CHAPTER III

RESEARCH METHODOLOGY

This chapter presents the methodology of this research including materials, apparatus and methods. The detail of each topic is described below.

Materials

1. Sericin powder (sericin A, B, and C) from Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.
2. Sericin and casein containing diets for animal study from Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Cell lines

1. Human colorectal adenocarcinoma cell lines (SW480, ATCC® No. CCL-228™, ATCC, Manassas VA, USA)
2. Normal human fetal colonic mucosal cell lines (FHC, ATCC® No. CRL-1831™, ATCC)

Animals

1. Male Spargue-Dawley rats from National Laboratory Animal Centre, Mahidol University, Nakhon Pathom, Thailand

Chemical and Reagents

1. Acetone (AR grade, RCI Labscan, Bangkok, Thailand)
2. Actin antibody (Cell signaling Technology®, Boston, USA)
3. 3,3-Aminopropyltriethoxysilane (APES, Sigma, Missouri, USA)
4. Ammonium persulphate (APS, Sigma, Missouri, USA)
5. Annexin V-FITC kit (Becton Dickinson, New Jersey, USA)
6. BCA protein assay kit (Thermo Scientific, Rockford, USA)

7. B-cell lymphoma 2 associated x protein antibody (Bax, Cell signaling Technology®, Boston, USA)

8. B-cell lymphoma 2 antibody (Bcl-2, Cell signaling Technology®, Boston, USA)

9. Bovine serum albumin (BSA, Sigma, Missouri, USA)

10. Caspase-3 fluorimetric kit (Sigma, Missouri, USA)

11. CD2, CD3, CD4, CD8a, CD11a, CD25, CD45, CD54, CD80 and CD86 antibodies (Becton Dickinson, New Jersey, USA)

12. Dimethylsulfoxide (DMSO, cell culture grade, Sigma, Missouri, USA)

13. Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4, Ajax finechem, Seven Hills, Australia)

14. 3,3-Diaminobenzidine (DAB, Becton Dickinson, New Jersey, USA)

15. 1,2-Dimethylhydrazine dihydrochloride (DMH, Sigma, Missouri, USA)

16. DPX mountant for microscopy (Ajax finechem, Seven Hills, Australia)

17. Dulbecco's modifide eagle's medium-low glucose (DMEM, Sigma, Missouri, USA)

18. Eosin (Merck, Darmstadt, Germany)

19. Ethanol (AR grade, RCI Labscan, Bangkok, Thailand)

20. Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, Molecular Biology grade, Sigma, Missouri, USA)

21. FACS buffer (Becton Dickinson, New Jersey, USA)

22. Fetal bovine serum (FBS, Gibco, California, USA)

23. Glycine (USP grade, Research Organics, Cleveland, USA)

24. Hematoxylin (Merck, Darmstadt, Germany)

25. Heparin sodium (Leo Pharma, Ballerup, Denmark)

26. Hydrogen peroxide (H_2O_2 (30%), Merck, Darmstadt, Germany)

27. Hydrochloric acid (HCl, Sigma, Missouri, USA)

28. Hydrocortisone (Cell culture grade, Sigma, Missouri, USA)

29. N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid) (HEPES, Cell culture grade, Sigma, Missouri, USA)

30. Insulin (Cell culture grade, Sigma, Missouri, USA)

31. Ki67 antibody (Abcam®, San Francisco, USA)

32. Mammalian protein extraction reagent (M-PER, Thermo Scientific, Rockford, USA)

33. Methanol (AR grade, RCI Labscan, Bangkok, Thailand)

34. Methylene blue (Sigma, Missouri, USA)

35. 2-Mercaptoethanol (Bio-Rad, Philadelphia, USA)

36. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, (MTT, Ultra pure grade, Amresco®, Solon, USA.)

37. Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Thermo Scientific, Rockford, USA)

38. Paraplast Plus (McCormick Scientific, Missouri, USA)

39. Penicillin and Streptomycin (Gibco, California, USA)

40. Phenylmethylsulfonyl fluoride (PMSF, Bio Basic, Markham Ontario, Canada)

41. Potassium chloride (KCl, RCI Labscan, Bangkok, Thailand)

42. Potassium dihydrogen orthophosphate (KH_2PO_4, Ajax finechem, Seven Hills, Australia)

43. Propidium iodide (PI, Molecular Probes, New York, USA)

44. Protease inhibitor cocktail (Sigma, Missouri, USA)

45. PageRuler Prestained Protein Ladder (Fermentas, Thermo Scientific, Rockford, USA)

46. RBC lysis buffer (Norgen BioTek Corp, Thorold, Canada)

47. RNase A (Amresco®, Solon, USA.)

48. Sodium bicarbonate (Na_2CO_3, Cell culture grade, Sigma, Missouri, USA)

49. Sodium chloride (NaCl, Ajax finechem, Seven Hills, Australia)

50. Sodium dodecyl sulfate (SDS, Sigma, Missouri, USA)

51. Thichloroacetic acid (TCA, Sigma, Missouri, USA)

52. Thiobarbituric acid (TBA, Sigma, Missouri, USA)

53. Tris (hydroxymethyl) amino-methane (Molecular biology grade, Research organics, Cleveland, USA)

54. Trypan blue (Cell culture grade, Sigma, Missouri, USA)

55. Trypsin/ EDTA (0.25%, Gibco, California, USA)

56. Tween-20 (Bio Basic, Markham Ontario, Canada)

57. Vectrastain Elit ABC kit (Vector Labs, Ontario, Canada)

58. Xylene (RCI Labscan, Bangkok, Thailand)

Instruments

1. Autoclave (HA-300P, Hirayama Manufacturing Corporation, Saitama, Japan)

2. Centrifuge (Hettich Mikro 120, Hettich Zentrifugen, DJB labcare Ltd, Buckinghamshire, England)

3. CO_2 –incubator (Forma series II, Thermo Fisher Scientific Inc., MA, USA)

4. FACSCalibur using CellQuestPro software (Becton Dickinson, New Jersey, USA)

5. Inverted microscope (Model TS100, Nikon Eclipse, Tokyo, Japan)

6. Laminar flow hood (Heal force®, HF safe 1200/c+, Shanghai, China)

7. Manual rotary microtome (Leica RM2235, Leica Microsystems, Nussloch, Germany)

8. Microplate Spectrophotometer (Multimode detector DTX 880, Becman Coulter, NSW, USA.)

9. Paraffin embedding station (Leica EG1160, Leica Microsystems, Nussloch, Germany)

10. pH meter (S20K, Mettler-Teledo GmbH, Schwerzenbach, Switzerland)

11. Semi-Dry blotting (Bio-Rad, Philadelphia, USA)

12. Stereo microscope (Olympus DF PLANAPO IX, Pennsylvania, USA)

13. Tissue processor (Lieca TP1020, (Bio-Rad, Philadelphia, USA)

14. Vertical gel electrophhroresis (Model Mini-PROTEAN Tetra Cell, Biorad laboratory, Philadelphia, USA)

Methodology

In the present study, we tested the effect of sericin on cell viability, cell apoptosis and cell cycle in SW480 colon cancer cell line in comparison with FHC normal colon cell line. In addition, we investigated the chemopreventive effect and potential mechanism of action of sericin as well as its immunoreguratory activity in DMH-treated rats (Figure 3).

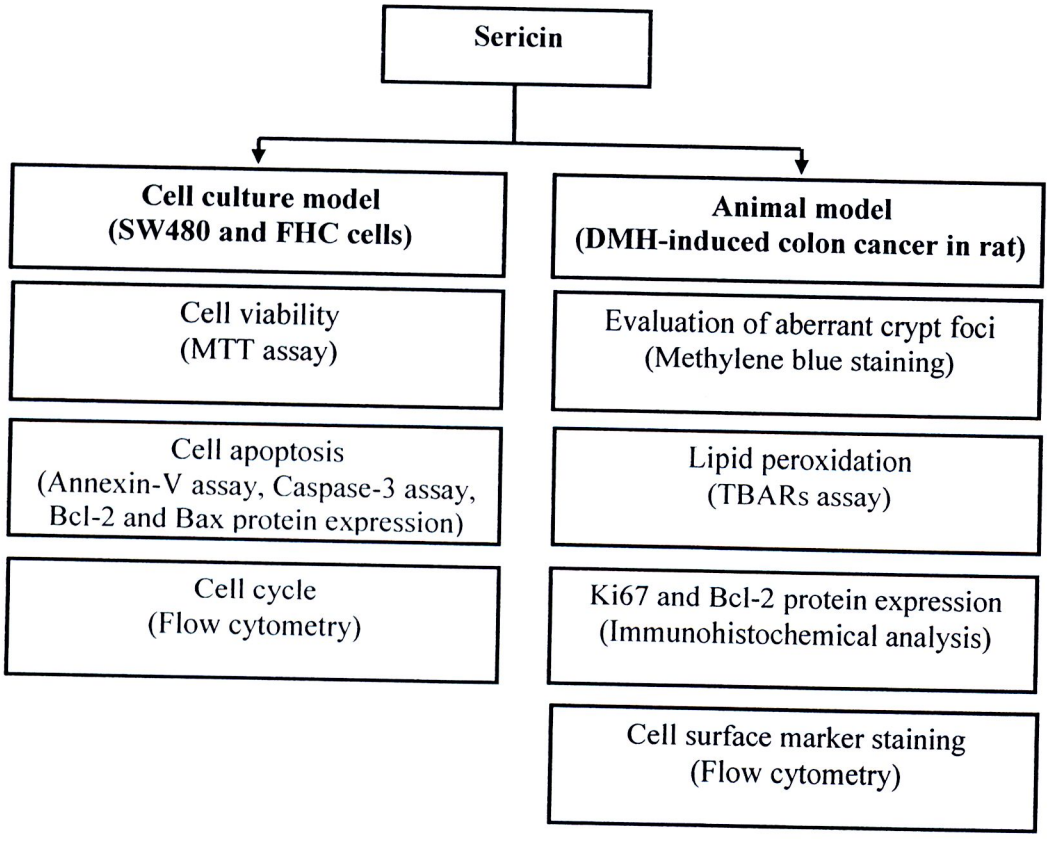

Figure 3 Experimental design in this study

1. Cell culture model

1.1 Preparation of sericin

Silk sericin was supplied by Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasrima, Thailand. Due to isolation techniques, the sericin was classified into 3 types according to its molecular weight ranges; sericin A, sericin B and sericin C (Table 1). Specific extraction condition was under Thai patent pending (application number 080595). Briefly, silk sericin was extracted with deionized water from raw silk yarns of silkworm *Bombyx mori*, under high pressure and temperature. The extract was dried at 130°C, under a sterile condition and dry mass was ground and sieved through 0.75 mm screen. Then the sericin powder was sealed in plastic bags and kept at 4°C until used. Table 2 and figure 3 show the amino composition and structure of sericin used in the present study.

Table 1 Type and molecular weight of sericin in this study

Sericin	Molecular weight (kDa)
A	191-339
B	76-132
C	61-113

Table 2 Composition of amino acids in sericin

Amino acid	Assay(g/100g)	Amino acid	Assay(g/100g)
Serine (Ser)	33.40	Valine (Val)	2.80
Aspartic acid (Asp)	16.70	Histidine (His)	1.30
Glutamic acid (Glu)	4.40	Leucine (Leu)	1.10
Glycine (Gly)	13.50	Isoleucine (Ile)	0.70
Threonine (Thr)	9.70	Phenylalanine (Phe)	0.50
Lysine (Lys)	3.30	Trytophan (Trp)	0.20
Tyrosine (Tyr)	2.60	Proline (Pro)	0.70
Arginine (Arg)	3.10	Cysteine (Cys)	0.20
Alanine (Ala)	6.00	Methionine (Met)	0.04

Source: Nantong Dongchang Chemical Industrial

1.2 Cell culture

The human colorectal cancer cells (SW480: ATCC® number CCL-228) and human fetal normal colonic mucosal cells (FHC: ATCC® number CRL-1831) were used in this study. These cell lines are widely used to test the chemopreventive effect against colon cancer in cell culture model. SW480 cells were cultured in Dulbecco's modified Eagle's medium with HamF-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 μg/ml streptomycin. FHC cells were cultured in DMEM/F-12 with 10% FBS, 25 mM N'-2-Hydroxyethylpiperazine-N'-2 ethanesulphonic acid (HEPES), 5 μg/ml insulin, 100 ng/ml hydrocortisone, 100 units/ml penicillin, and 100 μg/ml streptomycin. Both SW480 and FHC cells were cultured at 37°C in a humidified atmosphere of 5% CO_2, and fresh culture medium was replaced every 3-4 days.

1.3 Cell viability assay

Cell viability was determined by using MTT assay. The cells were plated in 96-well plates at 1×10^4 cells per well with complete culture medium. After an overnight incubation, the culture medium was replaced with fresh complete culture medium. The cells were exposed to various concentrations (25, 50, 100, 200, 400, 800, and 1600 μg/ml) of each type of sericin for 24, 48 and 72 h. Two hours before the end of the indicated treatments, cells were incubated with 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide (MTT). After culture medium was removed and cells were lysed with DMSO:ethanol (1:1), the absorbance was measured at 595 nm using a micro-plate reader. In viable cells, MTT is converted to the purple formazan dye by mitochondria dehydrogenases where the amount of formazan is proportional to the number of viable cells. No conversion occurs in dead cells. Cells treated with phosphate buffer saline (PBS) pH 7.4 were used as solvent control cells. Viability of treated cells was calculated as percentage of viable cells in comparing to un-treated cells.

1.4 Cell apoptosis assay

In this study, cell apoptosis was detected by an annexin V-FITC kit according to the manufacturer's instructions. Initially, the cells were plated in 60-mm petri dish at 1×10^6 cells per dish with complete culture medium. After an overnight incubation, the culture medium was replaced with fresh complete culture medium. The cells then were treated with each type of sericin at a concentration of 1600 μg/ml for 72 h. This high concentration was chosen because it gave the optimal result on cell viability. After treatment, the cells were collected, washed with cold PBS pH 7.4, and centrifuged 300g for 5 min at 4°C. After that, the cells were counted with hemocytometer and resuspended in 1X binding buffer at a concentration of 1×10^6 cells/ml and then 100 μl of cell suspension was transferred to a 5 ml test tube for flow cytometer. The suspended cells were mixed with 2 μl annexin V-FITC and 2 μl propidium iodide (PI), incubated for 15 min at room temperature (25°C) in the dark, mixed with 400 μl of 1X binding buffer, and then analyzed by FACSCalibur using CellQuestPro software within 1 h. The pattern of cell staining can be used to distinguish viable, apoptotic and necrotic cells as shown in table 3.

Table 3 Type of cell staining with annexin V-FITC and PI and analysis by flow cytometer

Type of cells	Annexin V-FITC	PI
Viable cells	- ve	- ve
Early apoptotic cells	+ ve	- ve
Late apoptotic cells	+ ve	+ ve
Necrotic cells	- ve	+ ve

Note: - ve: negative; + ve: positive

1.5 Caspase-3 activity assay

In this study, caspase-3 activity was detected by a caspase-3 assay fluorimetric kit. Initially, the cells were plated in 60-mm petri dish at 1×10^6 cells per dish with DMEM/F-12 with 10% FBS. After overnight incubation, the culture medium was replaced with fresh complete culture medium and then cells were treated with each type of sericin at concentration of 1600 μg/ml for 72 h. After treatment, the cells were scraped, washed with cold PBS pH 7.4 and centrifuged 300g for 5 min at 4°C. The cell pellets were suspended in mammalian protein extraction reagent (M-PER) solution at a concentration of 150 μl per 1×10^6 cells. The lysate was collected and transfered to a 1.5 ml microcentrifuge tube and then centrifuged at 14,000 g for 10 min at 4°C to pellet the cell debris. The supernatant was transferred to a new 1.5 ml microcentrifuge tube for measuring protein concentration by using BCA protein assay. For measuring caspase-3 activity, 5 μl of cell lysate, 5 μl of 1X assay buffer and 100 μl of reaction mixture were mixed in 96-well plate. The plate was covered and incubated at room temperature in the dark for 4 h. The caspase-3 activity was determined by using specific substrate for caspase-3 and measured fluorescent intensity (Ex 360 nm and Em 535 nm) by micro-plate reader. Cells treated with PBS pH 7.4 were used as control cells. Caspase-3 activity was calculated as a caspase-3 activity (pmol 7-amino-4-methylcoumarin, AMC/min/mg protein).

1.6 Western blotting

In this study, the expressions of Bcl-2 anti-apoptotic protein and Bax pro-apoptotic protein were investivated by western blotting. Cells were plated in 60-mm petri dish at 1×10^6 cells per dish with DMEM/F-12 with 10% FBS. After overnight incubation, the culture medium was replaced with fresh complete medium and then treated with each type of sericin at concentration of 1600 μg/ml for 72 h. Then, the cells were lysed in M-PER reagent at a concentration of 150 μl per 1×10^6 cells. The protein concentration was determined by BCA protein assay kit. Cell lysates at 20 μg were loaded and separated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and finally transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked by incubation in 1% bovine serum albumin (BSA) in 0.05% Tween-20 for 1 h at room temperature and after that washed

with TBS buffer containing 0.05% Tween-20 (TBS-T). Then the membrane was incubated overnight at 4°C with primary antibodies rabbit polyclonal anti-Bcl-2 (1/2,000 dilution), rabbit polyclonal anti-Bax (1/2,000 dilution) or rabbit monoclonal anti-β actin (1/4,000 dilution) in 5% skim milk in TBS-T. The membrane was washed 3 times with TBS-T and developed with secondary antibody goat anti-rabbit IgG conjugated with alkaline phosphatase (1/10,000) at room temperature for 1h. The protein bands were detected by using nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as a substrate. The intensity of protein bands of Bcl-2 and Bax were determined by using PhotoshopCS3 software. The intensity of β-actin was used as loading control.

1.7 Cell cycle analysis

The cell cycle was analyzed by using flow cytometry. The cells were plated in 60-mm petri dish at 1×10^6 cells per dish with DMEM/F-12 with 1% FBS for synchronization. After overnight incubation, the culture medium were replaced with fresh complete medium and then were treated with each type of sericin at concentration of 1600 μg/ml for 72 h. After treatment, the cells were harvested by trypsinization, washed twice with PBS pH 7.4, and then centrifuged at 300 g for 5 min at 4°C. The cells were counted with hemocytometer and fixed overnight with cold 70% ethanol by gradually dropping ethanol until reach the concentration of 1×10^6 cells/ 1 ml of 70% ethanol. After that, the cells were centrifuged 500 g for 5 min at 4°C, washed with cold PBS one time, and centrifuged 300 g for 5 min at 4°C again. The cells were treated with 100 μg/ml of RNase A in PBS pH 7.4 at 37°C for 30 min for lysis RNA. Then the cells were stained with 20 μg/ml of propidium iodide for 30 min at room temperature in the dark and washed with PBS 1 time. The distribution of cells in each cell cycle phase was performed by FACSCalibur using CellQuestPro software.

2. Animal model

2.1 Animals and diet

Male Spargue-Dawley rats in the weight range of 120-160 g were housed in stainless steel cages under hygienic conditions in the departmental animal house at room temperature of 24 ± 2°C and a humidity of 50 ± 10 % with a 12 hours light-dark cycle. The experimental protocol was approved by the ethical committee for animal care of Naresuan University. Before initiation of the experiments, rats were adapted to the laboratory condition for one week. Control casein diets and sericin diets were produced by Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The experimental diets were prepared by mixing standard rat diet with casein or sericin at the level of 4% w/w.

2.2 DMH administration

DMH was dissolved in 1 mM EDTA and the pH was adjusted to 6.5 to ensure the stability of the carcinogen. The preparation of DMH solution was followed by the study of Manoj [137]. The rats were given subcutaneous injections of DMH, once a week, for 10 weeks at a dose of 20 mg/kg bodyweight.

2.3 Experimental design

The rats were randomly distributed into five groups (six rats per group). The experimental design is shown in Figure 4. Group 1 and 3 were fed casein diet whereas group 2 and 4 were fed sericin diet. The last group (post-sericin) was received casein diet for the first 5 weeks and then replaced with sericin diet until the end of experiment. Group 3, 4 and 5 were subcutaneously injected with DMH (20 mg/kg bodyweight) once a week for 10 weeks. Group 1 and 2 were injected with 1 mM EDTA solution pH 6.5 used to dissolve DMH for the same period of time. Food consumption and body weight were recorded weekly throughout the experimental period. Ten weeks after the last DMH injection, all rats were sacrificed and colons were removed and flushed with cold phosphate buffer saline (PBS) pH 7.4. The colon was divided into three parts, proximal, middle and distal. Each part was then cut into three segments for aberrant crypt foci (ACF) analysis, lipid peroxidation and immunohistochemical analysis.

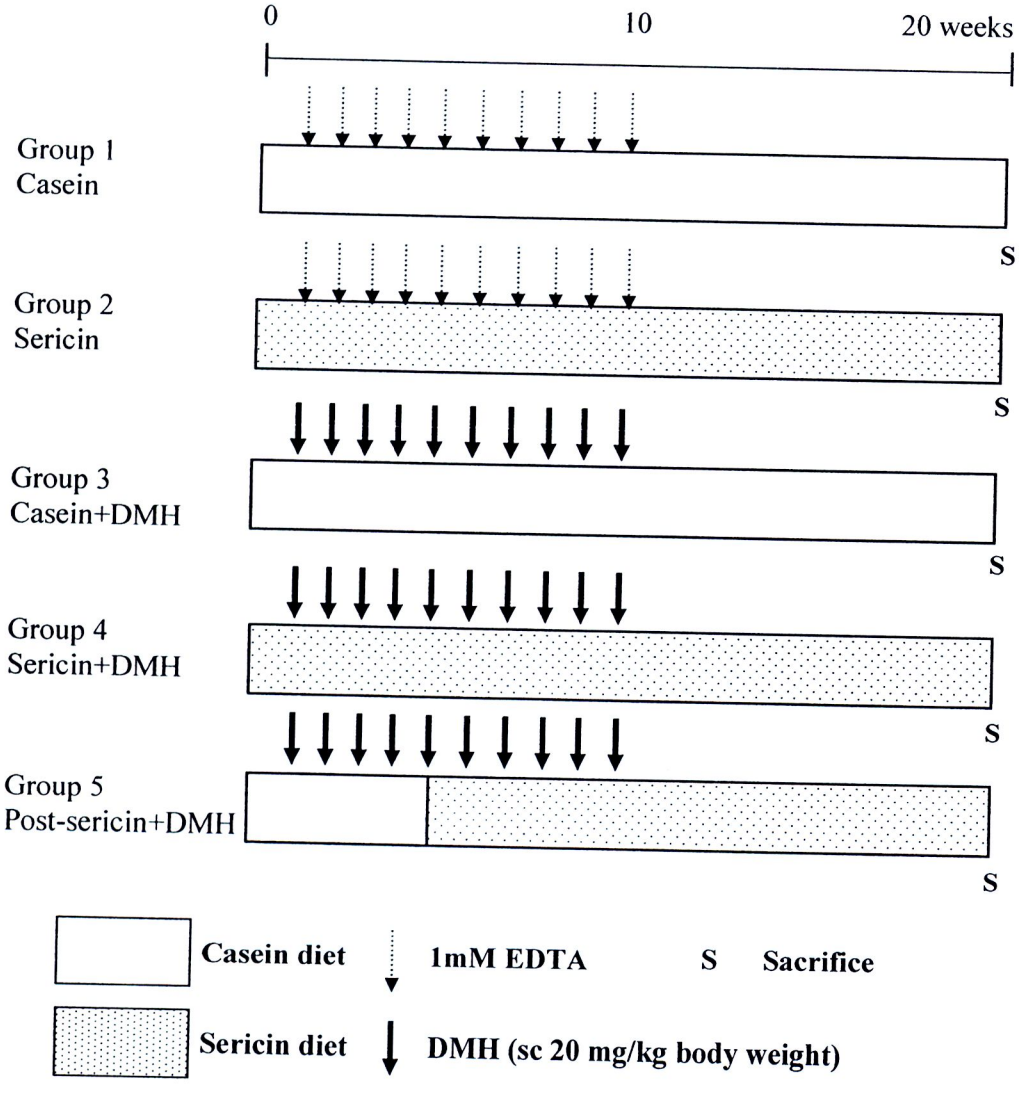

Figure 4 Experimental design for DMH-induced colon cancer in rat

2.4 Determination of aberrant crypt foci (ACF)

For determination of ACF, the colons were flushed with cold PBS, opened along the longitudinal median axis and fixed flat between two pieces of filter papers in 10% neutral formalin buffer for 6 h. The colonic tissues were stained with 0.2% methylene blue in PBS for 5 min, placed on microscopic slides, and observed ACF formation under a stereo microscope. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size and thicker epithelial lining compared to normal crypts. They usually gathered into a focus, consisting of one or more aberrant crypts. The number of ACF in each parts of colon was recorded. Crypt multiplicity was analyzed by determining the number of crypts in each focus (1, 2, 3, 4 and 5 or more aberrant crypts per focus) [138].

2.5 Lipid peroxidation of colon tissue

Lipid peroxidation was estimated by thiobarbituric acid reactive substances (TBARs) assay. The colon was homogenized with ice-cold PBS pH 7.4 containing 1 mM phenylmethylsulfonyl fluoride (PMSF), incubated with TBARs reagent (40% TCA: 1.4% TBA: 8% HCl; 1:2:1) and then heated at 90°C for 1 h. After that, the solution was centrifuged 5,000 rpm for 5 min at 25°C and the fluorescent intensity was measured at Ex 535 nm and Em 595 nm. The protein content of colon homogenate was determined by BCA protein assay kit.

2.6 Immunohistochemical analysis

After the colon was fixed in 10% neutral formalin buffer for 48 h, the segments was cut into serial strips, processed for paraffin-embedding and cut into 3 μm-thick sections. Immunostaining of Ki67 proliferation marker and Bcl-2 anti-apoptotic marker was performed by using avidin-biotin peroxidase complex system. Colon sections were deparaffinzed with xylene, dehydrated through a graded ethanol series (99.99%, 95% and 70% ethanol). Hydrogen peroxide at 0.5% was administered to the slides for 10 min. Antigen retrieval was accomplished by heating the sections in 10 mM citrate buffer pH 6.0 for 10 min. For blocking non-specific binding, the sections were incubated for 10 min with 1.5% normal goat blocking serum in Tris-buffer saline (TBS) pH 7.6 followed by incubation with avidin and biotin blocking solution for 15 min for each step, respectively. After that, the slides were incubated

with primary antibodies rabbit monoclonal anti Ki67 (dilution 1/150) or rabbit polyclonal anti Bcl-2 (dilution 1/2,000) for 1 h.

After washing twice in TBS, the sections were incubated for 30 min with biotinylated secondary antibodies anti-rabbit immunoglobulins, washed again in TBS, and incubated with 3, 3'- diaminobenzidine (DAB) peroxidase substrate to give a brown reaction product. The sections were counterstained with hematoxylin, dehydrated, cleared and mounted with DPX mounting solution. All sections were covered with cover slide and observed under a light microscope. Positive controls were performed in each staining (using rat spleen for cell proliferation and cell apoptosis investigation). Negative staining controls consisted of slides stained with omission of the primary antibody.

2.7 Collection of blood and detection of surface markers

Blood samples were obtained from these rats by cardiac puncture and were collected into two 1.5 ml micro-centrifuge tubes containing, heparin sodium. After that, 50 µl of each sample of the whole blood samples were transferred into two 5 ml test-tube for analysis by flow cytometer. The cells were stained with primary antibodies specific for CD2, CD3, CD4, CD8a, CD11a, CD25, CD45, CD54, CD80 or CD86 (Table 4) and the negative controls were performed for all primary antibodies. After incubation with the antibodies, the red blood cells were lysed with 1xRBC Lysis buffer and the cells (approximately 1×10^5 cells/tube) were added with FACS buffer. All samples were analyzed by FACSCalibur using CellQuestPro software (Becton Dickinson, New Jersey, USA).

Table 4 Cell surface markers for rats

Cell surface antigen	Antigen distribution	Function
CD2	Thymocytes, T lymphocytes in spleen and lymph node, dendritic epidermal T cells, splenic macrophages and NK cells	: A ligand for CD48 : Association with T-cell receptor complex, intercellular adhesion and signaling transduction
CD3	Thymocytes, peripheral T lymphocytes and dendritic epidermal T cells	: A critical component of the T-cell receptor (TCR) and a marker of the T-cell lineage : Induction of long-term specific tolerance to an organ allograft
CD4	Thymocytes, T lymphocytes, monocytes, macrophages, dendritic cells and microglia	: An antigen co-receptor on T cell surface that interacts with MHC class II molecules on antigen-presenting cells (APC) : Participation in T cell activation with T cell receptor complex and protein tyrosine kinases
CD8a	Thymocytes, T lymphocytes, NK cells, monocytes	: An antigen co-receptor on T-cell surface which interacts with MHC class I molecules on antigen-presenting cells (APC)
CD11a	Majority of leukocytes, but not on peritoneal macrophages or peritoneal mast cells	: Interaction with ICAM-1 (CD54) and ICAM-2 (CD102) : Inhibition of leukocyte infiltration in several the *in vivo* models of inflammation

Table 4 (Cont.)

Cell surface antigen	Antigen distribution	Function
CD25	T and B lymphocytes, NK cells, monocytes	: Association with CD122 (IL-2 receptor beta chain) and CD132 (common gamma chain) to form the high affinity IL-2 receptor : A role in lymphocyte differentiation and activation/proliferation
CD45	All hematopoietic cells, except erythrocytes and platelets	: A member of protein tyrosine phosphatase (PTP) family : T-cell and B-cell antigen receptor signal transduction
CD54	Thymocytes, peripheral lymphocytes, macrophages, dendritic cells, monocytes and granulocytes	: A ligand for LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) : A roles in trans-endothelial migration of leukocytes to sites of inflammation and in T cell interactions with antigen presenting cells
CD80	B lymphocytes, dendritic cells, macrophages	: High affinity to bind to two T cell surface antigens, CD28 and CD152 (CTLA-4) : T-B cell communication leading to in activation of T and B cell, respectively
CD86	B lymphocytes, dendritic cells and macrophages	: A counter-receptor for the T cell surface molecules CD28 and CD152 : T-B crosstalk, T cell co-stimulation, autoantibody production and Th2-mediated Ig production.

3. Statistical analysis

All data in cell culture were expressed as means ± standard error of the mean (SEM) and data in animal model were expressed as means ± standard deviation (SD). The data was analyzed by one-way analysis of variance (ANOVA) and student *t*-test. The p value ≤ 0.05 is considered significant.