CHAPTER III

MATERIALS AND METHODS

1. Animal Preparation

Male Sprague-Dawley rats weighing 220-260 grams from the National Laboratory Animal Center, Mahidol University, Salaya, Nakorn pathom were used. The animals were allowed to rest for a week after arrival at the Animal Center, Department of Physiology, Faculty of Medicine, Chulalongkorn University before used in the experiment. They were kept in a controlled temperature room at 25±1 C° under standard conditions (12 hour dark:12 hour light cycle), fed regular dry rat chow ad libitum, and had freely access to drinking water.

2. Experimental Protocols

Effect of 100% fat diet on changes of serum biochemical parameters, oxidative stress marker and liver histopathology in rats

Rats were randomly divided into two experimental groups (eight rats in each group)

Group 1 : fed ad libitum with regular dry rat chow and drinking water for 6 weeks

Group 2 : fed ad libitum with 100% fat diet and drinking water for 6 weeks to induce NASH

All rats were weighed weekly. After 6 weeks, each group was sacrificed and the samples were collected (diagram of the experiment 1 was shown in Figure 13).

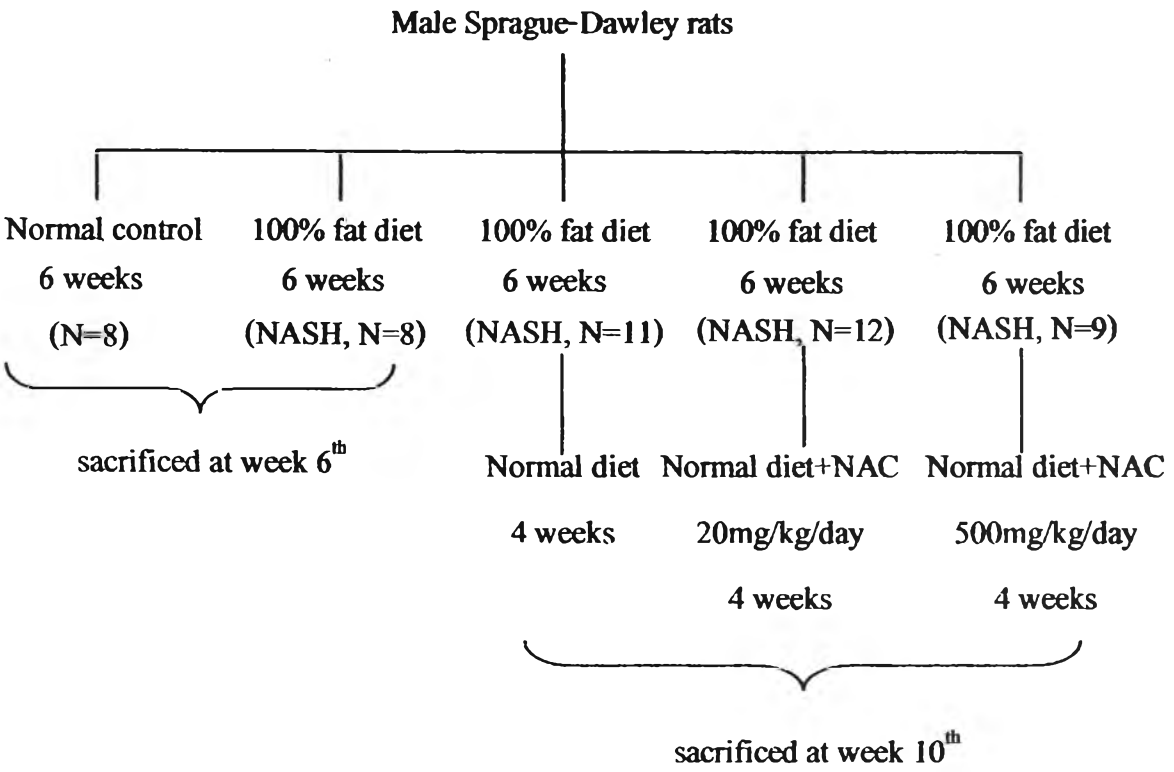
Effect of N-acetylcysteine on changes of serum biochemical parameters, oxidative stress marker and liver histopathology in rats with nonalcoholic steatohepatitis

Rats were randomly divided into three treatment groups (eight to twelve rats in each group)

Group 3-5 : rats (n=32) were fed with 100% fat diet for 6 weeks to induce NASH. After that, starting from week 7^{th}, rats were fed with regular dry rat chow (NASH+diet group) plus 20 mg/kg/day of NAC orally (NASH+diet+NAC_{20} group) or 500 mg/kg/day of NAC orally (NASH+diet+NAC_{500} group) respectitively, for other 4 weeks. All rats were weighed weekly. They were sacrified to collect blood, serum, and liver samples at the end of the study, 20 hours after the last NAC treatment (diagram of the experiment was shown in Figure 13).

3. Data Collection

At the end of the study, all rats were anaesthetized using intraperitoneal injection of an overdose (45 mg/kg BW) of sodium pentobarbital and then the abdominal walls were opened. Blood was drawn by cardiac puncture using a disposable syringe with needle NO.21 and collected into two microcentrifuge tubes (tube NO.1 for total glutathione assay and tube NO.2 for biochemical assay). Later, the animals were sacrificed by cutting the abdominal veins. The livers were excised quickly and cleaned in iced-cold NSS. Fat and fibrous tissues were removed before the liver was weighed and kept on ice afterward. One lobe of the liver was collected for MDA measurement and the remaining liver was fixed in 10% formalin solution for hispathology examination.



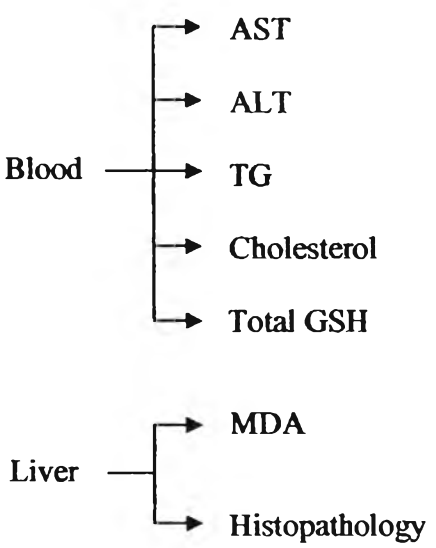


Figure13. Experimental diagram

3.1 Total Glutathione Determination

Total glutathione levels were measured using Cayman's GSH assay kit (Cayman Chemical Company, MI, U.S.A.). This assay uses glutathione reductase for the quantification of GSH (Figure 14). The sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 nm provides an accurate estimation of GSH in the sample.

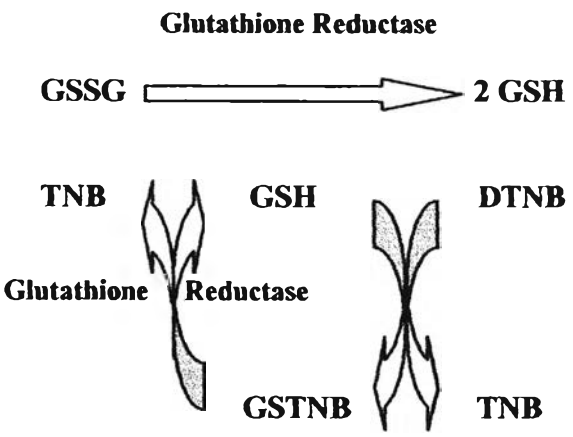


Figure 14. GSH recycling

3.1.1 Assay Preparation

3.1.1.1 Reconstitution of the Reagents

1. MES Buffer : The buffer was diluted with equal volume of double distilled water.

2. GSSG Standard : This standard was ready to use.

3. Cofactor Mixture : The contents of the vial were reconstituted with 0.5 ml of water and mixed well.

4. Enzyme Mixture : The Enzyme Mixture was reconstituted by adding 2 ml of diluted MES Buffer to the vial, replacing the cap, and mixing well.

5. DTNB : The contents of the vial were reconstituted with 0.5 ml of water.

3.1.1.2 Sample Preparation

1. 10%MPA reagent : 0.5 g of metaphosphoric acid was dissolved in 5 ml distilled water.

2. An amount of 600 µl of blood from tube NO.1 was added in a microcentrifuge tube containing 600 µl of 10%MPA reagent and mixed immediately on a vortex mixture at room temperature for 5 minutes and centrifuged 3,500 rpm at 4^0C for 10 minutes.

3. The supernatant was collected carefully without disturbing the precipitate.

4. TEAM reagent : 531 µl of 4 M solution of triethanolamic was added in 469 µl of double distilled water.

5. 10 µl of TEAM reagent per 200 µl of the supernatant was added in a microcentrifuge tube and vortexed immediately.

6. 15 µl of mixture from 5. was diluted with 1.485 ml of MES Buffer (1:100).

3.1.1.3 Performing the Assay

1. Preparation of the standards : Eight test tubes were marked A-H. The GSSG standard and MES Buffer were added to each tube as described below.

Tube	GSSG Standard (µl)	MES Buffer (µl)	Final concentration (µM GSSG)	Equivalent total GSH (µM)
A	0	500	0	0
B	5	495	0.25	0.5
C	10	490	0.5	1.0
D	20	480	1.0	2.0
E	40	460	2.0	4.0
F	80	420	4.0	8.0
G	120	380	6.0	12.0
H	160	340	8.0	16.0

2. 50 µl of standard (tubes A-H) per well was added in the designated well in the plate.

3. 50 µl of sample was added to each of the sample wells.

4. The plate was covered with the plate cover provided by the manufacturer.

5. Assay cocktail was prepared by mixing the following reagents in a 20 ml vial: MES Buffer (11.25 ml), reconstituted Cofactor Mixture (0.45 ml), reconstituted Enzyme Mixture (2.1 ml), water (2.3 ml), and reconstituted DTNB (0.45 ml).

6. The plate cover was removed and added 150 µl of the freshly prepared Assay cocktail was added to each of the wells containing standards and samples using an eight channel pipettor. The plate was covered and incubated in the dark on a plate shaker.

7. All standards and samples were analysed in duplicate.

8. The absorbance was measured in the wells at 405 nm using a plate reader at 5 minutes intervals for 30 minutes (a total of 6 measurements).

3.1.1.4 Calculating the Results

Total glutathione concentration of the samples in this study was determined by Kinetic Method.

1. The average absorbance values of the duplicate of each standard and sample were plotted as a function of time and the slope for each curve was determined. This was called i-slope.

2. The i-slope of each standard was plotted as a function of the concentration of total glutathione. The slope of this curve is called f-slope.

3. The values of total glutathione for each sample was calculated from their respective slopes using the slope versus GSH standard curve.

$$\left[\text{Total GSH}\right] = \left\{\text{(i-slope for the sample) - (y-intercept)}\right\} / \text{f-slope x 2 x sample dilution}$$

In this study, inter-assay coefficient of variations were 2.82% and 2.81% for 1723.99±48.56 µM and 1453.88±40.84 µM, respectively, and intra-assay coefficient of variations were 6.43% and 1.43% for 1683.33±106.07 µM and 1352.08±70.83 µM, respectively.

3.2 Serum Biochemical Determination

After leaving whole blood in tube NO.2 clotted for 30 minutes at room temperature, serum was separated by centrifuging at 3,500 rpm at 4°C for 10 minutes. Top serum layer was pipetted off into a microcentrifuge tube. The collected serums were kept in a -70^0 C freezer until analysis (2 months). Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglycerides and cholesterol were analyzed at the Laboratory Center, King Chulalongkorn Memorial Hospital.

3.3 Hepatic malondialdehyde (MDA) determination

One lobe of the liver was removed and weighed. Then 1 gram of the tissue was placed in a test tube containing 2.25 ml of homogenization buffer (1.15% KCl) and homogenized in an ice box using a homogenizer at a rotational speed of 12,000 rpm for 1 minute. Malondialdehyde was measured from the homogenized tissue by using thiobarbituric acid reaction as described by Ohgawa et al. (Ohgawa et al., 1979). The basic principle of the method is the reaction of one molecule of malonaldehyde and two molecules of TBA to form a red malonaldehyde-TBA complex, which can be quantitated spectrophotometrically (532 nm).

3.3.1 Reagents

1. 8.1% (w/v) Sodium dodecyl sulfate (SDS)

2. 20% (v/v) of Acetic acid solution (pH 3.5)

3. 0.8% (w/v) Thiobarbituric acid (TBA)

4. 1,1,3,3-Tetramethoxypropane (TMP) or malondialdehyde bis solution

5. 1.15% KCl

6. The mixture of n-butanol and pyridine (15:1, v/v)

3.3.2 Procedure

1. Standard curve preparation : TMP is used as an external standard. The level of lipid peroxide is expressed as nanomole (nmol) of MDA. A stock solution of 10^{-3} M TMP was prepared with distilled water and a series of standards (in duplicate) was set as described below.

Tube	Stock TMP (ml)	DW (ml)	Final TMP concentration (M)	TMP in 0.2 ml solution (nmol)
1	0.10	9.90	1.0×10^{-5} M	2
2	0.25	9.75	2.5×10^{-5} M	5
3	0.50	9.50	5.0×10^{-5} M	10
4	0.75	9.25	7.5×10^{-5} M	15
5	1.00	9.00	10.0×10^{-5} M	20

2. The following solutions and samples were pipetted into tubes with screw caps.

Solution	Blank:DW (ml)	Standard (ml)	Unknown (ml)
Sample	-	-	0.2
8.1% SDS	0.2	0.2	0.2
20% Acetic acid (pH 3.5)	1.5	1.5	1.5
0.8% TBA	1.5	1.5	1.5
TMP stock standard	-	0.5	-
Distilled water	0.8	0.3	0.3

Note: DW = Distilled water

3. The tubes were heated in the water-bath at 95°C for 60 minutes.

4. After cooling the tubes by immersion with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1, v/v) were added and shaken vigorously (at least 1 minutes).

5. After centrifugation at 4,000 rpm for 10 minutes, the absorbance of the organic layer (upper layer) was measured at 532 nm.

6. MDA levels in the samples were determined the linear regression equation from a standard curve. The content of lipid peroxide is expressed in terms of nmol of MDA/gram of wet weight and the total protein was determined by the Lowry method (Lowry et al., 1951) to correct the MDA level which is expressed in terms of nmol/mg protein.

3.4 Protein assay (Lowry Method)

The principle lines are in the reactivity of the peptide bond of protein with the copper II ion (divalention) under alkaline conditions and reduced to copper I ion (monovalention). Copper I ion and the radical groups of tyrosine, tryptophan and cysteine amio acid side chains of protein (the protein-copper complex) react with the folin phenol reagent (phosphomolybdic-phosphotungstic acid) to produce heteropolymolybdenum blue by the copper catalyzed oxidation of aromatic acid and change blue color which is analyzed by visible spectrophotometer (Lowry et al., 1980).

This assay is designed to quantify 1 to 100 μg/ml protein. A standard curve which was plotted an absorbance at 750 nm versus bovine serum albumin (BSA) concentration (μg/ml) was used for calculating the protein content of the samples based on their absorbance. The assay method was described as follows.

3.4.1 Reagents

1. Solution A (alkaline tartrate reagent)

Na_2CO_3	10.0	g
$Na_2C_4H_4O_6.2H_2O$	0.1	g
NaOH	1.2	g

These chemicals were dissolved in distilled water and made up 500 ml.

2. Solution B (0.5% copper sulfate): 0.5 g of $CuSO_45H_2O$ was dissolved in 100 ml distilled water.

3. Solution C (prepare fresh, within 1 day of use): 500 ml of solution A was added in 100 ml of solution B and mixed well.

4. Solution D (1 N folin phenol reagent): 30 ml of 2 N folin ciocalteu's phenol reagent was added in 30 ml of distilled water.

5. Standard protein solution (BSA 1 mg/ml)

3.4.2 Procedure

1. A series of standards (in duplicate) which contain: 10, 25, 50, 75 and 100 μg/ml of BSA were set as described below

The BSA was prepared as stock at concentration of 1 mg/ml.

Standard curve preparation

Tube	BSA (µl)	DW (µl)	Final BSA Concentration(µg/ml)
1	50	450	10
2	125	375	25
3	250	250	50
4	375	125	75
5	500	0	100

2. Samples were diluted with homogenization buffer (1:120) and mixed well. Then solutions and samples were added into tubes with screw caps as follows:

Solution	Blank:DW (ml)	Standard (ml)	Unknown (ml)
Distilled water	0.5	-	-
BSA	-	0.5	-
Diluted sample	-	-	0.5
Solution C	5.0	5.0	5.0

3. All tubes were mixed well and stood at room temperature for 10 minutes. Later, 0.5 ml of solution D was added and stood for 30 minutes at room temperature.

4. The absorbance of each solution was read and recorded at 750 nm. Protein concentration which multiply dilution factor is expressed in terms (µg/ml).

3.5 Histopathological examination

The remaining liver samples were fixed in 10% formalin solution at room temperature. They were processed by standard method, tissues were embedded in

paraffin, sectioned at 5 μm, and stained with Hematoxylin-Eosin (H&E) and then picked up on glass slides for light microscopy. An experienced pathologist evaluated all samples while blinded to the experiment. All fields in each section were examined for grading of steatosis and necroinflammation according to Brunt et al. criteria (Brunt et al., 1999).

The severity of steatosis was graded on the basis of the extent of involved parenchyma. Scored 1 = were those in which fewer than 33% of the hepatocytes were affected, scored = 2 were those in which 33% - 66% of the hepatocytes were affected, scored = 3 were those in which more than 66% of the hepatocytes were affected, and scored = 0 were those in which no hepatocytes were affected.

Hepatic necroinflammation was graded from 0 to 3 ; score 1 (mild) = sparse or mild focal zone 3 hepatocyte injury/inflammation, score 2 (moderate) = noticeable zone 3 hepatocyte injury/inflammation, score 3 (severe) = severe zone 3 hepatocyte injury/inflammation, and score 0 = no hepatocyte injury/inflammation.

4. Data Analysis

The data were expressed as mean ± standard error of the mean (SEM) using the SPSS version 11.5 for Windows program. Statistical comparisons between groups were analyzed by one-way analysis of variance (ANOVA) and post hoc comparisons were done with Bonferroni correction. Differences between two groups were analyzed by unpaired t-tests. P values less than 0.05 were considered significant.