ผลของเคอร์คูมินต่อความเสียหายของดีเอ็นเอ การเพิ่มจำนวนเซลล์และอัตราการเกิดมะเร็งกระเพาะอาหารที่ถูกเหนี่ยวนำโดยเกลือเข้มข้นร่วมกับเอ็นเมทิลเอ็นไนโตรโซยูเรีย ในหนูแรท

นางสาวกวีญา สินธารา

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EFFECTS OF CURCUMIN ON DNA DAMAGE, CELL PROLIFERATION, AND INCIDENCE OF GASTRIC CANCER INDUCED BY HIGH SALT AND *N*-METHYL-*N*-NITROSOUREA IN RATS

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Thesis Title	EFFECTS OF CURCUMIN ON DNA DAMAGE, CELL PROLIFERATION, AND INCIDENCE OF GASTRIC CANCER INDUCED BY HIGH SALT AND *N*-METHYL-*N*-NITROSOUREA IN RATS
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กวีญา สินธารา : ผลของเคอร์คูมินต่อความเสียหายของดีเอ็นเอ การเพิ่มจำนวนเซลล์และอัตราการเกิดมะเร็งกระเพาะอาหารที่ถูกเหนี่ยวนำโดยเกลือเข้มข้นร่วมกับเอ็นเมทิลเอ็นไนโตรโซยูเรีย ในหนูแรท. (EFFECTS OF CURCUMIN ON DNA DAMAGE, CELL PROLIFERATION, AND INCIDENCE OF GASTRIC CANCER INDUCED BY HIGH SALT AND *N*-METHYL-*N*-NITROSOUREA IN RATS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. พญ. ดวงพร ทองงาม, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. สุทธิลักษณ์ ปทุมราช, 123 หน้า.

โรคมะเร็งกระเพาะอาหารสามารถเกิดได้ทุกส่วนของกระเพาะอาหาร ด้วยการวินิจฉัยที่ล่าบากทำให้โรคนี้เป็นปัญหาทางสุขภาพที่สำคัญทั่วโลก เคอร์คูมินซึ่งเป็นสารออกฤทธิ์ของขมิ้นชันมีคุณสมบัติในการต้านอนุมูลอิสระและยับยั้งการเกิดมะเร็ง การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาว่าเคอร์คูมินสามารถลดอัตราการเกิดมะเร็งกระเพาะอาหารและระดับโปรตีนที่สำคัญในการเกิดมะเร็งกระเพาะอาหารที่ถูกเหนี่ยวนำโดยเกลือเข้มข้นร่วมกับ เอ็นเมทิลเอ็นไนโตรโซยูเรีย (เอ็มเอ็นยู) ในหนูแรทได้หรือไม่ หนูแรทสายพันธุ์วิสทาร์เพศผู้ถูกแบ่งออกเป็น 6 กลุ่ม คือ 1 กลุ่มควบคุม 2 กลุ่มควบคุมที่ได้รับเคอร์คูมิน 3 กลุ่มที่ได้รับการเหนี่ยวนำให้เกิดมะเร็งกระเพาะอาหาร 4 กลุ่มที่ได้รับการเหนี่ยวนำและได้รับการป้อนเคอร์คูมิน ขนาด 200 มิลลิกรัมต่อน้ำหนักตัว 1 กิโลกรัม ทุกวันในช่วง 3 สัปดาห์แรกที่มีการเหนี่ยวนำ 5 กลุ่มที่ได้รับการเหนี่ยวนำและได้รับเคอร์คูมินทุกวันในช่วง 17 สัปดาห์หลังจากการเหนี่ยวนำ และ 6 กลุ่มที่ได้รับการเหนี่ยวนำและได้รับเคอร์คูมินทุกวันเป็นเวลา 20 สัปดาห์ หนูในกลุ่มที่ได้รับการเหนี่ยวนำให้เกิดมะเร็งกระเพาะอาหารทุกกลุ่มจะได้รับการป้อนเอ็มเอ็นยูขนาด 100 มิลลิกรัมต่อน้ำหนักตัว 1 กิโลกรัม ในวันเริ่มต้นและวันที่ 14 ของการทดลองและได้รับการป้อนเกลือเข้มข้นสัปดาห์ละ 2 ครั้งในช่วงเวลาสามสัปดาห์แรก เมื่อสิ้นสุดการทดลองในสัปดาห์ที่ 20 กระเพาะอาหารถูกนำออกมาเพื่อศึกษาอัตราการเกิดมะเร็งด้วยวิธีทางเนื้อเยื่อวิทยา การแสดงออกของโปรตีนแปดไฮดรอกซี่สองดีออกซี่กัวโนซีนและโปรตีนไซคลินดีหนึ่งด้วยวิธีอิมมูโนพยาธิวิทยา และการแสดงออกของโปรตีนไอแคปปาบีแอลฟาที่ถูกเติมหมู่ฟอสเฟตและโปรตีนบีซีแอลสองด้วยวิธีเวสเทิร์นบล็อท

เกิดมะเร็งในส่วนก่อนกระเพาะอาหารของหนูทุกตัวในกลุ่ม 3 และ 5 การได้รับเคอร์คูมินในกลุ่มที่ 4 และ 6 ลดอัตราการเกิดมะเร็งได้ร้อยละ 40 และ 50 ตามลำดับ การแสดงออกของโปรตีนแปดไฮดรอกซี่สองดีออกซี่กัวโนซีน ไซคลินดีหนึ่ง และบีซีแอลสอง เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติในกลุ่มที่ 3 เทียบกับกลุ่มที่ 1 การแสดงออกของโปรตีนไอแคปปาบีแอลฟาที่ถูกเติมหมู่ฟอสเฟตมีแนวโน้มเพิ่มขึ้นในกลุ่มที่ 3 เทียบกับกลุ่มที่ 1 จำนวนเซลล์ที่มีการแสดงออกของโปรตีนแปดไฮดรอกซี่สองดีออกซี่กัวโนซีน ในกลุ่มที่ 5 และ 6 ลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มที่ 3 โดยค่าเฉลี่ยคือร้อยละ 46.43 ± 4.42 และ 46.38 ± 3.14 เทียบกับร้อยละ 53.06 ± 5.96 ($p = 0.012$ และ $p = 0.011$) ตามลำดับ ค่าเฉลี่ยของการแสดงออกของโปรตีนไอแคปปาบีแอลฟาที่ถูกเติมหมู่ฟอสเฟตมีแนวโน้มลดลงในกลุ่มที่ 4 เมื่อเทียบกับกลุ่มที่ 3 กล่าวโดยสรุปว่าการให้เกลือเข้มข้นร่วมกับเอ็มเอ็นยูนำไปสู่การเกิดมะเร็งในส่วนก่อนกระเพาะอาหารและการเพิ่มขึ้นของการแสดงออกของโปรตีน แปดไฮดรอกซี่สองดีออกซี่กัวโนซีน ไซคลินดีหนึ่ง และบีซีแอลสอง การได้รับเคอร์คูมินตั้งแต่เริ่มการเหนี่ยวนำให้เกิดมะเร็งช่วยลดอัตราการเกิดมะเร็งได้ร้อยละ 50 ร่วมกับการลดลงอย่างมีนัยสำคัญทางสถิติของการแสดงออกของโปรตีนแปดไฮดรอกซี่สองดีออกซี่กัวโนซีน

สาขาวิชา: สรีรวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา : 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5087754320 : MAJOR PHYSIOLOGY
KEYWORDS : CURCUMIN / GASTRIC CANCER / 8-OHdG / IκBα PHOSPHORYLATION / CYCLIN D1 / BCL-2

KAWIYA SINTARA : EFFECTS OF CURCUMIN ON DNA DAMAGE, CELL PROLIFERATION, AND INCIDENCE OF GASTRIC CANCER INDUCED BY HIGH SALT AND *N*-METHYL-*N*-NITROSOUREA IN RATS. ADVISOR : ASSOC. PROF. DUANGPORN THONG-NGAM, M.D., CO-ADVISOR : ASSOC. PROF.SUTHILUK PATUMRAJ, Ph.D., 123 pp.

Gastric cancer can generate in any part of the stomach. Because of poorly detected, gastric cancer causes the public health problem worldwide. Curcumin has anti-oxidant and chemopreventive properties. To determine whether curcumin could attenuate the gastric cancer incidence and the key proteins involved in carcinogenesis induced by saturated salt (s-NaCl) and *N*-methyl-*N*-nitrosourea (MNU) in rats. Male Wistar rats were divided into 6 groups: 1 control (CO), 2 control supplemented with 200 mg/kg curcumin (CC), 3 MNU+s-NaCl, 4 MNU+s-NaCl supplemented with 200 mg/kg curcumin daily for the first 3 weeks (MNU+s-NaCl+C3W), 5 MNU+s-NaCl rats supplemented with curcumin after induction (MNU+s-NaCl+C17W), and 6 MNU+s-NaCl supplemented with curcumin for 20 weeks (MNU+s-NaCl+C20W). To induce stomach cancer, rats except for group 1 and 2 were orally treated with 100 mg/kg MNU on day 0 and 14, and s-NaCl twice-a-week for the first 3 weeks. The experiment was finished and rats were sacrificed at the end of 20 weeks. The stomachs were removed for histological examination. The expressions of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and cyclin D1 were investigated by immunohistochemistry. Western blot technique was used to examine phosphorylated inhibitor kappaB alpha (IκBα), and Bcl-2 expressions

Cancers were found in the forestomach of all rats in group 3 and 5. Curcumin supplementations in group 4 and 6 showed 40% and 50% reduction of cancer incidence, respectively. Expressions of 8-OHdG, cyclin D1, and Bcl-2 significantly increased in group 3 compared with group 1. The phospho-IκBα expression had a tendency to increase in group 3 compared with group 1. Immunoreactive cells of 8-OHdG in group 5 and 6 significantly decreased when compared with group 3 (46.43% ± 4.42 and 46.38% ± 3.14 vs. 53.06% ± 5.96; $p = 0.012$ and $p = 0.011$, respectively). The relative intensity of phospho-IκBα in group 4 tended to reduce when compared with group 3. In conclusion, MNU and s-NaCl administrations lead to forestomach cancer and increase of 8-OHdG, cyclin D1, and Bcl-2 expressions in rats. Early supplementation of curcumin could attenuate the incidence of cancer at 50% with the significant reduction of 8-OHdG.

Department : Physiology Student's Signature ……

Field of Study : Physiology Advisor's Signature ……

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LIST OF ABBREVIATIONS

APS	=	Ammonium persulfate
AP-1	=	Activator protein
BaP	=	Benzo(a)pyrene
BCA	=	Bicinchoninic acid
Bcl-2	=	B cell CLL/lymphoma-2
BW	=	Body weight
CC	=	Control group supplemented with curcumin
CO	=	Control group
DAB	=	Diaminobenzidine
DMSO	=	Dimethylsulfoxide
DTT	=	Dithiothreitol
g	=	Gram
G	=	Gravity
GE	=	Gastric epithelium
H_2O_2	=	Hydrogen peroxide
H. pylori	=	*Helicobacter pylori*
H&E	=	Hematoxylin and Eosin
IARC	=	International Agency for Research on Cancer
IHC	=	Immunohistochemistry
IκB	=	Inhibitor kappa B
IKK	=	Inhibitor kappa B kinase
IL	=	Interleukin
iNOS	=	Inducible nitric oxide syntase
ip	=	Intraperitoneal injection
kDa	=	Kilo Dalton
kg	=	Kilogram
LP	=	Laminar propria
m	=	Meter
M	=	Muscle layer
MCL	=	Human mantle cell lymphoma

MDA	=	Malonaldehyde
mg	=	Milligram
mL	=	Milliliter
mM	=	Millimolar
MM	=	Muscularis mucosae
MMPs	=	Matrix metalloproteinases
MNNG	=	*N*-methyl-*N*-nitro-*N*-nitrosoguadinine
MNU	=	*N*-methyl-N-nitrosourea
MNU+s-NaCl	=	MNU and saturated NaCl induced group
MNU+s-NaCl+C3W	=	MNU and saturated NaCl induced rats supplemented with curcumin for 3 weeks
MNU+s-NaCl+C17W	=	MNU and saturated NaCl induced rats supplemented with curcumin for 17 weeks
MNU+s-NaCl+C20W	=	MNU and saturated NaCl induced rats supplemented with curcumin for 20 weeks
NDEA	=	*N*-nitrosodiethylamine
NDMA	=	*N*-nitrosodimethylamine
NF-κB	=	Nuclear factor-kappa B
NF-κB p65	=	Nuclear factor-kappa B subunit p65
nm	=	Nanometer
NMBA	=	N-nitrosomethyl-benzylamine
NO	=	Nitric oxide
O.D.	=	Optical density
O^6-MedGuo	=	O^6-methyldeoxyguanosine
PAGE	=	Polyacrylamide gel electrophoresis
PGs	=	Prostaglandins
rpm	=	Round per minute
RH	=	Rel homology
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
RS	=	Reactive species
SCC	=	Squamous cell carcinoma

SD	=	Standard deviation
SDS	=	Sodium dodecylsulfate
SM	=	Submucosa
s-NaCl	=	Saturated sodium chloride
TEMED	=	Tetramethylethylenediamine
TNF	=	Tumor necrosis factor
TPA	=	12-*O*-tetradecanoylphorbol-13-acetate
ºC	=	Degree Celsius
µg	=	Microgram
µL	=	Microliter
µm	=	Micrometer
µmol	=	Micromolar
8-OHdG	=	8-Hydroxy-2’-deoxyguanosine
8-oxodG	=	8-oxo-7,8-dihydro-2’-deoxyguanosine

CHAPTER I

INTRODUCTION

Background and Rationale

Gastric cancer can develop in any part of the stomach. With poor prognosis, gastric cancer causes almost one million deaths per year worldwidely [1-2]. *Helicobacter pylori (H. pylori)* infection is the major causative factor for gastric adenocarcinoma development. In the other hand, geographic differences and changes in patterns of cancer incidence collected from immigrants suggest that the cancer is also related to lifestyle factors, such as diet. There are evidences that consumption of high salt diets and *N*-nitroso compounds and low intake of fruits and vegetables raise the risk of gastric cancer [3-4]. High salt and nitrite dietary found in preserved meats are potentially carcinogenic. According to the case-control and the epidemiological studies in man, salt (sodium chloride, NaCl) and salted foods could be the risk factor of gastric carcinogenesis [5-6]. Moreover, many animal models provided evidences to support that high salt diet and hypertonic NaCl promoted gastric carcinogenesis when salt is given with *N*-nitroso carcinogen, such as "*N*-methyl-*N*-nitro-*N*-nitosoguadinine (MNNG)" and "*N*-methyl-*N*-nitrosourea (MNU)", induction [7-9].

Ingestion of hypertonic NaCl by rats led to damage of gastric mucosa, inflammation, loss of epithelium, and compensatory increase of cell proliferation [10-12]. Inflammatory responses and proliferative changes could increase the mutagenic effect of food-derived carcinogens. *N*-nitroso mutagens, potent carcinogen, can induce cell death, cellular stress, chromosomal aberrations, DNA damage, and mutations [13]. Thus, the association between hypertonic NaCl and *N*-nitroso carcinogen may promote gastric tumor in the rat by an increase of inflammation, mutation and compensatory cell proliferation.

Chronic inflammation belongs to a risk factor of many types of human cancers, including stomach cancer. The production of chemokines, inflammatory cytokines, and free radicals, as well as mediators of growth and angiogenesis are contributed to inflammatory responses [14]. Free radicals cause DNA damage and

disturb the mechanisms of DNA repair. They also induce genetic instability in the proliferative cells. Thus, free radicals are contributed to the initiation step of carcinogenesis [15]. "8-Hydroxy-2'-deoxyguanosine (8-OHdG)", also known as "8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG)" is a potential biomarker of DNA damage induced by oxidative stress. It also involves in a mediator of initiation and promotion stages of carcinogenesis [16]. Although the molecular pathways that link inflammatory responses to carcinogenesis are various, "nuclear factor kappa B (NF-κB)" is implied as a key protein. A range of stimuli such as "reactive oxygen species (ROS)" and cytokines from an inflammatory response can activate "inhibitor kappaB kinase (IKK) complex" resulting in "inhibitor kappaB (IκB)" phosphorylation. After that, the "phosphorylated IκB" is proteolysis allowing the translocation of "nuclear factor kappaB (NF-κB)" into the nucleus. The NF-κB in nucleus can activation target genes that contribute to the responses of inflammation and regulation of cell cycle and death [17]. In cancer progression, NF-κB-dependent genes include those encoding cell cycle regulator such as cyclin D1, and pro-survival proteins such as Bcl-xL and Bcl-2 [18]. These biochemical molecules playing a pivotal role in each of the carcinogenesis processes have supposed as therapeutic approaches.

Nowadays, traditional medicines are used for alternative treatment as chemoprevention and cancer therapy. Curcumin (diferuloylmethane), a polyphenol compound, is an active ingredient of tumeric (*Curcuma longa*). Curcumin has many biological activities including chemopreventive property. Interestingly, curcumin is safety for human and animals [19]. Curcumin shows the effects on various types of cancer including colorectal cancer, breast cancer, skin cancer, and oral cancer [20]. Curcumin protected DNA damage from reactive oxygen species (ROS) by increasing anti-oxidant enzymes and being potent scavenger of free radicals [21]. Curcumin prevents NF-κB activation. The studies revealed that curcumin acts on many components of NF-κB activation, including the "IκB phosphorylation" and degradation in some cancer cells [22-23]. Curcumin inhibited tumor cell growth through inhibition of NF-κB mediating cyclin D1 expression [24]. Curcumin down-regulated Bcl-2 in colon adenocarcinoma cells and breast cancer cells [25-26]. In gastric cancer cells, curcumin can induce apoptosis and inhibit proliferation by reducing cyclin D1

and Bcl-xL levels [27-28]. However, data concerning the effect of curcumin on *in vivo* study of gastric cancer development and key proteins involved in tumor initiation induced by high salt and MNU have not been documented.

Therefore, our study aims to examine the effects of curcumin supplementations on gastric cancer development in rats induced by MNU and saturated NaCl (s-NaCl). In addition, expressions of 8-OHdG, phospho-IκBα, cyclin D1, and Bcl-2 will be investigated.

Research Question

Whether curcumin supplementations attenuate high salt and MNU-induced gastric cancer, and reduce the biomarkers involving in carcinogenesis or not?

Hypothesis

Curcumin supplementations attenuate gastric cancer incidence, and reduce the biomarkers involving in carcinogenesis in high salt and MNU-induced rats.

Research Objective

To determine the effects of curcumin supplementations on high salt and MNU–induced rat gastric mucosal changes in:

1. Gastric cancer incidence
2. Oxidative stress-induced DNA damage (8-OHdG expression)
3. Activation of NF-κB (phospho-IκBα expression)
4. Expression of cyclin D1
5. Expression of Bcl-2

Expected Benefit and Application

The results from this work may support the understandings of pathogenesis of stomach cancer induced by MNU combined with high salt. In addition, the effects of

curcumin on the parameters involved in gastric tumor development may be beneficial for application as a chemopreventive substance of curcumin in patients.

CHAPTER II

LITERATURE REVIEWS

The Stomach

The stomach is a J-shaped tube, with two openings, lining between the esophagus and the duodenum. The lesser curvature along the medial to the superior aspect of the stomach is the short distance from the esophagus to the duodenum. The greater curvature on the lateral to the inferior aspect of the stomach is the longer distance. In human, the stomach has distinct anatomical subdivisions that have functional differences. First, “the cardia” is a small area presently inside the “cardiac” opening. Next, “the fundus” and “the body (corpus)” are the main parts of the stomach. The fundus consists of the dome of the stomach. They secrete hydrochloric acid and digestive enzymes such as pepsinogen. Last, the pyloric region is the distal end of the stomach, which is subdivided into antrum, pyloric canal, and pyloric sphincter. This region is responsible for secreting of mucus, gastrin, and pepsinogen [29]. The antrum is lined by cells that sense pH and secrete hormones that regulate acid production (such as gastrin and somatostatin).

The wall of the stomach is composed of four layers (figure 2-1) consisting of mucosa, submucosa, muscularis propria, and serosa.

The mucosa, surface of the stomach, is made up of epithelium and surrounding connective tissues called the lamina propria. The epithelium contains different types of cells, which secrete compounds to aid digestion and stomach protection. The lamina propria contains the supporting framework, such as capillary network and nerve fibers, for the epithelial cells.

The submucosa is consisted of the loose connectives tissues in which are embedded lymphatic vessels, blood vessels, and scattered mononuclear cells including mast cells.

The muscularis propria is composed of three layers of smooth muscles: the outer-lumen longitudinal muscles, middle circular muscles, and inner-lumen oblique

muscles. The nerves and ganglion cells are located between the outer longitudinal and middle circular muscle layers.

The serosa is a thin covering of loose connective tissues with blood vessels, lymphatic vessels, and nerve fibers. This layer is contiguous with the omentum and ligaments attaching the stomach to spleen and liver.

Whereas human stomachs have only glandular part, the rat stomachs have two parts: forestomach (non-glandular mucosa) and corpus (glandular mucosa) which is divided by the limiting ridge [30]. Forestomach is a thin-walled and non-glandular part that is next to the esophagus and serves as a chamber for food. Its wall is similar to that of the esophagus. Corpus is a thick-walled and glandular section. Its wall has secretory glands that produce mucus and enzymes involved in the digestion of food. Digestion begins in the corpus. The motion of food from the corpus to the intestines (specifically, the duodenum) is regulated by the pyloric sphincter. The stomach wall of two parts are composed of, mucosa, submucosa, muscle layer, and serosa as same as that in human. Whereas mucosa of the glandular part is lining by columnar epithelium; forestomach is lining by squamous epithelium [31].

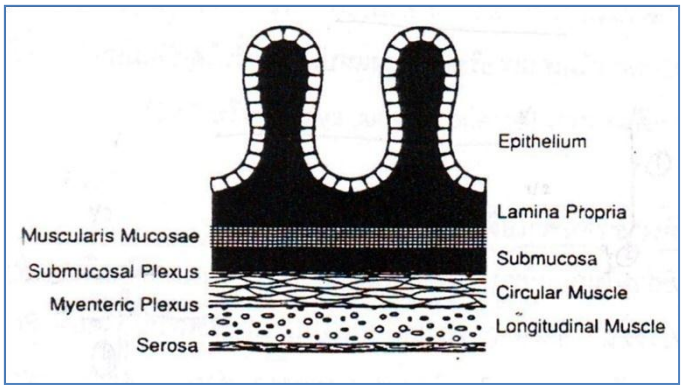


Figure 2-1: The structure of gastric wall [32].

Gastric Cancer

Gastric cancer can generate in any part of the stomach. With poor detection, gastric cancer causes nearly one million annual deaths worldwide. In every year, over 900,000 people are diagnosed with gastric cancer [1-2]. Gastric cancer showed

geographic variation. Almost two-thirds of gastric cancer patients were found in developing countries [33]. The highest rates of gastric cancer in the world are in Japan and Korea. The much lower rates are in Western countries including the USA. In addition, gastric cancer incidence rates are different in races, age, sex, and socio-economic groups. The genetic polymorphisms may cause the variability of gastric cancer incidence rates [34]. Stomach cancer is one of the most public health problems in Thailand. It made for 1-2% of all cancer incidences [35]. In "King Chulalongkorn Memorial Hospital", 119 patients with gastric cancer have been reported between "January 1994 and December 1998" [36]. In 2006, it has been reported that annual rate of gastric cancer incidence in Thai people is aroud "7.1 per 100,000 populations" [37].

In early stages, stomach cancer shows nonspecific symptoms. In the long time, the cancer has invaded and metastasized to other organs. Hence, stomach cancer remains a low prognosis disease but high mortality rate. Gastric cancer shows some signs and symptoms. At early, indigestion or burning sensation (heartburn) or loss of desire for food, especially for meat could be involved. At late, the most common symptoms observed are abdominal pain, nausea and vomiting, bleeding (vomiting blood), weight loss, and anorexia. In diagnosis, gastroscopy will be applied and biopsied some stomach samples. Then, the presence of abnormal cells will be determine by histology or immunohistochemistry [36]. The process of carcinogenesis is currently described by the following morphological changes: diffuse hyperplasia → focal proliferation and metaplasia → benign tumor→ grade I, II or III dysplasia → carcinoma *in situ* → invasive carcinoma.

Risk Factors Mediated Gastric Cancer

In gastric cancer, the pathogenesis is complex and incompletely understood. Multiple factors are involved in stomach carcinogenesis. Besides genetic polymorphisms, *Helicobacter pylori* (*H. pylori*) infections and diets are involved.

A. Dietary Factors (High Salt Diet and *N*-Nitroso Compounds)

Although infection of *H. pylori* is the major causative aspect for the development of gastric adenocarcinoma, geographic differences and alterations in

patterns of the cancer incidence obtained from immigrants suggest that gastric cancer is closely associated with modifiable factors, such as diet. There is evidence that consumption of salty foods and *N*-nitroso compounds and low intake of fresh fruits and vegetables increases the risk of gastric cancer [3-4].

High salt and nitrite compound diets found in the preserved food are potentially carcinogenic. Recently, information from "a joint World Health Organization/Food and Agriculture Organization Expert Consultation" fulfilled that high salt diet increase the risk of gastric cancer [38]. Substantial evidences from "case–control, and cohort studies" also indicate that the cancer risk could raise with a high consume of table salt and some preserved food. In the data of "INTERSALT study", urine samples from subjects aged 20–49 years were randomly taken from "39 populations in 24 countries (N = 5756)". The samples were analyzed in relation between the sodium level and the national mortality rate of gastric cancer [5]. In the 24 countries, there were a close correlation between gastric cancer mortality and sodium level. Coefficient value was 0.70 in men and 0.74 in women (both $P < 0.001$). Many "case–control studies" indicated that high salt diet was associated with the risk of gastric cancer [39-40]. In the cohort study [6], the selected population was in four areas in Japan (Iwate, Akita, Nagano, and Okinawa). All populations aged between 40 and 59 years were 18,684 men and 20,381 women. During 12 years of follow up, 358 men and 128 women were confirmed gastric cancer by histological examination. The data of daily salt intake was obtained from "multiplying the consumption frequency of each of the 44 food items by the salt content of their standard portions/units". "The Standard Tables of Food Composition (Science and Technology Agency, 1982)" specified the value of salt content and salt adding for all foods [41]. The 44 types of food included miso soup, pickled vegetables, dried fish, salted fish roe, and salted fish preserves. In both genders, the high frequency intake of salted fish roe and salted fish preserves was closely related to the risk of gastric cancer. The salt content in these food items is more than 5% of that in other high salt foods. Usually, salt content in the most food items is <5% (less than 0.85 M NaCl). In addition, the powerful association between intake of high salt food and risk of gastric cancer could be chemical carcinogens. During the process of food preservation and digestion, *N*-nitroso carcinogen can be formed by reactions of nitrate compounds. Moreover, the 9 case-

control and 6 prospective studies revealed that the increased risk of gastric cancer was associated with the population consuming processed meat, which contains high amounts of salt and *N*-nitroso carcinogen [42].

Humans are often exposed to the "*N*-nitroso compounds (NOCs)" from various sources. The major source of nitrate is vegetable, whereas nitrite is commonly found in food additive. Our digestive tract has circulation of nitrate and NOCs generation. In normal physiologic function, generations of some NOCs, such as nitric oxide show the beneficial effects [43]. However, some NOCs, such as *N*-nitrosamines are carcinogens [44]. Exogenous sources of nitrosamines are mainly in smoked preserved foods, salty preserved foods, processed foodstuff, pickled products, and cured meat [45]. Nitrosamines are from endogenously metabolites of nitrate and nitrite. Even though the utilization of sodium nitrites has reduced for a long time, it is commonly found in the process of food preservation. Nitrite is also generated from the digestive tract of human, especially from "oral reduction of salivary nitrate". In the stomach, nitric oxide is generated from nitrites by acid-catalysed reaction. This nitric oxide in the stomach can act as a "nitrosating agent of amines and amides", consequenly inducing DNA damage [45]. Under some conditions, such as chronic inflammation found in precancerous lesions of oesophageal and gastric cancer, "nitrosating agents" are overproduced [46]. Based on these data, "International Agency for Research on Cancer (IARC)" classified two important nitrosamines, known as "*N*-nitrosodiethylamine (NDEA) and *N*-nitrosodimethylamine (NDMA)" as possibly human carcinogen (group 2A). In Thailand, gastric cancer incidence in North and Northeast was associated with increased daily consumption of preserved foods containing of NDMA, nitrate, and nitrite. The data from 1988-1996 and from 1998-2005 indicated that there are significant differences in mean daily consumption of preserved foods between four areas of Thailand, and these corresponded to the variations in gastric cancer "age-standardized incidence rates (ASR)" values between areas [47].

Accordingly, many data indicate an important role of diet on etiology of gastric cancer. Several foods containing nitrate, nitrites, and nitrosamines produce an inflammatory responses resulting in the increase of mucosal damage and risk of stomach cancer.

Animal Models of Gastric Cancer and Rat Model Related to This Study

According to pathological events in gastric adenocarcinoma proposed by Correa [48], the cancer develops as a consequence from chronic inflammation to dysplasia and carcinoma. *H. pylori* infection, high salt, *N*-nitroso carcinogens, and reactive oxygen species (ROS) relate to gastric carcinogenesis. Various experiments of gastric cancer have established in rats with the risk factors mimicking those in human.

A. *N*-Nitroso Carcinogen-Induced Gastric Cancer

Many *N*-nitroso derivatives are used to induce gastric cancer. Sugimura and Fujimura [49] reported that adenocarcinomas are in the glandular stomachs of rats induced by "*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)". In the glandular stomach of the rat exposed to MNNG, there were abnormalities of glandular structure orientation, erosion, and hyperproliferation. "Atypical glands" and gastric cancer cells were also detectable. Finally, both intestinal and diffuse types of gastric carcinomas imitating the histologial type gastric carcinomas in human are observed in this model. In male F344 rats, administration of "*N*-methyl-*N*-nitrosourea (MNU)" dissolved in the drinking water for 25–42 weeks induced gastric carcinoma in the glandular stomach [50-51]. These carcinogens are also used to induce gastric cancer in many animal models, such as mouse [52] and dog [53].

Now, MNU is available for research purposes. MNU is pale yellow crystals. It exhibits hydrophilic property. The compound is highly reactive and sensitive to humidity and light. In aqueous solutions, its half-lives are depended on pH with ranging from 125 hours (pH 4) to about 2 minutes (pH 9). Explosive decomposition may occur when prolonged storage at room temperature. Carcinogenic ability of this compound is known to be related with DNA alkylation. They will react with various nucleophiles, especially with amines [54]. It can induce GC → AT transition, unsuccessfully addressed by the mismatch-repair system, which results in a cell-cycle arrest in G2 [55]. Rats given MNU by oral gavage showed gastric cancer, including genetic alterations, which had similar to human stomach cancers, such as mutations of oncogene, *ras*, and tumor suppressor gene, *p53* [56]. These mutations lead to uncontrolled cell proliferation. Recently report suggested that MNU also processed its

specific target on expression of *ras*, *c-myc*, and *p53*, which could take part in MNU-induced tumorigenesis [57].

B. Association Between Hypertonic NaCl and *N*-Nitroso Carcinogen

"Hypertonic sodium chloride" probably declines the viscosity of the gastric mucin, resulting in the reduction of the protective barrier [9, 58]. Ingestion of hypertonic NaCl by rats led to damage of gastric mucosa, inflammation, and subsequently increased cell proliferation [10-12]. The increase of inflammation and proliferation may promote carcinogen effects. Hypertonic-salt administration markedly increased the gastric tumors in the MNU-induced gastric cancer in mice [59]. In short-term induction, Ganapathy *et al*. [60] reported that rats given with 200 mg/kg MNNG by oral gavage for 2 times with saturated sodium chloride "twice a week for the first 3 weeks" produced a high incidence of gastric cancer at week 20^{th}. The studies in these rodents support the suggestion that high salt acts as a co-carcinogen.

This study developed the rat model of stomach cancer using the combination of MNU and hypertonic salt. The association between hypertonic NaCl and *N*-nitroso carcinogen may promote gastric tumor in rat by increase of inflammation, mutation and compensatory cell proliferation. The studies in analysis of pathological and molecular mechanisms of gastric cancer are necessary.

High Salt and MNU Induce the Production of "Reactive Oxygen Species (ROS)" That Involve in DNA Damage and Carcinogenesis.

Inflammatory milieu, epithelium loss, and increased the turnover rate cause the transformation of epithelial cells through damage and impairment of DNA. Inflammatory condition is generally associated with the excessive production of "reactive oxygen species (ROS)". Oxygen (O_2) is metabolized by enzymes such as "NAD(P)H oxidases" and "xanthine oxidase" or nonenzymically hydrolized by "redox-reactive compounds such as the semi-ubiquinone". These processes generate superoxide anion (O_2^-) [61]. Cytokines or other inflammatory mediators stimulate NAD(P)H oxidase. "Superoxide anion radical (O_2^-)", "singlet oxygen ($^1O_2$)", "hydrogen peroxide (H_2O_2)", and the highly reactive "hydroxyl radical (^-OH)" are

ROS. Normally, ROS exists in balance with endogenous antioxidants. Imbalance of the process, excess ROS and/or antioxidant depletion, is contributed to oxidative stress, and initiates carcinogenesis by damaging nucleic acids, lipids, and proteins [62].

In oxidative DNA damage, specifically oxidized purines and pyrimidines, the mutation may cause by breakage of single strand. Then, instability of the re-formation may result from the direct re-formation or repair processes. The previous study showed that mutations are usually "base pair substitutions", whereas "base deletions" and "insertions" are less frequent [63]. The occurrence of oxidative stress induced DNA alterations in cancer tissues supports that ROS causes the initiation of human cancer. Two to three fold higher accumulation of "8-Hydroxy-2'-deoxyguanosine (8-OHdG)" in lung cancer cells suggested that ROS induced DNA modifications, resulting in fibrosis and tumor development [64]. In breast cancer, it has been shown that high levels of 8-OHdG accumulation play an important role [65]. In hepatocellular carcinoma, 8–OHdG has been reported to increase [66-67]. Thus, investigation of 8-OHdG level in tissues could be the beneficial parameter in supporting an association between "free radical producing agents" and cancer risk.

Ingestion of "hypertonic sodium chloride solutions" caused injury of rat gastric epithelium, followed by the increased proliferation of gastric epithelial cells [68]. The previous report showed that of "hypertonic sodium chloride solutions (0.25-1.5 g/kg body weight)" induced the damage and proliferation by dose-dependent manner. They also suggested that "hypertonic sodium chloride solution" at the same dose of "0.8 g/kg body weight" induced different degrees of mucosal damage and cell proliferation by concentration-dependent manner [68]. A "high concentration of sodium chloride (1.3 M and greater concentrations)" caused initial damage of rat gastric epithelium and consequently regenerative cell proliferation suggesting that "high concentration of sodium chloride" may contribute to the further development of gastric cancer [11]. The previous study using Wistar rats intragastrically administrated MNNG, with or without 4.5 M NaCl (1 mL), salt induced squamous forestomach injury and increased the risk of tumors in forestomach [69]. In addition, they found that the number of proliferative cells in forestomach epithelium at 12 and 24 hours after salt administration significantly increased compared with the control animals. Hence, cellular damage from exposure of hypertonic NaCl induces activation of

inflammatory cells, consequently release of cytokines and production of reactive species. Tatemichi *et al.* [8] demonstrated that hypertonic NaCl solution at the concentrations of "higher than 3.4 M" induced alkalization in the stomach (elevation of gastric pH). They suggested that elevated pH may be due to the mucosal damage and loss of gastric gland functions, including dysfunction of parietal cells (acid secreting cells). In *ex vivo* study, 1 M NaCl administration elevated gastric pH, which may result from the increase of HCO_3^- level and "the inhibition of acid secretion" [8]. The damaged portion of gastric mucosa enhanced HCO_3^- flux and exposed gastric alkalization. The acid secretion was inhibited by the release of "endogenous prostaglandins (PGs)" and "nitric oxide (NO)" from the hypertonic NaCl-induced irritated gastric mucosa [70-71]. Tatemichi *et al.* [8] also found that elevated pH was prolonged by NaCl-induced "inducible nitric oxide synthase (iNOS)" expression. NaCl at "the concentrations higher than 1.7 M" induced iNOS expression mostly in the infiltrating cells within 4 hours. Moreover, the continuous iNOS induction in chronic inflammatory responses could be possibly mutagenic, through "NO-mediated DNA damage" or DNA impairment, and finally could be carcinogenic [72-74]. "NO" reacts with "superoxide (O_2^-)" to form "peroxynitrite ($ONOO^-$)", a highly reactive species causing "nitrative and oxidative DNA damage". $ONOO^-$ can induce the 8-OHdG and 8-nitroguanine formation [75]. Hence, hypertonic NaCl ingestion may increase gastric cancer development via induction of epithelial injury, "oxidative DNA damage", and increased cell proliferation that probably an important causes of the carcinogenic promoter of high salt.

N-nitroso mutagens in stomach, formed from dietary nitrates, also damage DNA [76]. *N*-methyl-*N*-nitrosourea (MNU) is an effective mutagen. It can induce cell death, cellular stress, chromosomal aberrations, DNA damage, and mutations. MNU can damage proliferating cells through alkylation of DNA bases and induction of ROS production [77]. The *in vitro* study demonstrated that ROS are the main factor in MNU mediated damage of DNA and transformation of gastric epithelial cells. Activation of transcription factors and cell-cycle regulated proteins are increased in MNU-treated cells [77].

Accordingly, increased ROS level from high salt and MNU administrations induces gastric cell or DNA damages, and relates to high epithelial cell turnover.

Increased cell proliferation enhances the accumulation of mutation and impairment of cell cycle regulation. Furthermore, increased ROS level is related to pathological consequences determining an irregularity of signal pathways and/or gene expressions shown in the following paragraph.

Nuclear Factor-Kappa B (NF-κB) Links Gastric Inflammation to Gastric Cancer Development.

Inflammation also induces protein called NF-κB as a key player. NF-κB dimers are composed of "REL-A (also known as p65), REL (also known as cREL) and p50 subunits". Hyperactivation of NF-κB plays vital roles in human cancer and inflammatory diseases [78].

There are two pathways of NF-κB activation: "the classical pathway" and "the alternative pathway" [17, 79]. Both pathways can affect tumor development, but most of evidences relate to "the classical pathway", which is activated by viral and bacterial infections, in addition to proinflammatory cytokines and ROS. These triggers activate "the IKK (inhibitor of NF-κB (IκB) kinase) complex". "The IKK complex" is composed of two "catalytic subunits", "IKK-α (also known as IKK1) and IKK-β (also known as IKK2), and a regulatory subunit, IKK-γ (also known as NEMO)". Once activation, this enzyme complex can phosphorylate "NF-κB-bound IκB", targeting them for "proteasomal degradation" and leaving "NF-κB dimmers" to go into the nucleus. In the nucleus, the activated NF-κB mediates target genes encoding. The "alternative NF-κB-activation pathway" implicate the upstream "kinase NF-κB-inducing kinase (NIK)", which activates "IKK-α homodimers", independently of either "IKK-β or IKK-γ". Activated IKK complex phosphorylates p100 subunits of NF-κB, in return to certain members of the "tumor necrosis factor (TNF) family" [17, 80]. These two different pathways activate different gene sets, resulting in the mediation of different immune functions [17]. The involvement of "the classical NF-κB-activation pathway" to inflammation and cell-survival mechanisms is well established, and continuous activation of NF-κB in various cancer types and malignancies has been described [81]. Thus, it seems that "the classical NF-κB

activation" may link inflammatory responses to tumor promotion and progression towards the regulation of gene expressions in death-survival pathways.

Inflammation can link to tumor development by means of three routes. In primary, inflammatory mediators, including ROS, cytokines, and growth factors induce DNA synthesis and cell proliferation. These processes lead to the raise of genetic instability due to "the accelerated DNA replication" [82]. At inflammatory sites, macrophage progenitors (monocytes) migrate and then mature and release several inflammatory mediators such as cytokines, growth factors, and angiogenic factor. All of these inflammatory mediators are encoded by "the IKK-β -dependent NF-κB-activation pathway" [83-84]. The cytokines can activate NF-κB in both target cells and macrophages themselves. ROS also activate NF-κB activation. The 300 mM hydrogen peroxide increased phosphorylation of IκBα in "EL4 T cells" [85]. After induction of oxidative stress in in JurkatT cells, "IκBα phosphorylation" and "activation of the IKKα" were induced [86].

Next, inflammation might promote tumor initiation through the generation of ROS and "reactive nitrogen species (RNS)". Activated neutrophils and macrophages from inflammatory responses can generate ROS and RNS, which somehow interact with genomic DNA in adjacent epithelial cells. Damage of DNA in epithelial cells caused mutations that drive tumor progression [87-88]. In the last mechanism, the activation of NF-κB pathway is an important mediator of gene expressions involving in the regulation of the cellular apoptosis and proliferation [89].

Anti-Apoptotic Protein Bcl-2 in Carcinogenesis

Apoptosis, programmed cell death, and proliferation of epithelial cells play a vital role in the homeostasis of the gastrointestinal mucosa. Two main pathways of apoptosis have been illustrated. "The extrinsic pathway" is composed of death receptors, such as "CD95 (APO-1/Fas), tumour necrosis factor (TNF) receptors, and TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1) and TRAIL-R2" [90-91]. The corresponding ligands "CD95 ligand (CD95L/Fas)" binds to its receptor resulting in the formation of "Fas-associated death domain (FADD)". This protein domain recruits caspase-8 and the activated CD95 receptor to form the "CD95 death-inducing

signaling complex (DISC)". The actived caspase-8 and "effector caspases (caspases-3, 6, and 7)" are the consequences of these cascades, resulting in apoptosis by cleaving cellular proteins and "DNA fragmentation factor ICAD (Inhibitor of Caspase-Activated DNase)" [92]. In the other pathway (intrinsic), many pro-apoptotic signals of the "Bcl-2 family" linked to permeabilization of the outer mitochondrial membrane, resulting in "the translocation of cytochrome c from the mitochondria to the cytoplasm". In the cytopklasm, released cytochrome c binds to "Apaf-1" and induces the recruitment of "procaspase-9". Then, "activated caspase-9" cleaves and activates "procaspase-3", which induces apoptosis [93]. The extrinsic pathway connects to the intrinsic one by the induction of "Bid, a BH3 domain-containing protein of the Bcl-2 family". Caspase-8 induces Bid cleavage, the subsequent cytochrome-c translocation, and thereby, caspase cascades [94].

Bcl-2 family can be divided into anti-apoptotic proteins "Bcl-2, Bcl-xL, Bcl-w, Mcl1, A1/BFL1, Boo/Diva, NR-13" and pro-apoptotic proteins "Bax, Bak, Bok/Mtd, Bcl-xS, Bid, Bad, Bik/Nbk, Blk, Hrk/DP5, Bim/Bod, Nip3, Nix, Noxa, Puma, Bmf". Most of anti-apoptotic members contain "the Bcl-2 homology (BH) domains 1, 2 and 4", whereas "the BH3 domain" seems to be essential for induction of apoptosis [95, 96]. The impact of "Bcl-2 family" proteins on apoptosis is well identified. Apoptotic stimuli activate mitochondrial release of cytochrome c, "AIF (apoptosis inducing factor)", and other apoptogenic mediators from "the intermembrane space" to the cytosol. Concurrently, "the mitochondrial transmembrane potential" drops. "Mitochondrial membrane permeabilization" involves "the permeability transition pore complex (PTPC), a multiprotein complex" that consists of "the adenine nucleotide translocator (ANT)" of the inner membrane, "the voltage-dependent anion channel" of the outer membrane and other proteins [95]. Bcl-2 proteins might cooperate with the PTPC and regulate the mitochondrial permeability. Proteins containing only BH3 domain act as "death sensors" in the cytosol. In response to death signal, these sensors interact with members of "the Bax subfamily". After that, "Bax proteins change the conformation, insert into the mitochondrial membrane, oligomerize, and form protein-permeable channels". "Anti-apoptotic Bcl-2 proteins can inhibit the conformational change or the oligomerization of Bax and Bak [96]. The localization of the pro-apoptotic Bad is regulated by

phosphorylation. Only non-phosphorylated Bad are able to antagonizing anti-apoptotic Bcl-2 or Bcl-xL on the mitochondrial membrane".

From the regulation of some Bcl-2 family proteins, it is probably to indicate the fate of cells when specific proteins are expressed. Cancer cells have developed several strategies to resist apoptosis, such as an augmentation of "anti-apoptotic proteins or a decrease or defective function of pro-apoptotic molecules.

Anti-apoptotic genes that are activated directly by NF-κB comprise of "the cellular inhibitors of apoptosis (c-IAP1, c-IAP2, and IXAP), the TNF receptor–associated factors (TRAF1 and TRAF2), the Bcl-2 homologue A1/Bfl-1, and IEX-IL" [97-98]. Inflammatory mediators can NF-κB pathway, subsequently enhance anti-apoptotic functions, which protect cancer cells from apoptosis. One of the best-examples that activate apoptosis is the study that induced cells with TNF-α. Application of TNF-α increases the expression of "TRAF1, TRAF2, c-IAP1, and c-IAP2" [97]. The increased expression of these proteins protected "RelA-deficient cells, which are highly sensitive to TNF-α–induced apoptosis", from cell death. These anti-apoptotic factors inhibit "the activation of caspase-8, an initiator protease, involved at an early step in stimulating the apoptotic pathway" [97]. In human gastric cancer cells, NF-κB mediated cyclooxygenase-2 (COX-2) expression is associated with cell proliferation [99]. COX-2 overexpression protected cells against apoptosis by increase of Bcl-2 production [100]. Gastric epithelial cells express cell-specific pro-survival and pro-apoptotic pathway. Overexpression of anti-apoptotic protein Bcl-2 is suggested in gastric adenocarcinoma and precancerous patients. The Bcl-2 expression is negatively correlated with apoptosis [101]. Bcl-2 is also overexpressed in MNNG-induced stomach tumor rats. The shift in the balance of "anti-apoptotic Bcl-2" and "pro-apoptotic Bax", as evidenced by an enhancement of Bcl-2/Bax ratio with decreased expression of cytochrome C and caspase 3, provide evidence for apoptosis avoidance in MNNG-induced gastric tumors [102].

Cell Cycle Regulator Cyclin D1 in Carcinogenesis

Activation of NF-κB in the regulation of cell cycle is an important factor in indicating the degree of cellular proliferation and apoptosis. The cell cycle is

composed of four different phases “G1, S, G2, and M phases”. The succession of a cell through the phases of cell cycle is mediated by CDKs, which are helapfully and unhelpfully regulated by cyclins and CKis, respectively. “Cyclin D isoforms” interact with “CDK4 and CDK6” to make the progression through the G1 phase. “Cyclin D/CDK4, 6 complexes” phosphorylate “retinoblastoma protein (pRb)”, which releases E2F to transcribe genes that are crucial for cell division [20]. As a very important regulator of G1 re-entry and progression, the turnover of cyclin D1 is tightly regulated by phosphorylation of a specific “threonine residue (Thr-286) located near its C-terminus, by Glycogen Synthase Kinase-3β (GSK-3β)” [103]. Cell cycle regulators, particularly G1 phase progression, are simple targets during carcinogenesis because this phase provides cells for growth advantage. Cyclin D1 is broadly studied because it frequently overexpressed in many types of human cancer, including gastric cancer [104]. In mouse model, cell proliferation and expression of in D1 are increased in MNU-induced stomach tumors [105]. Interestingly, experimental evidences showed that cyclin D1 played as an effector in the development of cancer. The overexpression of cyclin D1 in human cancers is regulated by multiple factors composing of “genomic alterations, post-transcriptional regulation, and post-translational protein stabilization” [106].

NF-κB is found to stimulate cyclin D1 transcription. Levels of cyclin D1 protein during phases of cell cycle are controlled by NF-κB. Inhibition of NF-κB activation causes a reduction of “serum-induced cyclin D1-associated kinase activity”, resulting in delayed pRb protein and cell cycle progression. NF-κB activation induced the transition stage of G1-to-S-phase. One of the target gene might be cyclin D1 [107]. Thus, the NF-κB activation could inhibit apoptosis by the regulation of multiple genes involved in growth control.

According to these reviews, NF-κB activation encodes multiple genes that are involved in the regulation of the immune and the inflammatory response. High salt and MNU administration could enhance inflammation and DNA damage, which is an important process for compensatory deregulated proliferation. NF-κB activation acts as a crucial role in the deregulation of cell proliferation. Its activation also enhances anti-apoptotic protein (Bcl-2) and positive cell cycle regulator (cyclin D1)

expressions. Our rat model may develop cancer through increased NF-κB activation, important for promotion of cell survival and proliferation. Inhibition of DNA damage accumulation and cell proliferation should be allowed the improvement of chemotherapy.

Curcumin (Diferuloylmethane)

Curcumin (diferuloylmethane), an active ingredient, is isolated from the rhizomes of *Curcuma longa* Linn. (*C. longa* L.). The rhizome, or root, is processed into turmeric powder, which is 2% to 8% curcumin [108]. The rhizomes are a household remedy in Nepal [109]. In several countries, turmeric, the bright yellow compound, has been used as a coloring mediator in food. It also has been used for centuries as various medicinal properties. The traditional Indian medicine suggests the usage of tumeric against "biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorder, rheumatism, and sinusitis" [110]. In the 19th century, there has been considerable interested in "the curcuminoids, the active compounds in turmeric". Tumeric extract contains "turmerin (a water-soluble peptide), essential oils (such as turmerones, atlantones and zingiberene), and curcuminoids. The major curcuminoid is called curcumin (diferuloylmethane), which makes up approximately 90% of the curcuminoid content in tumeric, followed by demethoxycurcumin and bisdemethoxycurcumin" [111]. The chemical structure of curcumin is determined by Roughley and Whiting (figure 2-2) [112].

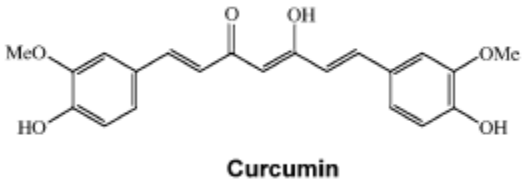


Figure 2-2: Chemical structure of curcumin (diferuloylmethane).

Curcumin is 368.37 g/mol and dissolved in organic solvents such as dimethylsulfoxide (DMSO), oil, alcohol, and petroleum agents. Curcumin has melting point of 183 °C. According to the global demand of turmeric as colouring, flavouring, and food preservative for consumer use, the pharmacokinetic properties of curcumin has been studied. Animal studies demonstrated that curcumin is rapidly metabolized and poorly absorbed in Sprague-Dawley rats. Wahlström and Blennow [113] published that orally given this compound in "a dose of 1 to 5 g/kg BW" to rats did not cause any "adverse effects" and it is excreted about 75% in the feces, whereas traces in the urine. Rats were orally administrated with curcumin indicated 60% absorption and showed evidence of glucuronide and sulphate conjugated forms in urine [114]. In a previous study, intraperitoneal injection of curcumin in the mouse (0.1 g/kg) indicated that curcumin was primary biotransformed to "dihydrocurcumin and tetra-hydrocurcumin". Then, these compounds were changed to "monoglucuronide conjugates" [115]. For orally administration of curcumin in rats, measurement of curcumin metabolites in blood plasma showed small measurements of "curcumin, hexahydrocurcumin, hexahydrocurcuminol, and hexahydrocurcumin glucuronide" with higher measurements of" glucuronide and sulphate conjugates" [116]. Ireson, *et al*. [117] also extended their work in isolated human hepatocytes and intestine microsomes. The data suggested very rapidly of metabolic reduction, in a matter of minutes. Accordingly, bioavailability of curcumin in rodents is poorly absorbed by the gastrointestinal tract. Absorbed curcumin is rapid metabolized and excreted in the bile.

Curcumin has been recommended the safety in animals and human even at very high dose. Without significant side-effects, human is able to stand for high doses of curcumin. In a phase 1 study, Cheng *et al*. [118] did not found any side-effects of curcumin consumption at doses up to 8000 mg/day for 3 months. In long-term treatment, rats are fed with curcumin "1.8 g/kg BW per day for 90 days" and monkeys are fed with curcumin "0.8 mg/kg BW per day for 90 days" show no adverse effects [119]. In the study of toxicity, oral administration of curcumin "up to 1 g/kg BW daily in two successive generations of Wistar rats" showed no toxicity or adverse effect on reproduction [120].

The biological properties of curcumin in preclinical models of carcinogenesis are extraordinary. Chemopreventive potential of curcumin has been revealed in the several different models of cancer (*in vitro* and *in vivo*), including colon [121], forestomach, duodenal [122], prostate [123], and breast carcinogenesis [124]. Vary doses of curcumin are used to prevent carcinogenesis. The effects of curcumin in diet "0.05–2.0%, approximately 50-2000 mg/kg" on colon carcinogenesis have been demonstrated in both chemical and genetic induction in rodent models [125-127]. In stomach cancer mouse model, administration of 2% dietary curcumin to "female A/J mice" for 14 days, caused a significant inhibition in "benzo[a]pyrene (BaP)-induced forestomach cancer" [128]. In addition, curcumin supplementation (0.05 and 0.2%, for 55 weeks) reduces, albeit without statistical significance, the total incidences of atypical hyperplasias combined with adenocarcinomas induced by 5% NaCl solution and MNNG in rats [129]. Extract solution from radix curcumae (1 and 2 g/ml, for 40 weeks) reduced incidence of gastric cancer induced by 10% NaCl solution and MNNG in rats [130]. These studies show the reduction of cancer incidence by curcumin, but the molecular targets of cancer that are modulated by curcumin have not been indicated.

A. Chemopreventive Effect of Curcumin - Antioxidant Activity

"Reactive oxygen species (ROS) and reactive nitrogen species (RNS)" from activated neutrophils and macrophages lead to "cancer-causing mutations" in epithelial cells [87]. Excesses ROS initiate carcinogenesis by damaging nucleic acids, lipids, and proteins [62]. Curcumin acts as a scavenger of free radicals. Curcumin suppressed the induction of "nitric oxide synthase (NOS)" in activated macrophages and had been shown to be an effective scavenger of free radicals like peroxynitrite, superoxide anion, hydroxyl radical and singlet oxygen [131-133]. The antioxidant mechanism of curcumin is contributed to its unique structure, including "two methoxylated phenols and an enol form of β-diketone". These conjugared structures show ability of typical radical-trapping [134]. In addition, curcumin increases the activities of antioxidant enzymes like "superoxide dismutase and glutathione peroxidase in lymphocytes" [135]. Curcumin also enhances the activities of antioxidant enzymes in rat liver to protect cells apoptosis from iron-induced lipid peroxidation

[136]. According to above mentions, curcumin could prevent oxidative-induced cancer initiation.

Opposing to the previous mention of antioxidant effect, curcumin exhibited "pro-oxidant DNA cleavage activity", which is the consequent binding between Cu (II) and various sites of curcumin structure [137]. Curcumin inducing the death of many cancer cells is associated with the generation of superoxide radicals and sensitivity of those cancer cells. Curcumin can generate ROS that causes mitrochondria damage. With the lipophilic structure, curcumin disturbed the energy coupling system in the mitochondria and induced the failure of mitochondrial membrane potential for the proton permeability. Through these actions, curcumin caused cells suffering from increased ROS levels [138]. According to the described above, curcumin exhibits the complexity of redox regulation. It is very difficult to provide one exactly mechanism for chemoprevention by curcumin.

B. Chemopreventive Effect of Curcumin – Inhibition of NF-κB Activation

Curcumin act on many components of "NF-κB signaling pathway". Bharti *et al*. [22] discovered that curcumin inhibit IKK complex blocking both IκB-α phosphorylation as well as NF-κB p65 translocation and thus leads to NF-κB inhibition. The inhibition of NF-κB activation is also related to the effect of curcumin to prevent the proteasome function in human colon cancer cells [139]. Inhibition of NF-κB activation by curcumin is certainly an interesting strategy against tumor development. It is notified that "NF-κB signaling pathway" plays a major role in tumorigenesis. The activated NF-κB binds to DNA and results in transcription of genes that contribute to cancer development, such as, oncogenes, anti-apoptotic protein (Bcl-2), positive regulator of cell proliferation (cyclin D1) [81], and invasion potential (matrix metalloproteinases (MMPs), adhesion molecules).

Curcumin blocks the "NF-κB signaling and inhibits IKK activation", thus suppressing proliferation of "head and neck squamous cell carcinoma" [140]. They also demonstrated that curcumin suppressed various cell survival and cell proliferative genes, including Bcl-2, cyclin D1, IL-6, cyclooxygenase-2 (COX-2), and MMP-9. In the regulation of NF-κB, curcumin induced apoptosis of "hepatic cancer cells" via

activated caspase 3 and 9 mRNA expression, decreasing Bcl-xL mRNA and increasing Bcl-xS and c-IAP-2 mRNAs [141]. Overexpression of cyclin D1 is a feature of "human mantle cell lymphoma (MCL)", an aggressive "B cell non-Hodgkin's lymphoma". Curcumin successfully repressed the survival and proliferation of MCL by inducing cell cycle arrest and apoptosis by downregulating NF-κB activation [142]. Inhibition of NF-κB activation by curcumin could alter the deregulation of cell cycle, which increase expression of cyclin D1 and Bcl-2.

1. Antiproliferative Effects-Down Regulation of Cyclin D1

Deregulation of cell cycle control results in the formation of tumor cells, in which growth and proliferation proceeds uncontrolled. A way for curcumin to counteract cancer cell proliferation includes in the cell cycle arrest.

Curcumin suppresses cell proliferative genes, including cyclin D1. Curcumin has been shown to reduce the expression of cyclin D1 in many cancers including "head and neck squamous cell carcinoma" [143]. Moreover, curcumin reduced growth of "head and neck squamous cell carcinoma" in xenograft model [144]. Curcumin decreased cyclin D1 in orthotopic model of human colon cancer [145] and human bladder cancer [146]. Curcumin also inhibit proliferation of breast cancer via down regulation of cyclin D1 transcription [147]. Curcumin downregulated cyclin D1 in cervical carcinoma cells [148] and pancreatic carcinomas [149]. Cai, *et al*. [28] showed that curcumin inhibited the proliferation and invasion of gastric cancer cells. Curcumin downregulated the expressions of cyclin D1 mRNA and protein and reduced transition of the cells from G1 to S phase.

The effects of curcumin on cell cycle regulation may attribute to inhibition of transcription factors and subsequent suppression of downstream gene products. Curcumin has been shown to upregulate the expression of "the Cip/Kip family of CDK inhibitors", thus inhibiting the formation of cyclin D1 and CDK4, 6 complex [150]. Curcumin also declined Rb phosphorylation and reduced transcription of E2F-regulated genes in the presence of curcumin has been demonstrated [151]. This leads to the disruption of cell cycle and to the death of cells by apoptosis.

2. Apoptotic Effects-Down Regulation of Bcl-2

Apoptosis resistance is induced by cancer cells. A way for curcumin to counteract resistance of apoptosis of cancer cells is to induce “pro-apoptotic signals” and inhibit “anti-apoptotic mechanisms”.

Curcumin was reported to induce apoptosis by the downregulation of “anti-apoptotic proteins” from the Bcl-2 family and up-regulation of pro-apoptotic proteins. Treatment with 50 μM curcumin on human renal cancer Caki cells resulted in the activation of caspase 3, and DNA fragmentation. Subsequently, apoptosis of the Caki cells is mediated. Curcumin caused dephosphorylation of Akt, down-regulation of the anti-apoptotic proteins, induction of cytochrome c release, and activation of caspase 3 [152]. Curcumin also inhibited the expressions of “Bcl-2, Bcl-xL, survivin and XIAP, and induced the expressions Bax, Bak, PUMA, Bim, and Noxa and death receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5)” in prostate cancer cell lines [153]. Recently, *in vitro* study of tongue squamous cell carcinoma (SCC-4 cells) demonstrated that curcumin significantly induced apoptosis with a diminution of the Bcl-2 level, reduction of mitochondrial membrane potential, and promotion of the caspase-3 active forms [154].

As above illustrated, the regulation of balance between cell proliferation and apoptosis may be the strategy of curcumin to reduce hyperproliferation in tumor development. These properties of curcumin could support the idea of implication for cancer prevention and therapy. From various doses of curcumin usage, the lowest dose could prevent carcinogenesis is approximately 50 mg/kg (0.05% curcumin in rat dietary). Our previous study, we demonstrated that oral gavage of 200 mg/kg curcumin reduced gastric inflammation from *H. pylori* infected rats [155]. With a limited number of studies addressed the oral administration of curcumin on gastric cancer development in rats, we decided to use curcumin at the concentration of 200 mg/kg/day in this study. Moreover, it is interesting to examine molecular targets of curcumin that prevent gastric carcinogenesis in animal model.

Considering the above literature view, it might be said that high salt promotes the progression of gastric cancer. Combined this factor with MNU are used to develop stomach cancer in the animal models. Our rat model is developed using saturated

NaCl (s-NaCl) and MNU. High salt and MNU trigger gastric epithelial cells damage directly or via “reactive oxygen species (ROS) production”. Oxidative DNA damage induces formation of 8-OHdG, which is related to carcinogenesis. Moreover, MNU directly increases DNA mutation. Damage or mutation of DNA is related to cellular apoptosis and compensatory increase of cell proliferation. Increased cell proliferation enhances the accumulation of mutation and impairment of cell cycle regulation resulting in carcinogenesis. Furthermore, gastric inflammation and cell proliferation by high salt administration in rats have been reported. Inflammatory cytokines and ROS can activate NF-κB which plays an essential role in deregulation of cell proliferation and gastric carcinogenesis. The expressions of positive regulator of cell cycle (cyclin D1) and anti-apoptotic protein (Bcl-2) are target of NF-κB in carcinogenesis.

Curcumin shows chemopreventive activity by scavenging free radicals that damage DNA and induce carcinogenesis. Curcumin also inhibits NF-κB activation and regulates balance between cell proliferation and apoptosis in various types of cancer cells. Moreover, curcumin supplementation also reduced incidence of gastric cancer in rat models. With the mechanisms of action including inhibition of NF-κB activation signalling pathways, curcumin could prevent gastric carcinogenesis and reduce gastric cancer incidence. Therefore, this present study is desired to evaluate the effects of curcumin on MNU and s-NaCl induced gastric cancer by monitoring incidence of gastric cancer and expressions of 8-OHdG, IκBα phosphorylation, cyclin D1, and Bcl-2 in gastric tissues as shown in the following conceptual framework and the research objective.

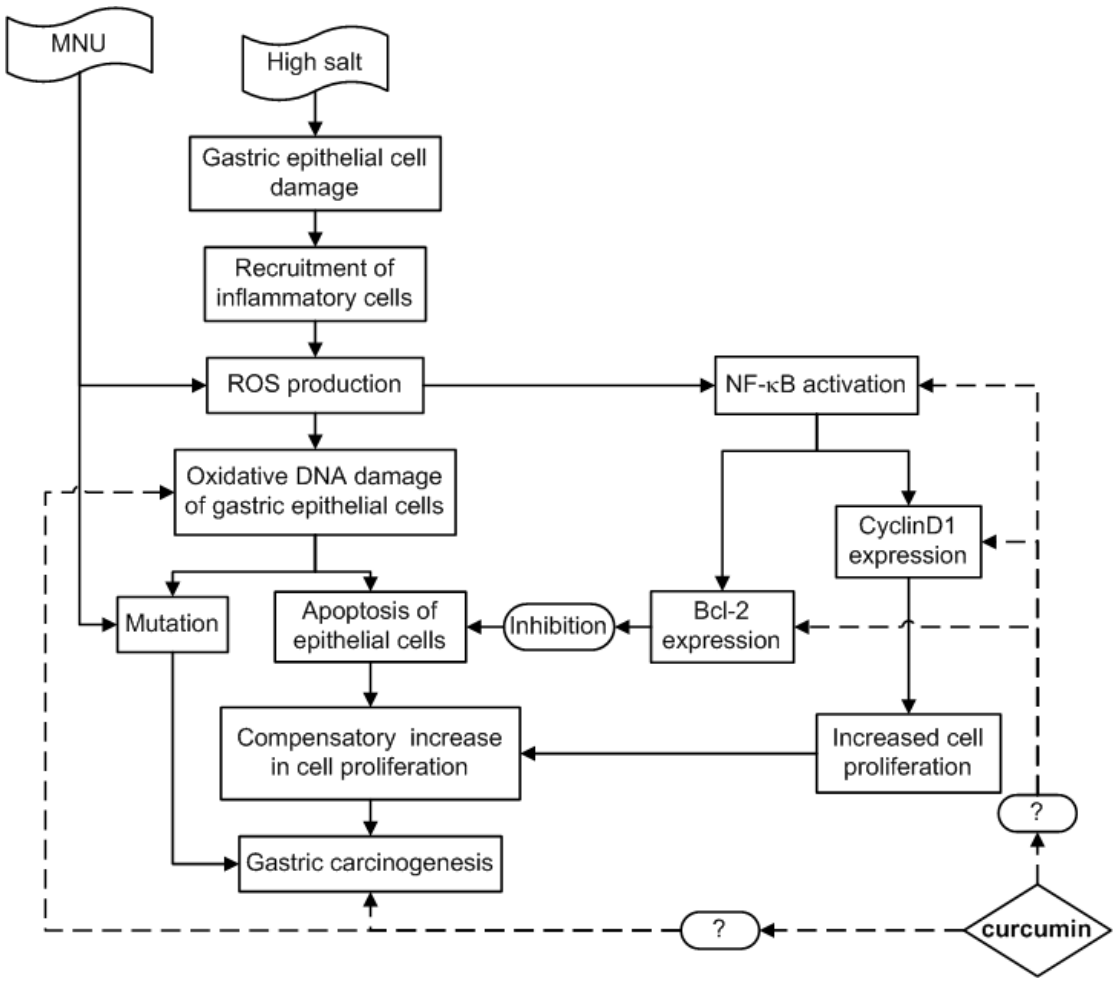


Figure 2-3 The conceptual framework.

To examine the effects of curcumin supplementations on high salt and MNU–induced rat gastric mucosal changes in:

1. Gastric cancer incidence
2. Oxidative stress-induced DNA damage (8-OHdG expression)
3. Activation of NF-κB (phospho-IκBα expression)
4. Expression of cyclin D1
5. Expression of Bcl-2

CHAPTER III

MATERIALS AND METHODS

Reagents

Acrylamide (Bio Basic Inc., Canada)

Ammonium persulfate (APS; Bio Basic Inc., Canada)

Anti-Bcl-2 antibody (sc-7382; Santa Cruz Biotechnology Inc., CA)

Anti-cyclin D1 antibody (Thermo Scientific, MI)

Anti-phospho-IκBα (Ser32/36) antibody (5A5; Cell Signaling Technology Inc., MA)

Anti-8-Hydroxy-2'-deoxyguanosine (8-OHdG) antibody (N45.1; Japan Institute for the Control of Aging, Japan)

Anti-β-actin antibody (sc-47778; Santa Cruz Biotechnology Inc., CA)

Antibody diluent (DAKO, CA)

95% Alcohol (Merck, Germany)

BCA Protein Assay Kit (Thermo Scientific, MI)

Bis-acrylamide (Bio Basic Inc., Canada)

Bromophenol blue (Bio Basic Inc., Canada)

Citrate buffer pH 4.5

95% Curcumin (Cayman Chemical Company, MI)

Developer (Kodak, NY)

Distilled water

Dithiothreitol (DTT; Bio Basic Inc., Canada)

ECL plus western blotting detection reagents (Amersham, GE Healthcare, UK)

Eosin (C.V. Laboratories, Thailand)

Fixer (Kodak, NY)

Fuji Electron Microscopic Film FG (Fujiflim, Japan)

Glycerol (Bio Basic Inc., Canada)

Glycine (Research Organics, OH)

Goat anti-mouse IgG HRP (Cayman Chemical Company, MI)

Hematoxylin (C.V. Laboratories, Thailand)

Hydrochloric acid (Merck, Germany)

Methanol (Sigma-Aldrich, MO)

N-methyl-*N*-nitrosourea (MNU; Sigma-Aldrich, MO)

NaCl (Merck, Germany)

NaOH (Merck, Germany)

Phosphate buffer saline (PBS) pH 7.4

Phosphatase inhibitor cocktail 2 (Sigma-Aldrich, MO)

Polyvinylidene difluoride (PVDF) membrane (Pall Corporation, FL)

Prestained protein molecular weight marker (#SM0441; Fermentas, Thermo Scientific, MI)

Protease inhibotor cocktail (Sigma-Aldrich, MO)

4% Para-formaldehyde in phosphate buffer saline

Radioimmunoprecipitation assay buffer (RIPA; Cell Signalling Technology Inc., MA)

Skim milk power (Fluka BioChemika, Switzerland)

Sodium dodecyl sulfate (SDS; Bio Basic Inc., Canada)

Sodium Thiopental (Abbott, Italy)

Sterile normal saline solution

TEMED (Bio Basic Inc., Canada)

Thick blotting papers (Bio-Rad Laboratories, PA)

Tris (Research Organics, OH)

Tween 20 (Bio Basic Inc., Canada)

Xylene (Zenith Science CO.LTD, Thailand)

Reagents Used in BenchMark® XT Instrument (Ventana, Medical System Inc., AZ)

EZ prep™ for deparafinization

Liquid Coverslip (LCS™) for reagent evaporation prevention

Sodium Chloride Sodium Citrate pH 6.5-7.5 (SSC™)

Reaction buffer pH 7.6

Universal buffer pH 8.4

1% Hydrogen peroxide (H_2O_2, UltraView^{TM} Inhibitor) for endogenous peroxidase inhibition

Goat anti-Mouse IgG (UltraView^{TM} HRP Multimer)

UltraView^{TM} DAB chromogen

UltraView^{TM} H_2O_2 (Substrate)

UltraView^{TM} Copper

Hematoxylin II

Lithium Carbonate (Bluing reagent)

Animal Preparation

5-week-old male Wistar rats were taken from National Laboratory Animal Centre, Mahidol University, Bangkok, Thailand. The animals were housed in a temperature-controlled room at 25 ± 1 ℃ under standard conditions (12-h dark-light cycle). They had freely access to food (regular dry food) and water. They were allowed to acclimate to the housing environment at least 7 days before experiments. All rats were concerned in accordance with the Ethical Committee, Faculty of Medicine, Chulalongkorn University, Thailand (approval No. 09/54).

The Preliminary Study: Development of Gastric Cancer Model in Rat

Male Wistar rats (6-week-old, N = 17) were used. Rats were given with "100 mg/kg MNU (dissolved in citrate buffer, pH 4.5) by oral gavage on days 0 and 14 of the experiment. In addition, rats were given s-NaCl (1 ml/rat) by oral gavage twice a week for the first 3 weeks of the experiment". Rats were sacrificed at week 3rd (N = 3), 6th (N = 3), 9th (N = 2), 13th (N = 3), 16th (N = 2), and 20th (N = 4) of the experiment [156].

Rat Model of Gastric Cancer Used in This Study

Male Wistar rats (6-week-old) were used. Rats were given "100 mg/kg MNU (dissolved in citrate buffer, pH 4.5) by oral gavage on days 0 and 14 of the

experiment. In addition, rats were given saturated NaCl (s-NaCl; 1 ml/rat) by oral gavage twice a week for the first 3 weeks of the experiment". All rats were sacrificed at week 20th of the experiment.

Chemical Preparations

N-methyl-*N*-nitrosourea (MNU) was dissolved in citrate-buffered saline, pH 4.5 [157] at a concentration of 100 mg/kg of rat body weight. In addition, high salt or saturated NaCl solution (5.2 M NaCl or 303.89 g/L NaCl or 30% NaCl) was prepared in distilled water and given to rats [60, 156]. Furthermore, 200 mg/kg curcumin was dissolved in corn oil.

Experimental Protocols

Thirty-six Wistar rats were randomized into six groups (six rats each) as follows.

"Control rats (CO) were fed citrate buffer, pH 4.5 (1 mL/rat) orally via intragastric tube on days 0 and 14 of the experiment. In addition, rats were fed normal saline (0.9% NaCl; 1 mL/rat) orally twice a week for the first 3 weeks of the experiment. Corn oil (2.5 ml/kg) was administrated by intragastric tube daily before administration of those vehicles for 20 weeks."

"Control rats supplemented with curcumin (CC) were fed citrate buffer, pH 4.5 and normal saline as same as previous described. 200 mg/kg curcumin (95% purified curcumin, Cayman Chemical, MI) was dissolved in corn oil and given to rats by intragastric tube daily before administration of those vehicles for 20 weeks."

"MNU and saturated NaCl induced rats (MNU+s-NaCl) were induced gastric carcinogenesis by MNU and s-NaCl according to the preliminary study. Briefly, Rats were fed 100 mg/kg MNU (dissolved in citrate buffer, pH 4.5; Sigma-Aldrich, MO) via intragastric tube on days 0 and 14 of the experiment. In addition, rats were fed saturated NaCl (30% NaCl, 1 ml/rat; Merck, Germany) orally twice a week for the first 3 weeks of the experiment. Corn oil (2.5 ml/kg) was administrated by intragastric tube daily before administration of MNU and s-NaCl for 20 weeks."

"MNU and saturated NaCl induced rats, supplemented with curcumin for 3 weeks (MNU+s-NaCl+C3W), were induced gastric carcinogenesis by MNU and s-NaCl according to previous describe. 200 mg/kg curcumin dissolved in corn oil was fed to rats by intragastric tube daily during administration of MNU and s-NaCl for the first 3 weeks."

"MNU and saturated NaCl induced rats, supplemented with curcumin for 17 weeks (MNU+s-NaCl+C17W), were induced gastric carcinogenesis by MNU and s-NaCl according to previous describe. After 3 weeks of the induction, 200 mg/kg curcumin dissolved in corn oil was administrated by intragastric tube daily for 17 weeks."

"MNU and saturated NaCl induced rats, supplemented with curcumin for 20 weeks (MNU+s-NaCl+C20W), were induced gastric carcinogenesis by MNU and s-NaCl according to previous describe. 200 mg/kg curcumin was dissolved in corn oil and fed to rats by intragastric tube daily for 20 weeks."

All rats were weighed weekly. After the end of the experiment (at week 20^{th}), the rats were anesthetized by intraperitoneal injection of Thiopental (60 mg/kg) after overnight fasting. Then, the rats were incised through the abdominal wall, and excised their stomachs. The rat stomach was divided into 2 parts symmetrically along greater and lesser curve. Stomach tissues were washed 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4. One part of the tissue was fixed in liquid nitrogen and kept in -80°C for western blot analysis of phospho-IκBα and Bcl-2 expression. Another part was fixed in 4% paraformaldehyde in PBS for histological study of gastric cancer incidence. In addition, this part of stomach was used for immunohistochemistry of 8-OHdG, and cyclin D1 expression. The study design was summarized in the diagram below (figure 3-4 and 3-5).

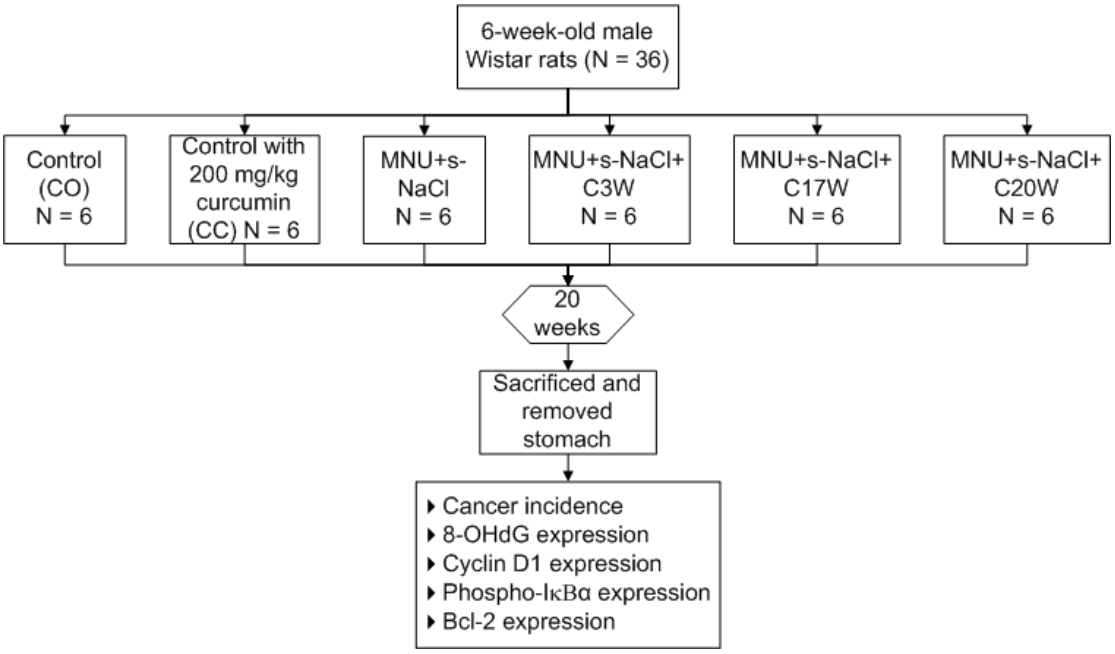
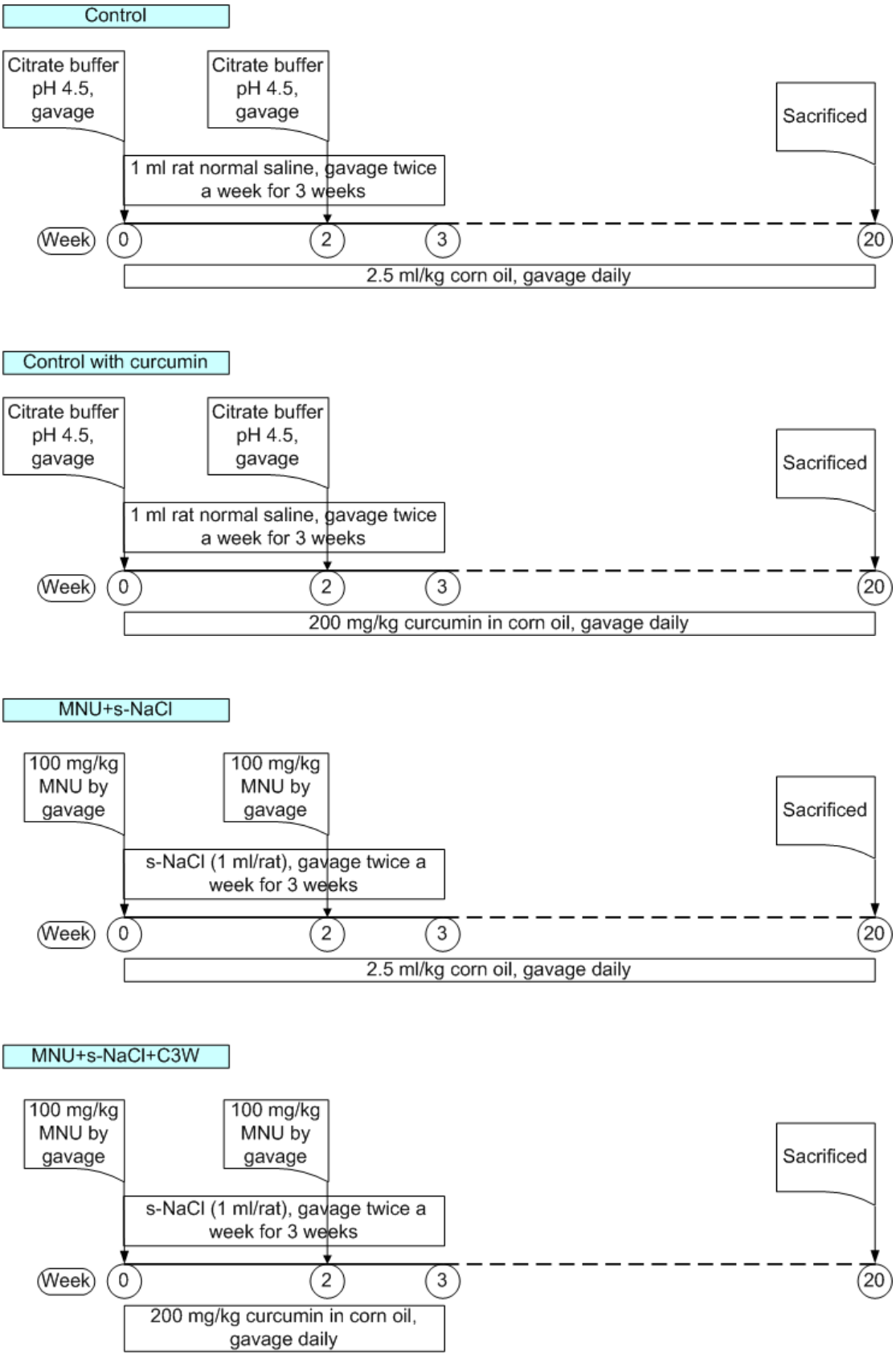


Figure 3-4: The diagram of the experimental design.


Control
Citrate buffer pH 4.5, gavage
Citrate buffer pH 4.5, gavage
Sacrificed
1 ml rat normal saline, gavage twice a week for 3 weeks
Week
0
2
3
20
2.5 ml/kg corn oil, gavage daily
Control with curcumin
Citrate buffer pH 4.5, gavage
Citrate buffer pH 4.5, gavage
Sacrificed
1 ml rat normal saline, gavage twice a week for 3 weeks
Week
0
2
3
20
200 mg/kg curcumin in corn oil, gavage daily
MNU+s-NaCl
100 mg/kg MNU by gavage
100 mg/kg MNU by gavage
Sacrificed
s-NaCl (1 ml/rat), gavage twice a week for 3 weeks
Week
0
2
3
20
2.5 ml/kg corn oil, gavage daily
MNU+s-NaCl+C3W
100 mg/kg MNU by gavage
100 mg/kg MNU by gavage
Sacrificed
s-NaCl (1 ml/rat), gavage twice a week for 3 weeks
Week
0
2
3
20
200 mg/kg curcumin in corn oil, gavage daily

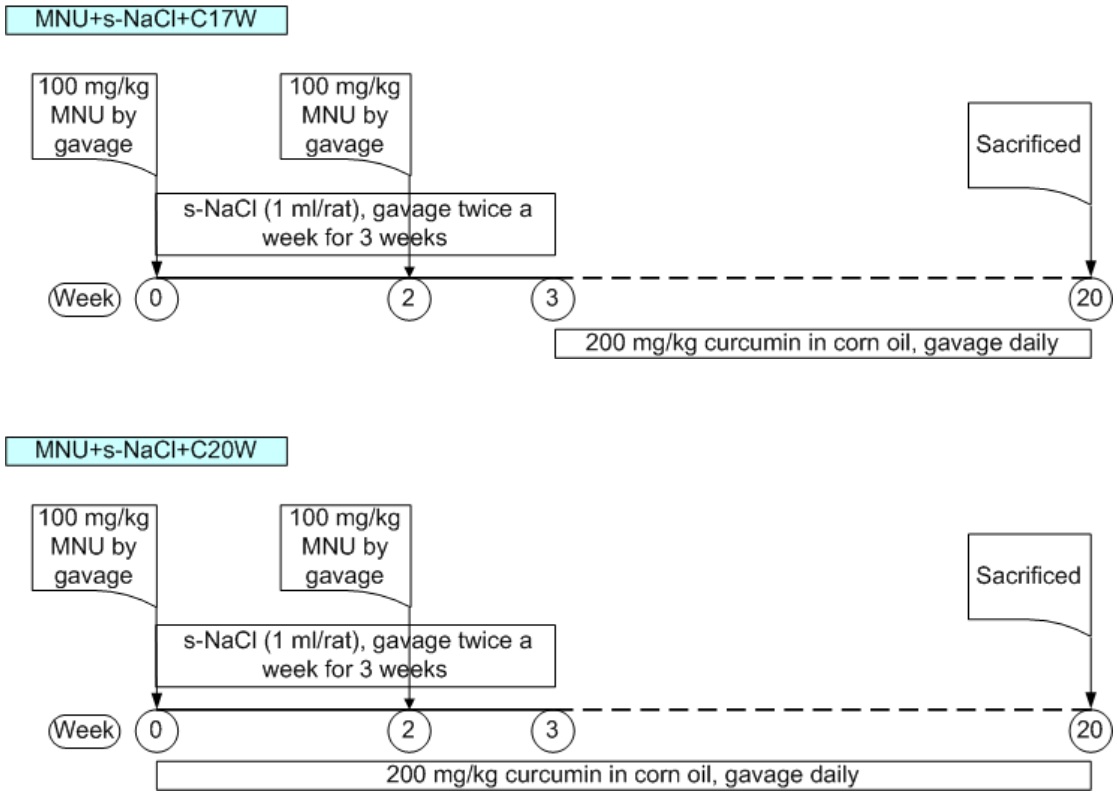


Figure 3-5: The timeline of chemical administration in all experimental groups.

Histopathological Examination

The stomach tissue in 4% paraformaldehyde was sliced along the longitudinal axis into 2-3 strips of equal width. Then, the tissue was fixed by 4% paraformaldehyde and embedded in paraffin. Multiple 2 μm-thick histological sections were put on the glass slides. The slides were deparaffinized in xylene for 3 times (5 minutes). Then, the slides were dehydrated with 95% alcohol for 3 times (5 minutes) and run with tap water for 3 minutes. After that, they were immersed in hematoxylin for 3 minutes at room temperature and washed in running tap water for 3 minutes. Then, the slides were immersed in eosin for 5 seconds at room temperature and dehydrated by moving the slide through 95% alcohol for 2 times and xylene for 2 times, respectively. After dehydration, the slides were mounted with the mounting media.

Histopathological analysis was performed to determine alterations of gastric epithelial cells and the incidence of gastric carcinogenesis by the experienced pathologist who blinded to the experiment. Under a microscope, all fields in each section were observed for alterations of gastric mucosa and identified the depth of carcinoma invasion.

Immunohistochemistry

Immunohistochemistry is the process of localizing proteins in the cells of tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues. The immunohistochemistry is wildly used for the diagnosis of cancer and the basic research. This technique is used for understanding the distribution and localization of biomarkers in a different part of tissues. Visualizing an antibody-antigen interaction can be provided by a number of ways. Classically, a secondary antibody is conjugated to enzyme, such as peroxidase, that can catalyse a color-producing reaction.

In this study, we used BenchMark® XT Instrument (Ventana, Medical System Inc., USA). Immunostaining for 8-OHdG or cyclin D1 was performed in paraffin-embedded sections by the following processes. "Briefly, the tissue sections were deparaffinized with EZ prep™. After that, the sections were retrieved the antigen (8-OHdG or cyclin D1) with Sodium Chloride Sodium Citrate pH 6.5-7.5 (SSC™). Next, 1% Hydrogen peroxide (H_2O_2, UltraView™ Inhibitor) was performed to block endogenous peroxidase activity. Then, the primary antibody used for 8-OHdG (Japan Institute for the Control of Aging, Japan, dilution 1:400) or cyclin D1 (Thermo Scientific, USA, dilution 1:200) were applied and incubated for 60 minutes or 32 minutes at 37°C, respectively. After that, the Goat anti-Mouse IgG (UltraView™ HRP Multimer) was used as secondary antibody. When the development of color with UltraView™ DAB chromogen, UltraView™ H_2O_2, and UltraView™ Copper was detected, the slides were counterstained with Hematoxylin II and Lithium Carbonate. Immunoreactive cells of 8-OHdG and cyclin D1 were dark brown stained in nuclei of gastric epithelial cells."

Western Blot Analysis

Western blot technique with specific antibodies is used to identifiy proteins that have been separated from one another depending on their size by gel electrophoresis. Western blotting refers to protein transferring and immunodetecting. The application of an electrical current induces the proteins in the SDS-PAGE gel to move to the membrane. Thus, the membrane is a copy of the gel's protein pattern and is subsequently detected by the specific antibody. The entire process of western blot consists of sample preparation, gel electrophoresis, protein transfer from gel to membrane, immunostain, and protein detection.

A. Sample Preparation

"Forestomach sample (0.05 g) was homogenized in 0.5 mL of iced-cold lysis buffer: RIPA buffer (Cell Signalling Technology Inc., MA), protease inhibitor Sigma-Aldrich), and phosphatase inhibitor (Sigma-Aldrich). The homogenate was sonicated for 15 seconds and centrifuged at 11,000 g for 10 minutes at 4°C. The supernatant was retained. Protein concentration was measured with the BCA Protein Assay Kit (23252, Thermo Scientific)."

This assay kit was used for quantitation of total protein in samples, based on the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (biuret reaction) and the subsequent sensitive and selective colorimetric detection of the cuprous ion (Cu^{1+}, purple) using bicinchoninic acid (BCA). The color produced from this reaction was stable, and increrases in a proportion over a wide range of increasing protein concentrations [158]. The assay protocols were performed as descriptions from the company. Briefly, all standards, negative control, and unknown samples were prepared as in the reagent preparation part of the protocol and assayed in duplicate. Each replicate of standard, control, and unknown sample was added to each microplate well and followed by the compatibility reagent solution. Mix on a plate shaker and incubate at for 15 minutes at 37°C. The working reagent (WR) was freshly prepared by mixing 50 parts of the BCA reagent A and 1 part of the BCA reagent B. Then, the WR was applied to each well the plate was incubated at 37°C for 30 minutes. After cool down at room temperature for 5 minutes, the optical density

(O.D.) was determined by using a microplate reader (Bio-Rad Model 680, Bio-Rad Laboratories) set to 570 nm.

The average 570 nm absorbance value of each standard and unknown samples were subtracted the average of the negative control optical density. The below table showed the calculation of results.

Table 3-1: The corrected O.D. plotted on y-axis of the standard curve was calculated from the mean subtract the average of zero standard O.D. Note: μg/mL = micogram per milliliter; nm = nanometer.

Standard albumin concentration (μg/mL)	Obtical density (O.D.) (570 nm)		Average O.D.	Corrected O.D.
	Data 1	Data 2		
0	0.209	0.206	0.2075	
62.5	0.273	0.263	0.268	0.0605
125	0.317	0.316	0.3165	0.109
250	0.416	0.421	0.4185	0.211
500	0.607	0.601	0.604	0.3965
1000	0.930	0.944	0.937	0.7295
2000	1.555	1.549	1.552	1.3445

Then, the standard curve was prepared by plotting the average blank-corrected 570 nm value for each standard vs. its concentration (μg/mL). The data were linearized with computer software and the best fit line can be determined by regression analysis. In addition, the standard curve was produced using curve-fitting algorithms for more accuracy. The concentration of unknown sample was converted to its corresponding concentration by the standard curve. If samples had been diluted prior to assay, the concentration read from the standard curve must be multiplied by the dilution factor.

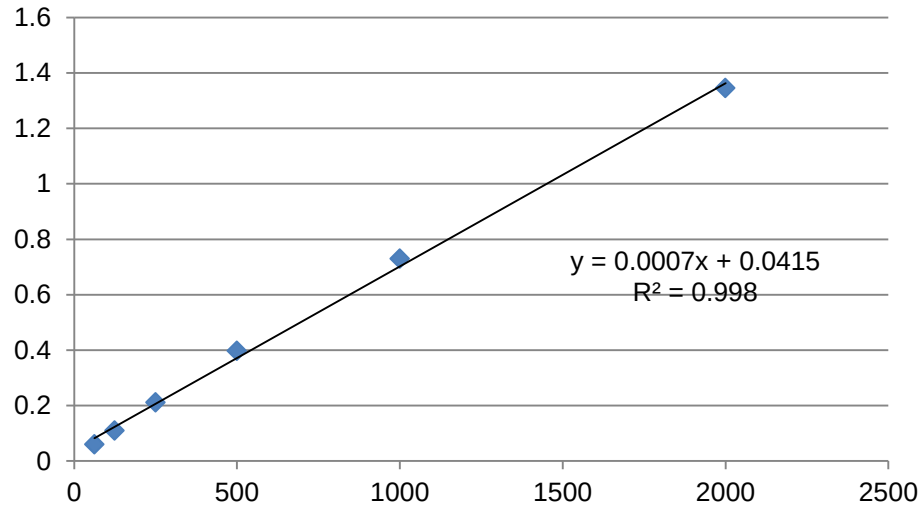


Figure 3-6: A standard curve of albumin concentration. The x-axis and y-axis of the standard curve represented standard albumin concentrations (μg/mL) and O.D., respectively. Strong correlation (R^2 = 0.998) was able to present by the linear equation of y = 0.000x + 0.041.

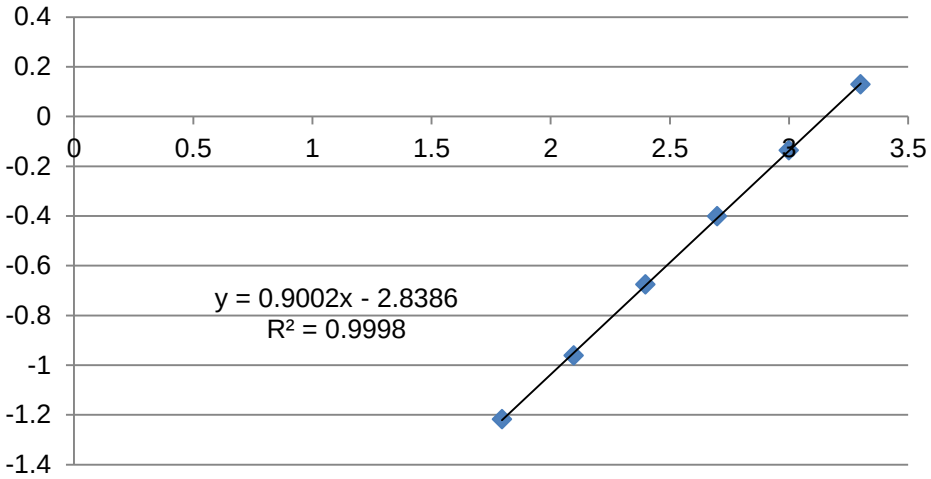


Figure 3-7: A Log scale curve of albumin concentration. Strong correlation (R^2 = 0.999) was able to present by the linear equation of y = 0.900x - 2.838.

After protein concentrations had determined, the samples were prepared for loading into the gels by mixing with 2X sample buffer (see Appendix). Then, they

were boiled for 5 minutes. Extracted proteins (35 μg/lane) were loaded to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

B. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Gel Electrophoresis (SDS-PAGE)

For the setting of the PAGE gels, the 10% acrylamide gel solution was prepared (see Appendix) and poured into the glass mold (Bio-Rad Laboratories, PA). A thin layer of water was carefully floated on the surface of the gel mixture before it polymerized. After that, the separating gel was performed, and the water was poured off. Then, the 4% gel solution was prepared (see Appendix) and poured on the top of the separating gel. A comb was inserted into the top of the mold to give the wells. After polymerization, the comb was removed. Then, gel was mounted in the electrophoresis apparatus and placed in the electrophoresis tank (Bio-Rad Laboratories, PA) as instructed by the company procedures. The running buffer (see Appendix) was filled into the gel assembly and the tank.

Loading tips were used to load the molecular weight marker (5 μL, Fermentas, Thermo Scientific, MI) and samples (20 μL) in each well. The marker can determine the protein size and also check the progress of an electrophoretic run. The protein samples should be evenly loaded (35 μg/lane). After the electrical leads were applied into a suitable power supply (Bio-Rad Laboratories, PA), the electric power (100 Volt) was set to begin electrophoresis. After 1 hour and 45 minutes, the power was turned off when the dye molecule was near the bottom of the gel. The gel was removed from the assembly, and part of stacking gel was cut away.

C. Protein Transfer and Staining (Western Blotting)

Transfer was done in semi-dry conditions using Trans-Blot® SD (Bio-Rad Laboratories, PA) for 3 minutes. Following electrophoresis, the gel was equilibrated in transfer buffer (see Appendix). The polyvinylidene fluoride membrane (PVDF, Pall Corporation, FL) and extra thick blotting paper (Bio-Rad Laboratories, PA) were also soaked in transfer buffer for 15 minutes. Three incubated sheets of blotting paper were placed onto the platinum anode. Carefully roll out all air bubbles, test tube was used like a rolling pin over the surface of blotting papers. The incubated membrane

was placed followed by the gel. The other three sheets of blotting papers were applied on the top of the gel. All air bubbles were removed from between each layer. The transfer buffer was added for protection of drying. Then, the cathode was placed on the top. The separated proteins were transferred to the PVDF membrane using electrical supply (15 Volt) for 1 hour.

Following transfer, the blot was incubated with 5% non-fat dry milk in TBS (see Appendix) for 1 hour at room temperature on a gentle shaker. This condition was used to prevent non-specific binding of the primary and/or secondary antibodies to the membrane. After blocking, the blotted membrane was incubated with TBS-T (see Appendix) for 2 times (5 minutes each) on a shaker.

The dilutions of anti-Bcl-2, anti-β-actin, and secondary antibodies were prepared in 1% non-fat dry milk in TBS. "Anti-Phospho-IκBα (Ser32/36) antibody" was prepared in 5% non-fat dry milk in TBS. Then, the membrane was probed with "mouse monoclonal anti-Phospho-IκBα (Ser32/36) antibody (1:1000; Cell Signalling Technology Inc.) or mouse monoclonal anti-Bcl-2 antibody (1:200; Santa Cruz Biotechnology Inc.)" overnight at 4°C. In addition, the membrane was also incubated with "mouse monoclonal anti-β-actin antibody (sc-47778, 1:5,000; Santa Cruz Biotechnology Inc.)" for 1 hour at room temperature. After incubation with the primary antibody, three-time washing in TBST were applied. Then, the membrane was incubated with "the secondary antibody (goat anti-mouse IgG HRP, 1:4,000; Cayman Chemical)" for 1 hour at room temperature. After that, the membrane was washed in TBST for 5 minutes 3 times.

D. Protein Detection

Protein band was developed with a commercial chemiluminescence detection kit (Amersham ECL plus western blotting system, GE Healthcare, UK). The detection solutions A and B were mixed in a ratio 40:1 in a dark room. The excess wash buffer was gently removed before the mixed reagent was applied on the membrane. After 5 minutes of incubation, the excess reagent was gently removed. Then, the membrane was placed, protein side up, in the film cassette and covered by a fresh piece of plastic. Then, the autoradiography film (Fuji Electron Microscopic Film FG, Fujiflim, Japan) was placed carefully on the top. The cassette was closed and exposed for a

suitable time (30 minutes for Phospho-IκBα and Bcl-2, and 10 minutes for β-actin). It is essential that these processes were performed quickly and in a dark room once the membrane had been exposed to the detection reagent. After exposition, the film was manually processing. After that, the processed film was scanned. The expression levels of proteins were quantified by "ImageJ (U.S. National Institutes of Health, Bethesda, MA)." The relative levels of Phospho-IκBα (Ser32/36) and Bcl-2 expressions were normalized by β-actin intensity.

Data Collection

A. Histological Study of the Stomach Tissue and Gastric Cancer Incidence

The stomach tissue was examined histologically for carcinogenesis and the depth of cancer invasion by the pathologist. The benign or precancerous tissues were also determined. In addition, the cancer incidence was calculated in the percentage (%) from the following equation:

The percentage of cancer incidence (%) = 100 × (number of cancer-bearing rats / number of induced rats).

B. Expression of 8-OHdG and Cyclin D1

By using immunohistochemistry, localization and distribution of the expressions were focused. For semi-quantification, nuclei stained of cyclin D1 or 8-OHdG in gastric epithelial cells was counted manually as positive cells from over 1,000 examined cells.

Under light microscope "(Nikon E50i, Nikon Corporation, Japan)", the expressions of 8-OHdG and cyclin D1 were nuclei-stained cells (figure 3-8). To verify the expression of 8-OHdG and cyclin D1, the digital images were taken in high magnification field (400×) from each sample using the microscope equipped with "digital camera (Nikon Digital Sight DS-Fi1, Nikon Corporation, Japan)". Ten images from two sections per animals were analyzed. The numbers of dark brown-stained in nuclei of epithelial cells were counted manually using Point tool in the IMAGE-PRO® PLUS software program (version 6.1) as shown in figure 3-9. Over one thousand of

gastric epithelial cells were counted for each rat. The digital images from each sample were also analyzed with blind. The data were shown as a percentage (%) of immunoreactive cells calculating from this equation:

The percentage of immunoreactive cells (%) = 100 × number of stained cells / the examined cells.

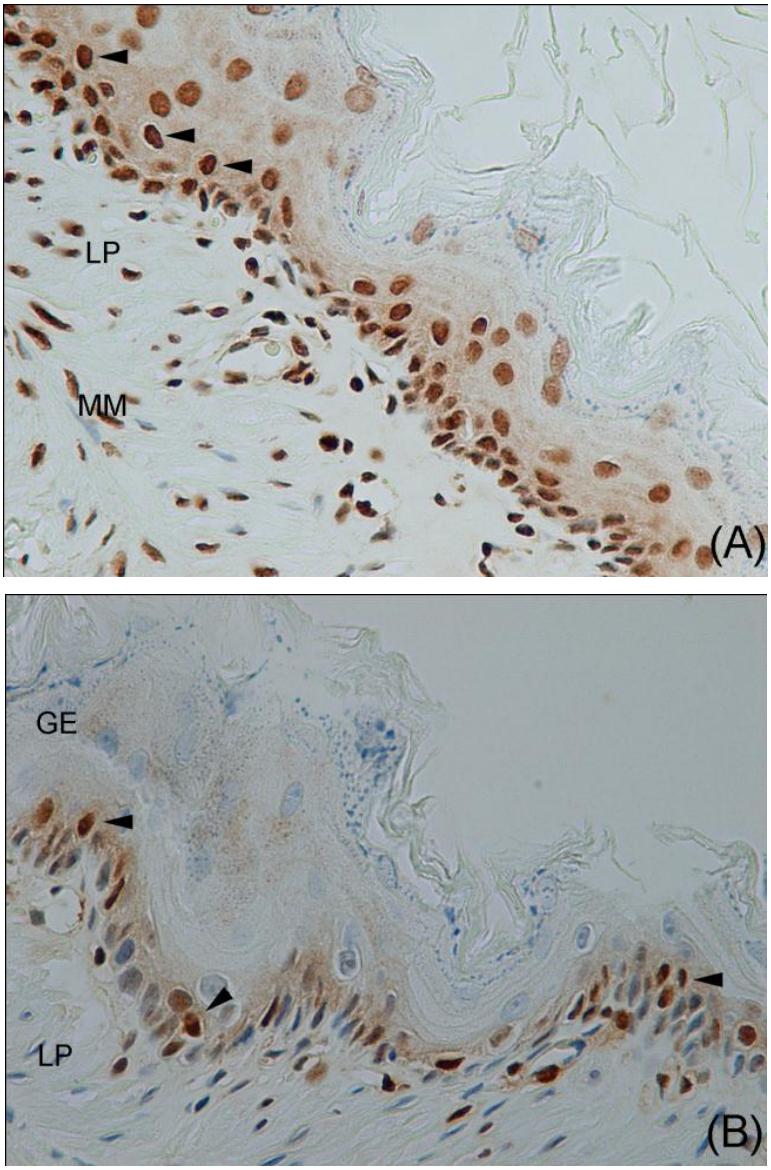


Figure 3-8: Immunohistochemical staining of 8-OHdG (A) and cyclin D1 (B) antibodies in control (CO) group (×40). The arrowheads identified the positive cells with dark brown-stained in the nuclei. GE: gastric epithelium, LP: lamina propria, and MM: muscularis mucosae.

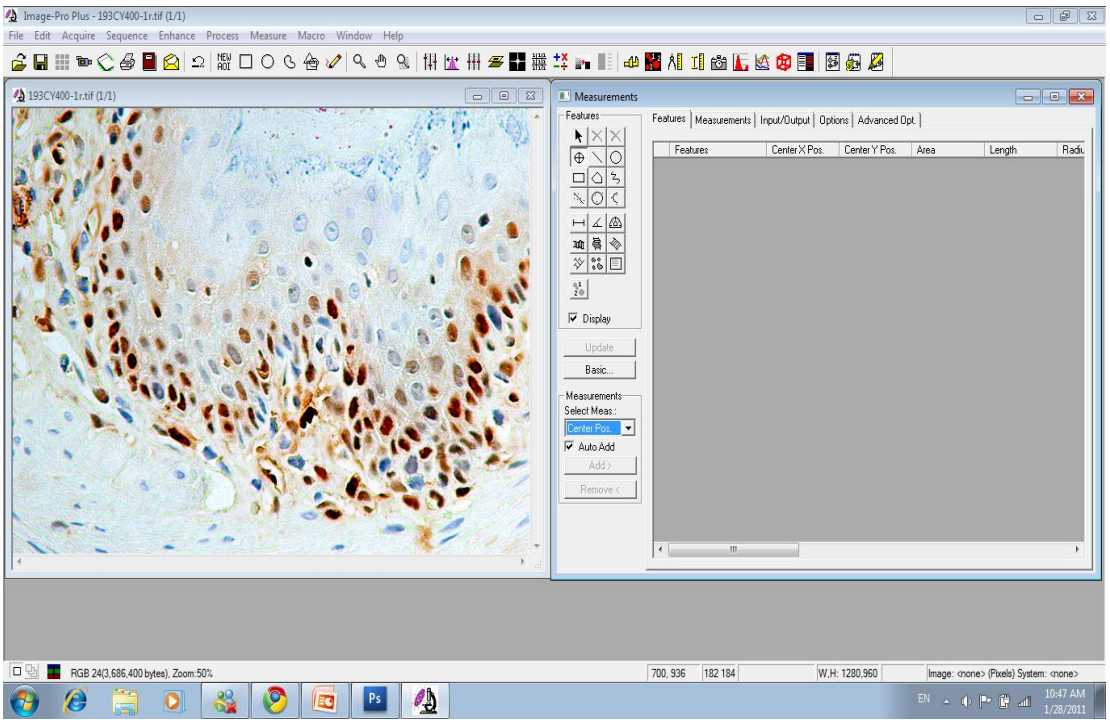


Figure 3-9: IMAGE-PRO® PLUS software program (version 6.1) is used for manually counting numbers of positive cells in the gastric epithelium.

C. Expressions of Phospho-IκBα and Bcl-2

By using western blot technique (also called immunoblotting), the specific proteins can be detected in a sample of tissue homogenate or extract. In this study, the expressions of "phospho-IκBα (Ser32/36) and Bcl-2" were determined. For quantification of protein expression, ImageJ program was used.

The digital images were brought from the scanner (UMAX model Astra 6850, China). The images were set as grayscale and 300 dpi resolution. The lane of the interesting protein was selected and opened with ImageJ program. The "process" and "subtract background" commands were selected to remove continuous backgrounds from the image. The light background was selected to allow the processing of images with bright background and dark objects. The appropriate background can be calibrated from the pixel value range in the radius of curvature of the paraboloid. Then, the rectangular tool was used for outlining the first lane (the left most lane). The "analyze", "gels", and "select first lane" commands were used for selecting lane

1. The rectangular selection was move right to the next lane by dragging. Then the command “select next lane” was used for outlining the next lane. Each lane was outlined by repeating the movement of the rectangular selection and the selection of the “select next lane” command. After that, the special menu, plot lane, was selected to generate the lane profile plots. To quantify the areas under the curve representing the intensity of each band in the lane, the straight line tool was used to draw base lines. Each peak of interest defines a closed area for the separation. Then, the magic wand tool was used for selection the separate areas consecutively. The data from the measurement were shown. Figure 3-10 displayed the results using ImageJ.

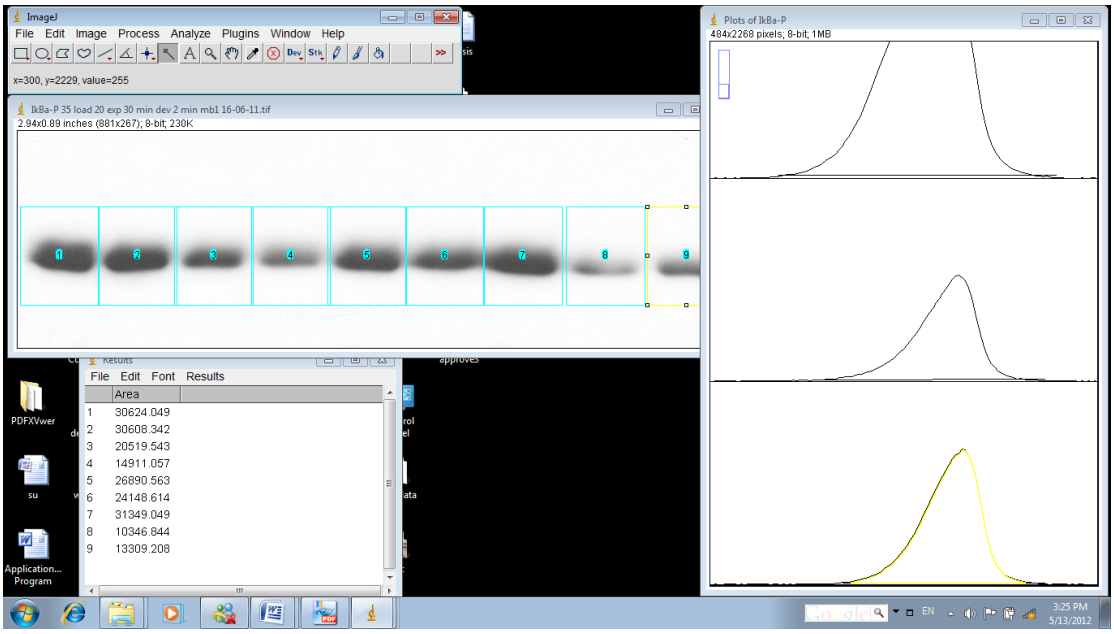


Figure 3-10: ImageJ program is used for studying intensity of each band in the selective lane.

Data Analysis

Data was presented as means ± standard deviation (SD) for continuous data and frequency for categorical data (alterations of gastric mucosa and gastric cancer incidence). Statistical analyses were conducted using χ^2-test for incidence of gastric cancer. However, Fisher’s exact test was used instead if the expected frequency less

than 5. Body weight change assessed every week was analyzed with one-way repeated-measures ANOVA and LSD Post Hoc Test. The percentage of immunoreactive cells and the relative intensity were analyzed with one-way ANOVA and LSD Post Hoc Test, if the data passed either normality or equal varience tests. A *p* value of less than 0.05 is considered to be statistically significant. All statistical data have been analyzed using SPSS version 13.0 programs.

CHAPTER IV

RESULTS

The Preliminary Study: Development of Gastric Cancer Model in Rat

Intragastric administrations of MNU and s-NaCl induced alerations of forestomach mucosa and squamous cell carcinoma (SCC). Histological study of normal stomach indicated that forestomach mucosa was composed of epithelial cells, laminar propria and muscularlis mucosae. Squamous cells lining in forestomach mucosa produce keratin (figure 4-11). Benign papillary growth displayed hyperplasia (increase of epithelial cells), hyperkeratosis (increase of keratinization), and no invasion into the deeper layer of the forestomach. The histological character of the papillary growth was shown in the figure 4-12. These lesions were not diagnosed as cancer. Gastric tissues obtained from six rats at the 3rd, 6th, 9th, 13th, and 16th week exhibited benign papillary growth with hyperkeratosis in the forestomach. Histological study of gastric tissues determining alterations of gastric epithelium and the incidence of cancer was shown in table 4-2.

Invasion and dyskeratosis (abnormality of keratinization) of cancerous tissue were characters used for identifying SCC [159]. The figure 4-13 showed SCC and morphologic characters. One rat from the 6th week, one from the 9th week, one from the 13th week, one from the 16th week, and four from the 20th week were SCC. The depth of invasiveness of SCC was shown in table 4-2. Cancer incidence rates at the 3rd, 6th, 9th, 13th, 16th, and 20th week were 0% (0/3), 33.33% (1/3), 50% (1/2), 33.33% (1/3), 50% (1/2), and 100% (4/4), respectively.

Table 4-2: Histological changes of gastric mucosa and the cancer incidence in the preliminary study.

Week of experiment	No. of rats					Cancer incidence (%)
	Normal	Benign papillary growth	Squamous cell carcinoma (SCC)			
			Submucosa invasion	Muscle layer invasion	Serosa invasion	
3	2	1	-	-	-	0
6	1	1	1	-	-	33.33
9	-	1	1	-	-	50
13	-	2	1	-	-	33.33
16	-	1	1	-	-	50
20	-	-	3	1-	-	100

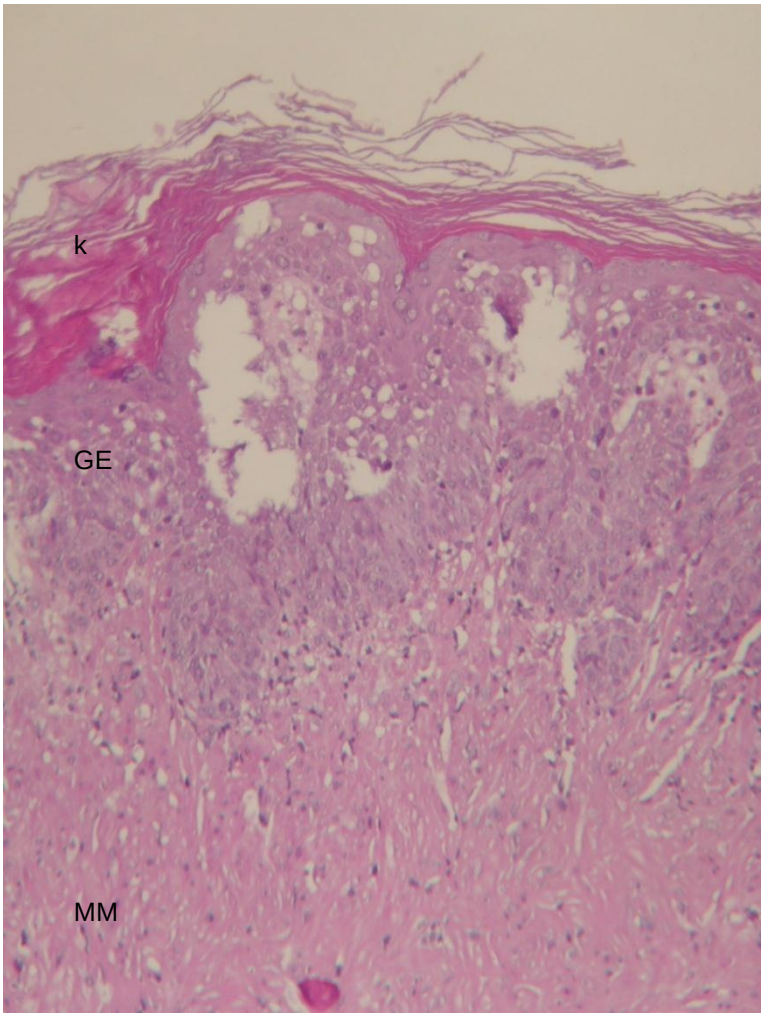


Figure 4-11: Histological study of normal forestomach (H&E staining, 200×). Squamous cells lining in forestomach mucosa produce keratin (k) for protection. GE: gastric epithelium and MM: muscularis mucosae

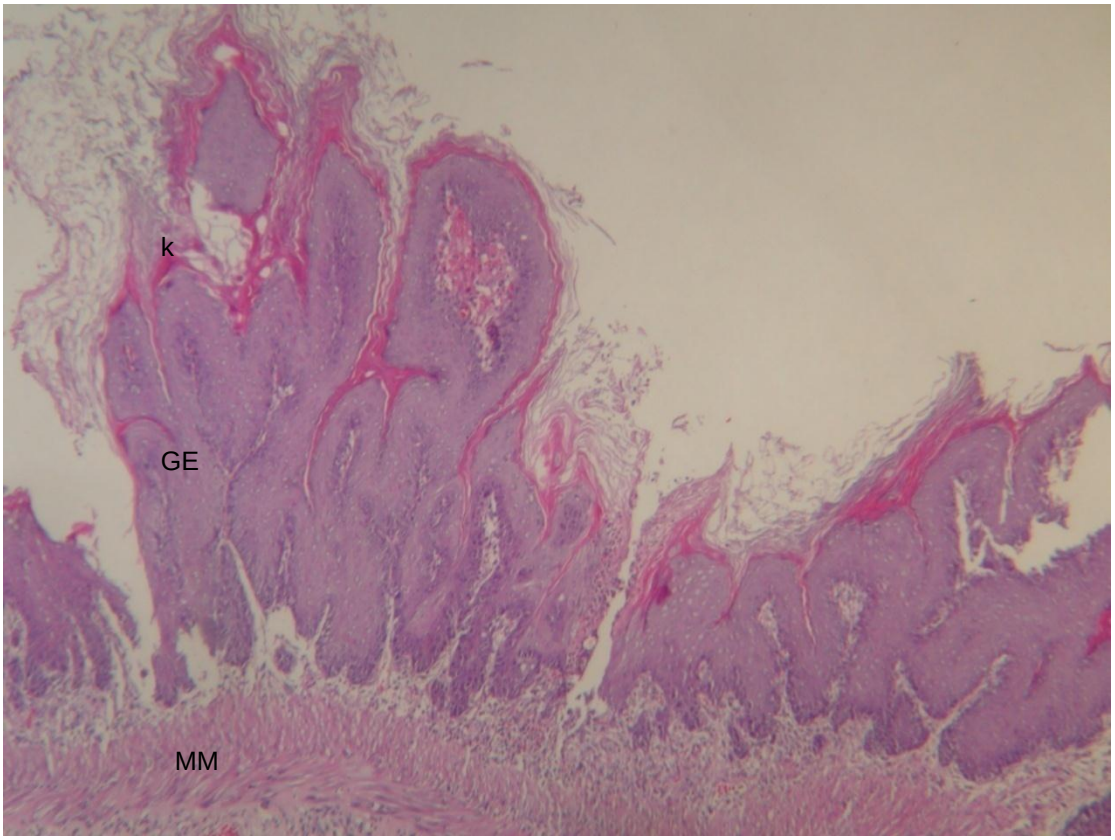


Figure 4-12: Histological study of benign papillary growth (H&E staining, 40×). After intragastic administration of MNU and s-NaCl, the mucosa of forestomach showed benign papillary growth in the 3rd week. This lesion is associated with increase keratinization. The papillary growth did not invade into the deeper layer of the stomach wall. GE: gastric epithelium, MM: muscularis mucosae, and k: keratin

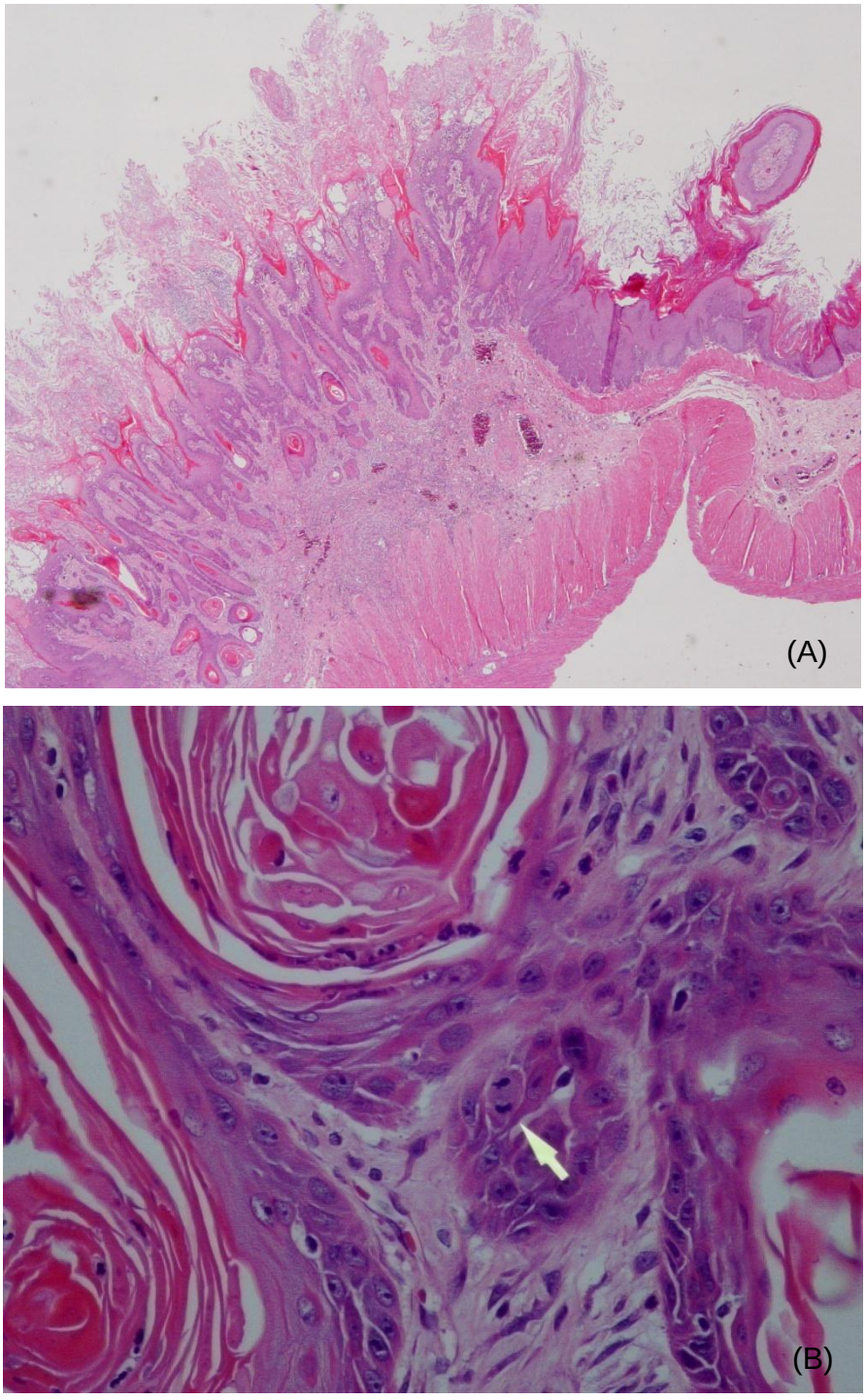


Figure 4-13: Histological study of SCC (H&E staining). After intragastic administration of MNU and s-NaCl, the mucosa of forestomach showed SCC with submucosal invasion in the 20th week (A, 40×). The high magnification showed dyskeratosis of cancerous tissue. The arrow indicated the mitotic cell (B, 400×).

Rat Model of Gastric Cancer Used in This Study

From the preliminary study, SCCs were developed at the 20th week. The incidence of cancer is 100%. Thus, we used the 20th week model for this study. From the experimental design (figure 3-4), 35 rats survived the 20 weeks of this study. During the whole study period, there was only one rat death in total. At 3 weeks, the rat in MNU+s-NaCl+C3W died from aspiration after feeding. The results of autopsy showed congestion and bleeding spots in both lungs. There was hyperkeratosis in the mucosa of forestomach, but no precancerous lesion detected by histological examination. Thus, we exclude the data from the rat died at 3 weeks.

Changes of Body Weight

Body weights of all rats were recorded every week and analyzed using one-way repeated-measures ANOVA. There was no significant difference in body weight among groups during the study period (p = 0.099) as shown in figure 4-14.

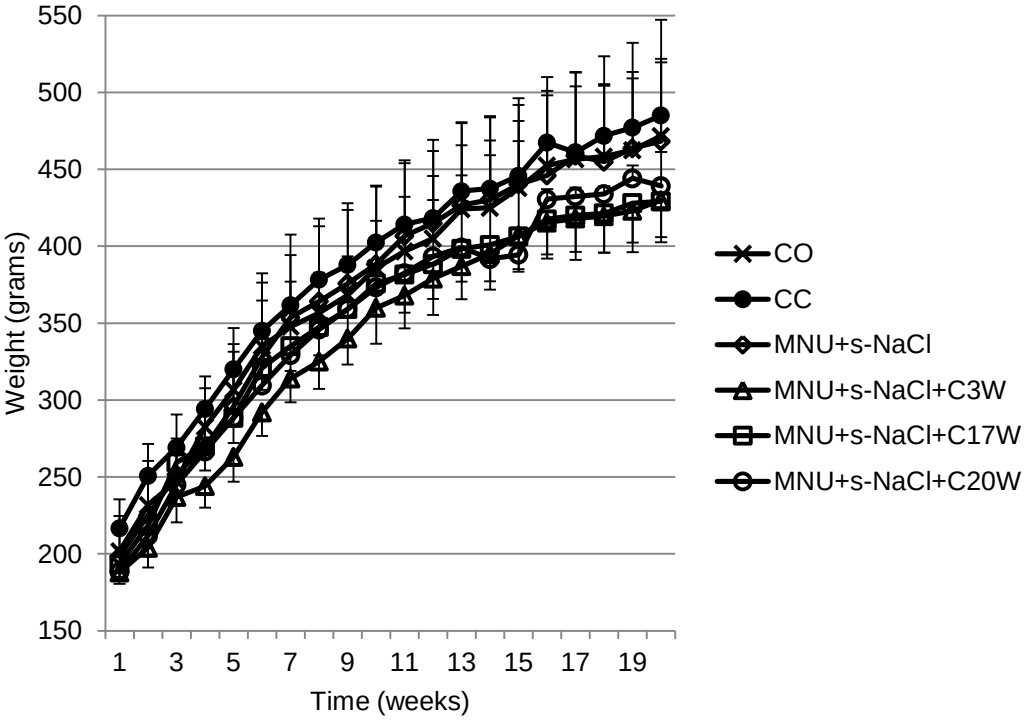


Figure 4-14: Line graph of the body weight changes (mean ± SD) assessed every week in all groups. CO: control group (N = 6), CC: control with curcumin supplementation group (N = 6), MNU+s-NaCl: MNU and saturated NaCl induced rats (N = 6), MNU+s-NaCl+C3W: MNU+s-NaCl induced rats supplemented with curcumin for 3 weeks (N = 5), MNU+s-NaCl+C17W: MNU+s-NaCl induced rats supplemented with curcumin for 17 weeks (N = 6), and MNU+s-NaCl+C20W: MNU+s-NaCl induced rats supplemented with curcumin for 20 weeks (N = 6).

Histological Study, Incidence of Gastric Cancer, and Roles of Curcumin

The excised stomachs of CO was shown in figure 4-15A. Rats have two parts of stomach: forestomach (non-glandular stomch) and corpus (glandular stomach) which is divided by the limiting ridge [30]. The multiple polypoid tumors developed in the forestomach after MNU and high salt administration (figure 4-15B).

Histological study of normal gastric mucosa in CO was shown in figure 4-16. Forestomach of rat is composed of mucosa, submucosa, muscle layer, and serosa.

These layers are similar to those in the corpus. Epithelial cells lining in the forestomach mucosa are squamous cells, whereas those in the corpus are columnar cells. From the histological study, the table 4-3 displayed the alterations of stomach mucosa and invasiveness of SCC in the stomach. Gastric cancer incidences were calculated in the percentage and also shown in the table 4-3. There is no abnormal finding throughout the stomach of CO. The incidence of cancer is 0%. Intragastric administration of MNU and s-NaCl induced SCC in the forstomach. At the 20^{th} week, SCC from this model showed cancerous tissue in the epithelium and invasion of cancerous tissue with dyskeratosis through the forestomach wall. Cancer with submucosal invasion was occurred in one rat. Three rats showed muscle layer-invasion, and one rat exhibited stomach perforation from serosal invasion. In MNU+s-NaCl group, SCC was found in forestomach of all rats (100%).

Curcumin supplementation in control rats (CC) did not exhibit any alterations including SCC. The cancer incidence of CC is 0%. Curcumin supplementation for 3 weeks during administrations of MNU and s-NaCl had preventive effect. In table 4-3, there was 3/5 (60%) rats in MNU+s-NaCl+C3W developed SCC. Two rats in this group showed benign papillary growth. Hyper-proliferation of squamous cells, hyperkeratosis, and no invasion into the deeper layer of the forestomach were characters of the papillary growth. Therefore, this lesion was not diagnosed as cancer. Two SCC rats were submucosa invasion. Another one was muscle layer invasion.

Curcumin supplementation for 17 weeks after administrations of MNU and s-NaCl was late treatment. Rats in MNU+s-NaCl+C17W group developed SCC for 100%. 5 rats were submucosa invasion. Another one showed stomach perforation (table 4-3).

Curcumin supplementation for 20 weeks was early treatment. In MNU+s-NaCl+C20W group, three rats developed SCC with submucosa invasion. The cancer incidence was 50%. Interestingly, one rat exhibited mucosal congestion, the congested blood vessels undering the mucosa. Another 2 rats developed the papillary growth (table 4-3).

However, there was no significant difference of gastric cancer incidence between MNU+s-NaCl and MNU+s-NaCl+C3W groups. In addition, there was no significant difference of gastric cancer incidence between MNU+s-NaCl and MNU+s-

NaCl+C20W groups. The papillary growth showed hyperplasia and hyperkeratosis of epithelium (figure 4-17A). SCC showed cancerous tissue in epithelium and invasion of cancerous tissue with dyskeratosis through submucosal layer (figure 4-17B), muscle layer (figure 4-17C), or serosa (figure 4-17D).

Table 4-3: Histological changes of gastric mucosa and the cancer incidence in experimental groups. Mucosal congestion represented congested blood vessels in the underlying the mucosa.

Group	No. of rats						Cancer incidence (%)
	Normal	Mucosal congestion	Benign papillary growth	Squamous cell carcinoma (SCC)			
				Submucosa invasion	Muscle invasion	Serosa invasion	
CO (N=6)	6	-	-	-	-	-	0
CC (N=6)	6	-	-	-	-	-	0
MNU+s-NaCl (N=6)	-	-	-	2	3	1	100
MNU+s-NaCl+ C3W (N=5)	-	-	2	2	1	-	60
MNU+s-NaCl+ C17W (N=6)	-	-	-	5	-	1	100
MNU+s-NaCl+ C20W (N=6)	-	1	2	3	-	-	50

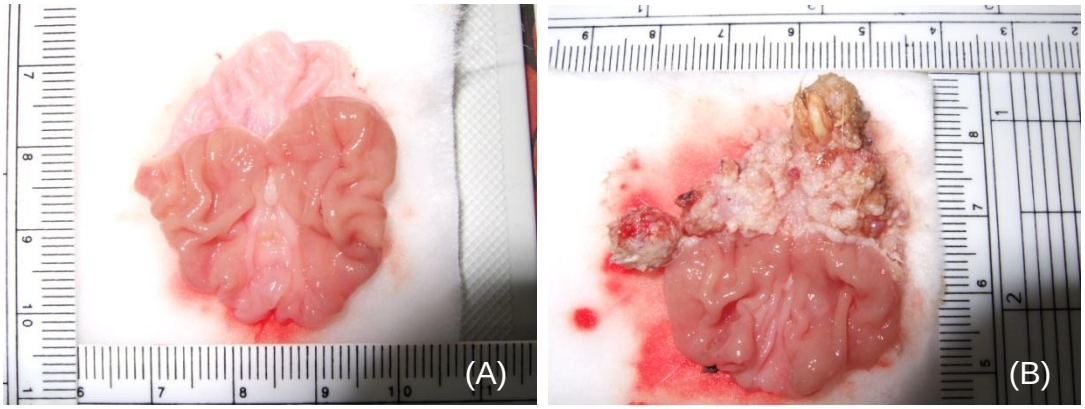


Figure 4-15: Macroscopic appearances of normal stomach (A) and SCC in the forestomach (B). Stomachs were divided along greater curve. Forestomach is a thin-walled part (upper). Corpus is a thick-walled section (lower). In MNU and s-NaCl administrated group, the multiple polypoid tumors developed in the forestomach.

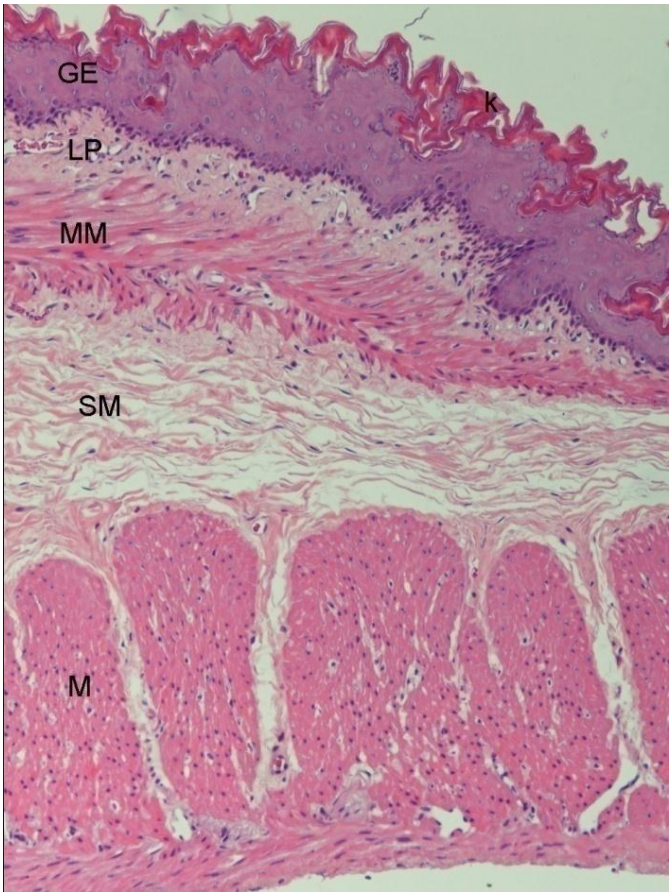


Figure 4-16: Microscopic view of rat forestomach in the control group (H&E straining, 100×); GE: gastric epithelium, LP: laminar propria, MM: muscularis mucosae, SM: submucosa, and M: muscle layer. In GE of forestomach, squamous cells are lining and producing keratin (k) above.

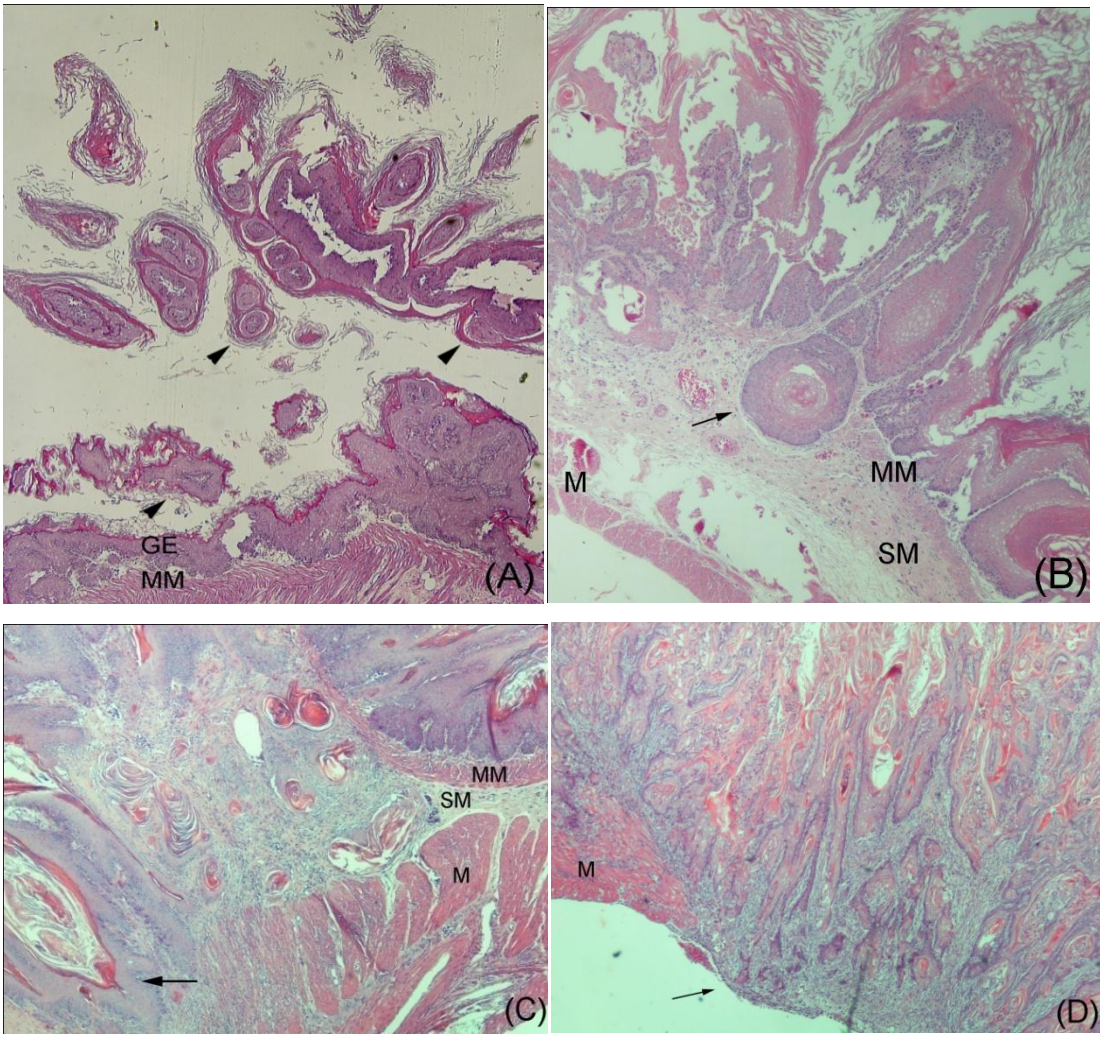


Figure 4-17: Microscopic views of the papillary growth (A) and SCCs (B-D) (H&E staining 40×). Papillary growth (arrowheads) of the stratified epithelium of forestomach showed hyper-proliferation and hyperkeratosis of epithelium (A). SCCs (arrows) showed cancerous tissue in the epithelium and invasion of cancerous tissue with dyskeratosis through submucosa (B), muscle layer (C), or serosa (D). GE: gastric epithelium, LP: laminar propria, MM: muscularis mucosae, SM: submucosa, and M: muscle layer.

Effects of MNU and Saturated NaCl Induction on 8-OHdG Expression and Roles of Curcumin

8-OHdG expression was studied by using immunohistochemical technique and shown as nuclei-stained cells (figure 4-18). 8-OHdG was expressed primarily in basal cells of the epithelium in normal mucosa (figure 4-18, A and B). This protein was upregulated in the mucosa of SCC (figure 4-18C), and was limited to a small number of epithelial cells in the mucosa that was supplemented by curcumin (figure 4-18D-F).

The numbers of 8-OHdG immunoreactive cells were manually counted using IMAGE-PRO® PLUS software program (version 6.1). The percentage of immunoreactive cells was not significantly different between CO and CC. The percentage of immunoreactive cells was significantly increased in MNU+s-NaCl compared with CO. Curcumin supplementation for 3 weeks during administration of MNU and s-NaCl tended to decrease the percentages of 8-OHdG expression, but did not reach the statistic significance when compared with untreated group. The late treatment (MNU+s-NaCl+C17W) and the early treatment (MNU+s-NaCl+C20W) of curcumin significantly decrease the percentages of 8-OHdG expression when compared with MNU+s-NaCl group. The average percentages of immunoreactive cells of all groups were shown in table 4-4 and figure 4-19.

From the histopathological result (table 4-3), 2 rats in MNU+s-NaCl+C3W were papillary growth. The other rats in this group were SCC. In MNU+s-NaCl+C20W group, 2 rats in were papillary growth and 1 rat was mucosal congestion. The others showed SCC. Rats in these groups were divided into subgroups: C3W benign group (N = 2), C3W cancer group (N = 3), C20W benign group (N = 3), and C20W cancer group (N = 3). The mean percentages of 8-OHdG immunoreactive cells were shown in table 4-5.

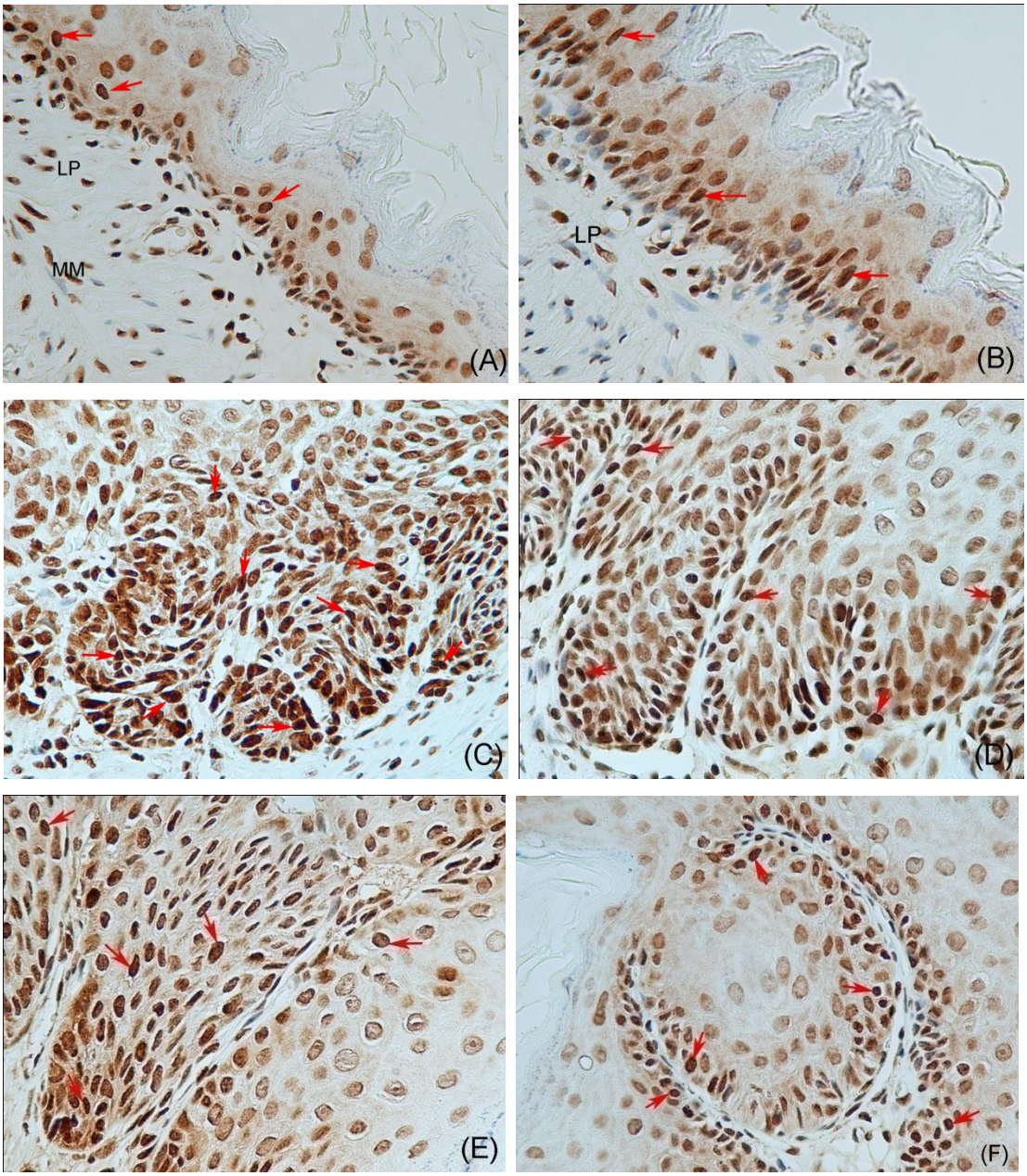


Figure 4-18: Immunohistochemical staining of 8-OHdG antibody in representative tissue specimens: CO (A), CC (B), MNU+s-NaCl (C), MNU+s-NaCl+C3W (D), MNU+s-NaCl+C17W (E), and MNU+s-NaCl+C20W (F). DAB stained immunoreactive cells (dark brown, arrows). Nuclear counterstaining was performed with Hematoxylin II and Bluing reagent (400×). LP: lamina propria and MM: muscularis mucosae.

Table 4-4: Means ± SD of 8-OHdG immunoreactive cells (%) in experimental groups.

Group	Immunoreactive cells (%)	*P* value (compared with CO)	*P* value (compared with MNU+s-NaCl)
CO (N=6)	44.42 ± 3.41##		0.001
CC (N=6)	40.39 ± 3.52##	0.112	0.000
MNU+s-NaCl (N=6)	53.06 ± 5.96**	0.001	
MNU+s-NaCl+ C3W (N=5)	48.55 ± 4.50	0.120	0.091
MNU+s-NaCl+ C17W (N=6)	46.43 ± 4.42#	0.421	0.012
MNU+s-NaCl+ C20W (N=6)	46.38 ± 3.14#	0.432	0.011

** represented significant difference compare with CO group ($p < 0.01$).

represented significant difference compare with MNU+s-NaCl group ($p < 0.05$).

represented significant difference compare with MNU+s-NaCl group ($p < 0.01$).

One-way ANOVA and LSD post hoc test

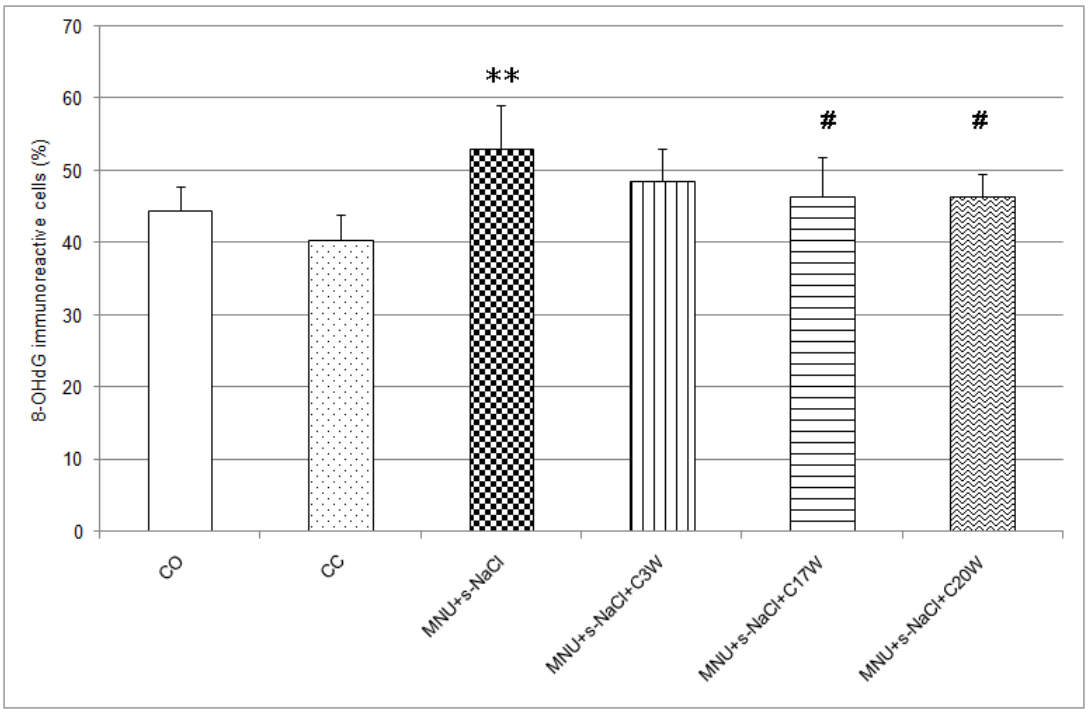


Figure 4-19: A bar graph showed the mean ± SD of 8-OHdG expression (%) in experimental groups. The sign ** represented significant difference compared with CO group ($p < 0.01$). The sign # represented significant difference compare with MNU+s-NaCl group ($p < 0.05$). One-way ANOVA and LSD post hoc test.

Table 4-5: Means ± SD of 8-OHdG immunoreactive cells (%) in all groups and subgroups.

Parameter/ Group	CO (N=6)	CC (N=6)	MNU+s-NaCl (N=6)	MNU+s-NaCl+C3W (N=5)		MNU+s-NaCl+C17W (N=6)	MNU+s-NaCl+C20W (N=6)	
				C3W benign (N=2)	C3W cancer (N=3)		C20W benign (N=3)	C20W cancer (N=3)
8-OHdG immunoreactive cells (%)	44.42±3.41	40.39±3.53	53.06±5.96	49.28±7.43	48.06±3.47	46.43±4.42	44.99±3.51	47.77±2.55

Effects of MNU and Saturated NaCl Induction on Cyclin D1 Expression and Roles of Curcumin

Cyclin D1 expression was studied by using immunohistochemical technique and shown as nuclei-stained cells (figure 4-20). Cyclin D1 was expressed primarily in basal cells of the epithelium in normal mucosa (figure 4-20, A and B). This protein was upregulated in the mucosa of MNU+s-NaCl (figure 4-20C). Expressions of cyclin D1 in MNU+s-NaCl+C3W, MNU+s-NaCl+C17W, and MNU+s-NaCl+C20W were shown in figure 4-20D, E, and F, respectively.

The percentage of cyclin D1 immunoreactive cells was not significantly different between CO and CC. The percentage was significantly increased in MNU+s-NaCl compared with CO. Curcumin supplementations for 3 weeks during MNU+s-NaCl induction and the late treatment of curcumin did not statistically reduce cyclin D1 expression when compared with MNU+s-NaCl. The average percentage of cyclin D1 positive cells in MNU+s-NaCl+C20W tended to reduce when compared with MNU+s-NaCl. The average percentages of immunoreactive cells of all experimental groups were shown in the table 4-6 and figure 4-21.

The average percentage of cyclin D1 stained cells in MNU+s-NaCl+C3W and MNU+s-NaCl+C20W groups were also divided into subgroups: C3W benign group (N = 2), C3W cancer group (N = 3), C20W benign group (N = 3), and C20W cancer group (N = 3). The mean percentages of cyclin D1 immunoreactive cells in all groups and subgroups were shown in the table 4-7.

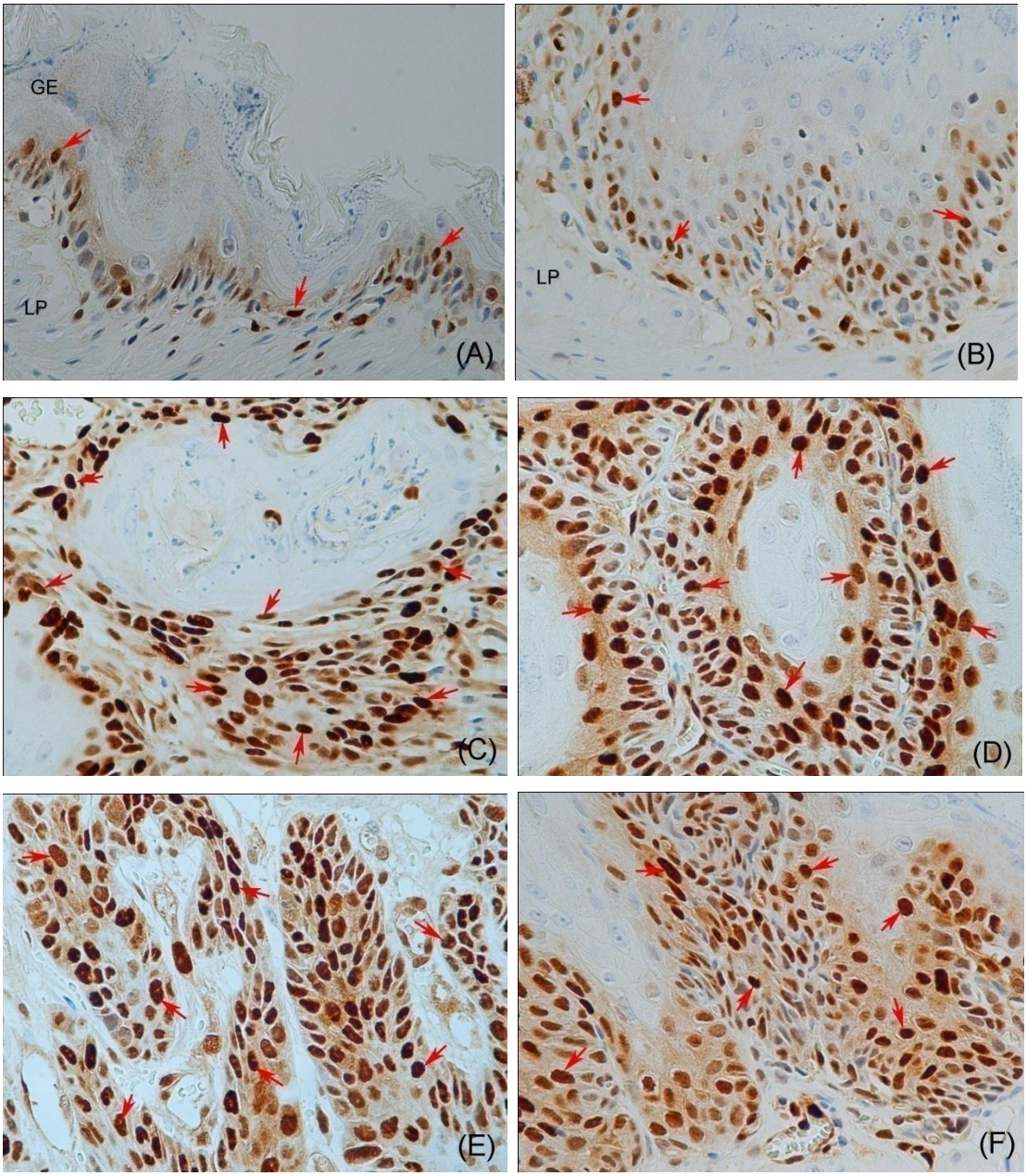


Figure 4-20: Immunohistochemical staining of cyclin D1 antibody in representative groups: CO (A), CC (B), MNU+s-NaCl (C), MNU+s-NaCl+C3W (D), MNU+s-NaCl+C17W (E), and MNU+s-NaCl+C20W (F). DAB stained immunoreactive cells (dark brown, arrows). Nuclear counterstaining was performed with Hematoxylin II and Bluing reagent (×400). GE: gastric epithelium and LP: lamina propria.

Table 4-6: Means ± SD of cyclin D1 immunoreactive cells (%) in experimental groups.

Group	Immunoreactive cells (%)	*P* value (compared with CO)	*P* value (compared with MNU+s-NaCl)
CO (N=6)	38.58 ± 5.37$^{\#\#}$		0.000
CC (N=6)	42.53 ± 6.90$^{\#\#}$	0.311	0.003
MNU+s-NaCl (N=6)	54.91 ± 7.93**	0.000	
MNU+s-NaCl+ C3W (N=5)	54.69 ± 2.83**	0.000	0.957
MNU+s-NaCl+ C17W (N=6)	56.86 ± 4.72**	0.000	0.613
MNU+s-NaCl+ C20W (N=6)	49.57 ± 9.27**	0.008	0.173

** represented significant difference compare with CO group ($p < 0.01$).

$^{\#\#}$ represented significant difference compare with MNU+s-NaCl group ($p < 0.01$).

One-way ANOVA and LSD post hoc test

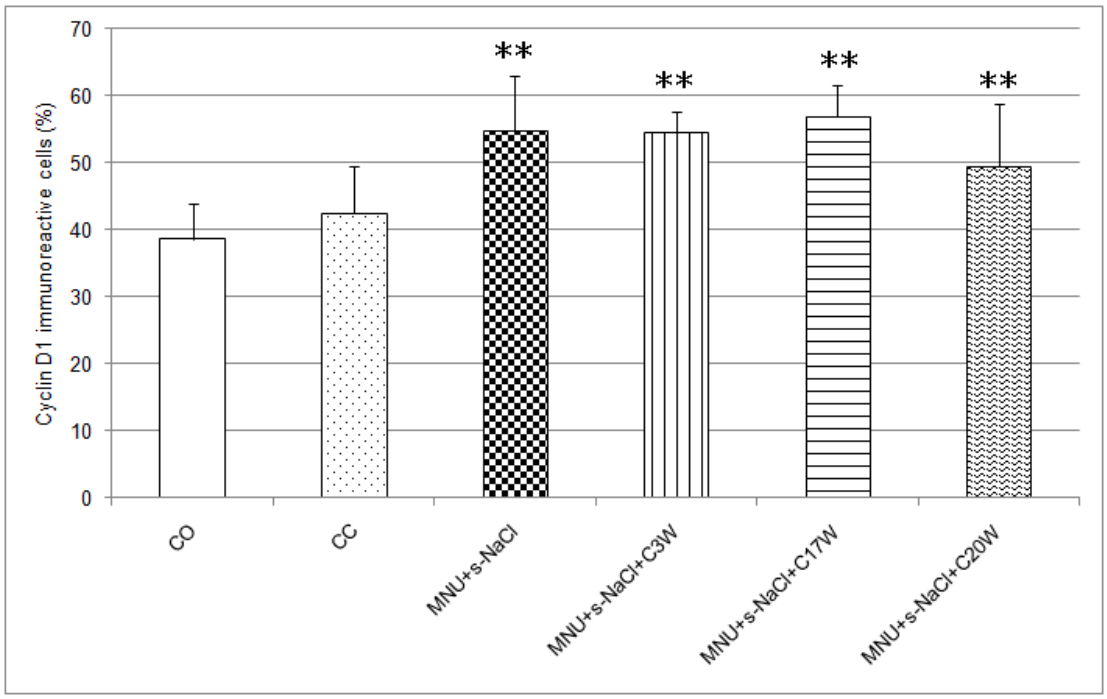


Figure 4-21: A bar graph showed the mean ± SD of cyclin D1 expression (%) in experimental groups. The sign ** represented significant difference compared with CO group ($p < 0.01$). One-way ANOVA and LSD post hoc test.

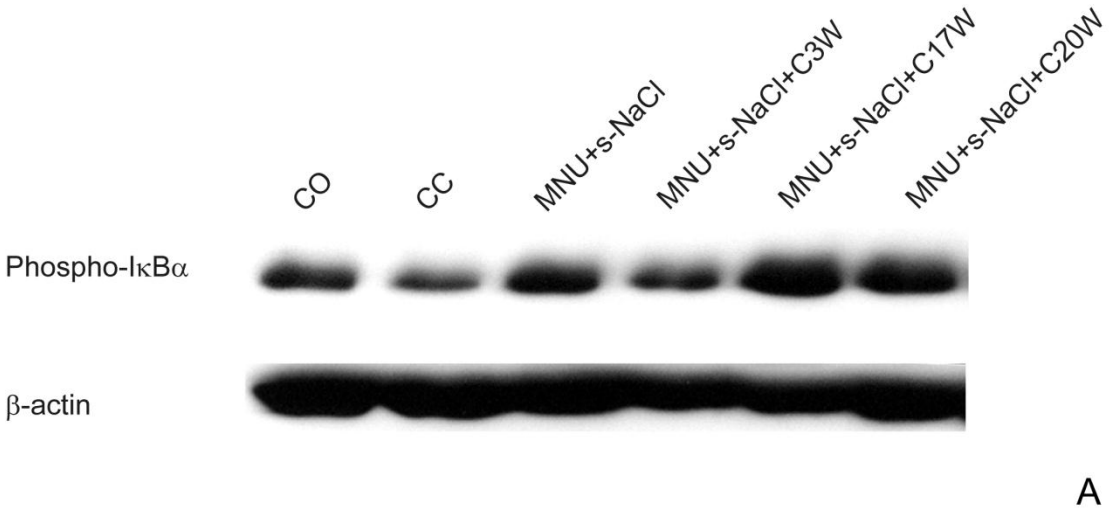
Table 4-7: Means ± SD of cyclin D1 immunoreactive cells (%) in all groups and subgroups.

Parameter/ Group	CO (N=6)	CC (N=6)	MNU+s-NaCl (N=6)	MNU+s-NaCl+C3W (N=5)		MNU+s-NaCl+C17W (N=6)	MNU+s-NaCl+C20W (N=6)	
				C3W benign (N=2)	C3W cancer (N=3)		C20W benign (N=3)	C20W cancer (N=3)
Cyclin D1 immuno-reactive cells (%)	38.58±5.37	42.53±6.90	54.91±7.93	54.33±1.27	54.92±3.87	56.86±4.72	45.31±6.29	53.82±11.01

Effects of MNU and Saturated NaCl Induction on Phospho-IκBα Expression and Roles of Curcumin

Phospho-IκBα Ser32/36 expression was determined by western blot technique. The expression of phosphorylated IκBα was normalized by β-actin expression of each rat. The immunoblot and the representative ratio of phosphorylated IκBα (~50 kDa) and β-actin (~42 kDa) expressions were shown in figure 4-22A. The results showed that the mean expression of phosphorylated IκBα were not significantly different between CO and CC. When compared with CO group, expression of phosphorylated IκBα in MNU+s-NaCl increased but not reached the statistic significance (p = 0.053). Curcumin supplementation for the first three weeks (MNU+s-NaCl+C3W) tended to decline the mean relative intensity of phosphorylated IκBα compared with MNU+s-NaCl (p = 0.079). The averages of relative intensity ratio of phosphorylated IκBα of all experimental groups were shown in the figure 4-22B and table 4-8.

We further analyzed effects of curcumin on cancer development. MNU+s-NaCl+C3W and MNU+s-NaCl+C20W groups were divided into subgroup C3W benign group (N = 2), C3W cancer group (N = 3), C20W benign group (N = 3), and C20W cancer group (N = 3). The averages of relative intensity ratio of phospho-IκBα in all groups and subgroups were displayed in the following table 4-9.



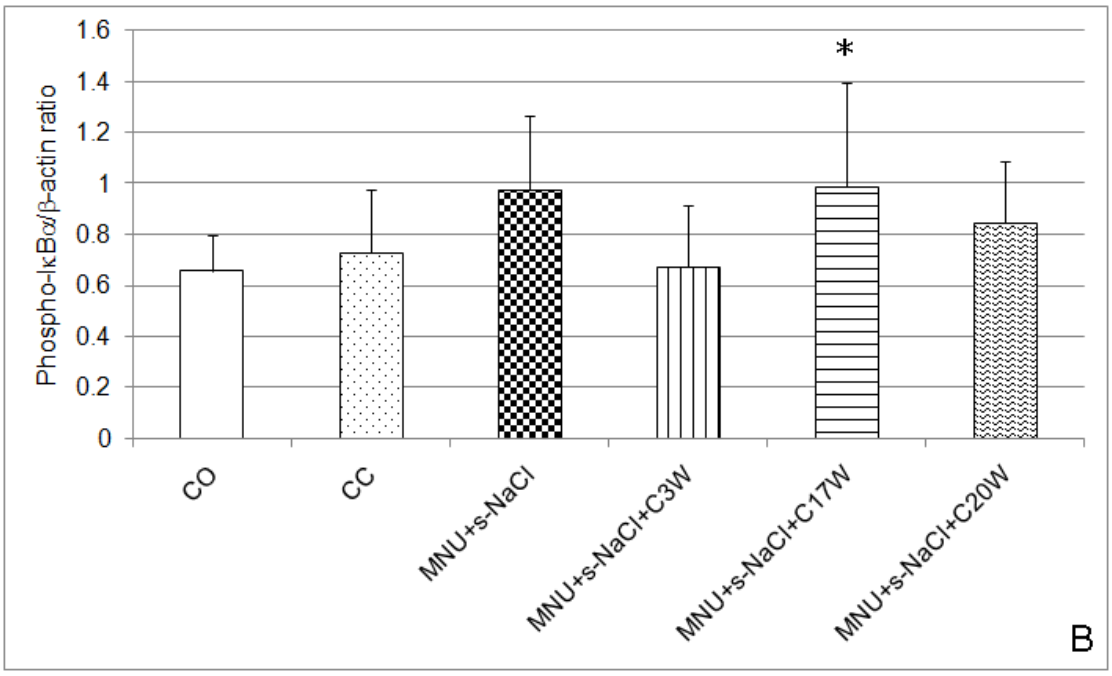


Figure 4-22: Representative blot of phospho-IκBα Ser32/36 and β-actin (A). A bar graph showed the mean ± SD of intensity ratio of phospho-IκBα Ser32/36 to β-actin in all groups (B). The sign * represented significant difference compared with CO group ($p < 0.05$). One-way ANOVA and LSD post hoc test.

Table 4-8: Means ± SD of intensity ratio of phospho-IκBα Ser32/36 to β-actin in all groups.

Group	Intensity ratio of phospho-IκBα Ser32/36 to β-actin	*P* value (compared with CO)	*P* value (compared with MNU+s-NaCl)
CO (N=6)	0.66 ± 0.14#		0.053
CC (N=6)	0.73 ± 0.25	0.664	0.125
MNU+s-NaCl (N=6)	0.98 ± 0.29	0.053	
MNU+s-NaCl+ C3W (N=5)	0.68 ± 0.24	0.920	0.079
MNU+s-NaCl+ C17W (N=6)	0.99 ± 0.41*	0.044	0.929
MNU+s-NaCl+ C20W (N=6)	0.85 ± 0.24	0.241	0.418

* represented significant difference compare with CO group ($p < 0.05$).

represented significant difference compare with MNU+s-NaCl group ($p < 0.05$).

One-way ANOVA and LSD post hoc test

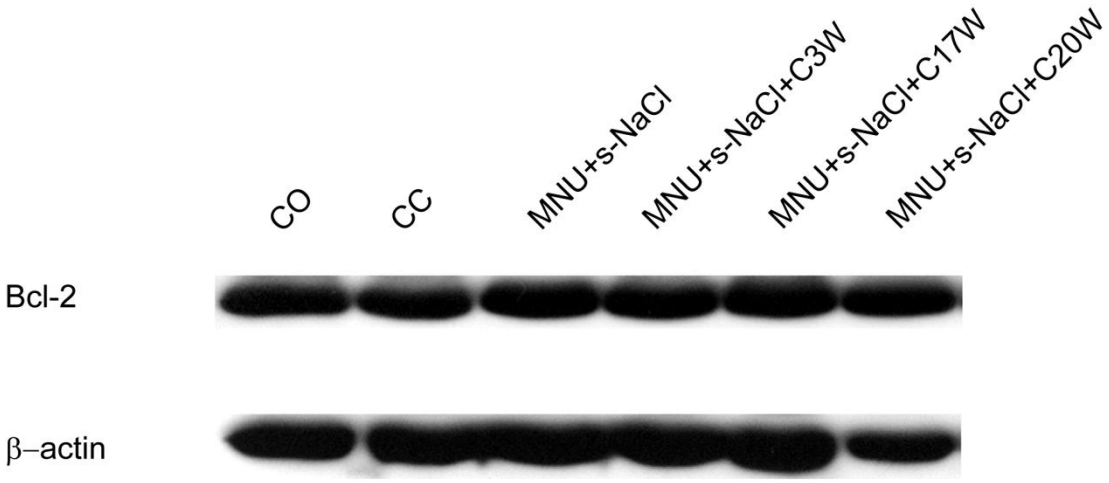
Table 4-9: Means ± SD of intensity ratio of phospho-IκBα Ser32/36 to β-actin in all groups and subgroups.

Parameter/Group	CO (N=6)	CC (N=6)	MNU+s-NaCl (N=6)	MNU+s-NaCl+C3W (N=5)		MNU+s-NaCl+C17W (N=6)	MNU+s-NaCl+C20W (N=6)	
				C3W benign (N=2)	C3W cancer (N=3)		C20W benign (N=3)	C20W cancer (N=3)
Phospho-IκBα/β-actin ratio	0.66±0.14	0.73±0.25	0.98±0.29	0.61±0.18	0.72±0.31	0.99±0.41	0.64±0.04	1.05±0.12

Effects of MNU and Saturated NaCl Induction on Bcl-2 Expression and Roles of Curcumin

Bcl-2 expression was determined by western blot technique. The relative ratio of Bcl-2 was normalized by β-actin expression of each rat. The immunoblot and the representative ratio of Bcl-2 (~26 kDa) and β-actin (~42 kDa) expressions were shown in figure 4-23A. The results showed that expressions of Bcl-2 were not significantly different between CO and CC. Bcl-2 expression in MNU+s-NaCl significantly increased compared with CO. Curcumin supplemented groups did not show the statistic significance when compared with MNU+s-NaCl. The averages of relative intensity ratio of Bcl-2 of all experimental groups were shown in the figure 4-23B and table 4-10.

We further analyzed effects of curcumin on cancer development. MNU+s-NaCl+C3W and MNU+s-NaCl+C20W groups were divided into subgroup C3W benign group (N = 2), C3W cancer group (N =3), C20W benign group (N = 3), and C20W cancer group (N = 3). The averages of relative intensity ratio of Bcl-2 of all groups and subgroups were shown in the table 4-11.



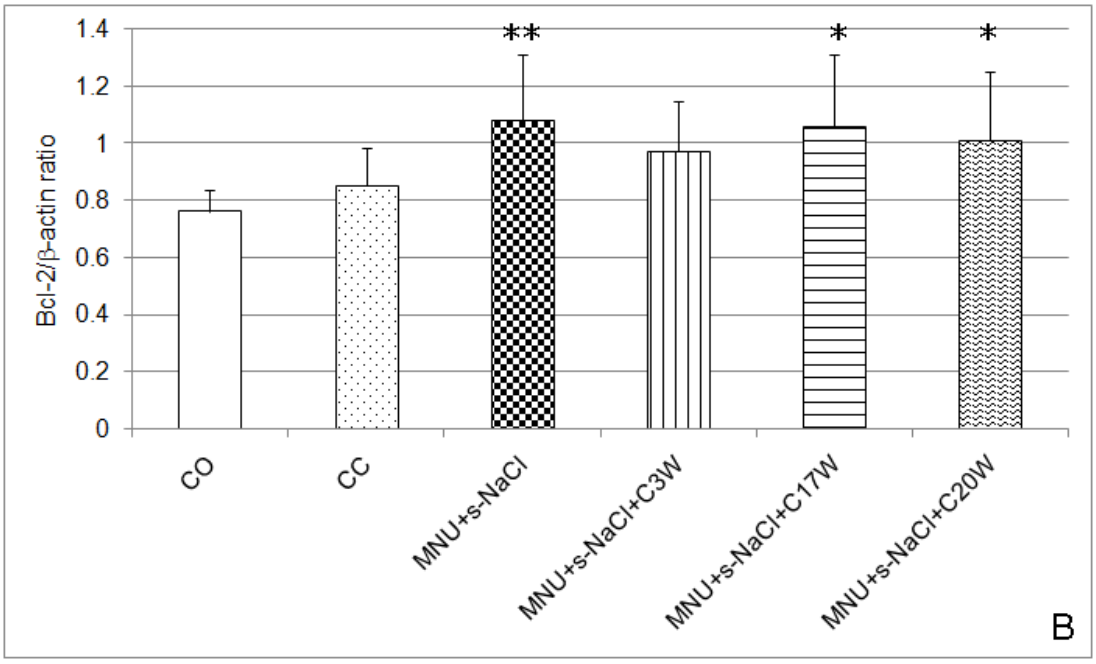


Figure 4-23: Representative blot of Bcl-2 and β-actin (A). A bar graph showed the mean ± SD of intensity ratio of Bcl-2 to β-actin in all groups (B). The sign * represented significant difference compared with CO group ($p < 0.05$). The sign ** represented significant difference compared with CO group ($p < 0.01$). One-way ANOVA and LSD post hoc test.

Table 4-10: Means ± SD of intensity ratio of Bcl-2 to β-actin in all groups.

Group	Intensity ratio of Bcl-2 to β-actin	*P* value (compared with CO)	*P* value (compared with MNU+s-NaCl)
CO (N=6)	0.76 ± 0.07##		0.008
CC (N=6)	0.85 ± 0.13	0.431	0.051
MNU+s-NaCl (N=6)	1.08 ± 0.23**	0.008	
MNU+s-NaCl+ C3W (N=5)	0.97 ± 0.17	0.083	0.370
MNU+s-NaCl+ C17W (N=6)	1.06 ± 0.25*	0.013	0.842
MNU+s-NaCl+ C20W (N=6)	1.01 ± 0.24*	0.035	0.536

* represented significant difference compare with CO group ($p < 0.05$).

** represented significant difference compare with CO group ($p < 0.05$).

represented significant difference compare with MNU+s-NaCl group ($p < 0.01$).

One-way ANOVA and LSD post hoc test

Table 4-11: Means ± SD of intensity ratio of Bcl-2 to β-actin in all groups and subgroups.

Parameter/Group	CO (N=6)	CC (N=6)	MNU+s-NaCl (N=6)	MNU+s-NaCl+C3W (N=5)		MNU+s-NaCl+C17W (N=6)	MNU+s-NaCl+C20W (N=6)	
				C3W benign (N=2)	C3W cancer (N=3)		C20W benign (N=3)	C20W cancer (N=3)
Bcl-2/β-actin ratio	0.76±0.07	0.85±0.13	1.08±0.23	1.12±0.15	0.88±0.12	1.06±0.25	0.86±0.09	1.16±0.26

CHAPTER V

DISCUSSION AND CONCLUSIONS

Gastric cancer is a multifactorial disease. Risk factors, including high salt diet and *N*-nitroso carcinogens, are involved in the pathogenesis of gastric cancer. Our preliminary study can establish rat model of gastric cancer using *N*-methyl-*N*-nitrosourea (MNU) and saturated NaCl (s-NaCl). In this study, the experiments were conducted to examine the effects of curcumin supplementations on incidence of gastric cancer. Curcumin was supplemented to cancer-induced rats at different time of experimental period: during the first 3 weeks of MNU and s-NaCl administrations, after administration of MNU and s-NaCl, and entire period of experiment. In addition, the effects of curcumin on the molecular markers of carcinogenesis, such as oxidative DNA damage and cell proliferation were investigated.

Rat Models of Gastric Cancer

Animal models on pathological and biological aspects of gastric cancer have been established to determine the potential risk factors, prevention, and treatment options. From our preliminary study, we demonstrated that intragastric MNU administration with s-NaCl induced squamous cell carcinoma (SCC) of forestomach. At week 20th, the incidence of forestomach SCC is 100% (table 4-2). In addition, we also showed 100% of gastric cancer incidence in rats induced by the same protocol (in MNU+s-NaCl group, table 4-3).

The target organ of MNU is not specificity depending on the route of administration. Intraperitoneally injection of 50 mg/kg MNU in female Sprague-Dawley rats provided the model of MNU-induced mammary tumors at week 5th [160, 161]. In gastric cancer models, exposure of the stomach to the carcinogen MNU induces carcinogenesis of stomach. After 42 weeks, both forestomach SCC and adenocarcinoma of the glandular stomach were induced in F344 rats treated with MNU 100 ppm in drinking water [50]. Administration of MNU 400 ppm in drinking water for 12 weeks induced gastric cancer in total 20 rats, at week 36th [162]. Chronic

oral administration of MNU (5 mg/kg/day/rat, totally 435 mg/kg/rat) in Wistar R rats resulted in 27 SCC of forestomach and 2 adenocarcinomas out of 53 rats [163]. Heterozygous p53+/- knockout mice were orally administrated of MNU (90 mg/kg dissolved in citrate-buffered saline saline pH 4.5). At week 13th, this model exhibited SCC in the non-glandular stomach and leiomyosarcoma (cancer of smooth muscle) in the glandular stomach [157]. Sugimura and Fujimura [49] reported that adenocarcinomas were in the glandular stomachs of rats treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). MNU and MNNG are genotoxic carcinogens. MNU is known as an alkylating agent and reacting with various nucleophiles, especially amines [54]. Alkylation of MNU is occurred after non-enzymatic hydrolysis and chemical rearrangements to the methyldiazonium ion, whereas the decomposition of MNNG is enhanced by thiol compounds (reduced glutathione and L-cysteine), which is found at high concentration in the glandular stomach [164-165]. However, MNNG is not available now. MNU is available used in other rodent models of gastric cancer. The glandular stomach of mice has generally been found to be relatively resistant to MNNG action. MNU was also used for gastric cancer induction in Mongolian gerbils [166]. MNU is stable under acidic condition. Hence, MNU is relatively stable in animal stomach and induce the greater incidence of SCC.

According to studies in human and report from a joint World Health Organization/Food and Agriculture Organization Expert Consultation, salt and salt-preserved food poses a possible risk to gastric carcinogenesis [38, 167]. The model in this study also used s-NaCl for promoting gastric carcinogenesis. From our previous study, SCC tended to develop earlier in rats receiving both MNU and s-NaCl than the one received MNU alone [156]. Some animal studies also demonstrated the same results. Tatematsu, *et al.* [7] found that NaCl increased occurrence of the glandular stomach cancer in rats treated with MNNG and 4-NQO. Takahashi, *et al.* [168] illustrated that NaCl could promote gastric carcinogenesis in a dose-dependent manner. Thus, the rat model of gastric cancer is developed by risk factors that mimic those in human. In this study, we used this rat model to examine the efficacy of curcumin supplementations on treatment options and the key proteins involved in the carcinogenesis.

Changes of Body Weight

The body weight may change in some pathogenic conditions. Cancers may alter this parameter, so we measured the body weight every week. From general observation, the experimental rats had the body weight gain in every week. In this study, we found that there was no significant difference in all groups ($p > 0.05$, figure 4-11). We speculated that the administrations of all chemicals and cancer-bearing did not affect the rats' body weight gain. Previous study supported that body weight gain in 80 mg/kg and 160 mg/ kg MNU-injected groups during the experiment (20 weeks) did not differ significantly from the control group [169]. In addition, curcumin supplementation did not affect the rats' body weight gain. This result was in aggrement with the study of Rao, *et al*. [170]. Rats were supplemented with 0.5% curcumin in diet for 7 weeks. There was no difference in body weight gain between rats fed the control diet and the curcumin-supplemented diet. Moreover, Kawamori *et al.* [127] demonstrated that the body weights of rats received the diet containing 0.6% curcumin for 58 weeks were comparable to those received the control diet. Rats supplemented with 0.2 or 0.6% curcumin in diet for 36 weeks after carcinogen-induced colonic cancer were also comparable to those supplemented with control diet after carcinogen-induced colonic cancer.

Effects of Curcumin on Histopathological Changes of Gastric Carcinogenesis and Incidence of Gastric Cancer

The histological results in this study (table 4-3) showed that cancers did not occur in CO and CC groups. Macroscopic view (figure 4-15A) and microscopic view (figure 4-16) of CO exhibited the normal gastric mucosa of forestomach, lining with squamous epithelial cells. There is no abnormal finding throughout the stomach. The rats receiving oral gavage of MNU and high salt exhibited squamous cell carcinoma (SCC) in the forestomach. From the macroscopic view (figure 4-15B), SCC was shown as multiple polypoid tumors scattering in the forestomach. The histopathology of SCC showed invasion of cancerous tissue through the deep layers of the stomach wall, such as submucosa, muscle layer, and serosa (figure 4-17). Oral gavage of MNU and high salt in MNU+s-NaCl induced a 100% cancer incidence in rats. Curcumin

supplementations could attenuate the gastric carcinogenesis induced by MNU and s-NaCl in rats. Curcumin supplementations during the first 3 weeks of chemical-induced cancer in MNU+s-NaCl+C3W and MNU+s-NaCl+C20W diminish the incidence of cancer. There were 3/5 (60%) rats in MNU+s-NaCl+C3W and 3/6 (50%) rats in MNU+s-NaCl+C20W developed SCC. However, curcumin supplementation after chemical-induced cancer in MNU+s-NaCl+C17W group did not reduce the cancer incidence (table 4-3).

Administrations of curcumin in MNU+s-NaCl+C3W and MNU+s-NaCl+C20W groups showed 60% and 50% cancer incidence, respectively. These observations indicated that early administration of curcumin (during the first 3 weeks of cancer induction) could prevent the initiation of carcinogenesis and reduce the incidence of cancer. This is in agreement with the previous studies. Feeding 0.5 and 2.0% of commercial grade curcumin in the diet during the initiation period (2 weeks before, during, and for 1 week after benzo(a)pyrene (BaP) administration) reduced the number of mice with forestomach tumors [171]. In addition, 1 and 2 g/mL radix curcuma extract solution daily feeding during MNNG administration for 40 weeks also showed the reduction of tumor incidences in 10% NaCl and MNNG-induced gastric cancer in rats [130]. According to our result, the late phase of curcumin supplementation (MNU+s-NaCl+C17W) did not reduce cancer incidence. It emphasized that curcumin could suppress the cancer incidence during the initiation period but not the post-initiation period. During initiation period, curcumin might prevent MNU-induced mutagenicity. Alkylation of MNU is occurred after non-enzymatic hydrolysis and chemical rearrangements to the methyldiazonium ion. The antimutagenic activity of curcumin on the direct mutagen MNU has been reported. Curcumin at a concentration of 300 μg/plate showed a protective effect against mutagenicity of MNU at a concentration of 10 μg/plate in the Ames test with *Salmonella typhimurium* TA100 strains [172]. This activity of curcumin could operate before MNU-induced DNA damage. Previous study provided evidence that phenolic compounds such as ellagic acid probably inhibited mutagenesis of MNU by blocking of nucleophilic centers in DNA [173]. In addition to an inhibitory effect on the initiation stage, the action of curcumin could affect on proliferative and invasive stages of cancer.

In SCC, we also demonstrated the invasions of cancerous tissues (table 4-3). The cancer invasions of MNU+s-NaCl were occurred in submucosa, muscle layer, and serosa. We did not determine tumor volume that could reflect the tumor growth. However, the invasiveness of cancerous tissues may suggest the proliferation stage and growth of cancerous tissues. The previous study suggested that tumor volume was significantly associated with tumor invasiveness in adjacent tissues [174]. Moreover, the development and growth of cancer require the proliferative and invasive characters. Cancer cells facilitate adaptation of signaling pathways and maintain growth and survival potential. From *in vitro* model, application of growth factor, such as hepatocyte growth factor/scatter factor (HGF/SF) resulted in cooperation of Myc and MAPK signaling pathways to confer both proliferative and invasive phenotypes of glioblastoma cells [175]. The expanded experiment of this study [175] indicated that highly proliferative and invasive cells promoted tumor growth in the xenografts.

The invasiveness could be reduced by curcumin supplementations. There was no cancer-bearing rat with serosa invasion in MNU+s-NaCl+C3W and MNU+s-NaCl+C20W groups. Moreover, there was no cancer-bearing rat with muscular invasion in MNU+s-NaCl+C20W. Although there was a rat exhibited stomach perforation in MNU+s-NaCl+C17W, most of them showed submucosa invasion. Many studies demonstrated that curcumin reduced growth and invasion of cancer cells. In human chronic myelogenic leukemia cells, K-562, curcumin (20 μg/mL) displayed 50% growth arrest [176]. Curcumin (40 μmol/L) reduced both viability and invasion of esophageal cancer cells (TE-8 and SKGT-4) [177]. In the case of xenograft model, head and neck squamous cell carcinoma (HNSCC), CAL27, were injected in the right flank of nude mice. Intravenous administration of liposomal curcumin (50 mg/kg, four times per week for 3.5 weeks) suppressed tumor volume [178].

Effects of MNU and Saturated NaCl Induction on 8-OHdG Expression and Roles of Curcumin

Oxidative stress has an essential role in carcinogenesis and cancer progression, because oxidative damage permanently occurs to lipids of cellular membranes, proteins, and DNA. The interaction of reactive oxygen species (ROS) with the

nucleobases of the DNA strand, such as guanine, leads to the formation of "8-hydroxy-2-deoxyguanosine (8-OHdG)" and structural alteration in DNA. DNA damage and inadequate repair have also been linked to mutation and carcinogenesis. 8-OHdG has been a potential biomarker of oxidative DNA damage and a factor of initiation and promotion of carcinogenesis [16]. In this study, we detect 8-OHdG expression in epithelial cells by immunohistochemical study. Then, the slide was counterstained with Hematoxylin II for nuclear detection. Thousand of gastric epithelial cells were counted for each rat to indicate a percentage of 8-OHdG expression.

As shown in the figure 3-8A, the 8-OHdG immunoreactive cells were dark brown-stained in the nuclei. In normal mucosa of CO (figure 4-18A), 8-OHdG was expressed in basal cells of the forestomach epithelium. In figure 4-18C, 8-OHdG positive cells increased in MNU+s-NaCl. The table 4-4 and figure 4-19 showed that 8-OHdG expression was significantly increased in MNU+s-NaCl (53.06% ± 5.96) compared with CO (44.42% ± 3.41). These data demonstrated that administration of MNU and s-NaCl led to stomach carcinogenesis with the significant increase of 8-OHdG expression. MNU+s-NaCl caused DNA damage that led to enhance of 8-OHdG expression. Normally, there are variations of steady-state cellular background level of 8-OHdG between cells types and animal organs [179]. 8-OHdG was detected in the glandular stomach and intestinal epithelia in male Fisher 344 rats. Glandular cells and cells present in the bottom of crypts of the stomach were stained positive [180]. If the endogenous defense systems do not properly repair the damage, these adduct and chain breaks may lead to oxidative DNA damage-induced mutation resulting in carcinogenesis.

In addition, reactive oxygen species (ROS) promoted growth of normal cells [181] and increased proliferative index of colonic crypts in colon cancer patients [182]. Some cancer cells, such as medulla thyroid carcinoma cells [183], human pancreatic carcinoma cell line (AsPC 1), and human breast carcinoma cell line (CAMA-I) [184] produced extensive amounts of ROS. Excess of ROS can adduct DNA and induce the production of 8-OHdG. Our observation confirmed many previous results obtained from various types of cancer in patients. Serum 8-OHdG of patients with colorectal cancer was significantly increased compared with the control group [185]. A recent

study found that levels of 8-OHdG was an important risk factor for hepatocellular carcinoma, particularly in patients with “hepatitis C virus infection” [186]. Salivary analysis of 8-OHdG level in patients with “oral squamous cell carcinoma” showed an increase of 65% compared with controls [187]. In gastric cancer patients, 8-OHdG positive cells were observed in the cancerous lesions using immunohistochemical technique [188]. In animal models, previous studies demonstrated that serum 8-OHdG level significantly increased in Mongolian gerbils infected with *Helicobacter pylori* and MNU administration-induced gastric cancer [189-190]. Considering the number of reports suggesting a close relation between 8-OHdG formation and carcinogenicity, as well as this study, the expression of 8-OHdG could participate in the development of cancer.

In this study, control rats were treated with 200 mg/kg curcumin. By the safety of curcumin, CC group showed no change of mean percentage of 8-OHdG immunoreactive cells compared with the control group (figure 4-18, table 4-4, and figure 4-19). 200 mg/kg curcumin supplementations for 17 and 20 weeks in MNU+s-NaCl+C17W and MNU+s-NaCl+C20W diminished 8-OHdG expression significantly compared with MNU+s-NaCl (figure 4-18, table 4-4, and figure 4-19). This finding is consistent with the previous report that dietary curcumin supplementation suppressed such an increase in 8-OHdG formations in ferric nitrilotriacetate (Fe-NTA)-mediated renal DNA damage [191]. In precancerous lesion of oral SCC patients, levels of malonaldehyde (MDA) and 8-OHdG in serum and salivary were decreased after intake of 1 g caplets of curcumin (900 mg curcumin, 80 mg desmethoxycurcumin, and 20 mg bismethoxycurcumin; from Sabinsa Corperation, NJ, USA; for 1 week) as compared to the pretreated levels [192].

Protective effect of curcumin could attenuate cancer incidence in MNU+s-NaCl+C3W according to the previous description [172]. However, 200 mg/kg curcumin supplementation for the first 3 weeks during MNU and s-NaCl administrations did not reduce 8-OHdG expression. It could be the problem of curcumin bioavailability. Curcumin showed poor absorption, high elimination from the body, and rapid metabolism. After oral administration of 400 mg curcumin to rats, no curcumin was found in heart blood. In the portal blood, a trace amount (less than 5 μg/mL) was found from15 minutes to 24 hours [193]. After 45 minutes of curcumin administration

(1 g/kg, orally) to rats, maximum level of serum curcumin (0.5 μg/mL) was determined [194]. Once absorbed, curcumin is metabolized in liver [195]. The elimination half-life values for oral administration (500 mg/kg) curcumin in rats was 44.5 ± 7.5 hours [196].

Curcumin supplementation after cancer induction (MNU+s-NaCl+C17W) significantly decreased 8-OHdG expression. Although cancer incidence in this group was not reduced, invasiveness of cancer could be diminished by curcumin supplementation. Most of SCC in this group invaded to submucosa. Only one rat showed stomach perforation (table 4-3). In MNU+s-NaCl+C20W, early treatment of curcumin reduced cancer incidence and showed a significant decrease of 8-OHdG immunoreactive cells. Moreover, in this group, there was no SCC invasion to muscle layer and serosa (table 4-3). According to previous explaination, some cancer produced ROS, which can promote growth of cancer cells [182]. Curcumin is a potent scavenger of superoxide anion, hydroxyl radical, singlet oxygen, nitric oxide and peroxynitrite [197]. Curcumin inhibited free radical-mediated DNA damage by physical quenching or chemical reaction with ROS [133]. They also demonstrated that aromatic substituents and a styryl ketone motiety were essential for preventing singlet oxygen-induced DNA damage. Curcumin may also provide additional protection against ROS by upregulation of antioxidant factors, especially reduced glutathione (GSH) and superoxide dismutase (SOD) [198]. From the above-mentioned reviews, curcumin acts as an antioxidant agent. Our data suggested that curcumin administration could protect carcinogenesis and cancer invasion against oxidative DNA damage.

Effects of MNU and Saturated NaCl Induction on Cyclin D1 Expression and Roles of Curcumin

In cell cycle regulation, a primary target of growth factor signaling is the cyclin D1-dependent kinase (D1-CDK4/6) whose activity promotes G1 phase progression. Cyclin D1 is the key protein for cell proliferation and carcinogenesis. Overexpression of G1-cell control protein, cyclin D1, has been associated with cancer development and malignancy. In this study, we detect cyclin D1 expression in epithelial cells by immunohistochemical study. The slide was counterstained with

Hematoxylin II for nuclear detection. In addition, thousand of gastric epithelial cells were counted for each rat to indicate a percentage of cyclin D1 expression.

As shown in the figure 3-8B and 4-20, the cyclin D1 immunoreactive cells is dark brown-stained in the nuclei. In normal mucosa of CO (figure 4-20A), cyclin D1 was limited to the basal cells of the forestomach epithelium. In figure 4-20C, cyclin D1 positive cells increased in MNU+s-NaCl induced SCC. The table 4-6 and figure 4-21 showed that cyclin D1 expression was significantly increased in MNU+s-NaCl (54.91% ± 7.93) compared with CO (38.58% ± 5.37). These data suggested that MNU+s-NaCl induced SCC in the forestomach with the statistic increase of cyclin D1 expression. Normally, cyclin D1 is known as a critical element in cell cycle regulation. Once it is induced, cyclin D1 interacts with CDK4 and CDK6 to make the protein binding complexes. These complexes phosphorylates pRb and drives the G1 phase [20]. As a very important regulator of G1 re-entry and progression, the turnover of cyclin D1 is tightly regulated by phosphorylation of a specific "threonine residue (Thr-286) located near its C-terminus, by Glycogen Synthase Kinase-3β (GSK-3β)" [103]. Cell cycle regulators, particularly G1 phase progression, are simple targets during carcinogenesis because this phase provides cells for growth advantage.

Our findings supported evidences that overexpression and nuclear accumulation of cyclin D1 are essential for the indication of its oncogenicity. Mouse models of mammary carcinoma where cyclin D1 deficiency were resistant to mammary tumors induced by either the *neu* or *ras* oncogenes. Markedly, the $D1^{-/-}$ mice were not resistant to the oncogenic effects of *myc* or *wnt* demonstrating that the coupling of oncogenic pathways to the cell cycle mechanism was highly specific [199]. In addition, mice, which endogenous CDK4 was deleted via homologous recombination also reported resistance to mammary tumor genesis [200-201]. Overexpression of cyclin D1 has also been associated with the development of endocrine resistance in breast cancer cells [202-203]. Cyclin D1 is a marker for the development and progression of many cancers including those of the pancreatic tumor cell lines [204], mouse model of hepatocellular carcinomas [205], patients with oral SCC [206], and gastric cancer [104].

The increased expression of cyclin D1 protein could be an early event in cancer development, and it tended to maintain stable throughout the progression of

invasion and metastasis. Previous studies showed that the positive expression of cyclin D1 was statistically associated with well-differentiated tumors, and was more prominent in early-stage tumors than in malignancy [207-208]. Cyclin D1 overexpression from gene amplification is a critical genetic alteration in esophageal squamous cell carcinoma [209-210]. Fong, *et al*., [211] showed that *N*-nitrosomethyl-benzylamine (NMBA)-induced tumorigenesis in cyclin D1 overexpression transgenic mice, together with dietary zinc deficiency (ZD), significantly increased incidence of forestomach tumor compared with wild-type mice. Cyclin D1 protein was positively expressed in gastric carcinoma patients. The amplification of cyclin D1 gene and overexpression of cyclin D1 mRNA were detected [104]. In addition, overexpression of cyclin D1 also results from defective regulation of cyclin D1 at the post-translational level [212-213].

The 200 mg/kg curcumin supplementation on control rats (CC) did not change cyclin D1 expression compared with the control group (CO). 200 mg/kg curcumin supplementation for the first 3 weeks during MNU and s-NaCl administrations did not reduce cyclin D1 expression (table 4-6 and figure 4-21). These results suggested that curcumin supplemetation during the induction of cancer did not showed the improvement of cyclin D1 expression. This design of curcumin supplementation was different from the study of anti-cancer strategy of curcumin. In the both of breast and prostate cancer lines, curcumin suppressed proliferation of cancer cells and certainly downregulated cyclin D1 mRNA and protein expressions [214]. However, we did not examine cyclin D1 expression at the end of the week 3rd to evaluate the protective effects of curcumin on cyclin D1 expression and carcinogenesis.

Curcumin supplementation after cancer induction (MNU+s-NaCl+C17W) did not decrease cyclin D1 expression. Moreover, curcumin supplementation in MNU+s-NaCl+C20W tended to diminish cyclin D1 expression (table 4-6 and figure 4-21). Our results in the table 4-7 also demonstrated the mean percentage of cyclin D1 positive cells in C20W benign and C20W cancer groups. These results might suggest that curcumin supplementations did not suppress cyclin D1 expression, once SCC had been developed. These results were different from the previous report. *In vivo* study, human colon cancer cells (HCT 116) were injected into the cecum of nude mice. The expression of cyclin D1, which is associated with growth and proliferation, was

downregulated by curcumin (1 g/kg, once daily, orally) administration for 4 weeks [145]. It could be result from different cancer model or doses of curcumin.

From many *in vitro* study, curcumin diminished cyclin D1 relating to cancer cell proliferation [28, 140, 215]. In head and neck squamous cells (HNCCs), curcumin suppressed cancer cell growth and cyclin D1 expression [140]. In a human colon cancer-derived cell line (Moser cells), curcumin reduced cyclin D1 expression, resulting in inhibition of cell growth [215]. Curcumin also downregulated the mRNA and the protein expression of cyclin D1 in human gastric cancer cells [28]. In this study, we did not determine which cell was the cancer cell, when we counted the cyclin D1 positive cells in forestomach tissues. The co-localization of carcinogenesis marker, such as 8-OHdG positive cell and cyclin D1 might help the clearified results. Moreover, isolation of SCC and culture the cancer cells could clearly determine the effects of curcumin on cyclin D1 expression and cancer cell growth.

Effects of MNU and Saturated NaCl Induction on Phospho-IκBα Expression and Roles of Curcumin

A range of stimuli such as ROS and cytokines activate IκB kinase (IKK) complex resulting in IκBs phosphorylation and proteolysis. Phosphorylation of IκBα elicits IκBα degradation, allowing the nuclear translocation of NF-κB. In nucleus, NF-κB proteins bind on the promoters and lead to activation of target genes, regulating physiological processes, which involve in the control of the cellular proliferation and apoptosis [89, 216]. Hyperactivation of NF-κB proteins has strong correlation with chronic inflammation, carcinogenesis, and chemotherapeutic resistance [217-218]. In NF-κB activation, one of the most important steps is the dissociation of IκB, which is mediated through phosphorylation and subsequent proteolytic degradation of IκB. Phosphorylation of IκBα could imply the activation of NF-κB [18, 79]. In this study, we examine the expressions of phosphorylated IκBα by western blot technique.

Western blotting of phosphorylated IκBα expression in all experimental groups were shown in figure 4-22A. β-actin expression was used as an internal

control. In the figure 4-22B and table 4-8, relative intensity of phospho-IκBα tend to increase in MNU+s-NaCl compared with CO (p = 0.053). Our results suggested that MNU+s-NaCl induced forestomach SCC with the tendency to increase of phospho-IκBα expression. These results also suggested the limitation of our study. For the further observation of phospho-IκBα expression, the numbers of rats should be more than 6 rats to increase the power of statistical significance. These findings might be coincided with other reports. Tropical application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on dorsal shaven area of mice induced IκBα phosphorylation and degradation. This finding was associated with increase of NF-κB DNA binding [219]. Activation of NF-κB seems to be a key step of keratinocyte transformation into SCC in mice [220]. In gastric carcinoma patients, NF-κB activation was correlate with phosphorylation and degradation of IκBα [221-222]. 49% of prostate adenocarcinoma patients showed NF-κB overexpression that correlated with advanced tumor stage [223].

The 200 mg/kg curcumin supplementation on control rats (CC) showed no alteration of phospho-IκBα expression compared with the control group (CO). The protective effect of curcumin supplementation during the first 3 weeks tended to reduce phospho-IκBα expression compared with MNU+s-NaCl (p = 0.079, table 4-8 and figure 4-22). We also showed the averages of phospho-IκBα relative intensity in C3W benign and C3W cancer subgroups in table 4-9. These results indicated that curcumin supplementations for 3 weeks had a tendency to repress phosphorylated IκBα, which may result from suppression of carcinogenesis. The tendency in our study might cause of curcumin concentration. In prostate cancer cell line, low concentration of curcumin (12.5 μmol/L) did not suppress phospho-IκBα expression in NF-κB mediated resistance of prostate cancer cell line to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), whereas high concentration (25 and 50 μmol/L) suppressed [224].

The late treatment of curcumin (MNU+s-NaCl+C17W) and early treatment of curcumin in MNU+s-NaCl+C20W did not significantly decrease phospho-IκBα expression (table 4-8 and figure 4-22). Our results in the table 4-9 also demonstrated the averages of phospho-IκBα relative intensity in C20W benign and C20W cancer

groups. These results may suggest that curcumin treatments did not suppress phospho-IκBα expression. These results were different from the previous report. Oral administration of 1 g/kg curcumin treatment displayed the antitumor effects on the xenograft model of colorectal cancer. This dose of curcumin suppressed NF-kappaB activation and NF-kappaB-regulated gene expressions, such as cyclin D1 and Bcl-2 [145]. Many studies revealed that curcumin acts on several components of NF-κB activation pathway. Curcumin was shown to suppress activation of IKK, subsequently phosphorylation and degradation of IκBα in some cancer and premalignant cell types [22, 23, 144, 225]. Hence, the effects of curcumin on the others components in NF-κB activation pathway might be further verified. In addition, curcumin acts at or above the level of IKK in the NF-κB pathway. The previous study has suggested that curcumin modulated serine/threonine protein kinase Akt, which is known to activate NF-κB by phosphorylating NF-κB subunit p65. Curcumin attenuated both Akt activation and Akt–IKK association [226]. The identification of other signalling molecules, which are modulated by curcumin, further suggests the effects of curcumin on carcinogenesis pathways.

Effects of MNU and Saturated NaCl Induction on Bcl-2 Expression and Roles of Curcumin

In the homeostasis of the gastrointestinal mucosa, apoptosis and proliferation of epithelial cells play a vital role. The BCL-2 family controls the mitochondrial pathway of apoptosis. Cancers have developed several strategies to resist apoptosis. Either overexpression of anti-apoptotic proteins or loss of pro-apoptotic molecules can be carcinogenesis. This study demonstrated the expressions of an anti-apoptotic member, Bcl-2 by western blot technique. Immunoblotting of Bcl-2 in all experimental groups were shown in figure 4-23A. In the figure 4-23B and table 4-10, relative intensity of Bcl-2 significantly increase in MNU+s-NaCl compared with CO. This result was in common with other studies suggesting that Bcl-2 overexpression could play some role in carcinogenesis. After 20 weeks of 4-Nitroquinoline 1-oxide (4NQO)-induced oral squamous cell carcinoma in rat, overexpression of bcl-2 was found in some cells of tumor islands [227]. Overexpression of Bcl-2 has been observed

in patients with severe gastric dysplasia [228] and oral SCC [229]. In small cell carcinoma of lung, expression of Bcl-2 was correlated with clinical prognosis [230]. Nonetheless, overexpression of Bcl-2 in some cases did not correlate with progression of cancer. Lower expression of Bcl-2 had been associated with poorly prognostic outcomes in patients with metastatic breast carcinoma [231]. These findings could be related to the influence of Bcl-2 on the pro-survival outcome, cell cycle progression, and chemotherapeutic resistance in cancer development [232].

In CC group, supplementation of 200 mg/kg curcumin on control rats did not alter Bcl-2 expression compared CO. The protective effect of curcumin supplementation in MNU+s-NaCl+C3W did not significantly reduced Bcl-2 expression compared with MNU+s-NaCl (table 4-10 and figure 4-23). We also showed the averages of Bcl-2 relative intensity in C3W benign and C3W cancer subgroups in table 4-11. These results indicated that curcumin supplenetation during the induction of cancer did not showed the improvement of Bcl-2 expression. Our design of curcumin supplementation was dissimilar to the *in vitro* study of cancer cells. Curcumin induced Bcl-2 reduction and apoptosis of human ovarian cancer cells [233] and prostate cancer cells [153]. Curcumin inhibited Bcl-2 expression and proliferation in breast cancer cells [234]. However, we did not examine Bcl-2 expression before the end of the experiment to evaluate the protective effect of curcumin on Bcl-2 expression and carcinogenesis.

The late treatment of curcumin (MNU+s-NaCl+C17W) and early treatment of curcumin in MNU+s-NaCl+C20W did not significantly decrease Bcl-2 expression compared with MNU+s-NaCl (figure 4-23, table 4-10, and table 4-11). These results indicated that curcumin could not alter anti-apoptotic property found in cancer as indicated by Bcl-2 expression. It is in accordant with curcumin treatment on human cervical cancer cell line (Ca Ski). 50 and 100 μM curcumin did not change in Bcl-2 expression after treatment for 24 hours [148]. Bcl-2 transfected human acute myelogenous leukemia (HL-60) cells were stable cell line expressing Bcl-2. They found that Bcl-2 transfected cells were relative resistance to curcumin-inhibited growth [235]. In other studies, high doses of curcumin repressed Bcl-2 expression. 20 μg/mL curcumin significantly decreased Bcl-2 expression in human breast cancer MDA-MB-231 cells, whereas 5 and 10 μg/mL did not alter the expression [234]. In

ovarian cancer cell line Ho-8910, Bcl-2 and Bcl-xL were downregulated in the 40 μM curcumin-treated cells. This concentration inhibited cell growth and inhibitory rate was almost 60% [233]. Furthermore, curcumin downregulated the expression of NF-κB-dependent anti-apoptotic Bcl-2 and Bcl-xL in prostate cancer cell lines [236]. Treatment of mentle cell lymphoma (MCL) with curcumin inhibited the phosphorylation of IκBα and p65. Curcumin treatment also suppressed the expression of IκBα, Bcl-2, Bcl-xL, cIAP-1, xIAP, TRAF-1, survivin, cyclin D1, TNF, IL-6, and COX-2 [142]. From our result of phospho-IκBα expression, curcumin had a tendency to suppress the constitutively active NF-κB. This propensity might not be sufficient for reducing Bcl-2 expression.

In consider to this study, it suggested that MNU and s-NaCl, risk factors of stomach cancer, induced squamous cell carcinoma (SCC) by triggering damage of gastric epithelial cells, production of reactive oxygen species (ROS), and formation of 8-Hydroxy-2'-deoxyguanosine (8-OHdG, an oxidative DNA damage marker) in the epithelial cells. Breakage or mutation of DNA was induced by MNU directly or MNU-induced ROS production. It was related to cellular apoptosis and compensatory increase of cell proliferation. Increased cell proliferation enhances the accumulation of mutation and impairment of cell cycle regulation resulting in gastric carcinogenesis. In addition, activation of NF-κB as shown as increased level of phospho-IκBα expression could be related to ROS production. Activation of NF-κB regulated expression of positive regulator of cell cycle (cyclin D1) and anti-apoptotic protein (Bcl-2) involving in cancer development. Furthermore, these molecules may be able to enhance cancer growth and invasion. Cancer cells could produce ROS, which increase DNA damage or cancer cell proliferation and progression. Excess of ROS also induced hyperactivation of NF-κB following by overexpressions of cyclin D1 and Bcl-2.

Interestingly, curcumin at dose 200 mg/kg improved gastric carcinogenesis induced by MNU and s-NaCl in rats. In this present study, using of curcumin at different time of supplementations indicated that the cancer incidence was suppressed by early administration (during the first 3 weeks of cancer induction). Curcumin could

diminish mutagenicity of MNU and protect DNA base against alkylation. Curcumin supplementations in MNU+s-NaCl+C3W and MNU+s-NaCl+C20W might exert the protective effects on the cancer development. Bioavailability of curcumin is poor. Curcumin has a limited absorption, a high elimination from the body, and a rapid metabolism. Continuous supplementations suggested that curcumin exerted the antioxidant effect on cancer development and progression. Early treatment of curcumin (MNU+s-NaCl+C20W) could attenuate incidence of cancer via the reduction of 8-OHdG. Moreover, late treatment of curcumin (MNU+s-NaCl+C17W) might reduce oxidative DNA damage and invasiveness of cancerous tissues by suppressing 8-OHdG expression.

Curcumin supplementations for the first 3 weeks had a tendency to repress phospho-IκBα expression, which could be the initiation factor of cancer development and progression. However, once SCC had been developed, these effects of curcumin may not be sufficient for considerably reduce cyclin D1 and Bcl-2 expressions as shown in the following figure.

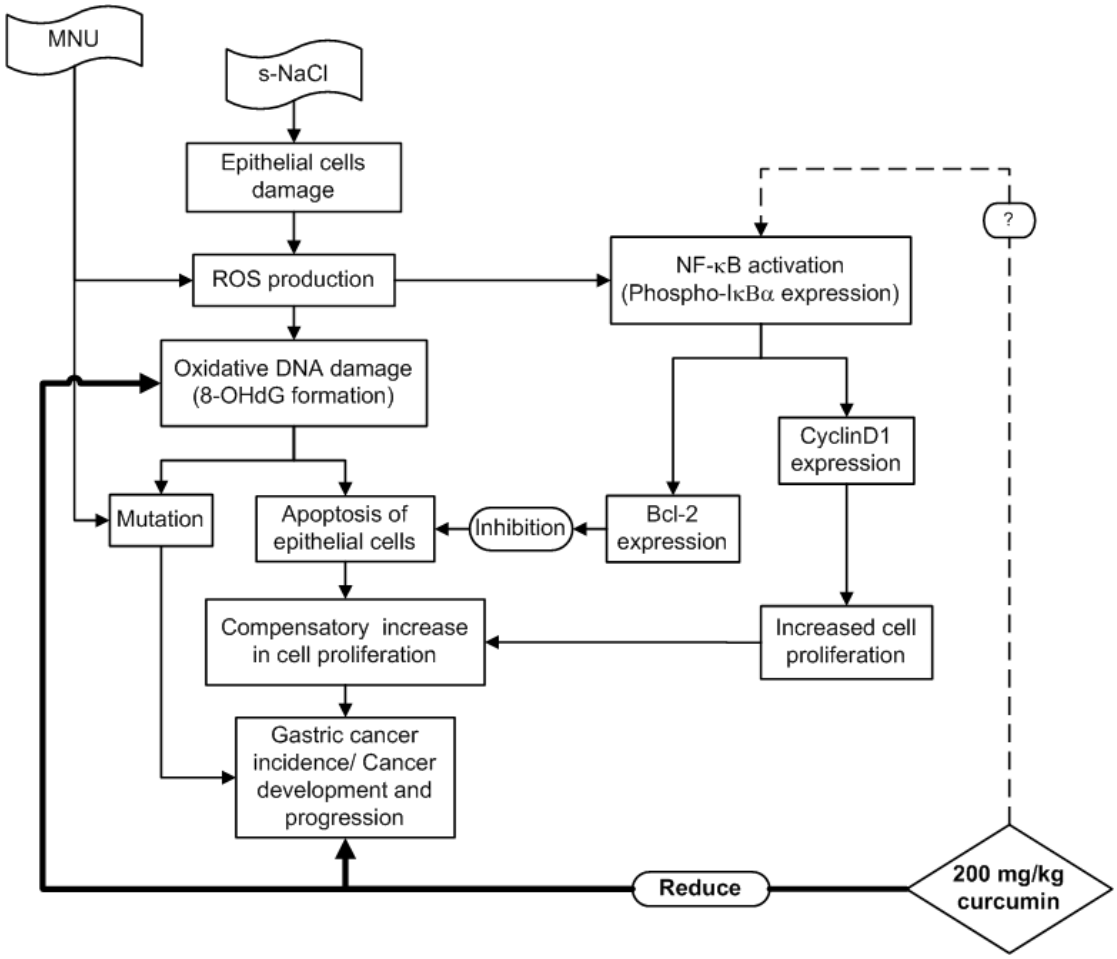


Figure 5-24: Diagram of this study.

In this study, gastric cancer was developed by MNU and s-NaCl. MNU and s-NaCl significantly increased ROS and then causing 8-OHdG formation as well as NF-κB activation [221-222]. Both cyclin D1 and Bcl-2 were expressed by NF-κB activation [100, 107]. Consequently, the inhibition of cell apoptosis and increased cell proliferation were resulted by the expression of cyclin D1, and Bcl-2, respectively. Finally, together with ROS-induced mutation, this lesion area was turned to forestomach SCC and cancer progression in rats (as it showed by the present study that MNU and s-NaCl could induced gastric carcinogenesis by 100%).

It is noted that continuous supplementations of curcumin (MNU+s-NaCl+C17W and MNU+s-NaCl+C20W) could reduce epithelial expressions of 8-OHdG significantly. Therefore, the treatment effect of curcumin (200 mg/kg) on

attenuating cancer progression may be due to its antioxidant potential as which it could inhibit free radical-mediated DNA damage by physical quenching or chemical reaction with ROS [133]. Futhermore, our study also showed that once SCC had been developed, curcumin could not sufficiently inhibit phospho-IκBα, cyclin D1, and Bcl-2 expressions. Therefore, the actions of curcumin on NF-κB induced cyclin D1 and Bcl-2 expressions still need to be confirmed.

Conclusions

The effects of curcumin on cancer incidence in MNU and s-NaCl induction the forestomach SCC in rat and biomarkers, including 8-OHdG, cyclin D1, phospho-IκBα, and Bcl-2 expressions were determined in this study. 200 mg/kg curcumin was supplemented to the rats with the different periods. Curcumin supplementation during the first 3 weeks of MNU and s-NaCl induction was designed to examine the protective effect. In the early and late treatments, curcumin was supplemented for the entired period of the experiment (20 weeks) and 17 weeks after the chemical induction, respectively.

Our findings could be summarized as the follows

1. The oral administration of MNU and s-NaCl lead to the development of forestomach SCC and cancer progression in rats. At week 20th, the cancer incidence of this model is 100%.
2. The expressions of 8-OHdG, cyclin D1, and Bcl-2 in the forestomach of MNU+s-NaCl are significantly increased compared with CO. Moreover, phosphorylated IκBα expression tends to increase in the forestomach tissue of MNU+s-NaCl compared with CO.
3. 200 mg/kg curcumin supplementation for 20 weeks has no effect on control rats. The biomarkers, 8-OHdG, cyclin D1, phospho-IκBα, and Bcl-2 expressions in the forestomach of CC were not significant difference compared with CO.
4. Early administration of curcumin for the first 3 weeks shows the protective effect against the cancer development. The cancer incidence of MNU+s-NaCl+C3W is reduced to 60%. This effect of curcumin has a tendency to

decrease phospho-IκBα expression. However, there are no significant difference in the expressions of 8-OHdG, phospho-IκBα, cyclin D1, and Bcl-2 when compared with MNU+s-NaCl.

5. The early and continuous administration of curcumin for 20 weeks also shows the protective effect against the cancer development. The cancer incidence of MNU+s-NaCl+C20W is reduced to 50%. In addition, 8-OHdG expression significantly reduces when compared with MNU+s-NaCl. Nevertheless, phospho-IκBα, cyclin D1, and Bcl-2 expressions are not statistic difference compared with MNU+s-NaCl.
6. The late treatment of curcumin for 17 weeks cannot reduce the cancer incidence, whereas significantly decreases 8-OHdG expression compared with MNU+s-NaCl. The expressions of phospho-IκBα, cyclin D1, and Bcl-2 are not statistic difference compared with MNU+s-NaCl.

Thus, early administration of curcumin could protect the development of gastric cancer. Continuous treatments of curcumin could attenuate gastric cancer development and progression by suppressing 8-OHdG expression. However, 200 mg/kg curcumin supplementations might not be sufficient for significantly reduce phospho-IκBα, cyclin D1, and Bcl-2 expressions in the cancer progression.

According to these findings, the results suggest that curcumin is a useful agent to prevent the cancer development induced by MNU and s-NaCl in rats. Moreover, it might be beneficial for application as a chemopreventive agent in people exposing to risk factors of carcinogenesis.

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APPENDIX

The Solutions for Western Blotting

Lysis buffer (fresh preparation)

1X RIPA buffer (Cell Signalling Technology Inc., MA)

20 mM Tris-HCl (pH 7.5)

150 mM NaCl, 1 mM Na2EDTA

1 mM EGTA

1% NP-40

1% Sodium deoxycholate

2.5 mM Sodium pyrophosphate

1 mM β-glycerophosphate

1 mM Na_3VO_4

1 μg/mL Leupeptin

Distilled water

Protease inhibitor cocktail (Sigma-Aldrich, MO)

Phosphatase inhibitor cocktail (Sigma-Aldrich, MO)

30% Acrylamide / 0.8% bisacrylamide (Dark bottle)

1.5 M Tris pH 8.8

1 M Tris pH 6.8

10% Sodiun dodecylsulfate (SDS)

10% Ammonium persulfate (APS, fresh preparation on ice)

Tetramethylethylenediamine (TEMED)

10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Reagent	**10% Separating gel (mL)**	**4% stacking gel (mL)**
dH_20	4	2.1
30%Acrylamide	3.3	0.5
1.5 M Tris pH 8.8	2.5	-
1 M Tris pH 6.8	-	0.38
10% SDS	0.1	0.03
10% APS	0.1	0.03
TEMED	0.004	0.004
Total	**10**	**3**

Quickly add and fill solution into glass plate assembly

1. 2X sample buffer (storage at -20°C) 10 mL

0.5 M Tris pH 6.8	2.5	mL
Glycerol	2	mL
Bromophenol blue dissolved in ethanol	10	μL
10% SDS	4	mL
Dithiothreitol (DTT, MW = 154.25 g/mol)	0.3085	g
Distilled water	1.49	mL

2. Running buffer pH 8.3 (storage at 4°C) 1000 mL

Tris base	3.03	g
Glycine	14.42	g
SDS	1	g

Adjust pH to 8.3 with 1M HCl

3. Transfer buffer (storage at 4°C) 1000 mL

Tris base	3.03	g
Glycine	14.42	g

Adjust volume to 800 mL

Add 100% methanol 200 mL

4. Tris buffer saline (TBS) pH 7.6 (storage at 4°C) 1000 mL

1M Tris pH 7.6	20	mL
NaCl	8	g

Adjust pH to 7.6 with 1M HCl

5. Wash buffer (TBS-T) pH 7.6 (storage at 4°C) 1000 mL

TBS pH 7.6	999	mL
Tween 20	1	mL

6. Stripping buffer 1000 mL

0.2 M NaOH

BIOGRAPHY

Miss Kawiya Sintara was born on November 10, 1982, in Chonburi, Thailand. In 2004, she graduated Bachelor degree of Science (B.Sc.) in Biology (Second Class Honors), Faculty of Science, Chulalongkorn University. In 2007, she graduated Master of Science Degree (M.Sc.) in Physiology, GPAX 3.81, Faculty of Medicine, Chulalongkorn University. She has received Development and Promotion of Science and Technology Talent Project (DPST) scholarship since 2002. She has publication as lists below.

Original articles:

1. **Sintara, K**., Thong-Ngam, D., Patumraj, S., and Klaikeaw, N. Curcumin attenuates gastric cancer induced by *N*-methyl-*N*-nitrosourea and saturated sodium chloride in rats. JBB *In press*.
2. **Sintara, K**., Thong-Ngam, D., Patumraj, S., Klaikeaw, N., and Chatsuwan, T. 2010. Curcumin suppresses gastric NF-κB activation and macromolecular leakage in *Helicobacter pylori*-infected rats. World J Gastroenterol 16(32): 4039-4046.
3. Thong-Ngam, D., **Sintara, K.**, Chayanupatkul, M., Klaikeaw, N., and Chatsuwan, T. 2010. The rat models of gastric cancer using *Helicobacter pylori* infection, *N*-methyl-*N*-nitrosourea, and high salt induced carcinogenesis. Thai J Gastroenterol 11(1): 41-48.
4. **Sintara, K.** and Thong-Ngam, D. 2008. Gastric cancer: The experimental models. TJPS 21(1): 33-36.
5. Chanchao, C., **Sintara, K.**, and Wongsiri, S. 2006. Comparison of antibiotic and organoleptic properties of honey from various plant sources in Thailand. J Apic Sci 50(2): 13-18.

She has “The first prize” award from the physiological society of Thailand at the 36th Annual Scientific Meeting in 2007, and “Very good” award in graduated research competition at Chula Medical Expo 2007.