CHAPTER III

MATERIALS AND METHODS

Animals

Eighty female Sprague-Dawley rats, 30 days of age, body weight of 120-140 g, obtained from the National Laboratory Animal Center, Thailand, were used. They were housed 5 animals per cage in a room with controlled lighting (lights on 06.00-20.00 h) in which the temperature was maintained at 25 $\pm$ 1°C at the Primate Research Unit, Chulalongkorn University, Thailand. In order to control the phytoestrogens content in the rat diets, the animals were fed with the same lot of rat chow diet (lot no. 070, Pokaphan Animal Feed Co, Ltd, Thailand) throughout the study period. Diet and water were supplied *ad libitum*. The rats were acclimated for 2 weeks before the onset of study.

The preparation of NMU, tamoxifen and genistein solutions

N-nitroso-N-methylurea (NMU) (Sigma, St. Louis, MO) was dissolved in a 100 μl of 3% acetic acid and diluted with distilled water to give a stock concentration of 20 mg/ml. The freshly prepared NMU, within 2 hours after preparation, was intravascularly injected into the rats via tailed vein. The NMU-induced rat mammary tumor was used as a subject for this study because it has been proved that the mammary tumors induced by NMU are hormone dependent and closely resembled to the human breast cancer (Arafah *et al.*, 1982, Gottardis and Jordan, 1987).

Genistein (98% purity, lot no.049H0521, Sigma, St. Louis, MO) was dissolved in 2% dimethylsulfoxide (DMSO) (Sigma, St Louis, MO), then mixed with peanut oil (Sigma, St.

Louis, MO) to give a stock concentration of 3 mg/ml, and kept in the dark bottle at 4 °C. Genistein stock solution was adjusted with peanut oil to a concentration to 1 mg/kg BW/0.1 ml before administration to rats.

Tamoxifen (Sigma, St. Louis, MO) was dissolved in a few drops of ethanol and the required concentration of 100 μg/0.1 ml was adjusted by peanut oil. The ethanol was evaporated overnight at room temperature under the ventilation hood.

Experiment 1: The effects of genistein on NMU-induced tumorigenesis

The rats (45 days old) were induced mammary tumors before randomized into 2 groups. They were injected a single dose of 40 mg/kg BW of freshly prepared NMU via tailed vein at the first day of the study period. The rats were randomized into 2 groups (20 rats/group) as follows;

Vehicle treated group

The rats were daily subcutaneously injected with 0.1 ml of 2% of DMSO in peanut oil for 20 weeks.

Genistein treated group

The rats were daily subcutaneously injected with 1 mg/kg BW of genistein in 0.1 ml of 2 % of DMSO in peanut oil for 20 weeks. This genistein dosage is equated with the daily genistein consumption in Asian people on a mg (kg^{-1}) BW basis (Hilakivi-Clarke *et al.*, 1999)

Rats in both groups were checked changes of vaginal epithelium cells daily. Body weight, diet intake and tumorigenesis in rats were measured weekly. One-millilitter of blood sample was monthly collected from all animals on the day of diestrous phase. Blood sera were separated and kept at –20 °C for analysis of serum E_2 and genistein concentration. The rats were euthanized by ether at the end of study period or when the tumor size reached 3.5 cm in diameter. The tumor tissues, livers, uteri and ovaries were dissected, trimmed off the fat, weighed, and only the tumor tissues were fixed in 10% neutral buffered formalin for histopathological study. All rats were also examined for metastases of the tumor to the other organs, e.g. thoracic and abdominal cavities during necropsy.

Experiment 2: The effects and mechanism of genistein and tamoxifen on NMU- induced mammary tumor growth

The rats (45 days old) were given injection of a single dose of 40 mg/kg BW of NMU via tailed vein. After 4 weeks of NMU injection, all animals were palpated weekly to detect the mammary tumors. Tumor sizes were measured weekly by digital vernia calipers. When the tumor reached a 1-cm diameter (approximately after 8-10 weeks of NMU injection), rats were randomized into four treatment groups (10 rats/group) as follows;

Vehicle group

The rats were daily subcutaneously injected with 0.1 ml of 2% of DMSO in peanut oil for 10 weeks.

Tamoxifen group

The rats were daily subcutaneously injected with 100 μg of tamoxifen in 0.1 ml of peanut oil for 10 weeks. This dose of tamoxifen was chosen for the present study because the daily injection of 100 μg of tamoxifen in 0.1 ml of peanut oil could inhibit tumor appearance completely in female Sprague-Dawley rats (Gottardis and Jordan, 1987).

Genistein group

The rats were daily subcutaneously injected with 1 mg/kg BW of genistein in 0.1 ml of 2 % of DMSO in peanut oil for 10 weeks.

Tamoxifen and genistein treated group

The rats were received injections of both tamoxifen and genistein for 10 weeks. The doses of tamoxifen and genistein administered were the same as tamoxifen and genistein groups.

In these four groups, the tumorigenesis of rats were determined weekly until the end of treatment period or when the tumor diameter reaching 3.5 cm. The rats on the diestrous phase were euthanized thereafter under ether anesthesia. Blood samples were collected and the blood sera were separated and kept at –20 °C for E_2 assay. The tumor tissues, livers, uteri and ovaries were dissected, trimmed off the fat and weighed. The livers, uteri and ovaries were fixed in 10%

neutral buffered formalin for histopathological study. The tumor tissues were processed and determined the expression of cancer related genes. All rats were also examined for metastases of the tumor to the other organs, e.g. thoracic and abdominal cavities during necropsy.

Vaginal cytology assay in rats

Estrous cycle of rats was monitored daily by vaginal cytology assay. The glass-rod was inserted into the vagina of the rats and gently touched against the vaginal wall. The vaginal cells were smeared into a drop of 0.9% normal saline on the glass slide. The vaginal cells were observed under light microscope (Olympus, Japan) (X100) and classified. The estrous cycle was separated based on the vaginal cell-type into 4 stages: proestrus, estrus, metestrus and diestrus. Proestrus was identified when the vaginal cells were nucleated epithelial cell type, and occasionally contaminated with cornified cells. Estrus was predominated with large squamous cornified cells. Metestrus had a large number of leukocytes and cornified cells. Mostly leukocytes and occasional nucleated cells were found in diestrus (Norris, 1997).

Monitoring the mammary tumorigenesis in rats

The rats were palpated weekly at the breast and abdomen to detect the appearance of mammary tumors (Figure 6). The location and size of firstly detected tumor were recorded in each animal. Most of firstly detected tumor was very small (0.5-0.6 cm in diameter, it was thereafter detected by rolling up the rat skin and pinching it between the fingers. The tumor sizes, the tumor length and the tumor width, were monitored once a week thereafter by a digital vernia caliper (Starrett, England). The length (L) is the longest axis and the width (W) is perpendicular to the longest axis (Figure 7). The tumor cross-sectional area was calculated according to a formula of L/2 x W/2 x π (mm^2) (Ju *et al.*, 2002). The data of a) the latency of tumor appearance, b) the tumor incidence (calculated from 100 x number of tumor-rats per number of total rats), c) the tumor multiplicity or tumor numbers per rat and d) the tumor cross-sectional area in each group of rats were calculated.

Figure 6. Detection of small size tumor (0.5-0.6 cm in diameter) by rolling up the rat skin and pinching it between the fingers.

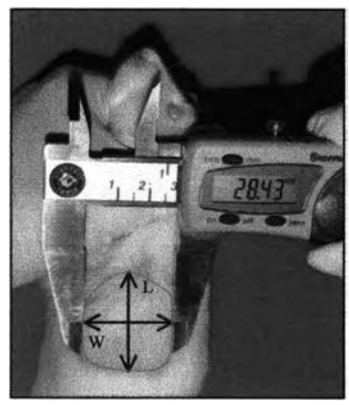

Figure 7. Measurement of tumor sizes by a digital vernia caliper. The length (L) is the longest axis and the width (W) is perpendicular to the longest axis.

Serum estradiol determination

Serum E_2 concentration was analysed by a double-antibody RIA system using I^{125}-labeled radioligands (Diagnostic Systems Laboratories, Inc, Texus, USA) with a slight modification. To minimize the inter-assay variation, all samples in each group were run in the same assay. The intra-assay coefficients of variations were 8.9, 6.8 and 8.9%, respectively, for the high, medium and low E_2 concentrations.

Serum genistein determination

Free and total genistein concentrations in the serum were analysed by HPLC technique. Analysis of free genistein level, 80-300 μl of rat serum was pipetted into 1.5 ml of microfuge tube and added 4 volume of acetone for deproteinization. The tubes were stood for 10 min at room temperature after vortexed and then centrifuged at 12,000 rpm for 5 min. The supernatant was collected and then dried up using centrifugal concentrator (Tomy, Japan) and redissolved with 120 μl of 0.4% acetic acid in ultrapure distilled water and finally stored at -20 °C until analysis. The samples were centrifuged at 12,000 rpm for 5 min just before starting the HPLC analysis. The supernatant was collected and 50 μl of the supernatant was injected into a column.

For total genistein levels determination, 80 μl of serum sample was pipetted into 1.5 ml of microfuge tube and adjusted to the volume of 200 μl with ultrapure distilled water. Genistein conjugated with glucoronic acid and sulfate were converted to free form by enzymatic hydrolysis with a 100 μl of β-glucuronidase (200 u/ml in 1M ammonium acetate buffer, pH 6.8) or a 100 μl of arysulfatase (6 u/ml in 1M ammonium acetate buffer, pH 5.0), at 37 °C for 3 hrs. The following steps were performed as similar as the procedure of the free form.

Analyses were performed using a high performance liquid chromatograph (LC-9A, Shimadzu, Japan). Chromatography was carried out on a column of 4.6 x 150 mm (ODS-80 TM, TOSOH, Japan) under the temperature of approximately 17 °C. The mobile phase consisted of solution A (100:0.4 v/v of ultra pure distilled water: acetic acid) and solution B (100:0.4 v/v of

acetonitrile: acetic acid). A linear gradient was performed for 60 min from 20% to 100% of solution B in solution A at a flow rate of 1 ml / min. Genistein eluted at 39 min, monitoring at 260 nm by UV spectrophotometric detector (Shimadzu, Japan)(Figure 8). An area of the peaks was determined using the Chromatopac machine (Shimadzu, Japan). Serum genistein concentration was quantified by the standard curves of peak areas of standard genistein (Figure 9). Glucoronized and sulfated genistein were quantified by the increased amount over the free level after each enzymatic digestion. Total genistein was calculated in the sum of free, glucoronized and sulfated forms.

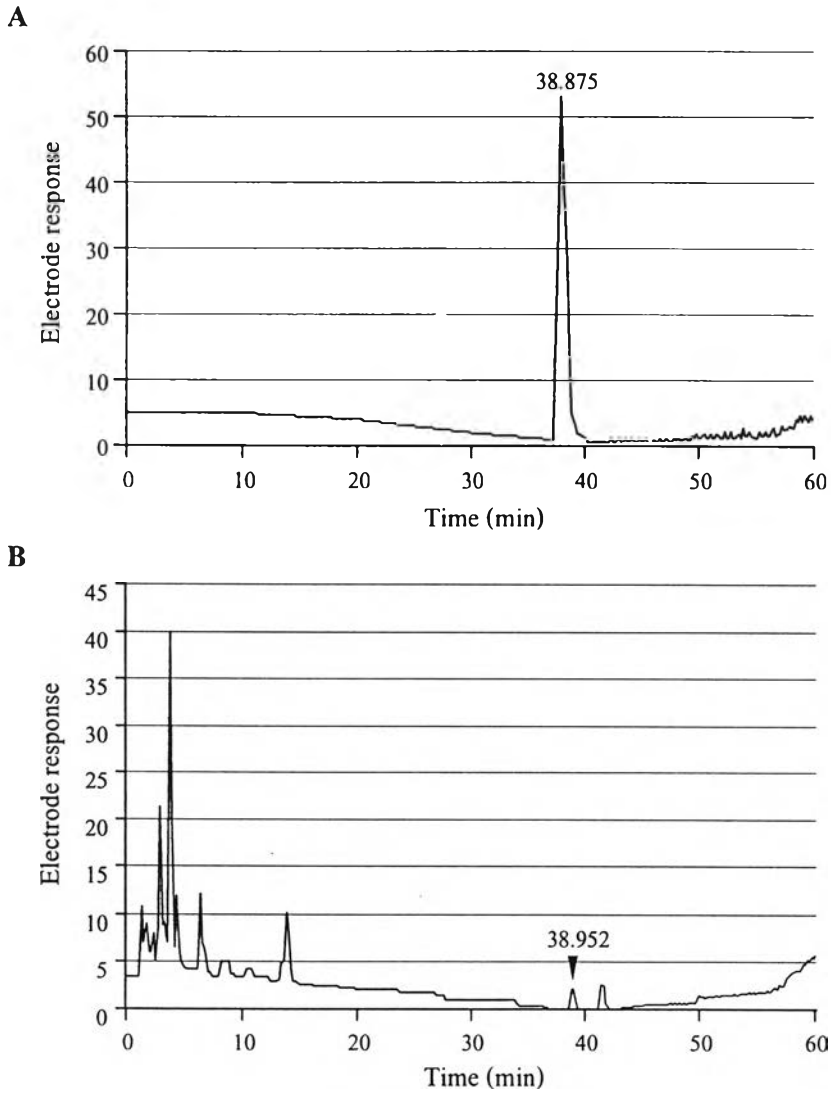

Figure 8. HPLC profiles of genistein standard (A) and serum sample of genistein treated NMU-rats (B). "Electrode response" refers to the electrical response of the chromatopac machine. Genistein peaks appear at approximately 39 minute.

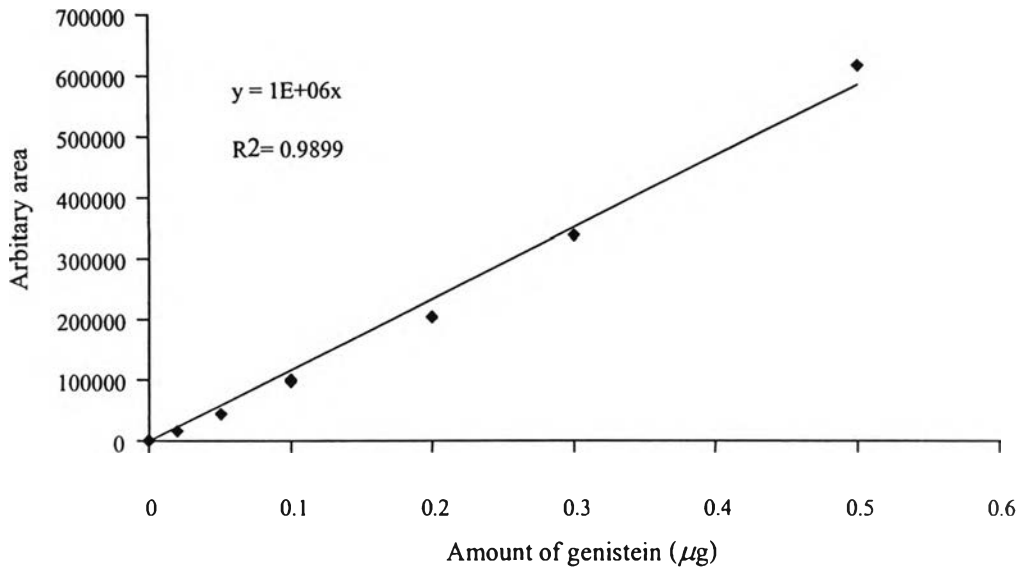

Figure 9. Standard curve of peak areas of genistein.

Histopathological study of tumor tissues, liver and reproductive organs

Tumor tissues of rats from experiment 1 and livers, uteri and ovaries of rats from experiment 2 were randomly selected for histopathological study. After overnight fixation in 10% buffer formalin, the tissues were dehydrated in series of ethanol gradient and clearing in xylene. They were then embedded in paraffin, cut into sections of 5 μm thickness, and stained with hematoxylin and eosin (H&E). The slides were analyzed under Olympus microscope and photographed using a Nikon camera. The histopathological criteria for identification of mammary tumor types and tumor subtypes were followed Russo and Russo, (2000) as shown in Table 3.

Table 3. Classification of rat mammary gland tumors

I. Epithelial neoplasms
- **A. Benign lesions**
  1. Intraductal papilloma
  2. Papillary cystadenoma
  3. Adenoma
     (a) Tubular
     (b) Lactating
- **B. Precancerous lesions**
  Intraductal proliferation (IDP)
- **C. Malignant lesions**
  1. *In situ* ductal carcinoma (DCIS)
     (a) Ductal papillary
     (b) Ductal solid and cribriform
     (c) Ductal comedo
  2. Invasive carcinoma
     (a) Papillary
     (b) Cribriform
     (c) Comedo
     (d) Tubular

II. Stromal neoplasms
- **A. Benign**
  Fibroma
- **B. Malignant**
  Fibrosarcoma

III. Epithelial-stromal neoplasms
- **A. Benign**
  Fibroadenoma
- **B. Malignant**
  Carcinosarcoma

IV. Nonneoplastic lesions
- **Cystic changes**
  (a) Ductal
  (b) Lobular

(Russo and Russo, 2000)

Determination of cancer related gene expression

The expression of cancer related genes in tumor tissues was determined via the mRNA levels by PCR technique. mRNA was extracted from the tumor tissue, reversely transcribed to cDNA, amplified the DNA and determined the DNA concentration by electrophoresis through 2 % agarose gel. The protocol for each step was explained below.

Fresh tissue preparation

After removing tumor tissues from the rats, the tumors were washed three times in sterile normal saline solution (0.9% (w/v) NaCl containing 1000 units/L of penicillin, 10 μg/L of streptomycin and 50 μg/L of amphotericin B). Tissues were cut into small pieces of 1-2 mm in sterile RPMI 1640 solution with pheno! red containing 10% (v/v) heat inactivated fetal bovine serum, 1000 units/L of penicillin, 10 μg/L of streptomycin and 50 μg/L of amphotericin B. The tissue suspensions were then centrifuged at 120 x g for 2 min. Supernatant was discarded and tissues were then resuspended in sterile RPMI 1640 solution containing 10% (v/v) filtered (0.22 μm) DMSO and 10% (v/v) heat inactivated fetal bovine serum. The tissue samples were aliquoted into 1-ml cryotubes, frozen at –20 ^{o}C for 4 hours, transferred to –80 ^{o}C, kept overnight and then transferred and stored in liquid nitrogen until the RNA extraction.

RNA extraction and cDNA synthesis

Tumor tissues were randomly selected from five rats in each group and assessed for mRNA expression levels. Total RNA was extracted from the fresh tumor tissue preparation using the ISOGEN kit (Nippon Gene, Tokyo, Japan) as per manufacturer instructions. The RNA pellet was dissolved into 20 μL of RNase free water and yield of total RNA was estimated by spectrophotometric absorbance at incident wavelengths of 260 and 280 nm. Five μg of total RNA was reverse transcribed to cDNA in a total volume of 20 μl of reaction mixture comprised initially of 1 μM of oligo(dT), 0.1 mM dNTP mix and distilled water (total volume of 13 μL) which was first incubated at 70 ^{o}C for 5 minutes, then supplemented with 4 μL of 5X First-Strand buffer (250 mM Tris-HCl, pH 8.30, 375 mM KCl, 15 mM MgCl2), 1 μL of 0.1 MDTT, 1

μL of RNase inhibitor (30 U) and 1 μL of Superscript Reverse Transcriptase (200 U) (Invitrogen) prior to incubation at 50 °C for 1 hour and the reaction then terminated at 70 °C for 15 minutes.

DNA amplification

To assess the expression levels of cancer related genes, we evaluated the mRNA expression levels of relevant marker ER - (ERα, ERβ and pS2), growth factor - (IGF-1 and *neu*) and metastasis suppressor - (GPR54) related genes by semi-quantitative RT-PCR using gene sequence specific primers for PCR amplification of the cDNA. PCR primers used to this end in this study (Table 1) were designed by Primer Express program (Applied Biosystems, USA) from reported gene sequences of rats (Rattus norvegicus) in EMBL database, and synthesized by Sigma Genosys (Japan). Amplification reactions were performed in a final volume of 20 μL containing GeneAmp 1x PCR buffer, 160 μM of dNTP, 1U of DNA polymerase (AmpliTaqGold, Applied Biosystem, Japan), 250 nM of each gene specific primer and 1μl 1/10 dilution of RT cDNA solution from the previous step. The optimal numbers of amplification cycles for each gene product were obtained within the linear logarithmic phase of each amplification curve. To determine the linear range of amplification reactions for each target gene, a series of 5 amplification reactions of 20, 25, 30, 35 and 40 cycles were performed, and found that 40 cycles for all amplicons except GAPDH (33 cycles) was within the linear range.

Thus forty cycles of DNA amplification of each gene, except 33 cycles for the housekeeping gene, glyceradehyde-3-phospate dehydrogenase (GAPDH), were carried out in Gene Amp PCR System, consisting of denaturation at 94 °C for 15 sec, annealing at 58-60 °C for 15 sec, and extension at 72 °C for 15 sec. The main PCR amplification cycle was preceded by an initial denaturation step of 94 °C for 10 min and followed by an additional extension period at 72 °C for 7 min. PCR product(s) were separated by 2% (w/v) agarose TAE gel electrophoresis and visualized with UV transillumination after ethidium bromide staining (100 ng/ml gel). Optical densities of ethidium bromide-stained PCR product were quantified by NIH image software (Japan), and calibrated with the PCR product of a housekeeping gene, GAPDH. Three replicates of each PCR were performed to validate the variation between experiments. For the amplification of ERβ and GPR54 genes, no amplicon was detected after initial PCR and two-

stage nested PCRs were performed, where one μl of the first round PCR product was added to the second nested reaction. The reaction components and conditions were as described above.

Table 4. Primers used for PCR

Primers	Sequence	Accession no.	Primer position	Product size (bp)	References
ER-α	5′-AGGTCCAATTCTGACAATCG-3′	NM 012689	(675-694)	318	Koike *et al.*, 1987
	3′-TCGTATCCCGCCTTTCAT-5′		(975-992)		
ER-β	5′-GATCAAAAACTCACCGTCGA-3′	NM 012754	(288-308)	373	Kuiper *et al.*, 1996
	3′-TCCTCTTCAGTGTCTCTCT-5′		(641-660)		
	5′-TCTACATCCCTTCCTCCTACG-3′		(370-390)	291	
pS2	5′-CCAGAACCAGGAAGAAACAT-3′	D 83231	(93-112)	348	Itoh *et al.*, 1996
	3′-CCGAATTTGAGGGTTAAAA-5′		(422-440)		
neu	5′-CAACTGGCTCCTGTCGAT-3′	AY116182	(649-666)	500	Watson *et al.*, 2002
	3′-CCATAGCACACTCGAGCAC-5		(1130-1148)		
IGF-1	5′-GACCTGCTGTGTAAACGACC-3′	NM 178866	(730-749)	393	Roberts *et al.*, 1987
	3′-ACTTCCTTCTGAGTCTTGGG-5′		(1103-1122)		
GPR54	5′-CAATTTCTACATCGCTAACC-3′	NM 023992	(231-250)	347	Ohtaki *et al.*, 2001
	3′-CACTGCAGTAGGTGTGAGG-5′		(559-577)		
	3′-CAGATGCTAAGGCTGACAGT-5′		(481-500)	270	
GAPDH	5′-ACTCAGAAGACTGTGGATGG-3′	AF 106860	(1393-1412)	313	Zheng and Ramirez, 2000
	3′-TGTTGAAGTCACAGGAGACA-5′		(1686-1705)		

Statistical Analysis

The tumor incidence was statistically analyzed by chi-square test. The median latency period, tumor multiplicity, tumor, body and organ weights, food intake and serum genistein concentration between groups were determined by unpaired t-test. Tumor cross-sectional area, serum E_2 concentration and density of cancer related genes between groups were determined by one-way ANOVA and confirmed the significant by the least significance difference (LSD) testing. The Statistical Packages for Social Science (SPSS)(version SPSS/PC 11.0, Chicago IL) was used. Data were expressed as mean $\pm$ SEM. The statistical significance was considered at $P < 0.05$.