# CHAPTER 2

# Materials and Methods

## 2.1 Methodological approach for research specific aims

**Aim 1:** To investigate the effects of estrogen deprivation on metabolic parameters in obese-insulin resistant condition

**Study protocols for aim 1**

All experimental protocols were approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. Thirty-two female Wistar rats weighing about 200-220 g (aged about 6 weeks old) were acquired from the National Animal Center, Salaya Campus, Mahidol University, Thailand. All animals were housed with n=2 in a temperature-controlled room and maintained on a light-dark cycle of 12:12 h (lights on at 6 a.m.) for 1 week prior to the following experiments for acclimatization. One week after arrival, female rats were randomly assigned to feed either normal diet (ND) or high-fat diet (HF). The normal diet (ND; n=16) group was given a standard laboratory chow (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand), which has energy content of 4.02 kcal/g, and 19.77% of total energy (%E) of the food was from fat. The high-fat diet (HF; n=16) group was consumed high-fat diet, which has energy content of 5.35 kcal/g and contains fat mostly from lard (59.28% E). The composition of the ND and HF were shown in Tables 2.1 and 2.2, respectively. All animals were given ad libitum access to food and water. The daily amount of calorie intake and weekly body weight were monitored. Blood samples were taken from the tail veins after fasting for at least 5 hours. Plasma was collected after centrifuged at 6,000 rpm, 4°C for 10 minutes and kept at -80$^{o}$C until needed for determined plasma glucose, insulin, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglyceride levels. After 13 weeks of either ND or HF consumption, each diet group was divided into sham-operated (S) and bilateral ovariectomized (O) groups for 7 weeks. Therefore, animals were subdivided into four

subgroups, including sham-operated female rats fed with normal diet (NDS), ovariectomized female rats fed with normal diet (NDO), sham-operated females rats fed with high-fat diet (HFS), and ovariectomized female rats fed with high-fat diet (HFO). The daily amount of calorie intake and weekly body weight were recorded. Blood samples were taken for determined plasma glucose, insulin, cholesterol, HDL, LDL, and triglyceride levels. After that, the oral glucose tolerance test (OGTT) was investigated. Visceral fat and uterus were removed and weighed. Blood was collected and stored at -80$^{o}$C for determined serum MDA level and estradiol level. Experimental protocol for aim 1 was illustrated in Figure 2.1.

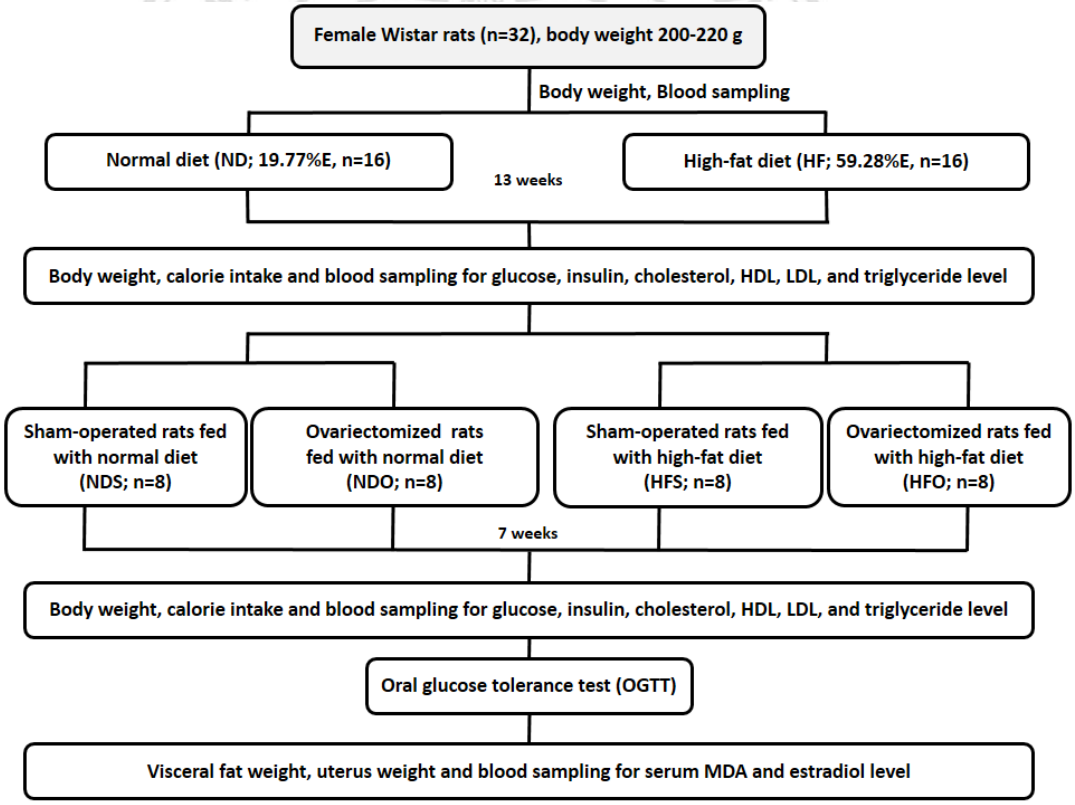


**Figure 2.1** Represents the flow charts of experimental protocol 1

**Aim 2:** To investigate the effects of estrogen deprivation on hippocampal and cortical reactive oxygen species and hippocampal synaptic plasticity in the obese-insulin resistant condition

**Study protocols for aim 2**

All experimental protocols were approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. Thirty-two female Wistar rats weighing about 200-220 g (aged about 6 weeks old) were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand. All animals were housed with n=2 in a temperature-controlled room and maintained on a light-dark cycle of 12:12 h (lights on at 6 a.m.) for 1 week prior to the following experiments for acclimatization. One week after arrival, female rats were randomly assigned to feed either normal diet (ND) or high-fat diet (HF). The ND (n=16) group was given a standard laboratory chow (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand), which has energy content of 4.02 kcal/g, and 19.77%E of the food was from fat. The HF (n=16) group was consume high-fat diet, which has energy content of 5.35 kcal/g and contains fat mostly from lard 59.28%E. All animals were given ad libitum access to food and water. After 13 weeks of either ND or HF consumption, each diet group was grouped into sham-operated (S) and bilateral ovariectomized (O) groups for 7 weeks. Therefore, animals were subdivided into four subgroups, including sham-operated female rats fed with normal diet (NDS), ovariectomized female rats fed with normal diet (NDO), sham-operated females rats fed with high-fat diet (HFS), and ovariectomized female rats fed with high-fat diet (HFO). At the end of the experiment, the rats were anesthetized with isoflurane and sacrificed by decapitation. The brain was rapidly removed for brain slice preparation to determine hippocampal synaptic plasticity by electrical induced LTP, hippocampal ROS production, cortical ROS production, hippocampal apoptosis, cortical apoptosis, hippocampal estradiol level, and cortical estradiol level. Experimental protocol for aim 2 was illustrated in Figure 2.2.

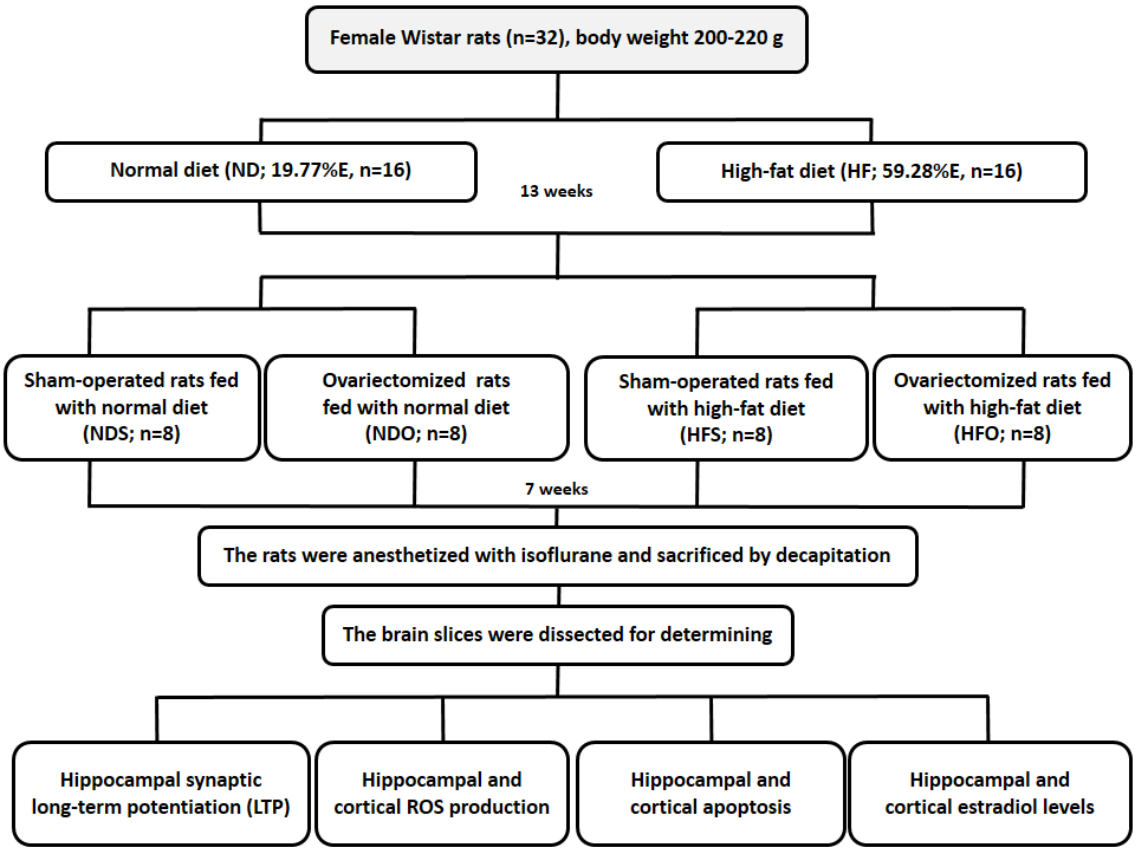


**Figure 2.2** Represents the flow charts of experimental protocol 2

**Aim 3:** To investigate the effects of estrogen deprivation on cognitive function (both hippocampal-dependent and hippocampal-independent memory) in the obese-insulin resistant condition

**Study protocols for aim 3**

All experimental protocols were approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. Thirty-two female Wistar rats weighing about 200-220 g (about 6 weeks old) were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand. All animals were housed with n=2 in a temperature-controlled room and maintained on a light-dark cycle of 12:12 h (lights on at 6 a.m.) for 1 week prior to the following experiments for acclimatization. One week after arrival, female rats were randomly assigned to feed either normal diet (ND) or high-fat diet (HF). The ND (n=16) group was given a standard laboratory chow (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand), which has energy content of 4.02 kcal/g, and 19.77%E of the food

was from fat. The HF (n=16) group was consume high-fat diet, which has energy content of 5.35 kcal/g and contains fat mostly from lard 59.28%E. All animals were given ad libitum access to food and water. After 13 weeks of either ND or HF consumption, each diet group was divided into sham-operated (S) and bilateral ovariectomized (O) groups for 7 weeks before was sacrificed. Therefore, animals were subdivided into four subgroups, including sham-operated female rats fed with normal diet (NDS), ovariectomized female rats fed with normal diet (NDO), sham-operated females rats fed with high-fat diet (HFS), and ovariectomized female rats fed with high-fat diet (HFO). At week 14$^{th}$ and 20$^{th}$, all animals were taken to test their locomotor activity with an open-field test (OFT), hippocampal-independent learning and memory with a novel object recognition (NOR) and, hippocampal-dependent learning and memory with a MWM test. Experimental for aim 3 was demonstrated in Figure 2.3.

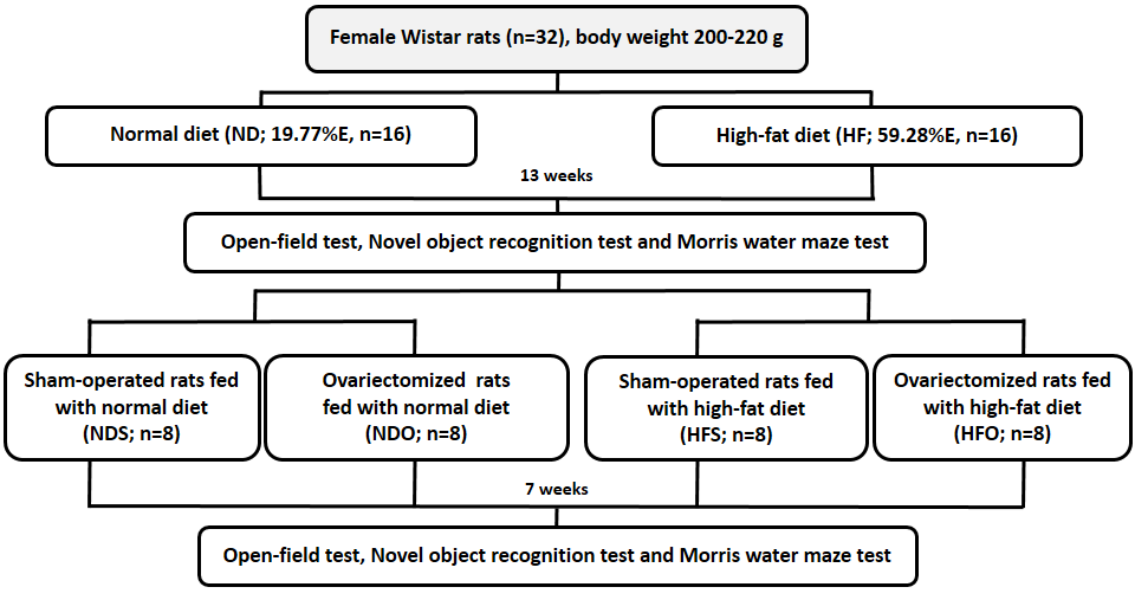


**Figure 2.3** Represents the flow charts of experimental protocol 3

**Table 2.1** Composition of the normal diet (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand) (9)

| Composition | Normal diet (CP 082) | | |
|---|---|---|---|
| | **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 the high-fat diet (9, 155)

| 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 | 90 | 1.68 |
| Vitamins | 85.19 | - | - |
| DL-Methionine | 3 | - | - |
| Fiber | 13.21 | - | - |
| Yeast powder | 1 | - | - |
| Sodium chloride | 1 | - | - |
| Total | 1000 | 5347.60 | 100 |
| Kcal/g | | 5.35 kcal/g | |

Diets ingredients and nutrient analyses were modified from Srinivasan et al (155).

Energy (kcal) per gram: carbohydrate 4; fat 9; protein 4.

### 2.2 Ovariectomy procedure

Ovariectomy was a surgery to remove the ovaries. Randomized female rats were anesthetized with isoflurane with oxygen support in side lying position, hair shaving and skin cleaning to receive sham operation or ovariectomy. The ovariectomized group was performed by bilateral flank incisions. The 2-cm-long incisions of both sides were centered between the inferior crest of the rib cage and superior base of the thigh. The skin was separated from the underlying muscle and the abdominal wall was exposed. Abdominal-pelvic cavity was accessed, then the uterine tubes and the ovaries were identified. Ligations of the blood vessel between the uterine tubes and uterine horns inside the peritoneal cavity were carried out. The ovaries were completely removed and the uterine horns were returned to the cavity. For the sham-operated group, rats were received the same surgical procedure as the ovariectomized group, but the ovaries of the rats were not removed. Then, the abdominal wall was sutured and the incisions were closed (156). After the operation, each rat was individually housed in a clear cage with dry bedding for 1 week prior being divided into the same groups for n=2 per cage.

### 2.3 Oral glucose tolerance test (OGTT) procedure

After fasting overnight for at least 12 hours, rats were given a bolus of glucose for 2 g/kg by gavage feeding. Blood samples were collected into microcentrification tubes with sodium fluoride (NaF) for 5 times points at 0, 15, 30, 60 and 120 minutes after glucose feeding from tail tip. After that, blood samples were centrifuged at 4$^{o}$C, 6,000 rpm for 10 minutes to collect the plasma (9). Plasma glucose levels were determined using a colorimetric assay kit (ERBA Mannheim, Mannheim, Germany).



### 2.4 Determination of metabolic parameters

After fasting (at least for 5 hours), blood was collected from the tail veins, then centrifuged at 6,000 rpm, 4$^{o}$C for 10 minutes and the plasma was kept at -80$^{o}$C until the metabolic parameters were measured.

#### 2.4.1 Determination of plasma insulin levels

The plasma insulin concentrations were determined by using Sandwich ELISA (Millipore, MI, USA). The principle of this method was the capture of insulin molecules from sample to wells of microtiter plate and coated by pre-titered amount of monoclonal

mouse anti-rat insulin antibodies. The binding of biotinylated polyclonal antibodies captured insulin, then washed away of unbound material from samples. Binding of horseradish peroxidase to the immobilized biotinylated antibodies, washed away of free enzyme conjugated and quantification of immobilized antibody enzyme conjugated by monitoring horseradish peroxidase activities to oxidized the substrate 3,3,5,5-tetramethybenzidine. The enzyme activity was determined by a spectrophotometer (BioTek Instrument, VT, USA) at λ450 nm, with the corrected wave length 590 nm. The increase in absorbance was directly proportion 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 reference standard of known insulin concentration of rat.

### 2.4.2 Determination of plasma glucose levels

Plasma glucose levels were measured by a colorimetric assay kit (ERBA Mannheim, Mannheim, Germany). The principle was that glucose in sample was oxidized by the glucose oxidase (GOD) into gluconic acid and hydrogen peroxide ($H_2O_2$). The enzyme peroxidase catalysed the oxidative coupling of 4-aminoantipyrine (4AAP) with phenol to yield a coloured quinoneimine complex. Sample was measured by spectrophotometer (BioTek, Winooski, VT, USA). The absorbance at of λ505 of colored quinoneimine complex was proportional to the concentration of glucose.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{phenol 4AAP} \xrightarrow{\text{Peroxidase}} \text{Red dye} + 2H_2O$$



### 2.4.3 The Homeostasis Model Assessment (HOMA index)

The HOMA index was calculated from fasting plasma insulin and fasting plasma glucose concentrations for determining the degree of insulin resistance. A higher HOMA index represented a higher degree of insulin resistance (157). The HOMA index was calculated by the following equation

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

### 2.4.4 Determination of plasma total cholesterol levels

Plasma total cholesterol levels were measured by a colorimetric assay kit (ERBA Mannheim, Mannheim, Germany). The principle was that cholesterol ester in plasma was hydrolysed by cholesterol esterase (CHE). The free cholesterol was then oxidized by cholesterol oxidase (CHO) to corresponding ketone liberating hydrogen peroxide, which was then converted to water and oxygen by enzyme peroxidase (POD). Para-aminophenazone was taken up the oxygen and together with phenol to form a pink colored quinoneimine, which can be measured at λ505 nm.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{CHE}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHO}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + 4\text{AAP} + \text{4-HBA} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4H_2O$$

### 2.4.5 Determination of plasma triglyceride levels

The plasma triglycerides concentration was measured by enzymatic colorimetric method using a commercially available kit (ERBA diagnostic, Mannheim, Germany). The method was that triglyceride in the sample was hydrolyzed by LPL into glycerol and free fatty acids. In the presence of ATP and glycerol kinase (GK), glycerol forms glycerol-3-phosphate which was further oxidized by glycerol-3-P-oxidase (GPO) into dihydroxyacetone-phosphate (DAP) and $H_2O_2$. The enzyme POD in the substrate-chromogen solution catalysed the reaction between $H_2O_2$ and 4-aminoantipyrine (4-AAP) to form the color complex (quinoneimine). The absorbance of reaction mixture was measured by a spectrophotometer (BioTek Instrument, VT, USA) at λ505 nm.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{LPL}} \text{glycerol} + \text{Free Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphare} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$$

$$H_2O_2 + 4\text{AAP} + \text{3,5-DHBS} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 2H_2O$$

### 2.4.6 Determination of plasma HDL and LDL levels

The plasma HDL level were determined by enzymatic colorimetric method using a HDL Cholesterol Quantification Kit (Biovision Inc, Milpitas, CA, USA). In the assay, cholesterol oxidase specifically recognized free cholesterol and produced products which react with probe to generate color and fluorescence. Cholesterol esterase hydrolyzed cholesterol ester into free cholesterol, therefore, cholesterol ester and free cholesterol could be detected separately in the presence and absenced of cholesterol esterase in the reaction. The plasma LDL was estimated from the following Friedewald's equation (158):

| Plasma LDL = Plasma total cholesterol – Plasma HDL – Plasma TG / 5 |
|---|

### 2.4.7 Determination of serum, hippocampal and cortical estrogen concentrations

Serum, hippocampal and cortical estrogen concentrations were detected by using the competitive enzyme immunoassay (EIA) kit (Cayman Chemical Company, MI, USA). This method used the competitive between estradiol and estradiol-acetylcholinesterase (AChE) conjugated (estradiol tracer) for limited amount of estradiol antiserum. The concentration of estradiol tracer was held constantly while the concentration of estradiol was varies, the amount of estradiol tracer which was able to bind the estradiol antiserum, was inversely proportional to concentration of estradiol in the well. After that, antiserum-estradiol complex bound to mouse monoclonal anti-rabit TgG which had previously attached to the well. The plate was washed to remove unbound reagents and Ellman's reagent (which contains the substrate to AChE) was added to the well. The product of this enzymatic reaction was a yellow color and was strongly absorbed at λ412 nm by using a microplate reader (BioTek Instrument, VT, USA). The intensity of the color was measured spectrophotometrically, and was proportional to the amount of estradiol tracer bound to the well, which was inversely proportional to amount of free estradiol. These free estradiol presented in the well during incubation.

## 2.5 Preparation of brain slices

At the end of the experimental period, the rats were anesthetized with isoflurane and decapitated by guillotine. Each whole brain was quickly removed on ice. Then, the

brains were immersed in ice-cold high sucrose artificial cerebrospinal fluid (aCSF) as source of energy, the high sucrose aCSF containing (mM): NaCl 85; KCl 2.5; $MgSO_4$ 4; $CaCl_2$ 0.5; $NaH_2PO_4$ 1.25; $NaHCO_3$ 25; glucose 25; sucrose 75; kynurenic acid 2; ascorbate 0.5, saturated with 95% $O_2$, 5% $CO_2$ (pH 7.4). This solution enhanced the neuronal survival during the slicing procedure. The hippocampal slices were cut using a vibratome (Vibratome Company, MO, USA). Following a 30 minutes post-slice incubation in high sucrose aCSF, all slices were transferred to a standard aCSF solution which was daily prepared, including NaCl 119; KCl 2.5; $CaCl_2$ 2.5; $MgSO_4$ 1.3; $NaH_2PO_4$ 1; $NaHCO_3$ 26; and glucose 10 were contained in mM, saturated with 95% O2, 5%CO2 (pH 7.4) for an additional 30 minutes at room temperature before the extracellular recording process (22-24$^{o}$C) started (157).

### 2.6 Extracellular recording of hippocampal slices for LTP

For the determination of electrical-induced LTP, brain slices were transferred to a submersion recording chamber and continuously perfused the standard aCSF at rate about 3-4 ml/min with controlling of temperature between 28-29$^{o}$C. The fEPSPs was evoked by stimulating the Schaffer collateral-commissural pathway with a bipolar tungsten electrode, while the fEPSPs recordings was taken from the stratum radiatum of the hippocampal CA1 region with micropipettes (3 MΩ) filled with 2M NaCl. LTP was induced by delivering a high-frequency tetani (high-frequency stimulation (Hfs); 4 trains at 100 Hz; 0.5 s duration; 20 s interval) at 1.5 times the baseline stimulation intensity. Experiments were performed for at least 40 minutes after high frequency stimulation. The significance from baseline in each group was taken to compare and values at different time points which were represented as means±SEM. Data was filtered at 3 kHz, digitized at 10 kHz, and recorded by using pClamp 10.6 software (Axon Instruments, CA, USA) (101). The initial slope of the fEPSPs was measured and plotted against the time.



### 2.7 Protein preparation for hippocampal and cortical ROS production measurement

At the end of the experiment, all animals were anesthetized and decapitated. Hippocampal and cortical tissue were rapidly removed and transferred into 1 ml of ice-cold MSE solution. Then, the solution was homogenized at 600 rpm with homogenizer. Then, the hippocampal and cortical homogenate was centrifuged at 2,000 g, 4$^{o}$C for 4

minutes. The supernatant was collected for protein concentration measurement. The supernatant of hippocampal and cortical protein were collected in microcentrifuge tube on ice. Then, 50 µl of hippocampal and cortical proteins concentrations were taken with 1 ml of Bicinchoninic (BCA) and incubated at 60$^{o}$C for 30 minutes prior to determination of the protein level concentration by the spectrophotometer (158). Finally, the protein concentration was calculated and converted into the concentration of 0.1 mg/ml protein.

### 2.8 Hippocampal and cortical ROS assay

Dichloro-hydrofluoresceindiacetate (DCFH-DA) dye was used to determine hippocampal and cortical ROS production (157). The concentration 0.1 mg/ml of hippocampal and cortical protein was applied with or without 2 mM of $H_2O_2$. $H_2O_2$ was used to accelerate more hippocampal and cortical ROS production. After that, it was stained with 2 µM DCFH-DA dye at 25$^{o}$C for 20 minutes in dark light. The principal was that the $H_2O_2$ oxidized DCFH to DCF; the more DCF determined the more hippocampal and cortical ROS produced. Fluorescence was used to determine an excitation wavelength λ485 nm and emission wavelength λ530 nm which was the spectral characteristic of DCF. Fluorescent microplate reader (Bio-tek Instrument, Inc. Winooski, Vermont USA) was to indicate the fluorescence.

Hippocampal and cortical ROS production were calculated as a percentage change from the following equation:

$$\frac{(\text{ROS with } H_2O_2 - \text{ROS})}{\text{ROS}} \times 100$$



### 2.9 Immunoblotting for hippocampal and cortical apoptotic protein expressions and ratio

The subsequent brain homogenates for immunoblotting were prepared as described in our previous study (9). Hippocampal and cortical homogenates for electrophoresed and immunoblotted were prepared to determine apoptotic protein expressions and ratio (Bax, Bcl-2 and Bax/Bcl-2 ratio). The rabbit antibodies were taken for the Bax (1:200, Santa Cruz Biotechnology, CA, USA) and Bcl-2 (1:1000, Abcam, MA, USA). All blots were incubated with a horseradish peroxidase conjugated anti-rabbit secondary antibody (1:2000, Cell Signaling Technology, MA, USA). The

membranes were exposed to ECL Western blotting substrate, and densitometric analysis was carried out by using ChemiDoc TouchImaging system (Bio-Rad Laboratories, CA, USA).

### 2.10 Serum level measurement using HPLC-based assay of MDA concentration

Serum oxidative stress was determined by measuring MDA concentrations, using a high-performance liquid chromatography (HPLC) method (100). A 0.5 ml aliquot of serum was mixed with 1.1 ml of 10% trichloroacetic acid (TCA) containing butylated hydroxytoluene (BHT) (50 ppm), heated at 90 °C for 30 minutes and cooled down to room temperature. The mixture was centrifuged at 6,000 rpm for 10 minutes. The supernatant (0.5 ml) was mixed with 0.44 M $H_3PO_4$ (1.5 ml) and 0.6% thiobabituric acid (TBA) solution (1.0 ml), and then were incubated at 90°C for 30 minutes, to develop a pink-color products, called thiobarbituric acid reactive substances (TBARS). The solution was filtered through a syringe filter (polysulfone type membrane, pore size 0.45 μm, Whatman International, Maidstone, United Kingdom) and analyzed by a HPLC system. The TBARS were fractionated in the adsorption column (Water Spherosorb ODS2 type, 250×4.3 mm, 5 μm diluted with mobile-phase solvent of $KH_2PO_4$ 50 mM: methanol at flow rate 1.0 ml/minute) and then detected at λ532 nm. Data was analyzed with BDS software (BarSpec Ltd., Rehovot, Israel). A standard curve was measured from the peak of the height of standard 1, 1, 3, 3-tetramethoxypropane (standard reagent for malondialdehyde) at different concentrations (100 μM). TBARS concentrations were determined directly from the standard curve and data was reported as the equivalent concentration of MDA.



### 2.11 Behavioral assessment

#### 2.11.1 Open-field test, as modified from Arakawa et al., 2005 (159)

Open-field test was developed to evaluate the locomotor activity in rodents. In this method, the apparatus consisted of a square-based box opened from above (90x90 cm of base, 45 cm of height). Each animal was placed into the box at the middle and allowed 10 minutes for exploration. After 10 minutes of exploration time, the animals were taken out. The distance and speed in the area of the open-field was detected as activity through the camera and analyzed with program Smart version 3. This parameter was used to exclude the different locomotor activity among groups.

### 2.11.2 Determination of hippocampal-dependent learning and memory ability via the MWM test

In the present study, the protocol of MWM was modified (22). MWM was set up with a circular water pool with a diameter of 200 cm and water height of 50 cm. Animals were placed in different directions for each trial of the day, including north (N), east (E), south (S) and west (W) and separate 4 quadrants including north east (NE), north west (NW), south west (SW) and south east (SE). Each day the animals were given the 4 different starting points according to table 2.3. The transparent round platform with a diameter of 10 cm was placed at the middle of the target quadrant (SW). To determine hippocampal-dependent learning and memory ability, there were 2 phases of the experiment; the Acquisition test (hidden platform) and the Probe trial test (remove the platform from the pool). The water was filled with blue color to blind and above each of the four quadrants of the pool, the different shape markers were placed to be external cues.

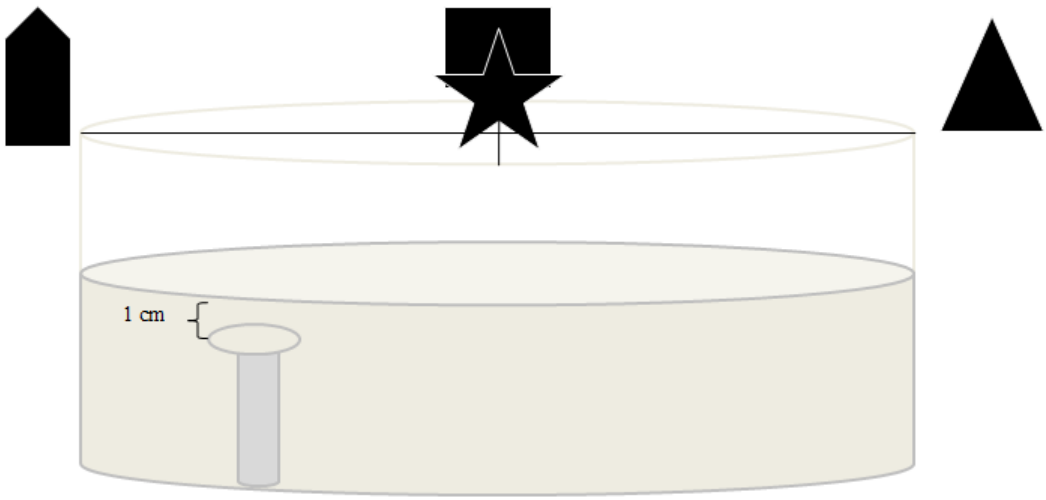


**Figure 2.4** The water pool set up

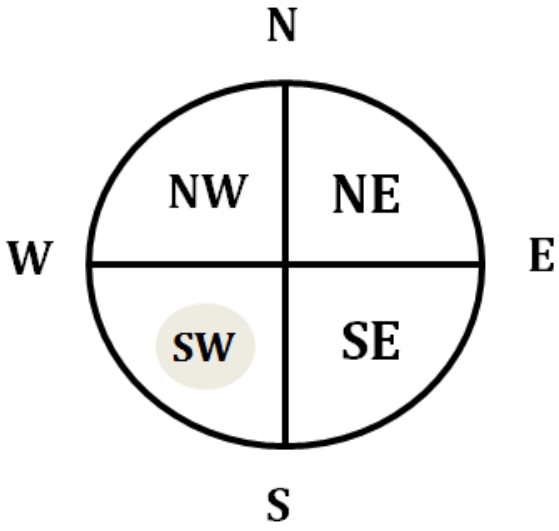


**Figure 2.5** Four directions and four quadrants of water pool

**Acquisition test (hidden platform)**

All animals were tested from day 1 to day 5 at similar times. The animals were rested in the testing room for at least 30 minutes before starting the experiment. After that, the animals were placed in the water at one of the different starting points, turning their heads towards the border of the pool. Time recording was start as soon as the animal was placed in the water automatically by the program and was end when the animal finds the submerged platform. Each animal was left on the platform for 15 seconds after finding the platform. If the animal taken more than 120 seconds to find the platform, it was guided to the platform and left for 15 seconds before removing them from the pool and allowed to rest for 15 seconds. After that, the animal was placed at the other 3 starting points with the same procedure. Data was analyzed by Smart version 3 program. Time to reach platform, speed and distance to platform were determined. The starting points in each trial per day was shown in table 2.3.

**Probe trial procedure (removal platform from the water)**

All animals were undergo this procedure on day 6. The animals were rested at the testing room for at least 30 minutes before starting the experiment. After that, the animal was placed in the water in NE with their head turned towards the pool. All data was recorded when the animal entered the water and the experiment was ended after 90 seconds in the pool. Data was analyzed using Smart version 3 program. The time spent

in the pool was automatically stopped and analyzed by this program. Time in target quadrant and distance in target quadrat were determined.

**Table 2.3** Starting points in each trial per day

| **Acquisition** ( platform locate in SW quadrant) | | | | |
|---|---|---|---|---|
| **Day** | **Trial 1** | **Trial 2** | **Trial 3** | **Trial 4** |
| 1 | N | E | SE | NW |
| 2 | SE | N | NW | E |
| 3 | NW | SE | E | N |
| 4 | E | NW | N | SE |
| 5 | N | SE | E | NW |
| 6 (**Probe**) | NE (remove the platform from SW) | | | |

### 2.11.3 Determination of hippocampal-independent learning and memory ability via the NOR test

This test was modified to determine hippocampal-independent learning and memory (23, 25, 160). Novel object recognition test was performed in a rectangular arena size 90x90 cm box. The task procedure consisted of three phases; habituation, familiarization, and test phase. The first 2 days were the habituation phase, each animal was allowed to freely explore the open-field arena without any object for 10 minutes. Distance and speed were analyzed to determine locomotor activity. On the third day, which was the familiarization phase, an animal was placed in the open-field arena containing two same objects and allowed to explore for 10 minutes. Exploration defined as orienting the object within a distance of equal or less than 2 cm. Between each trial, the open field and the objects were cleaned with a 70% ethanol solution and distilled

water. Time of exploration was calculated as percent exploration time. The last day, during test phase, two different objects were placed in the box; one was the object from the familiarization phase and another one was a novel object with a different texture, color, and shape to test for learning and memory. The animal was allowed 10 minutes to explore. Time spent during exploring the novel object was calculated and presented as a percentage of index preference by comparing to the familiar object. During both the familiarization and the test phase, objects were located in opposite and symmetrical corners of the arena and location of novel versus familiar object were counterbalanced. The time of exploration was manually counted, speed and distance were analyzed using the Smart version 3 program, which was connected to the camera that was located above the rectangular arena. Percentage of index preference was calculated from the following equation:

$$\% \text{ index preference} = \frac{\text{Time on object 2}}{\text{(Time on object 1 + time on object2)}} \times 100$$

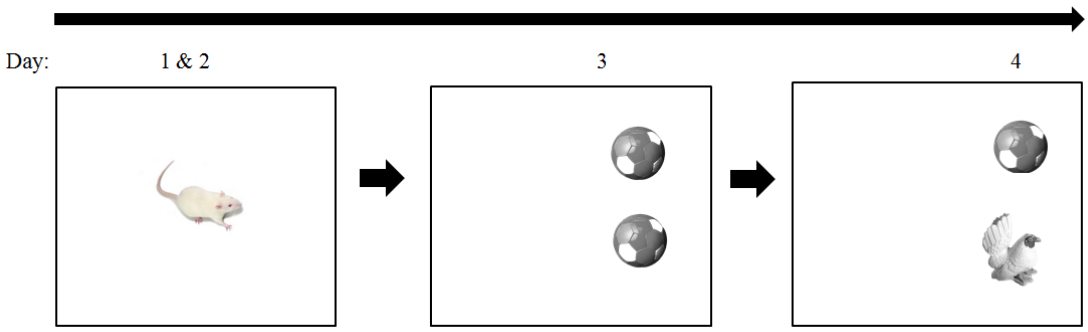


**Figure 2.6** The Novel object recognition set up

## 2.12 Statistical analysis

The data from all experiment were presented as mean ± SEM. The mean values between two groups were compared using unpaired student's t-test. For all comparisons, the significantly difference between four groups were calculated by a one-way ANOVA followed by post-hoc LSD. The comparisons among groups in the acquisition test for MWM tests were performed using a two-way ANOVA, followed by post-hoc LSD. P-value < 0.05 was considered as significant.