-200 g body weight; n=20) were used in this study. All animals were obtained from the National Animal Care, Salaya Campus, Mahidol University, Thailand. The animals were housed in a temperature-controlled room (25 ± 0.5 °C) in individual cages at Laboratory Animal House, Faculty of Medicine, Chiang Mai University. Food and water were given *ad libitum* while animals were maintained on a light/dark cycles (12:12 hours). The experimental protocol was adhered to the "Guide for the Care and Use of Animals" and followed appropriate Chiang Mai University Standard Operating Procedure. After 1 week of acclimation before the beginning of the experiment, Wistar rats were randomly divided into two dietary groups (n=10 per group): the normal diet (ND) and the high-fat diet (HFD) group. The animals in the ND group received standard laboratory rodent diet (normal diet), in which has energy content of 4.02 kcal/g and 19.77% of total energy (%E) of the diet (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand) was from fat for 12 weeks. The animals in the HFD group received fat-enriched diet (high-fat diet), in which has energy content of 5.35 kcal/g and 59.28 %E of the diet was form fat for 12 weeks. The compositions of the normal and high-fat diet are shown in Table 2-1 and 2-2, respectively.

Table 2-1 Composition of normal diet (ND).

Composition	Normal diet		
	g	kcal	%E
Carbohydrate	495.30	1981.20	51.99
Fat	83.70	753.30	19.77
Protein	269.00	1076.00	28.24
Vitamins	65.40	-	-
Fiber	34.30	-	-
Total	947.70	3810.50	100
Kcal/g	4.02 kcal/g		

Table 2-2 Composition of high-fat diet **(**HFD).

Composition	High fat diet		
	g	kcal	%E
Carbohydrate	190.76	763.04	14.27
Fat	342.24	3080.16	57.60
Protein	353.60	1414.40	26.45
Cholesterol	10.00	90	1.68
Vitamins	85.19	-	-
DL-Methionine	3.00	-	-
Fiber	13.21	-	-
Yeast powder	1.00	-	-
Sodium chloride	1.00	-	-
Total	1000	5347.60	100
Kcal/g	5.35 kcal/g		

Composition and description of diets and nutrients were modified from Srinivasan et al. (Srinivasan et al., 2005). Total energy from diet was represented as %E. The energy value of food (kcal/g) was as follows: carbohydrate 4, fat 9, and protein 4.

The experimental animals were maintained in individual cages, in which had free access to fresh water and diet. Body weight and food intake were recorded every day. At the end of experimental studies, blood samples were collected from the tail after fasting for at least 5 hours. Blood samples for glucose assay were kept on ice in test tube precoated with sodium fluoride (NaF). Blood samples for insulin, free fatty acid (FFA), and triglyceride (TG) assay were kept on ice in test tube precoated with ethylene diamine tetraacetic acid (EDTA). Plasma glucose, insulin, FFA, and TG were separated and stored at -80 ºC for peripheral insulin resistance assay. Subsequently, the animals were deeply anesthetized with isoflurane and sacrificed by decapitation. Brains were rapidly removed and collected. Brain tissues were used to investigate 1) the neuronal morphology in the hippocampus by histological method, 2) the nNOS expression in the hippocampal CA1 regions by immunohistochemical analysis, and 3) the brain oxidative stress by measuring antioxidant/oxidant status. Finally, the visceral fat was removed and weighed. The summarized experimental protocol is shown in Figure 2-1.

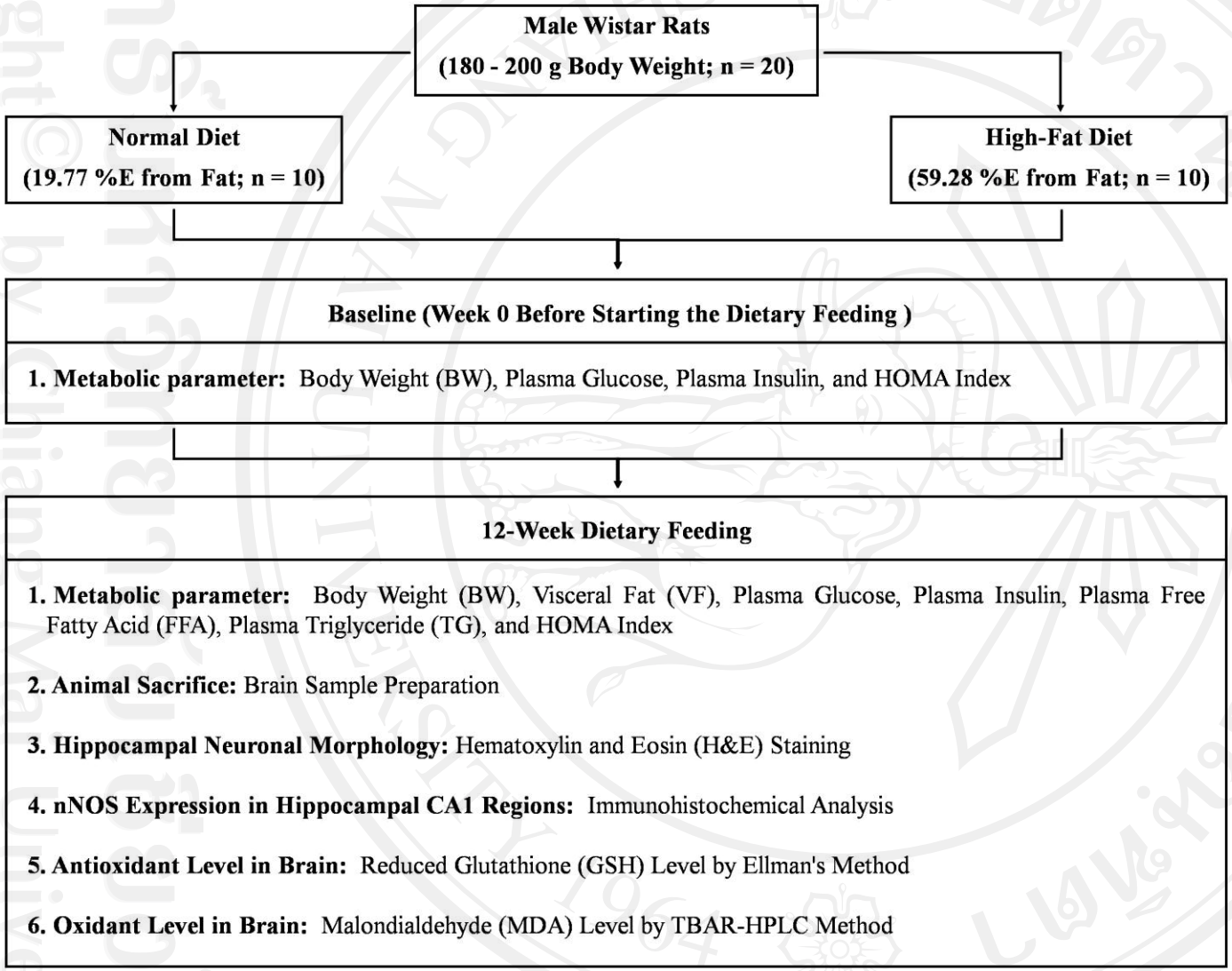

Figure 2-1 Schematic representation of the summarized experimental protocol.

2.2 Measurement of plasma glucose concentration

The plasma glucose concentration was determined by enzymatic colorimetric method using commercially enzymatic kit (Biotech, Bangkok, Thailand). The principle of this method is based on the fact that glucose in the sample is oxidized by the glucose oxidase (GOD) into the gluconic acid (gluconate) and hydrogen peroxide (H_2O_2). The amount of H_2O_2 formed is proportional to the glucose content of the sample. In the presence of peroxides (POD), the H_2O_2 formed affects the oxidative coupling of phenol and 4-aminoantipyrine (4-AAP) to form a red-colored quinoneimine. The absorbance of the quinoneimine was using Trinder indicator reaction by a spectrophotometer (UV-1700, UV-visible spectrophotometer, Shimadzu) at 505 nm against the blank.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{GOD} \text{Gluconic acid} + H_2O_2 \quad \text{..... (1)}$$

$$2H_2O_2 + \text{4-AAP} + \text{Phenol} \xrightarrow{POD} \text{Quinoneimine} + 4H_2O \quad \text{..... (2)}$$

2.3 Measurement of plasma insulin concentration

Plasma insulin concentrations were determined using a rat/mouse insulin ELISA kit (Millipore, MI, USA). The principle of this method is based sequentially on 1) the capture of insulin molecules from samples to the wells of a microtiter plate coated by the pre-titered amount of a monoclonal mouse anti-rat-insulin antibodies and the binding of the biotinylated polyclonal anti-rat-insulin antibodies to the capture insulin, 2) the washing away of the unbound materials from the samples, 3) the binding of horseradish peroxidase (HRP) to the immobilized biotinylated antibodies, 4) the washing away of the free enzyme conjugates, and 5) the quantification of

immobilized antibody-enzyme conjugates by monitoring HRP activities in the presence of the substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB). The methods for measuring plasma insulin concentration is shown in Figure 2-2.

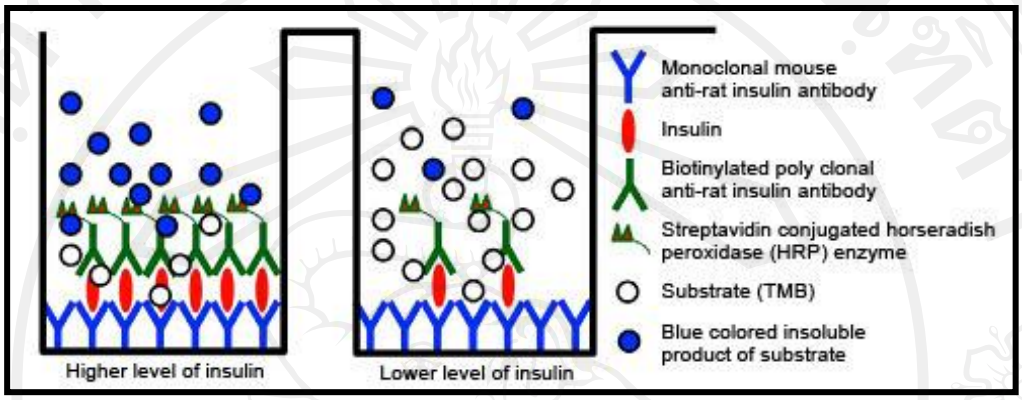

Figure 2-2 Schematic representation for the estimation of insulin concentration in the samples using rat/mouse insulin ELISA kit.

The enzyme activity was then measured by a microplate reader (BioTek Instrument, VT, USA) at 450 nm, corrected from the absorbance at 590 nm, after the acidification of the formed products. Since that increase in absorbance is directly proportional to the amount of captured insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with a reference standard of known concentrations of rat insulin.

2.4 Measurement of plasma FFA concentration

The plasma FFA (non-esterified fatty acid, NEFA) concentrations were determined using a commercially enzymatic kit (NEFA test, Wako Pure Chemical, Osaka, Japan). The principle of this method is that the FFA in the sample is converted to acetyl-coenzyme A (acyl-CoA), adenosine 5' monophosphate (AMP),

and pyrophosphoric acid (PPi) by the action of Acyl-CoA synthase (ACS) under coexistence with coenzyme A (CoA) and adenosine 5' triphosphate (ATP). Subsequently, the acyl-CoA is oxidized and yields 2, 3-trans-Enoyl-CoA and H_2O_2 by the action of Acyl-CoA oxidase (ACOD). In the presence of peroxidase, the H_2O_2 that is formed yields a blue purple pigment by quantitative oxidation condensation with 3-methyl-N-ethyl-N-(β-hydrooxyethyl)-aniline (MEHA) and 4-APP. The absorbance of the solution was measured by a spectrophotometer (UV-1700, UV-visible spectrophotometer, Shimadzu) at 550 nm against the blank.

$$\text{R-COOH} + \text{ATP} + \text{CoA} \xrightarrow{ACS} \text{Acyl-CoA} + \text{AMP} + \text{PPi} \quad \text{..... (1)}$$

$$\text{Acyl-CoA} + O_2 \xrightarrow{ACOD} \text{2, 3-trans-Enoyl-CoA} + H_2O_2 \quad \text{..... (2)}$$

$$2H_2O_2 + \text{4-AAP} + \text{MEHA} \xrightarrow{POD} [\text{Pigment}]OH^- + 3H_2O \quad \text{..... (3)}$$

2.5 Measurement of plasma TG concentration

The plasma triglyceride concentrations were determined using a commercially enzymatic kit (Biotech, Bangkok, Thailand). The principle of this method is that the TG in the sample is hydrolyzed by the lipoprotein lipase into glycerol and three FFAs. In the presence of ATP and glycerolkinase (GK), glycerol forms glycerol-3-phosphate that is further oxidized by glycerol-3-phosphate-oxidase (GPO) into dihydroxyacetone phosphate (DAP) and H_2O_2. The enzyme peroxidase in the substrate-chromogen solution catalyses the reaction between H_2O_2 and 4-APP to form a red colored quinoneimine complex. The absorbance of the quinoneimine was measured by a spectrophotometer (UV-1700, UV-visible spectrophotometer, Shimadzu) at 505 nm against the blank.

$$\text{Triglyceride} + 3H_2O \xrightarrow{Lipase} \text{Glycerol} + 3\ \text{FFA} \quad \text{..... (1)}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{GK} \text{Glycerol-3-Phosphate} + \text{ADP} \quad \text{..... (2)}$$

$$\text{Glycerol-3-Phosphate} + O_2 \xrightarrow{GPO} \text{DAP} + H_2O_2 \quad \text{..... (3)}$$

$$2H_2O_2 + \text{4-AAP} \xrightarrow{POD} \text{Quinoneimine} + HCl + H_2O \quad \text{..... (4)}$$

2.6 Peripheral insulin resistance detection

The Homeostasis Model of Assessment (HOMA) index reflects the peripheral insulin resistance in the fasting state (Appleton et al., 2005; Haffner et al., 1997). The HOMA index was calculated from fasting plasma glucose and fasting plasma insulin concentration (de Vinuesa et al., 2006). A higher HOMA index indicates a higher degree of peripheral insulin resistance. The HOMA index was calculated by the following equations (Muniyappa et al., 2008; Satirapoj et al., 2011):

$$\text{HOMA index} = \frac{[\text{fasting plasma glucose (mmol/l)} \times \text{fasting plasma insulin (}\mu\text{U/ml)}]}{22.5}$$

2.7 Hippocampal morphological analysis

In the present study, we used paraffin-embedded hematoxylin and eosin (H&E) sections for evaluation of the morphological appearance of neurons after 12 weeks of dietary consumption. For H&E staining, brains of the each experimental group were fixed in 4% paraformaldehyde. Subsequently, fixed brains were routinely processed and embedded in the coronal plane in paraffin wax. The paraffin-embedded brain tissues were serially sectioned at 5 μm thickness. Afterward, the paraffin cross sections containing hippocampus were mounted on glass slides and stained with hematoxylin and eosin according to the standard procedure. The

neuromorphological analysis of hippocampal tissue was studied under a light microscope (Gibthai, Bangkok, Thailand). Finally, images were captured and processed by a computerized microscope system (Thai Microscopy Co., Ltd, Bangkok, Thailand).

2.8 nNOS immunohistochemical analysis

Brains were pre-fixed in 4% paraformaldehyde and subsequently post-fixed in ice-cold 30% sucrose overnight. After that, the brain at the hippocampal CA1 regions was frozen and cut on a Thermo Scientific Microm HM 430 Sliding Microtome (A. S. Science, Bangkok, Thailand) into 40 µm coronal sections. The hippocampal slices were rinsed in TBS/TX containing 2.7% NaCl, 0.3% Triton-X, and 50 mM Tris Base (pH 7.5-7.6) for 5 minutes (3 times). Afterward, the hippocampal slices were incubated with 250 µl/well of 4% normal goat serum (NGS) in TBS/TX for 1 hour. After blocking, the hippocampal slices were randomly divided into 2 groups: negative control and nNOS-positive group. The negative control groups were incubated with 300 µl/well of 4% NGS in TBS/TX overnight at 4 ºC. The nNOS-positive groups were incubated with 300 µl/well of rabbit polyclonal anti-nNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 4% NGS (1:400) overnight at 4 ºC.

Subsequently, the hippocampal slices were transferred into 24-well plate and rinsed in TBS/TX for 5 minutes (3 times). The hippocampal slices were incubated with 250 µl/well of goat anti-rabbit IgG biotin conjugated secondary antibody in 4% NGS (1:200) for 1 hour and then rinsed in TBS/TX for 5 minutes (3 times). Afterward, the hippocampal slices were incubated with 250 µl/well of vectastain ABC

peroxidase kit (Vector Laboratories, Burlingame, CA, USA) for 1 hour and rinsed in TBS/TX for 5 minutes (3 times). The hippocampal slices were incubated with 250 µl/well of DAB solution (Peroxidase Substrate Kit, Vector Laboratories, Burlingame, CA, USA) for 5 minutes. After the slices were brown color, the hippocampal slices were rinsed in TBS/TX for 5 minutes (3 times).

The hippocampal slices were placed onto glass slide and air dry overnight. Subsequently, the hippocampal slices were dehydrated in an alcohol series, 50% (30 seconds), 70% (30 seconds), 90% (1 minute), 100% (30 seconds), cleared in toluene (30 seconds) and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ, USA). Finally, the total number of nNOS-positive cells in CA1 regions of hippocampal slices were observed using an eyepiece micrometer (Olympus CH30RF200, Olympus, Tokyo, Japan) and photographed with a computerized microscope system (Thai Microscopy Co., Ltd, Bangkok, Thailand). The summarized experimental protocol for nNOS immunohistochemical method and slide preparation are shown in Figure 2-3 and 2-4, respectively.

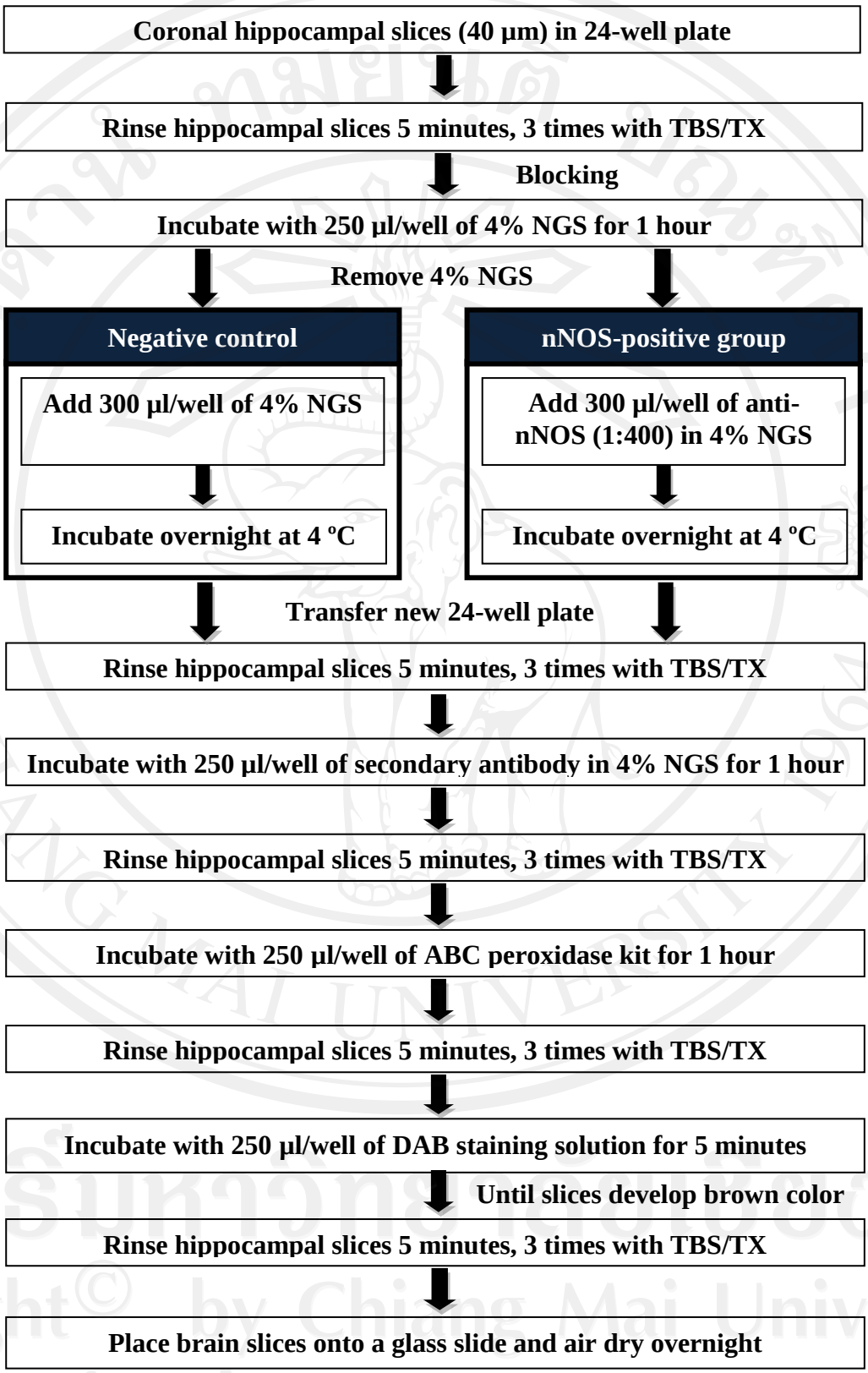

Figure 2-3 Schematic representation for nNOS immunohistochemical method.

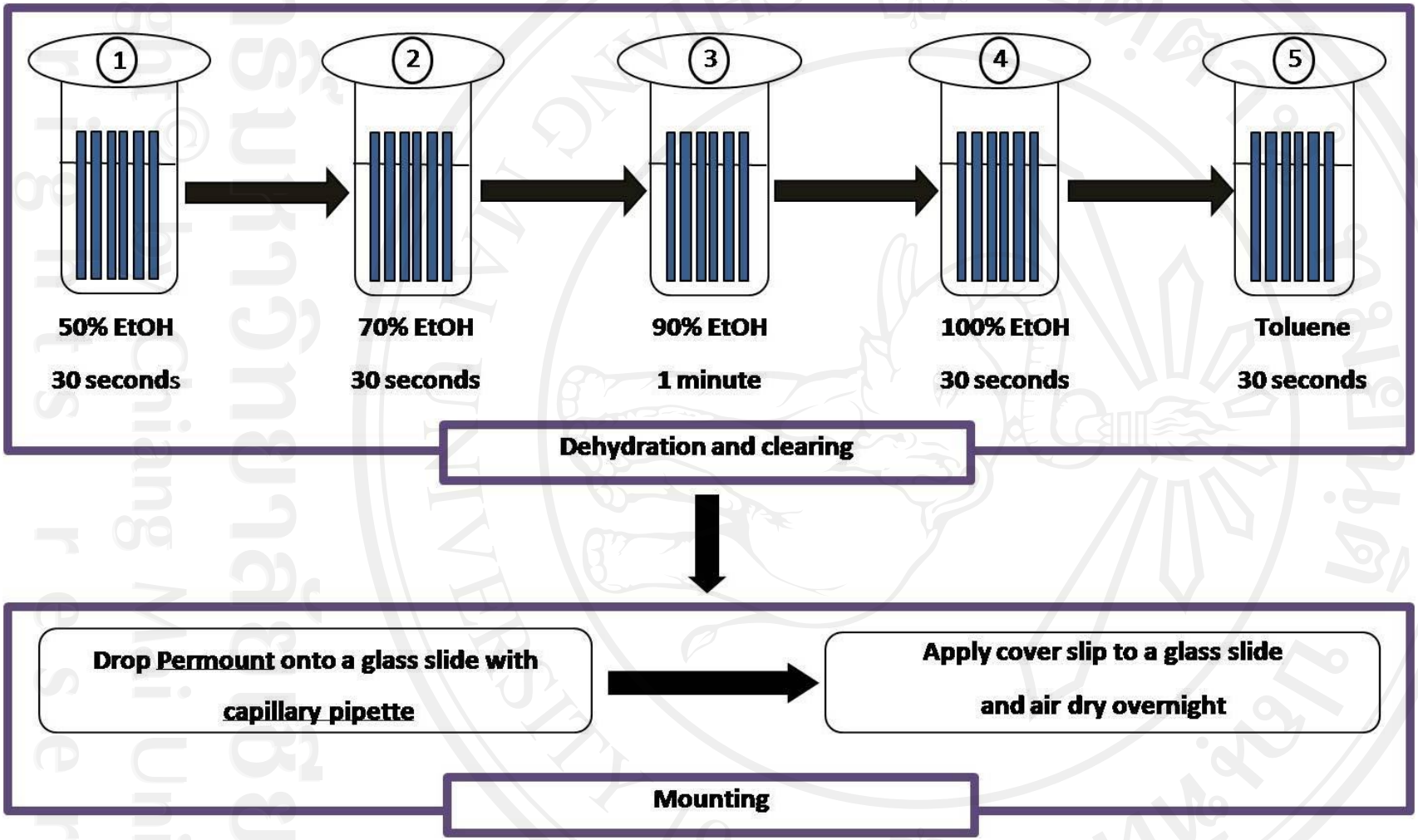

Figure 2-4 Schematic representation for slide preparation.

2.9 Brain homogenate preparation

Brain tissue was homogenized with a Teflon-glass homogenizer at 800 rpm (10 strokes) in 500 µl of ice-cold brain lysis buffer containing 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% nonyl phenoxypolyethoxylethanol (NP-40), 1% Triton X-100, and a protease inhibitor cocktail (Roche complete mini-tablets, Roche Molecular Biochemical, IN, USA). Finally, the brain homogenate was collected and frozen at -80 ºC for analysis of oxidative stress status.

2.10 Protein concentration assay in brain homogenate

The total protein concentrations were assessed by determining the Bradford protein assay (a colorimetric protein assay) in rat brain homogenate. The method is based on the proportional binding of the red cationic Coomassie Brilliant Blue (CBB) G-250 dye (λ_{max} = 470 nm) to negatively charged proteins and stabilize blue anionic complex (λ_{max} = 595 nm) under acidic condition.

For Bradford protein assay, the brain homogenates (10 µl) were incubated with 200 µl of Coomassie Brilliant Blue G-250 dye (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 25 °C for 10 minutes. The absorbance of the blue anionic complex of sample was measured by a spectrophotometer (UV-1700, UV-visible spectrophotometer, Shimadzu) at 595 nm against the blank. Furthermore, protein concentration of each sample was constructed by plotting the absorbance against the respective concentrations of standard protein, bovine serum albumin (BSA), ranging 0-1000 µg/ml. The protein concentration in each unknown sample was read out by interpolation of its absorbance on the standard curve. The summarized experimental protocol for protein quantification is shown in Figure 2-5.

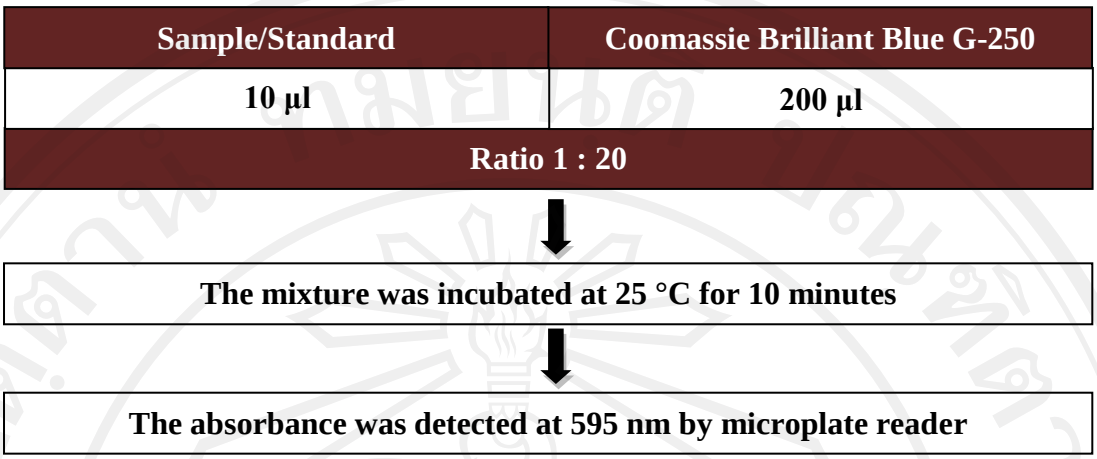

Figure 2-5 Schematic representation for protein quantification.

2.11 Determination of antioxidant level in brain homogenate

The antioxidant level in brain homogenate was determined by measuring reduced glutathione (GSH) level. The GSH is a tripeptide (γ-glutamyl-cysteinyl-glycine) with a reactive sulfhydryl group (Awasthi et al., 2009). It plays a vital role in the several cellular functions including cell differentiation, cell proliferation and regulation of the thiol-redox states of the cell (Ballatori et al., 2009). Moreover, it can also regulate defense mechanisms against reactive oxygen/nitrogen species (ROS/RNS) and the environmental pollutants (Awasthi et al., 2009). Therefore, intracellular GSH concentrations are an indicator of the healthy cells.

The Ellman's method was used for the quantification of GSH level. The brain homogenate (50 µl) was precipitated with 50 µl of 10% trichloroacetic acid (TCA). The mixture was centrifuged at 1,500 rpm for 15 minutes at 25 ºC to achieve clear supernatant. The supernatant (50 µl) was incubated with 200 µl of Tris HCl (pH 8.9) and 20 µl of a colorless 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB or Elman's reagent). The reaction of DTNB and GSH produces a yellow 5-thio-2-nitrobenzoic

acid (TNB) (Ellman, 1959; Fujioka et al., 1987). The absorbance of the TNB was measured by a spectrophotometer (UV-1700, UV-visible spectrophotometer, Shimadzu) at 415 nm against the blank. The GSH level was determined from the standard curve and expressed in µmol/mg protein. A standard curve of GSH was constructed by plotting the absorbance against the standard GSH, ranging 0-1000 µM. The summarized experimental protocol for determination of GSH level in brain homogenates is shown in Figure 2-6.

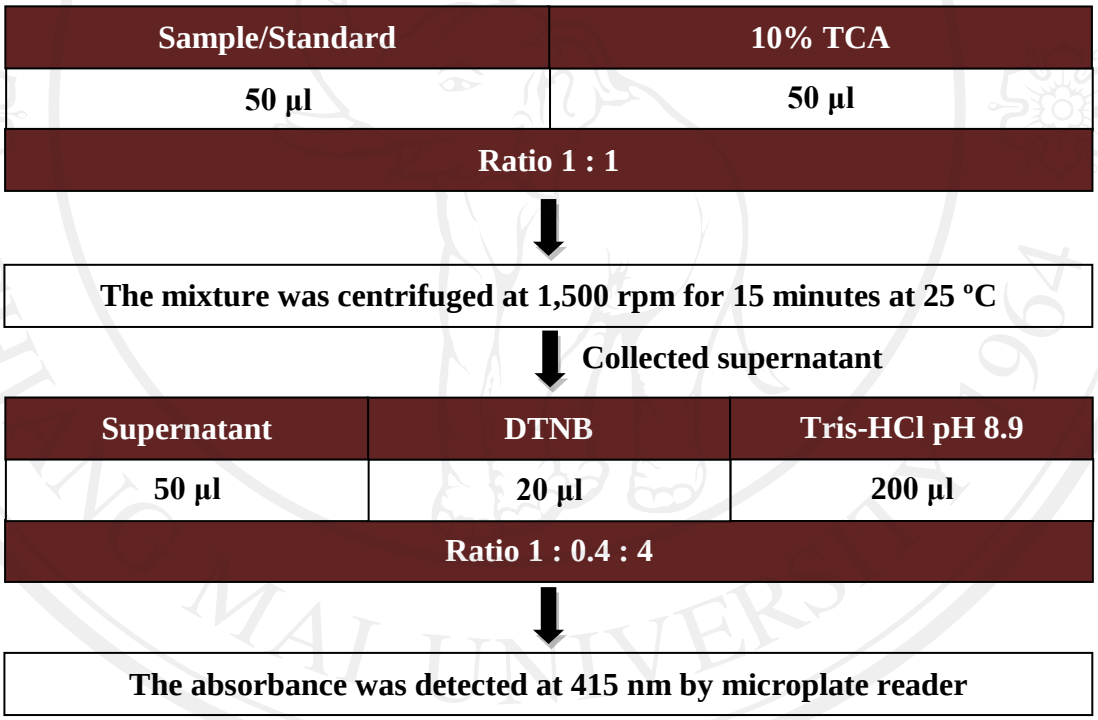

Figure 2-6 Schematic representation for determination of antioxidant level.

2.12 Determination of oxidant level in brain homogenate

Malondialdehyde (MDA) is a potential biomarker most frequently used to determine oxidative damage to lipid in cells and tissues (Meng et al., 2011). MDA is measured in the form of thiobarbituric acid reactive substances (TBARS) using high

performance liquid chromatography (HPLC). In the present study, the oxidant level in brain homogenate was determined by measuring MDA level.

An aliquot (40 µl) of brain homogenate was incubated with 88 µl of 10% TCA containing 50 ppm of butyrated hydroxytoluene (BHT) at 90 °C for 30 minutes and cooled down to room temperature (RT). The mixture was centrifuged at 6,000 rpm for 10 minutes to achieve clear supernatant. Supernatant (90 µl) was incubated with 270 µl of 440 mM phosphoric acid (H_3PO_4) and 180 µl of 0.6% thiobarbituric acid (TBA) solution at 90 ºC for 30 minutes to produce pink-colored products called thiobarbituric acid reactive substances (TBARS). The solution was passed through a syringe filter (polysulfone type membrane, pore size 0.45-µm, Whatman International, Maidstone, United Kingdom) and analyzed with the HPLC system. The TBARS was fractionated on the adsorption column (Water Spherosorb ODS2 type, 250×4.3 mm, 5 µm), eluted with mobile-phase solvent of 50 mM potassium dihydrogen phosphate (KH_2PO_4) : methanol (65 : 35 *v*/*v*) at flow rate of 1.0 ml/minute and on-line detected at 532 nm. Data was analyzed with BDS software (BarSpec, Rehovot, Israel).

A standard curve was constructed from the high peak of standard reagent for MDA, 1, 1, 3, 3-tetramethoxypropane (TEP), at different concentrations (0-100 µM). The TBARS concentration was determined directly from the standard curve and reported as MDA equivalent concentration. MDA concentration was expressed in nmol/mg protein for brain homogenate. The summarized experimental protocol for determination of oxidant level in brain homogenates is shown in Figure 2-7.

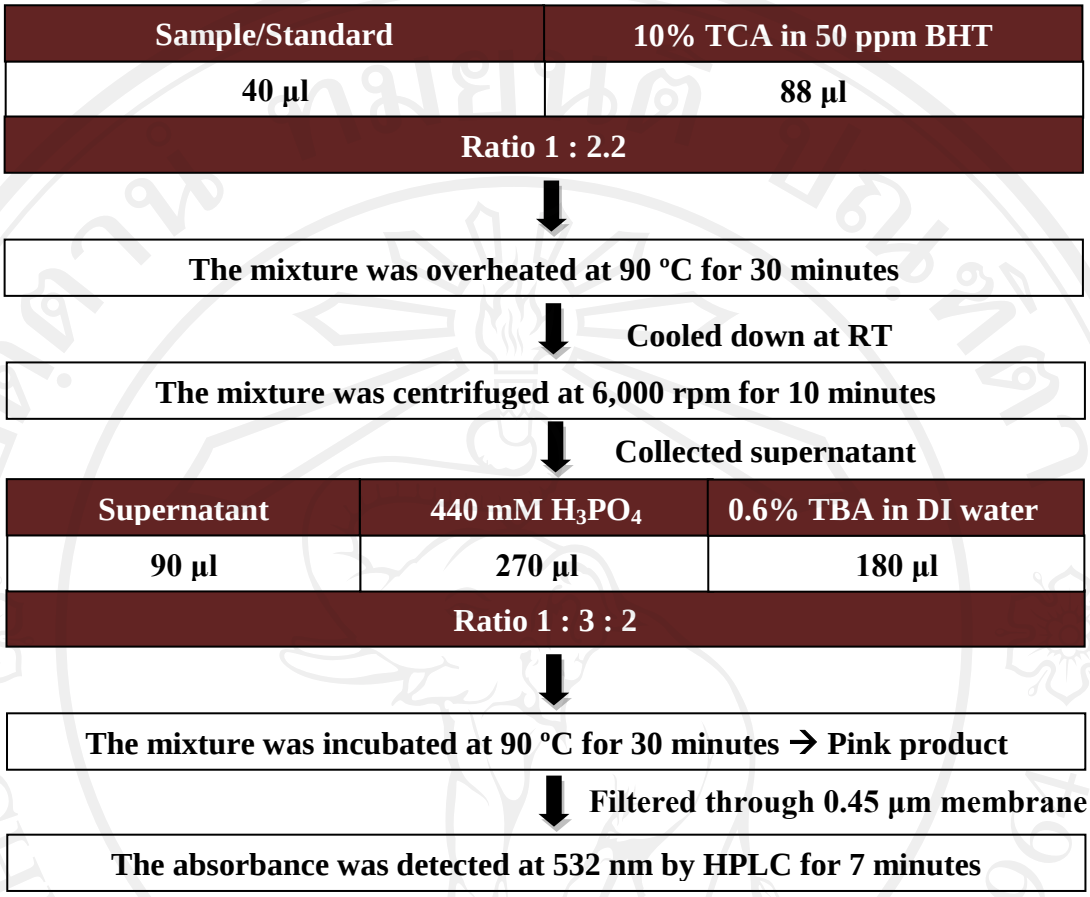

Figure 2-7 Schematic representation for determination of oxidant level.

2.13 Statistical analysis

All experimental data were presented as mean ± SEM. Statistical analyzes were performed using the SPSS software (version 20, IBM SPSS Statistics, Chicago, IL, USA). Comparisons between dietary groups were analyzed using independent sample *t*-test. Pearson's correlation analysis was used to determine the relationship among peripheral insulin resistance parameters, hippocampal nNOS expression, and brain oxidative stress in all experimental animals. *P* value < 0.05 was considered statistically significant difference.