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THE EFFECTS OF MODERATE HYPOTHERMIA ON INTESTINAL ISCHEMIA-REPERFUSION INJURY IN RATS

Miss Nuchanan Leawhiran

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By	Miss Nuchanan Leawhiran
Field of Study	Medical Science
Thesis Advisor	Assistant Professor Paisarn Vejchapipat
Thesis Co-advisor	Professor Yong Poovorawan

Accepted by the Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master 's Degree

..... Dean of the Faculty of Medicine
(Professor Pirom Kamolratanakul)

THESIS COMMITTEE

..... Chairman
(Associate Professor Vilai Chentanez)

..... Thesis Advisor
(Assistant Professor Paisarn Vejchapipat)

..... Thesis Co-advisor
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..... Member
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..... Member
(Professor Sootiiporn Chitimittrapap)

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ภาวะลำไส้ขาดเลือดและมีเลือดกลับคืนเป็นภาวะร้ายแรงที่มีอัตราการเสียชีวิตสูง การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของอุณหภูมิร่างกายต่ำระดับปานกลางต่อภาวะการบาดเจ็บจากลำไส้ขาดเลือดและมีเลือดกลับคืนในหนู โดยแบ่งสัตว์ทดลองออกเป็น 4 กลุ่ม กลุ่มละ 8 ตัว ดังนี้ กลุ่มที่ 1 เป็นกลุ่มควบคุมที่อุณหภูมิร่างกายปกติ (36 ถึง 38 องศาเซลเซียส) กลุ่มที่ 2 ทำให้ลำไส้ขาดเลือด 30 นาทีและมีเลือดกลับคืน 90 นาทีที่อุณหภูมิร่างกายปกติ กลุ่มที่ 3 เป็นกลุ่มควบคุมที่อุณหภูมิร่างกายต่ำระดับปานกลาง (32 ถึง 34 องศาเซลเซียส) และกลุ่มที่ 4 ทำให้ลำไส้ขาดเลือด 30 นาทีและมีเลือดกลับคืน 90 นาทีที่อุณหภูมิร่างกายต่ำระดับปานกลาง วัดระดับ Tumor necrosis factor-α (TNF-α) Interleukin 1-β (IL-1β) และ soluble intercellular adhesion molecule-1 (sICAM-1) ในซีรั่มโดยใช้ ELISA และจัดระดับการบาดเจ็บของเนื้อเยื่อลำไส้ ผลการศึกษาพบว่าระดับ TNF-α ระดับ sICAM-1 ในซีรั่ม และการบาดเจ็บของลำไส้เพิ่มขึ้นอย่างมีนัยสำคัญภายหลังการเกิดภาวะลำไส้ขาดเลือดและมีเลือดกลับคืนที่อุณหภูมิร่างกายปกติ ส่วนอุณหภูมิร่างกายต่ำระดับปานกลางลดการเพิ่มขึ้นของระดับ sICAM-1 ในซีรั่มและลดการบาดเจ็บของเนื้อเยื่อลำไส้ ทุกกลุ่มมีระดับ IL-1β ในซีรั่มไม่แตกต่างกัน นอกจากนี้ยังพบว่าไม่มีความแตกต่างระหว่างระดับ TNF-α ระดับ sICAM-1 ในซีรั่ม และลักษณะของเนื้อเยื่อลำไส้ในกลุ่มควบคุมที่อุณหภูมิร่างกายปกติเทียบกับกลุ่มควบคุมที่อุณหภูมิร่างกายต่ำระดับปานกลาง โดยสรุปอุณหภูมิร่างกายต่ำระดับปานกลางลดการบาดเจ็บของลำไส้และลดการเพิ่มขึ้นของระดับ sICAM-1 ในซีรั่มภายหลังการเกิดภาวะลำไส้ขาดเลือดและมีเลือดกลับคืน อุณหภูมิร่างกายต่ำระดับปานกลางสามารถนำมาใช้ในสัตว์ทดลองที่เป็นกลุ่มควบคุมได้ โดยไม่มีผลกระทบต่อระดับ TNF-α ระดับ IL-1β ระดับ sICAM-1 ในซีรั่ม และลักษณะของเนื้อเยื่อลำไส้

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2547 ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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NUCHANAN LEAWHIRAN : THE EFFECTS OF MODERATE HYPOTHERMIA ON INTESTINAL ISCHEMIA-REPERFUSION INJURY IN RATS. THESIS ADVISOR : ASST. PROF. PAISARN VEJCHAPIPAT, THESIS COADVISOR : PROF. YONG POOVORAWAN, 61 pp. ISBN 974-17-5966-5.

Intestinal ischemia-reperfusion (IR) is a serious condition with high mortality. The aim of this study was to investigate the effects of moderate hypothermia on intestinal IR in rats. Four groups of rats were studied, n=8 per group: A) sham at normothermia (36°-38°C); B) 30 min intestinal ischemia followed by 90 min reperfusion at normothermia; C) sham at moderate hypothermia (32°-34°C); and D) 30 min intestinal ischemia followed by 90 min reperfusion at moderate hypothermia. Serum levels of tumor necrosis factor-α (TNF-α), interleukin 1-β (IL-1β), and soluble intercellular adhesion molecule-1 (sICAM-1) were determined using ELISA and intestinal histological injury was graded. The results showed that serum TNF- α and sICAM-1 levels were increased significantly together with intestinal injury following intestinal IR at normothermia. The elevation of serum sICAM-1 levels and tissue injury were attenuated by moderate hypothermia. There was no difference in serum IL-1β among all groups. In addition, there was no difference in serum TNF- α, sICAM-1 or intestinal histology between sham animals at normothermia and sham animals at moderate hypothermia. In conclusion, moderate hypothermia ameliorates intestinal injury and the elevation of serum sICAM-1 following intestinal IR. Moderate hypothermia can be applied in sham animals without affecting serum TNF-α, IL-1β, sICAM-1 levels and intestinal histology.

Field of study Medical Science Student's signature.....
Academic year 2004 Advisor's signature.....
Co-advisor's signature.....

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My life is fulfilling because of my adorable father and mother, my classmates, and my dear friends who have given me the good wishes (by phone). I miss all of you.

In my opinion, the real objective of this thesis is to find a cure for patients who suffered from intestinal ischemia-reperfusion. Further studies must be needed to decide whether hypothermia is a useful clinical treatment or not. This decision may take a long time, a year or a decade. The value of the journey exists along the way, not the destination. Thanks everybody for coming with me on this journey.

Table of Contents

Page

Table Page

Figure Page

List of Abbreviations

°C	degrees Celsius
CA	celiac axis
cm	centimeter
ELISA	enzyme linked immunosorbent assay
H and E	hematoxylin and eosin
IL-1b	interleukin-1 beta
IMA	inferior mesenteric artery
IR	ischemia-reperfusion
KD	kilodalton
kg	kilogram
M	molar
min	minute
mm	millimeter
NEC	necrotizing enterocotitis
SD	standard deviation
sICAM-1	soluble intercellular adhesion molecule-1
SIRS	systemic inflammatory response syndrome
SMA	superior mesenteric artery
SMV	superior mesenteric vein
TNF-a	tumor necrosis factor-alpha
mm	micron

CHAPTER I

INTRODUCTION

1.1 Background and Rationale

Intestinal ischemia-reperfusion (IR) is a serious condition. It can occur from the neonatal period (necrotizing enterocolitis) through the adult period (acute mesenteric arterial occlusion). Despite improvement in critical care, the morbidity and mortality of patients with intestinal IR is high. The mortality rate reported for patients undergoing surgery for acute intestinal ischemia is as high as 85%, although with more aggressive approach, mortality rates may be reduced to 25%.[1]

Moderate hypothermia, which is cooling the body below the normal physiologic temperature, is an interesting therapeutic strategy. Clinical trials of moderate hypothermia are currently being conducted in five conditions; birth asphyxia,[2] traumatic brain injury,[3] stroke,[4] acute fulminant liver failure with increased intracranial pressure,[5] and post-cardiac arrest.[6] These conditions have ischemic characters. Therefore, moderate hypothermia may have beneficial effects on intestinal IR injury.

Intestinal IR can lead to multiple organ failure,[7] therefore, the systemic response of intestinal reperfusion might be considered as a form of systemic inflammatory response syndrome (SIRS). The levels of serum inflammatory mediators, which have been demonstrated to be involved in the pathogenesis of SIRS such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and soluble intercellular adhesion molecule-1 (sICAM-1),[8-10] can assess the severity of SIRS.[11] The investigation of these serum inflammatory mediators following intestinal IR will increase our knowledge of the mechanism of intestinal IR. In addition, intestinal IR causes the intestinal tissue damage and histological change, thus the intestinal injury following IR can be evaluated by histologic features of tissue sections.

1.2 Research Question

Does moderate hypothermia have an effect on intestinal IR injury in rats?

1.3 Objectives

To investigate the effects of moderate hypothermia on serum tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and soluble intercellular adhesion molecule 1 (sICAM-1), together with the histopathologic changes, following intestinal IR in rats.

1.4 Hypothesis

1.4.1 Whole-body-moderate hypothermia reduces serum levels of TNF-α, IL-1β, and sICAM-1 following intestinal IR.

1.4.2 Moderate hypothermia ameliorates the effects of intestinal IR injury.

1.5 Briefly Materials and Methods

1.5.1 Animals

Four groups of male Sprague-Dawley rats were studied. (n=8/group)

Group A 120 min sham operation at normothermia (36°-38°C);

Group B 30 min ischemia followed by 90 min reperfusion at normothermia;

Group C 120 min sham operation at moderate hypothermia (32°-34°C); and

Group D 30 min ischemia followed by 90 min reperfusion at moderate hypothermia.

1.5.2 Surgical Procedure

The animals were anesthetized with oxygen and 1.0-1.5% halothane by inhalation via a nose cone. Respiratory rate and rectal temperature were monitored. Exploratory laparotomy via midline incision was carried out. Intestinal IR was performed by occlusion (ischemia) and de-occlusion (reperfusion) of the superior mesenteric artery for the assigned duration. Moderate hypothermia or normothermia was induced by

adjusting the environmental temperature to achieve the target rectal temperature using a lamp, and was maintained throughout the experiment.

At the end of the experiment, blood samples were collected via cardiac puncture technique. In addition, terminal ileum (2-3 cm in length immediately next to the ileocecal junction) was fixed in 10% formalin for histological evaluation. The animals were be sacrificed by exsanguinations.

1.5.3 Measurement of Serum Inflammatory Mediators

Samples of blood were collected, immediately separated and stored at –80°C until they could be assayed. Enzyme linked immunosorbent assay (ELISA) was performed to determine the levels of serum TNF-α, IL-1β and sICAM-1.

1.5.4 Histological Study

Segments of terminal ileum were stored in 10% neutral buffered formalin. They were embedded in paraffin and cut into 3-4 micron (μm) sections. Paraffin sections were then stained with hematoxylin-eosin, and the sections of terminal ileum were evaluated under a microscope using 40x magnification. Using a grading scheme as per Farber *et al.* (1999),[12] the histological sections were analyzed and scored.

1.5.5 Data Analysis

All data are presented as mean $\pm$ SD. Statistical analysis of serum cytokine levels was performed using One-way analysis of variance (ANOVA) with post-hoc Tukey comparisons. For the histological study, nonparametric statistical analysis (mean score statistics) was used. Significant differences are established at $p<0.05$. SPSS and Prism software were used for all statistical analyses.

1.6 Key Words

Moderate hypothermia

Intestinal IR

TNF-α

IL-1

sICAM-1

1.7 Expected Benefits and Application

1.7.1 Development of an animal model of intestinal IR.

1.7.2 A further understandings of the pathophysiology of intestinal IR.

1.7.3 Another step towards the possibility to consider the use of moderate hypothermia as a treatment of patients with intestinal IR in the future.

1.8 Ethical Considerations

Rats were used as an animal model of intestinal IR. Since the ultimate goal of medical research is to be able to apply the knowledge obtained from the study to clinical practise, it is necessary to use an *in vivo* model. The minimum number of animals required to obtain valid scientific results was used in research. The intentional killing, for all purposes, is a buddhistic sin. For the least guilty conscience, the animals will be generally anesthetized throughout the experiment. Therefore, the animals will not suffer from any painful procedures.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction

Intestinal ischemia-reperfusion (IR) is a condition resulted from functional or mechanical reduction of blood flow (ischemia)[13] of the intestine followed by restoration of blood flow (reperfusion).[14] Intestinal IR may occur in infants, children and adults. These events can happen with malrotation with midgut volvulus, necrotizing enterocotitis, intussusception, acute mesenteric occlusion, shock, intestinal transplantation and sepsis. Intestinal-ischemia-induced tissue injury involves necrosis and apoptosis. Although early reperfusion can salvage tissue, reperfusion also increases cell mortality by increasing the inflammatory response. Intestinal IR can lead to further tissue damage in intestine and several organs such as liver, heart, lung and kidney, namely multiple organ dysfunction syndrome. The clinical presentation and management of individual cases are uncertain and non-specific. Moreover, mortality of intestinal IR is still high.

2.2 Splanchnic Circulation

The celiac axis (CA), superior mesenteric artery (SMA), and inferior mesenteric artery (IMA) are three major visceral arteries that supply almost all of the blood flow to the splanchnic organs, including liver, stomach, small intestine, large intestine, spleen and pancreas. The intestines are supplied by a series of parallel circulations via the branches of the superior and inferior mesenteric arteries.[15] The position and distribution of SMA are illustrated respectively in figure 2.1 and figure 2.2. At rest, splanchnic circulation accounts for one quarter of cardiac output, and three quarters of splanchnic blood flow is the blood flow to the intestine.[16] The intestinal blood flow to the mucosa is greater than that to the other layers of the intestinal wall.[17]

The regulation of intestinal blood flow is unclear. Systemic and local factors affect mesenteric blood flow. Systemic factors consist of neural and humoral control. Parasympathetic nervous system increases intestinal motility and obstructs blood flow.

Sympathetic nervous system is responsible for maintaining resting splanchnic arteriolar tone.[18] Sympathetic stimulation produces vasoconstriction and decrease blood flow. Circulating vasoconstrictor hormones include vasopressin, norepinephrine, epinephrine and angiotensin II whereas urokinase, acetylcholine, glucagon and prostaglandin E-1 are vasodilator hormones. Vasodilators are used in the treatment of acute nonocclusive mesenteric ischemia.[19] Local factors can affect mesenteric blood flow. Inadequate oxygen increases mesenteric flow. Potassium, osmolality, pH, and gastrointestinal hormones can affect the vascular tone of the splanchnic bed. Local factors may change the precapillary sphincter mechanism. Autoregulation is the particular aspect of regulation of the mesenteric circulation. Autoregulation is in almost all organs and tissues in the body. After moderate hemorrhage, autoregulation can maintain the steady blood flow. However, severe reduction in blood flow can defeat autoregulation.

2.3 General Histology of the Small Intestine

The small intestine is the part of completion of digestion, nutrient absorption, and endocrine secretion. It consists of three segments: the proximal duodenum, the middle jejunum, and the distal ileum. Although the small intestine is structurally and functionally differentiated from other parts of the digestive system, it is composed of the same four principal layers as are present in the other parts. These four layers are mucosa or mucous membrane, submucosa, muscularis externa, and adventitia or serosa. (Figure 2.3) Most of the content in this section is adapted from Basic Histology, L. Carlos[20] and Histology, Ronald A.[21]

2.3.1 Mucosa

The obvious feature of the small intestine is a series of permanent folds on the intestine lining. These folds are known as plicae circulares, or Kerckring's valves, consisting of mucosa and submucosa. Plicae circulares are absent in the proximal part of the duodenum and the distal part of the ileum. They are tallest in the jejunum. The entire intestinal mucosa forms villi, which project into the lumen of the intestine. The surface of the villi is formed by a simple columnar epithelium. The core of the villi is

formed by the blood vessels within the lamina propria. Absorptive cells, or enterocyte, are tall columnar cells. Each cell forms the layer called the striated (brush) border, which are composed of microvilli. The microvilli increase the surface area enormously. The muscularis mucosae is the smooth muscle fibers that separate the mucosa from the submucosa. It consists of an inner circular layer and an outer longitudinal layer. Between the villi are the openings of simple tubular glands called the crypts (or glands) of Lieberkühn. The cells, which are dispersed between the absorptive cells, are called the goblet cells. They can form the mucus for protect and lubricate the lining of the intestine.

2.3.2 Submucosa

In the submucosa layer, there are blood vessels, lymphatic vessels, and nerves. The submucosa contains glands, called Brunner's glands, only in the duodenum. Their secretion is mucous which protects the duodenal mucosa.

2.3.3 Muscularis Externa

The muscularis externa comprises two layers of smooth muscle: an inner circular layer and an outer longitudinal layer. Between the two muscular layers is the myenteric nerve plexus (Auerbach's plexus), which is the part of the autonomic nervous system.

2.3.4 Serosa

The serosa is the outer layer of loose connective tissue containing blood vessels, lymphatic vessels, lymphoid tissue, and adipose tissue.

The three segments of small intestine have histological differences. The duodenum has Brunner's glands. The ileum has Peyer's patches. Peyer's patches are the large aggregations of lymph nodules in the lamina propria of the ileum. The jejunum has only villi without Brunner's glands or Peyer's patches.

2.4 Clinical Relevance of Intestinal Ischemia-Reperfusion

Intestinal IR is a dominant character of many clinical conditions. These conditions include malrotation with midgut volvulus, necrotizing enterocotitis, intussusception, acute mesenteric occlusion, shock and sepsis, all of which are considered to be life menace. Understandings of intestinal IR are useful for patients with these serious conditions.

2.4.1 Malrotation with Midgut Volvulus

Malrotation is an abnormal or incomplete rotation of the midgut, resulting from interruption of the embryologic sequence of bowel development and fixation. Although the clinical presentations of malrotation vary, the most prominent is midgut volvulus, or the twisting of the midgut around its axis. The small bowel mesentary is shortened and the ligament of Treitz and cecum are poorly fixed. Malrotation with midgut volvulus around the SMA causes intestinal ischemia and necrosis. Malrotation usually occurs in the first year of life, most occur within the first month,[22, 23] and rarely present at the older age. Surgical correction is the treatment of choice.

2.4.2 Necrotizing Enterocotitis

Necrotizing enterocotitis (NEC) is a clinical condition in which intestinal tissue (mucosa) become inflamed and may progress to necrosis. This can lead to a perforation and cause sepsis. NEC most commonly occurs in newborns weighing less than 1.5 kg.[24] The etiology of NEC is still unclear. Its risk factors are immature gastrointestinal mucosal barrier, immature immune system, formula milk feeding, and infection. Experiments in animals have shown that hypoxia, combined with an formula milk feeding, can induce the pathologic equivalent of NEC by causing a reduction of blood flow in the mesenteric vessels.[25] NEC can be often treated without surgery, by using intravenous feeding and medication. However, patients will need an operation if they develop an intestinal perforation or refractory sepsis.

2.4.3 Intussusception

Intussusception is a condition in which part of the intestine telescopes in on itself. It usually results in intestinal obstruction, inflammation and decreased blood flow. It is a common cause of bowel blockage in children. This condition often occurs in children between 3 months and 3 years of age.[26] The causes of intussusception are not known exactly. It could be linked with a viral intestinal infection and the introduction of solid foods to a young digestive system of a weanling baby. Intussusception causes progressive edema formation and inflammation leading to bowel obstruction and ischemia. Patients with intussusception often have intense abdominal pain. An enama is generally tried first for treatment. If the enema is not satisfying, patients will need to have an operation. Mortality from intussusception is rare in modern practice.

2.4.4 Acute Mesenteric Arterial Occlusion

Acute mesenteric arterial occlusion is the most important cause of acute intestinal ischemia, which is a clinical problem in adults. The detail of this condition will be mentioned in 'Acute Intestinal Ischemia' part.

2.4.5 Shock

Shock is a pathologic state that results from inadequate tissue perfusion. There are four basic categories of shock.[15]

1) *Hypovolemic shock* results from low intravascular volume due to loss of blood or other body fluids, usually through the gastrointestinal tract.

2) *Distributive shock* occurs when vasodilation or capillary fluid leak causes a relative loss of fluid from the intravascular space. A severe infection or an allergic reaction can cause this type of shock.

3) *Cardiogenic shock* arises when the heart cannot pump blood effectively. It caused by all types of cardiac failure.

4) *Obstructive shock* results when an obstruction impedes blood flow through the heart.

The common feature of all shock syndromes is hypoperfusion. Intestinal ischemia usually occurs in shock syndromes. The aims in the treatment of shock are to restore perfusion and adequate oxygen delivery to tissue. However, the intestinal reperfusion may cause a greater injury than that of ischemia alone.

2.4.6 Sepsis

Sepsis (Alternative name is systemic inflammatory response syndrome or SIRS) is a severe illness caused by systemic response to infection. It associated with organ dysfunction, hypoperfusion or hypotension. Mediators, such as endotoxin and cytokines, may initiate cellular toxicity. Sepsis is also associated with an increase in oxygen consumption in the splanchnic organs.[27, 28] These imbalances can be considered as an intestinal ischemic event. Reperfusion of the microcirculation leads to the production of oxygen free radicals leading to tissue damage, especially to the gut mucosa. Appropriate therapeutic response and removal of the causes of infection are also very important. The principles of treatment include immediate resuscitation, stabilization and definitive therapy for underlying disease.[29] However, sepsis is still the leading cause of mortality in intensive care units (ICU).[30]

2.5 Acute Intestinal Ischemia

Intestinal ischemia can be divided as acute or chronic. Acute intestinal ischemia is a sudden reduction in the intestinal blood flow. This acute condition is critical and threatening. Chronic intestinal ischemia, or intestinal angina, differs from the acute ischemia in serveral parts. Chronic ischemia takes a long-standing period, but it takes the form of recurrent temporary episodes of ischemia. Chronic intestinal ischemia does not require urgent therapy. Intestinal ischemia in this study is the acute type. The arterial flow of the animal model was totally occluded during ischemia. Acute mesenteric ischemia is much more common than the chronic type.[13]

2.5.1 Etiology

Ischemia can be caused by an interruption in blood circulation through an artery or vein. There are a variety of factors that can cause the obstacle of blood delivery to the intestines. The usual cause of acute intestinal ischemia is occlusion of the SMA by atherosclerosis or by embolus. Atherosclerosis is a condition that the wall of the artery has been thick. An embolus is a migrating blood clot, which can form a blockage. Embolic occlusion most often occurs in the distal vessel of the SMA. The another cause is mesenteric artery thrombosis. A thrombus is a stationary clot attached to the wall of a blood vessel, which can prohibit blood passage. Thrombosis usually occurs at the origin of the SMA.[1] The next cause is the low-flow state, or nonocclusive mesenteric ischemia. This state can be caused by shock, sepsis, hemorrhage, and cardiac decompensation. Although the SMA is not occluded, mesenteric blood flow is largely decreased. Another cause is sudden occlusion of the superior mesenteric vein (SMV)[31] Thrombus in the portal and superior mesenteric venous system can cause intestinal ischemia. Unusual causes of acute mesenteric ischemia include trauma, aneurysms, collagen vascular disease, and abdominal aortic operations.[19]

2.5.2 Pathophysiology

1) Ischemic injury

Ischemic injury of the intestine results from deprivation of oxygen and nutrients necessary for the cells. The ischemia results in tissue injury with release of intracellular contents and byproducts of anaerobic metabolism to the circulation. The earliest change is in the submusoca and may be observed by electron microscopy as soon as 10 minutes after ischemia.[32] Light microscopy can observed the structural change after 30 minutes. Histologic changes follow with the process of inflammation. The damaging intestinal wall leads to bacterial translocation and releasing of blood into the lumen. Necrosis of the tips of the villi was found 1 hour after SMA occlusion.[33] The severity of intestinal injury reflects both the degree and the duration of the obstruction of blood flow. The prolonged obstruction of blood flow leads to necrosis of the muscularis and serosa.

2) Reperfusion injury

Reperfusion or restoration of blood flow can recover viable cells. However, the reperfusion mechanism, which is still unclear, may cause further damage. The return of blood flow introduces undesirable metabolites, such as high concentrations of calcium, and causes a cellular injury.[34] Reperfusion of injured cells results in an extensive inflammation and high levels of oxygen-derived free radicals. These free radicals can be identified as mediators of reperfusion injury. Oxidant formation occurs immediately after reperfusion, lasting for a few minutes.[35, 36]

2.5.3 Histopathology

Histologically, the first change is slough on the tips of the villi in the mucosa. Epithelial layer lifts up and forms the subepithelial space. Next, the villi denude. Edema appears with hemorrhage into the submucosa. Finally, the lamina propria has been digested and disintegrated. The intestinal wall becomes progressively thinned and ulcerated.

The severity of injury can be ranged in three levels, which are transmural infarction, mural infarction, and mucosal infarction, if the extension of lesion is not deeper than the muscularis mucosae.[37] The three levels of severity is illustrated in figure 2.4. Intestinal injury following ischemia reperfusion is often evaluated by histologic analysis of hematoxylin and eosin (H and E) stained tissue sections. Variety of grading systems for intestinal injury have been described but there is no consensus on how this injury should be graded at present.[38]

2.6 Roles of Cytokines and Intercellular Adhesion Molecule

Intestinal IR triggers a cascade of mediator production and leukocyte-endothelial interactions resulting in systemic inflammatory response syndrome.[14] Cytokines have important effects in inflammatory response. Cytokines are a family of proteins that mediate many of the responses of the immune system.[39] These cytokines are the messenger molecules of short range between the cells of the immune system.

Each cytokine is secreted by particular cell types in response to a variety of stimuli and produces characteristic effects on the growth, mobility, differentiation, or function of target cells.

The another of essential mediators in inflammation are adhesion molecules. Adhesion molecules are transmembrane glycoproteins that play a role in cell adhesion. Migration of cells into inflammatory sites is controlled by the expression of adhesion molecules. Different groups of cells preferentially localize to particular tissues. This depends on adhesion molecules on endothelium reacting with counter-receptors on the leukocytes. Expression of the adhesion molecules has been induced by inflammatory cytokines.

This study will investigate serum levels of tumor necrosis factors-α (TNF-α), interleukins-1β (IL-1β), and soluble intercellular adhesion molecules-1 (sICAM-1). Following intestinal IR, TNF-α and IL-1 have been shown to increase in both intestinal mucosa and the systemic circulation.[40-42] TNF and IL-1 are extremely potent inflammatory cytokines that mediate acute inflammation induced in animals.[43] After intestinal IR, systemic ICAM-1 expressions have been shown to increase with marked organ variability.[44]

2.6.1 Tumor Necrosis Factor

Tumor necrosis factors comprise two subclasses, TNF-α (cachectin) and TNF-β (lymphotoxin). TNF-β is 30% homologous to TNF-α.[45] TNF-α and β play critical roles in normal host resistance to infection and to the growth of malignant tumors, serving as immunostimulants and as mediators of the inflammatory response.

TNF-α (17 kD) is produced mainly by macrophages and, to a lesser extent, by other cell types including T and B cells, natural killer cells (NK cells), and mast cells. TNF-α is the one of the first cytokine appearing in the circulation following intestinal ischemia.[41, 46] This circulating molecule's activity is concentration-dependent. At low

concentrations ($<10^{-9}$ M), TNF-α induces increased expression of adhesion molecules on endothelial cells, increased neutrophil adhesion, acts on leukocytes and endothelium to induce acute inflammation, and stimulates macrophages to produce IL-1, IL-6, chemokines, and TNF-α itself. At moderate concentrations, TNF-α mediates the systemic effects of inflammation. It induces fever, acute phase protein synthesis in liver cells, and leukocytes. At high concentrations ($\geq 10^{-7}$ M), TNF-α causes the pathologic abnormalities of septic shock. High circulating levels of TNF-α cause severe metabolic disturbances, such as hypoglycemia. The ability of TNF to cause necrosis of tumors, which is the origin of its name, is mainly a consequence of thrombosis of tumor blood vessels.[39]

2.6.2 Interleukins-1

Interleukins-1 (IL-1) is a general name for two distinct proteins, IL-1α (membrane form) and IL-1β (secreted form). IL-1α most often remains on the cell surface. Unlike IL-1α, IL-1β is most of the IL-1 found in the circulation. This study will investigate serum cytokine, so only IL-1β will be investigated.

IL-1α and IL-1β are peptides that are encoded by separate genes and share only 26% amino acid sequence similarity.[47] IL-1α and IL-1β bind to the same cell surface receptors and share biological functions. Their major cellular sources are mononuclear phagocytes, fibroblasts, keratinocytes, and T and B lymphocytes. IL-1 production by macrophages is induced by bacterial products such as lipopolysaccharide (LPS) and by other cytokines such as TNF.[39] Both IL-1α and IL-1β are approximately 17 kD. IL-1 possesses a variety of biological activities. IL-1α and IL-1β are key inflammatory cytokines playing central roles in the immune response. Although normal production of IL-1 is obviously critical for initiation of normal host responses to injury and infection, inappropriate or prolonged production of IL-1 has been implicated as playing a role in the production of a variety of pathological conditions. At low concentrations, IL-1 is a mediator of local inflammation. IL-1 acts on

endothelial cells to increase expression of surface molecules that mediate leukocyte adhesion. When secreted in larger concentrations, IL-1 enters the blood circulation and exerts endocrine effects. Systemic IL-1 and TNF share several properties, which are causing fever, induction of synthesis of acute-phase plasma proteins by the liver, and to initiation of metabolic wasting.

2.6.3 Intercellular Adhesion Molecules-1

Intercellular adhesion molecules-1 (ICAM-1), member of the immunoglobulin superfamily, is a transmembrane glycoprotein that is expressed on a variety of cell types. ICAM-1 (CD54) is a generally inducible transmembrane molecule that plays a role in cell migration, antigen presentation, and leukocyte activation. It is receptors for lymphocyte function associated antigen type 1 (LFA-1). ICAM-1 can be cleaved from the cell surface. A soluble form of ICAM-1 (sICAM-1), a shedding form of ICAM-1, can be detected in serum.[48] sICAM-1 is regarded as a useful parameter in the diagnosis and monitoring of various inflammatory, neoplastic, and immune disorders.[49] Cells known to express ICAM-1 include endothelium, activated T cells, B cells and NK cells. ICAM-1 may be also induced on a variety of tissue cells, particularly at sites of immune reactions. The role of ICAM-1 in transendothelial migration depends on its induction by TNF and IL-1. The experiments by K.J. Kelly et al. demonstrated that ICAM-1deficient mice are markedly protected from ischemic injury to the kidney.[50]

2.6.4 Roles of TNF-α, IL-1β and ICAM-1 in inflammation

Inflammation is a protective response that occurs in the injury tissue. It can be divided into two patterns, which are acute and chronic. Intestinal ischemia in this study is the acute type that leads to SIRS, therefore this section describes the acute inflammation. Acute inflammation is the immediate and early response that relates with short duration. This process comprises two portions,[51] vascular changes and cellular events.

1) Vascular changes

After injury, vasodilation occurs quickly resulting in increased blood flow. Acute inflammation induces leakiness of endothelial monolayers and structural changes. These events allow plasma proteins to leave the circulation and vascular permeability increase. Cytokines, including TNF and IL-1, induce a structural reorganization of the endothelial cytoskeleton. The cytoskeleton is made up of actin filaments, intermediate filaments, and microtubules. These changes develop endothelial cell retraction from each other and cell junctions disruption.[51] Moreover, IL-1 elicits the release of histamine from mast cells at the inflammatory site. Histamine is a mediator that triggers early vasodilation and increase of vascular permeability.[43]

2) Cellular events

The major cellular event of inflammation is the leukocyte emigration from the vascular lumen to the extravascular space, which is divided into many processes. At first, the leukocyte accumulates at the periphery of vessels, called marginate. After that, leukocytes tumble on the endothelial surface and momentary adhesion, called rolling. Selectins, including E-selectin, P-selectin and L-selectin, are receptors that account for rolling process. E-selectin is induced after stimulation by TNF and IL-1. Next process, leukocytes firmly adhere to endothelial cells. TNF and IL-1 induce the expression of ICAM-1. ICAM-1 is the member of the immunoglobulin superfamily that mediated the adhesion of leukocytes. Finally, leukocytes transmigrate into extravascular space.

2.7 Therapeutic Role of Moderate Hypothermia

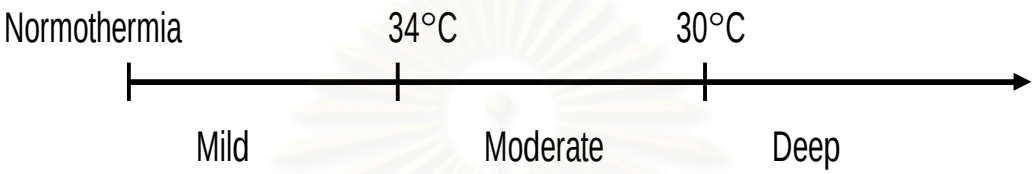
Many methods to prevent and treat intestinal IR have been developed. These strategies include augmentation of intestinal blood flow and oxygen consumption,[52, 53] selective digestive decontamination,[54-56] nutritional support with immuno-modulatory substrates,[57-59] use of monoclonal antibodies directed against specific pro-inflammatory cytokines[60] and against leukocyte adhesion molecules,[61] antioxidant therapies,[62, 63] and moderate hypothermia.[64, 65] Moderate hypothermia is an interesting strategy because of ease of use, safety, and low cost.

Hypothermia is cooling the body below the normal physiologic temperature. Hypothermia can divide into three categories, following broad definitions:

1) Mild hypothermia: down to 34°C

2) Moderate hypothermia: between 30° and 34°C

3) Deep hypothermia: less than 30°C



Spontaneous, uncontrolled hypothermia starts with potentially deleterious shivering, thermogenesis, catecholamine release, and vasoconstriction, whereas therapeutic, controlled hypothermia is potentially beneficial.[66] Whole body metabolic rate, during moderate hypothermia, is decreased by approximately 8% per °C, and is about half the normal rate at 28°C. Oxygen demand is concurrently reduced along with oxygen consumption.[67]

Whole body cooling was first introduced to treat patients with terminal stages of advanced cancer more than fifty years ago. Reports of use of hypothermia as a treatment for brain injury appeared as early as 1943.[68] Hypothermia has been used for special surgical procedures since the 1950s. Hypothermia is used most often during cardiac surgery with cardiopulmonary bypass, as a means of protecting the brain from ischemic injury. Hypothermia is also used during some neurosurgical procedures and is being investigated as a treatment for ischemic stroke and traumatic brain injury.[69] But clinical applications of hypothermia were not routinely established because of its harmful effects. At present, clinical trials of moderate hypothermia are currently being conducted in five conditions: birth asphyxia, traumatic brain injury, stroke, acute fulminant hepatic failure with increased intracranial pressure, and post-cardiac arrest. No clinical studies on intestinal IR have been reported.

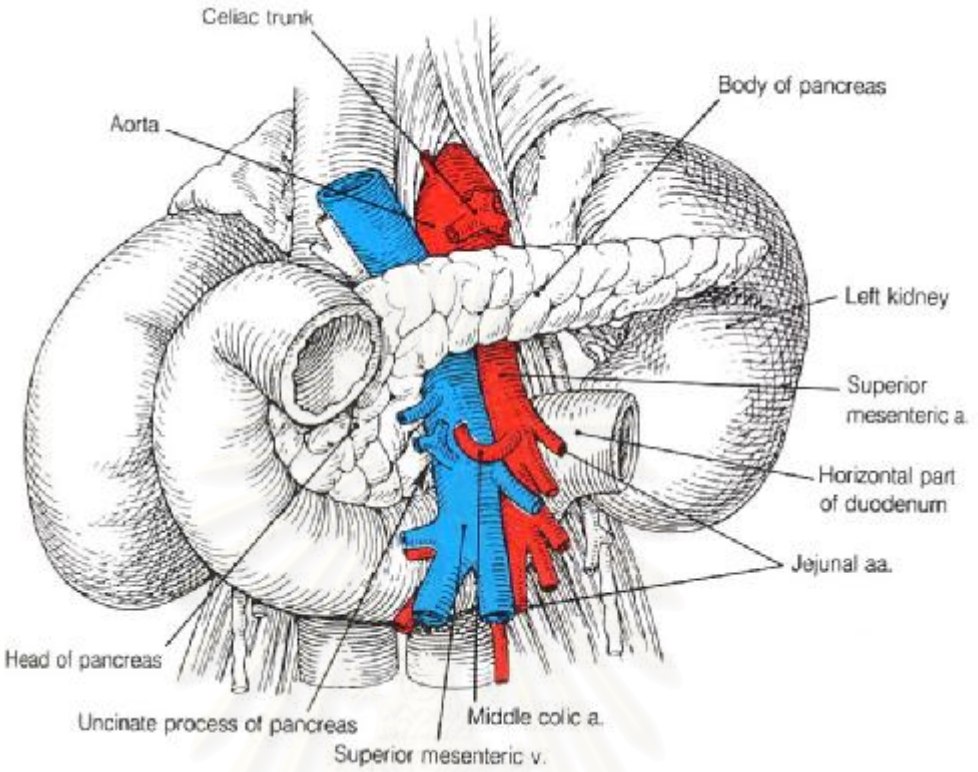


Figure 2.1 The relationships of structures assembled around the transpyloric plane.[70]

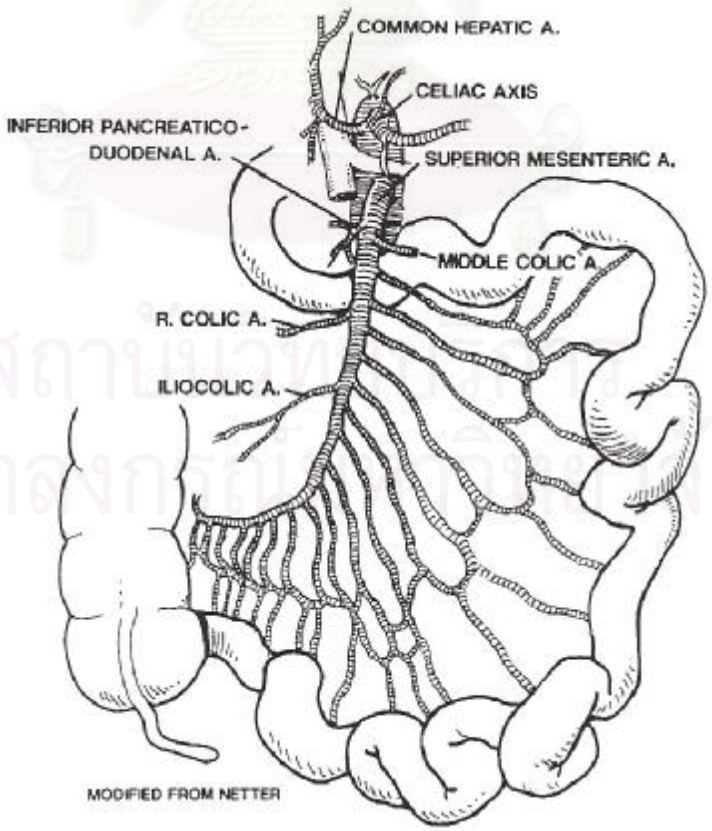


Figure 2.2 SMA distribution to the small intestine.[71]

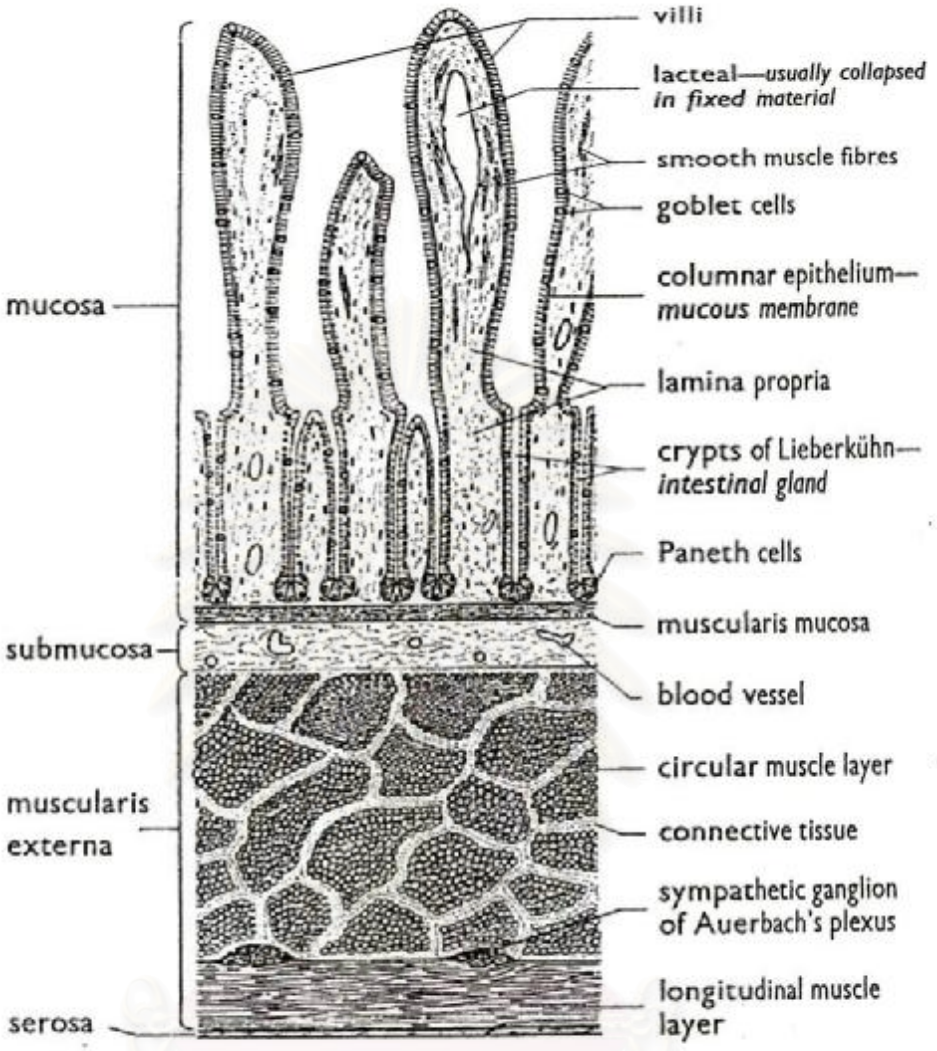


Figure 2.3 Drawing of ileum.[72]

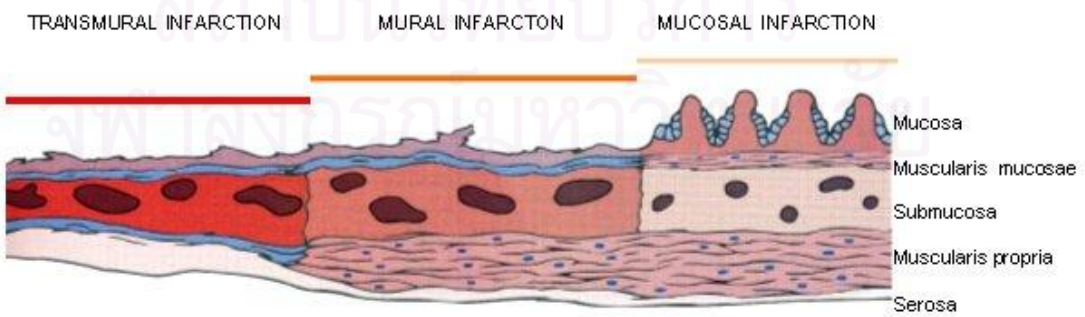


Figure 2.4 Acute ischemic bowel disease. Schematic of the three levels of severity, diagrammed for the small intestine.[37]

CHAPTER III

MATERIALS AND METHODS

3.1 Animals

The *in vivo* models are the best for investigating new therapeutic strategies.[73] Several species have been used to be animal models of intestinal IR, including rats, sheep, pigs, cats, and dogs. Rats are choosen for this study because of convenience and suitable size.

Male Sprague-Dawley rats (*Rattus norvegicus*) weighing between 250-350 g were studied. The animal model in this study was considered to be representative of various conditions, not of any specific disease. Intestinal IR can occur in all age groups, from the neonatal period (necrotizing enterocolitis) through the adult period (acute mesenteric arterial occlusion), therefore adult rats was chosen in this study.

The animals were divided into four groups:

Group A sham operation for 120 minutes at normothermia, n=8

Group B 30 minutes intestinal ischemia followed by 90 minutes reperfusion at normothermia, n=8

Group C sham operation for 120 minutes at moderate hypothermia, n=8

Group D 30 minutes intestinal ischemia followed by 90 minutes reperfusion at moderate hypothermia, n=8

Normothermia and moderate hypothermia were defined as rectal temperature between 36°-38°C and between 32°-34°C respectively. Naturally, a laparotomy in the experiment induces hypothermia. Moderate hypothermia or normothermia was induced by adjusting the environmental temperature to achieve the target rectal temperature using a lamp, and was maintained throughout the experiment.

3.2 Surgical Procedure

The animals were anesthetized with oxygen and 1.0-1.5% halothane by inhalation via a nose cone as shown in diagram (Figure 3.1). Rectal temperature (Digital Thermometer, eXeter, Taiwan) was continuously monitored. The animals were stilled on board using masking tape. In Group A and C (sham) animal, a midline laparotomy was performed. At this stage, the body temperature may decrease. A lamp and a blanket were used to control the body temperature. The abdomen was closed in order to maintain the temperature. In Group B and D (ischemia-reperfusion) animal, laparotomy via midline incision was carried out and the SMA was identified. (Figure 3.2) The SMA was occluded using a silicon loop. (Surg-I-Loop; Scanlan, Minnesota) (Figure 3.3) Position of the SMA is near the left kidney. The abdomen were be reopened and a silicone loop will be removed after 30 minutes. After that, the abdominal wall was closed again until the end of the experiment.

At the end of the experiment, blood samples were collected via cardiac puncture technique. The syringe was pierced at the apex of heart and blood was drawn. Volume of collected blood is approximately 10 ml. Terminal ileum (2-3 cm in length immediately next to the ileocecal junction) were removed and fixed in 10% formalin for histological evaluation. The animals were be sacrificed by exsanguinations.

3.3 Enzyme Linked Immunosorbent Assay

Samples of blood were collected and centrifuged for 10 min at approximately 3000 x g. Serum was separated and stored at –80°C until they could be assayed. Commercially available sandwich ELISA kits was performed to determine the levels of serum TNF-α (Quantikine, R&D Systems, USA), IL-1β (Endogen, Pierce, USA), and soluble ICAM-1 (Quantikine, R&D Systems, USA) according to the manufacturers' instructions.

An outline of the ELISA procedure used in laboratory for determining serum cytokines and adhesion molecules follows. (Schematically, this is illustrated in Figure

3.4) Assay procedures of TNF-α, IL-1β and sICAM-1 immunoassay are different in details, such as the concentration of the standard solution, but each step of the outline of the procedure are the same.

1) A monoclonal antibody specific for rat cytokine has been pre-coated onto the wells of microtiter plate. Any component that can be attached to the solid phase can be used. This flexibility is the one of the advantages of ELISA.[74]

2) Standard solution with known concentrations of antigen, control solution and serum containing antigen (cytokine) at an unknown concentration are added into the wells and allow antigen to bind the immobilized antibody. Wash off unbound antigen.

3) Add an enzyme-linked polyclonal antibody specific for rat cytokine to the wells. The antigen serves as a bridge, it looks like a “sandwich”. Therefore, the more antigens in the test or standard solutions, the more enzyme-labeled specific antibody will bind. Following a wash to remove any unbound antibody-enzyme reagent.

4) Add chromogenic substrate for enzyme in the well that will be convert to a colored product. The enzyme reaction yields a colored product that turns another color when the stop solution is added to stop reaction.

5) Determine the optical density of each well, using a microplate reader or spectrophotometer. The spectrophotometer is an instrument that measures the proportions of light of different wavelengths absorbed and transmitted by a pigment solution.[75] The intensity of the color measured is in proportion to the amount of enzyme-linked antibody that binds, which is directly related to the amount of antigen that was present to bind the immobilized antibody. From the results of standard solutions, a standard curve can be constructed which will allow the amount of unknown antigen to be determined.

3.4 Histological Technique

Terminal ileum, which was fixed in 10% formalin, was prepared for histological evaluation. The interesting tissues were studied with the light microscope. In order to be examined with a microscope, the tissue must be sufficiently thin to be transparent and must possess sufficient contrast to allow the resolution of structural detail. The procedures of preparation of tissues for microscopic examination are fixation, tissue processing, embedding in paraffin, paraffin section, and tissue staining. (Figure 3.5) The contents in this section were extracted and modified from Roy's web page.[76]

3.4.1 Fixation

The tissues, which were removed from the body, underwent a process of tissue digestion by enzymes (autolysis) or bacteria. In order to avoid tissue damage and to preserve cells and tissue constituents, the tissue should be fixed immediately as soon as possible after removal from the body. This treatment, called fixation, is the first and the most important step of the procedure of tissue preparation. The chemical substances used to preserve tissues are called fixatives. It should preserve tissues permanently in as a life-like state as possible and should not damage the tissues. The period of fixation depends on size and density of specimens. Penetration of tissues depends upon the ability of diffuse of each individual fixative. Penetration into a small specimen will occur more rapidly than a large specimen. One of the most widely used fixatives for routine light microscopy is 10% buffered formalin. Formalin can preserve tissues for long time. It is suitable for transit to another laboratory. In this study, segments of terminal ileum were stored in 10% neutral buffered formalin for 24 hours. The ratio of volume of fixative to tissue was 10:1.

3.4.2 Tissue Processing

After fixation, tissue was processed into a form in which it can be made into thin sections. Tissue processing is the technique of getting fixed tissue into a solid medium, usually paraffin, which supports the tissue and gives it enough rigidity to cut into thin sections, and yet soft enough not to damage the knife or tissue. The fixed tissues and

the thin paper card with writing an identical number of tissues in soft pencil were put in the cassettes. (Figure 3.7C) These cassettes were loaded in the automated tissue-processing machine. (Figure 3.6) An automated processing schedule is an overnight schedule of 18 hours. (Table 3.1) The automated tissue processing has the same principle as in the manual tissue processing. There are four stages of tissue processing.

1) Fixation

The tissues were fixed in fixative again, for the best conservation.

2) Dehydration

The fixed tissue that contains high water content must be dehydrated in order that an embedding medium can enter tissue. Dehydration is the removal of aqueous fixative fluids and tissue fluid from the tissues by chemical compounds, called dehydrants. Dehydration is usually done with a series of dehydrants, usually alcohols, from low to high concentrations. The period of immersion in the dehydrants varies with the size and penetrability of the tissue.

3) Clearing

Most of dehydrants are immiscible with embedding medium. Clearing agents, therefore, which are miscible with both, are used to facilitate the transition between dehydration and infiltration. The term “clearing agent” is called because the tissues, which immerse in these agents, are rendered more transparent. The properties of a suitable clearing agent are quickly removal of dehydrating agent, ease of removal by molten embedding medium and minimal tissue damage. Clearing agent in this study is dioxane. Dioxane is slower than ethanol as a dehydrating agent but the process of clearing and impregnation is faster.[77]

4) Infiltration

Infiltration or impregnation with wax is the method of infiltrating embedding medium, always is liquid wax, into the tissue in high temperature. When the solvent

evaporates, the space is filled with embedding medium. Paraffin wax is the most general embedding medium. It is a mixture of hydrocarbons produced in the cracking of mineral oil. It also has a wide range of melting points (40°-70°C).[77] Therefore, it can be used in the different climatic regions.

Table 3.1 The overnight schedule of the automated processing

Container	Fluid	Time (hours)	Stage
1	Formalin	1	Fixation
2	95% Alcohol	1	Dehydration
3	95% Alcohol	1	
4	95% Alcohol	1	
5	95% Alcohol	1	
6	Absolute ethanol	1.5	
7	Absolute ethanol	1.5	
8	Dioxane	2	Clearing
9	Dioxane	2	
10	Dioxane	2	
11	Hot paraffin	2	Infiltration
12	Hot paraffin	2	

After that, the cassette containing processed tissue was put in a vacuum cabinet at 60°C for 1 hour, for the best result of infiltration.

3.4.3 Embedding in Paraffin

The infiltrated tissues were embedded in paraffin, which was in embedding molds. (Figure 3.7B) Embedding medium supports tissues for sectioning. This process uses an embedding center, (Figure 3.8) which comprises three modules: wax dispenser, cold plate, and heated storage area for moulds.

Paraffin embedding followed these steps:

1) Put the cassette on the heated area, and opened the cassette.

2) Droped little paraffin into an embedding mold.

3) Warm forceps were used to put the tissue, from the cassette, on the bottom of the embedding mold. The tissue was aligned, or oriented, properly in the embedding mold.

4) Embedding ring (Figure 3.7A) was pressed on the embedding mold. Filled paraffin in the embedding ring fully. Thin card with an identical number of tissues was put on the embedding ring.

5) Brought the embedding mold on the cold plate. The tissue, which was in the embedding mold, would be moved into the correct orientation before paraffin wax was rigid.

6) Cracked the embedding mold and the tissue block with embedding ring would come off. This block would be sectioned with a microtome.

3.4.4 Paraffin Section

Once the tissues have been embedded, they must be cut into sections that can be placed on a slide. This step was done with a microtome, usually rotary microtome. (Figure 3.9)

Methods of paraffin sectioning were:

1) Placed the blocks on to a cold plate or ice to harden the face to be section for a while. Prolong cooling can cracks the surface of the paraffin block.

2) Installed a blade in the microtome. Set the correct clearance angle, normally 5 to 10°, and the desired thickness (3 to 4 μm)

3) Trimmed or cut away excess parts of the paraffin block and leave 2 to 3 mm of wax surrounds the tissue.

4) Fit the trimmed paraffin block into the block holder and progressed the block until it just touches the knife-edge.

5) Cut the block coarsely until the full face has been trimmed and cooled the block with ice for a minute.

6) Cut the paraffin section by rotate the drive wheel. Rotation of the drive wheel of rotary microtome moved the block holder up and down and the paraffin block passed over the knife-edge. The sections were cut at the fixed distance. (3 to 4 μm) The sections adhered to each other, producing a ribbon of six to eight sections.

7) The first section was held by forceps and the last section separated from the knife-edge by a hair brush. After that, the ribbon was floated on the surface of a warm water bath.

8) Sections could be separated while floating on the water by using the tips of forceps press softly.

9) Selected a clean glass slide and held it vertical and mostly beneath the surface of the water. When the slide was lift, sections were picked up on to a slide.

10) Slides were dried on heat area of an embedding center to ensure the section is firmly attached. Overnight drying at 60°C is essential for maximum section adhesion.

3.4.5 Tissue Staining

The observing colorless tissue in the light microscope is difficult, so that the tissues must be stained. The routine stain is the combination of hematoxylin and eosin (H and E). Each dye has a different ability to stain various cellular components. Hematoxylin stains the cell nucleus and other acidic structures blue. Eosin stains the cytoplasm red and collagen pink.[20]

In the staining process, (Table 3.2) the paraffin wax could be get out of the tissue sections and water-soluble dyes were allowed to penetrate the tissue sections. Deparaffinization was running the slides through xylenes to alcohols to water. After staining, the slides could be dehydrated in order to get rid of water and leaved dyes alone in the sections. The next step was clearing by running the slides through xylenes.

Table 3.2 A schedule of tissue staining

Container	Fluid	Time (min)	Stage
1	Xylene	5	Deparaffinization
2	Xylene	5	
3	Absolute ethanol	5	Hydration
4	95% Alcohol	5	
5	95% Alcohol	5	
6	Run water	10	
7	Hematoxylin	3	Staining
8	Run water	5	
9	Blueing solution	1	
10	Run water	3	
11	Eosin	1	
12	95% Alcohol	dip	Dehydration
13	95% Alcohol	dip	
14	Absolute ethanol	dip	
15	Absolute ethanol	dip	
16	Xylene	dip	Clearing
17	Xylene	dip	
18	Xylene	dip	
19	Xylene	dip	

The last step of slide preparation was mounting. Mounting media was dropped on the slide and a coverslip was covered on the slide to protect the tissue from being scratched. The tissue slide was ready to examination with the light microscope.

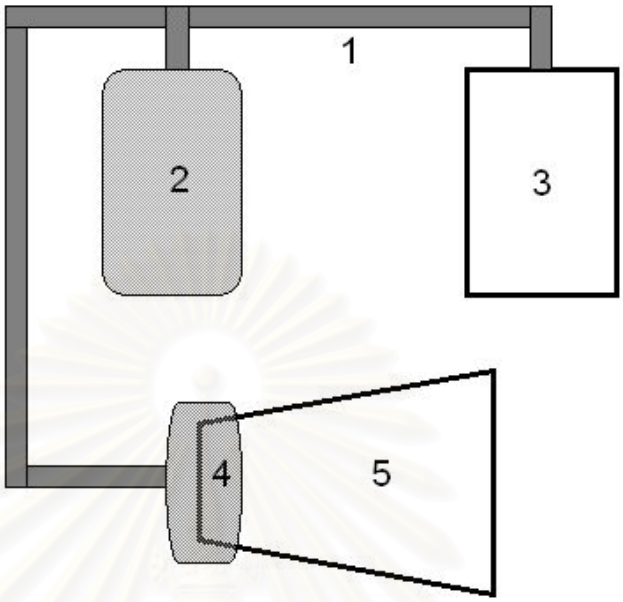


Figure 3.1 Schematic diagram of a circuit of anesthesia includes (1) pipeline, (2) vaporizer containing a volatile liquid anesthetic (halothane), (3) oxygen analyzers, (4) nose cone, and (5) animal.

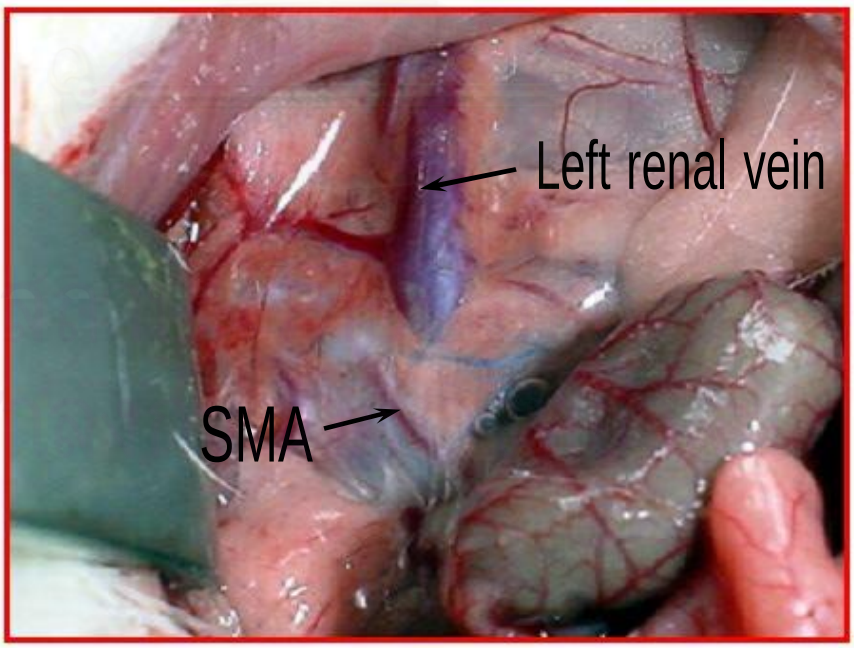


Figure 3.2 Position of superior mesenteric artery (SMA) in rat. A head is on the left side of the figure. A tail is on the right side of the figure.

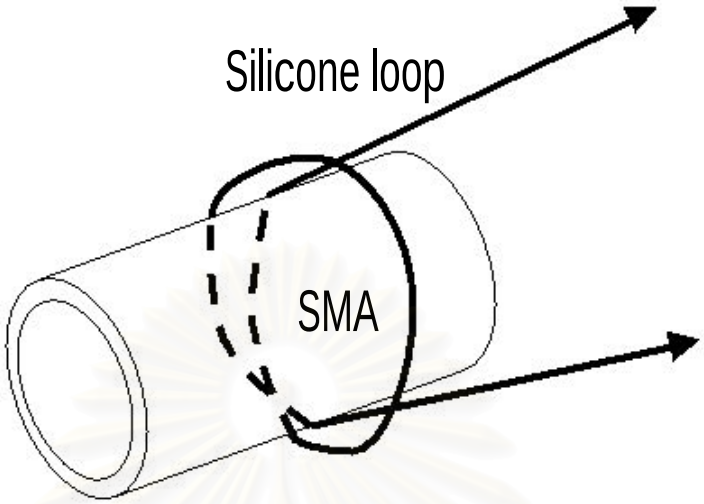


Figure 3.3 Diagram of the way of superior mesenteric artery occlusion using a silicone loop.

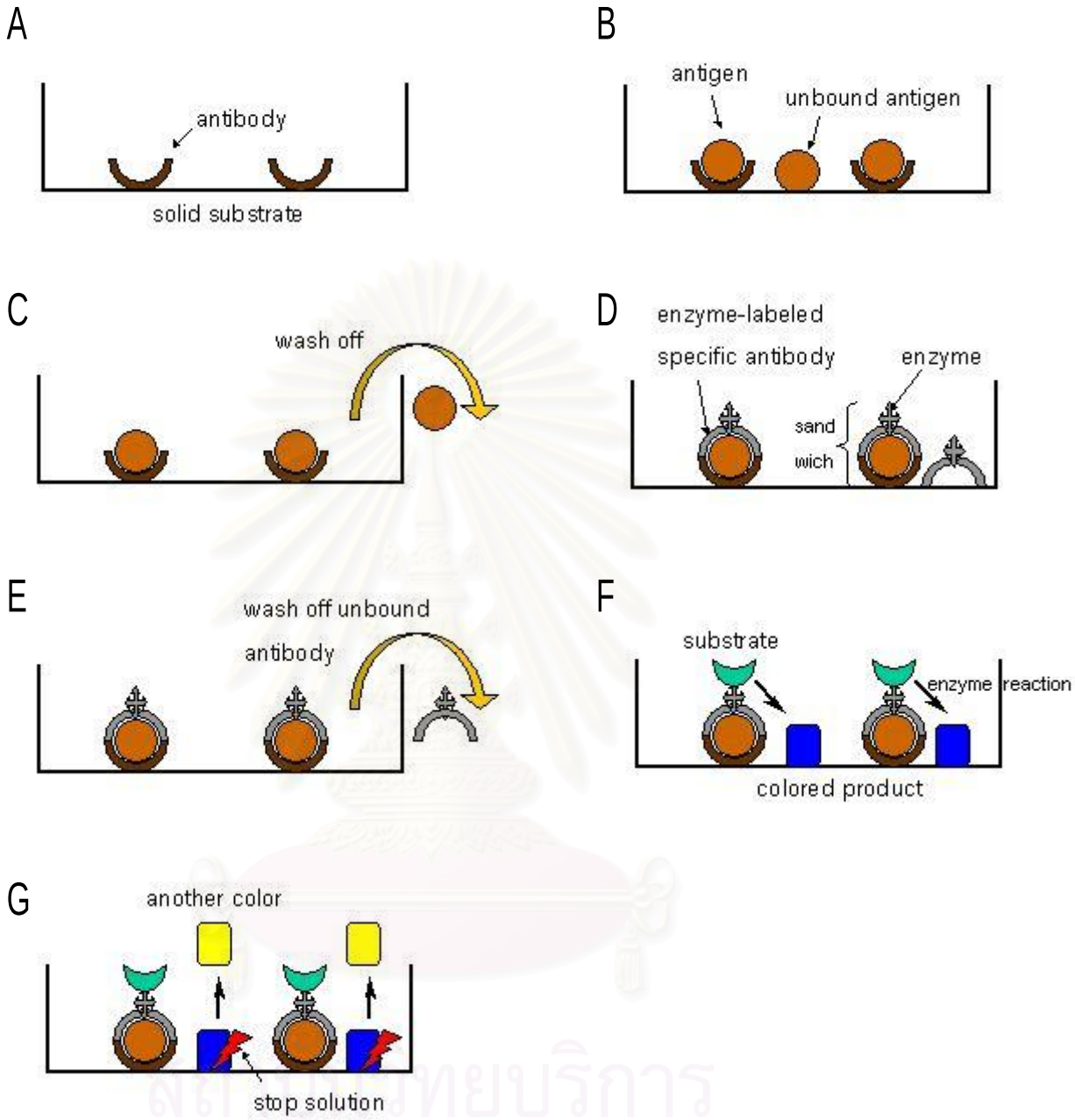
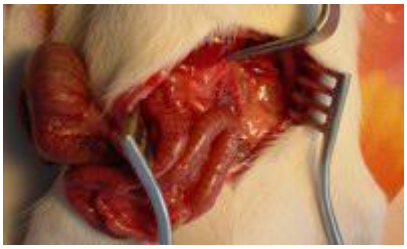


Figure 3.4 Steps of sandwich ELISA.

A



Removal of fresh tissue

B

Fixation by stored in 10% formalin

C

Automated tissue processing

D

Put tissue in the embedding mold

E

Paraffin tissue blocks

F

Paraffin section

G

Tissue staining

H

Completed tissue slide

Figure 3.5 Steps in preparing tissue slides for light microscopy.

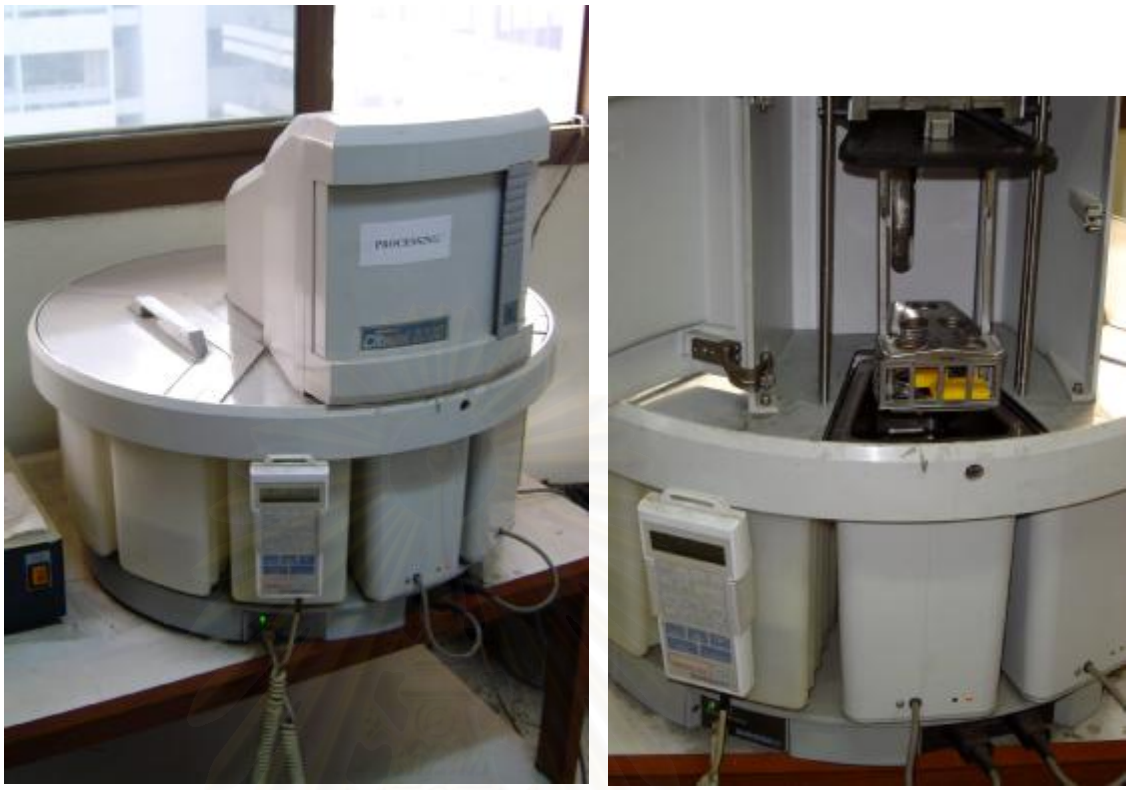


Figure 3.6 Automated tissue-processing machine.

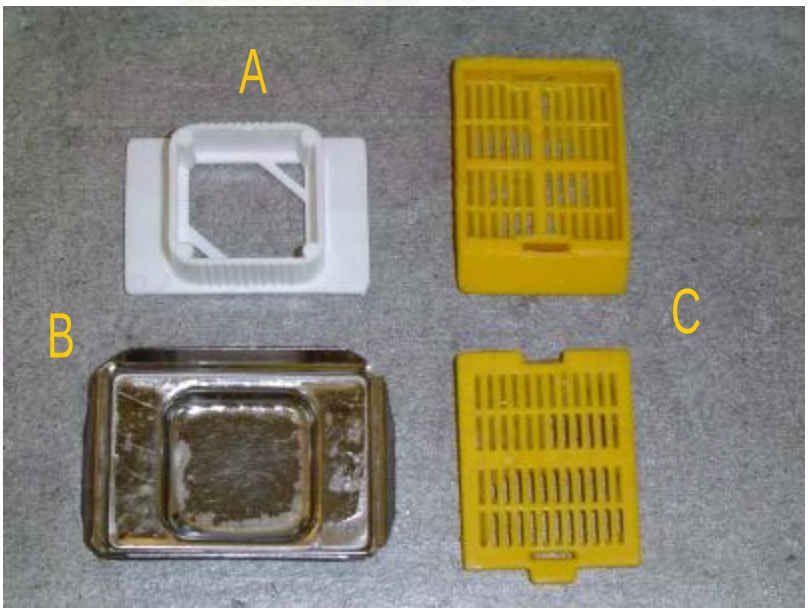


Figure 3.7 (A) Embedding ring, (B) embedding mold, and (C) cassette.

Figure 3.8 Embedding center.

Figure 3.9 Cutting paraffin section.

CHAPTER IV

RESULTS

The animals in this study were divided into four groups. Each group had eight rats (n=8). There was no mortality during the experiment.

Group A 120 min sham operation at normothermia (36°- 38°C);
Group B 30 min ischemia followed by 90 min reperfusion at normothermia;
Group C 120 min sham operation at moderate hypothermia (32°- 34°C); and
Group D 30 min ischemia followed by 90 min reperfusion at moderate hypothermia.

4.1 Measurement of Serum Inflammatory Mediators

Sandwich ELISA was performed to determine the levels of three mediators: serum TNF-α, serum IL-1β, and soluble ICAM-1. Intestinal IR at normothermia (Group B) results in significant increase in serum TNF-α ($p<0.05$) and soluble ICAM-1 ($p<0.05$) compared with sham-operated animals (Group A). Intestinal IR at moderate hypothermia (Group D) results in significant decrease in soluble ICAM-1 ($p<0.05$) compared with Intestinal IR at normothermia (Group B). There are no significant difference between the serum levels of TNF-α, IL-1β, and soluble ICAM-1 in sham-operated animals at normothermia (Group A) and sham-operated animals at moderate hypothermia (Group C). All of the four experimental groups had no significantly difference in serum IL-1β levels. The data of each group are shown in table 4.1, table 4.2, figure 4.1, figure 4.2, and figure 4.3.

4.2 Histological Study

The sections of terminal ileum were viewed under a light microscope with 40x of magnification. The histological sections were categorized into grade 1 to 5 by using a well-established grading scheme as per Farber *et al.* (1999).[12] This system scored by

progression of intestinal injury from the tips of the villi to the gut wall in 5 grades. (Figure 4.4)

Grade 1 represented normal mucosal villi.

Grade 2 represented development of mucosal slough at villous tips.

Grade 3 represented extension of the subepithelial space with the epithelial layer lifting up in sheets, presence of a few denuded villous tips, and mild capillary congestion.

Grade 4 represented denuded villi with exposed lamina propria; dilated, exposed capillaries with evidence of haemorrhage; and increased cellularity of the lamina propria.

Grade 5 represented digestion and disintegration of the lamina propria in villi and presence of hemorrhage and ulceration.

All of the animals undergoing sham operation at normothermia (Group A) and hypothermia (Group C) display normal mucosal villi. Animals undergoing intestinal IR at moderate hypothermia (Group D) have less histological injury ($p<0.05$) compared with intestinal IR at normothermia (Group B). Representative histological features in each group are shown in figure 4.5. The histological scores of each group are shown in table 4.3 and figure 4.6.

	Group	N	Minimum	Maximum	Mean	Std. Deviation
TNF-a	A	8	22.98	29.97	24.78	2.32
	B	8	30.80	125.80	60.82	32.84
	C	8	22.16	39.02	30.13	5.50
	D	8	31.21	110.99	60.00	28.39
	Total	32	22.16	125.80	43.93	26.79
IL-1b	A	8	55.27	118.85	80.63	21.51
	B	8	59.31	112.80	78.10	16.69
	C	8	71.42	105.73	88.95	13.33
	D	8	86.56	124.91	100.43	12.65
	Total	32	55.27	124.91	87.03	17.95
sICAM-1	A	8	26943.55	38328.04	30856.97	4558.82
	B	8	37883.96	56272.72	45529.12	6642.54
	C	8	32030.24	39680.45	35158.95	2724.93
	D	8	29042.82	43394.54	36238.86	6236.62
	Total	32	26943.55	56272.72	36945.98	7392.47

Table 4.1 Determine the levels of three mediators: serum TNF-α, IL-1β, and sICAM-1

TNF-a

Group	A	B	C	D
A	1.000	0.014*	0.961	0.016*
B	0.014*	1.000	0.043*	1.000
C	0.961	0.043*	1.000	0.050
D	0.016*	1.000	0.050	1.000

lL-1b

Group	A	B	C	D
A	1.000	0.990	0.743	0.098
B	0.990	1.000	0.558	0.051
C	0.743	0.558	1.000	0.511
D	0.098	0.051	0.511	1.000

sICAM-1

Group	A	B	C	D
A	1.000	0.000*	0.378	0.197
B	0.000*	1.000	0.003*	0.008*
C	0.378	0.003*	1.000	0.976
D	0.197	0.008*	0.976	1.000

Table 4.2 Tables of p-value

(* The mean difference is significant at the 0.05 level.)

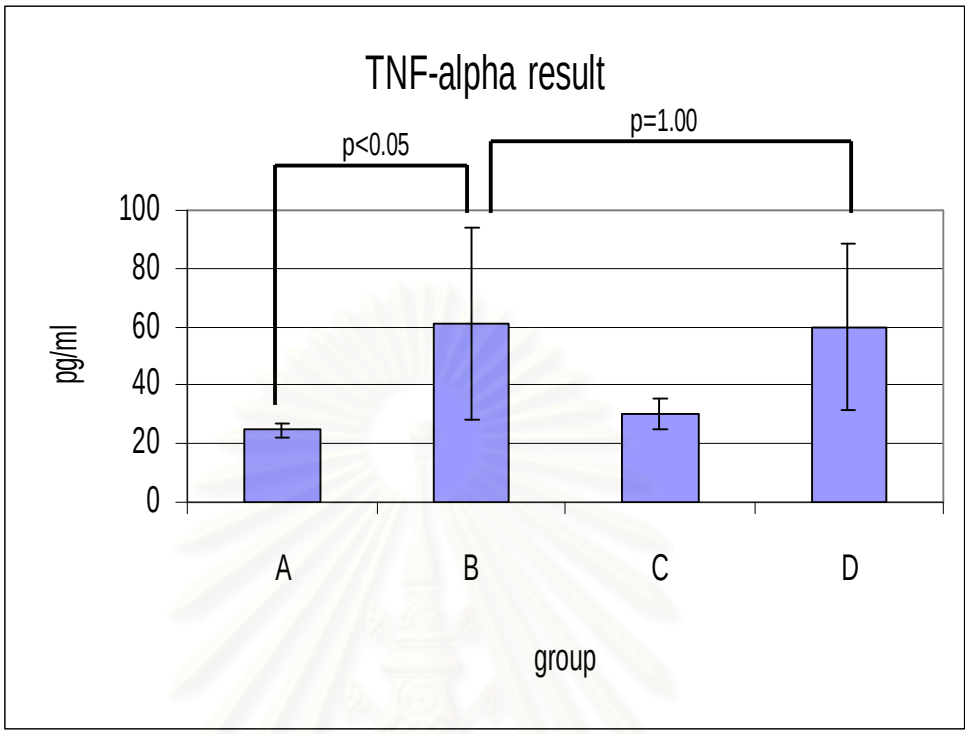


Figure 4.1 Comparison of the mean ± SD of the TNF-α levels.

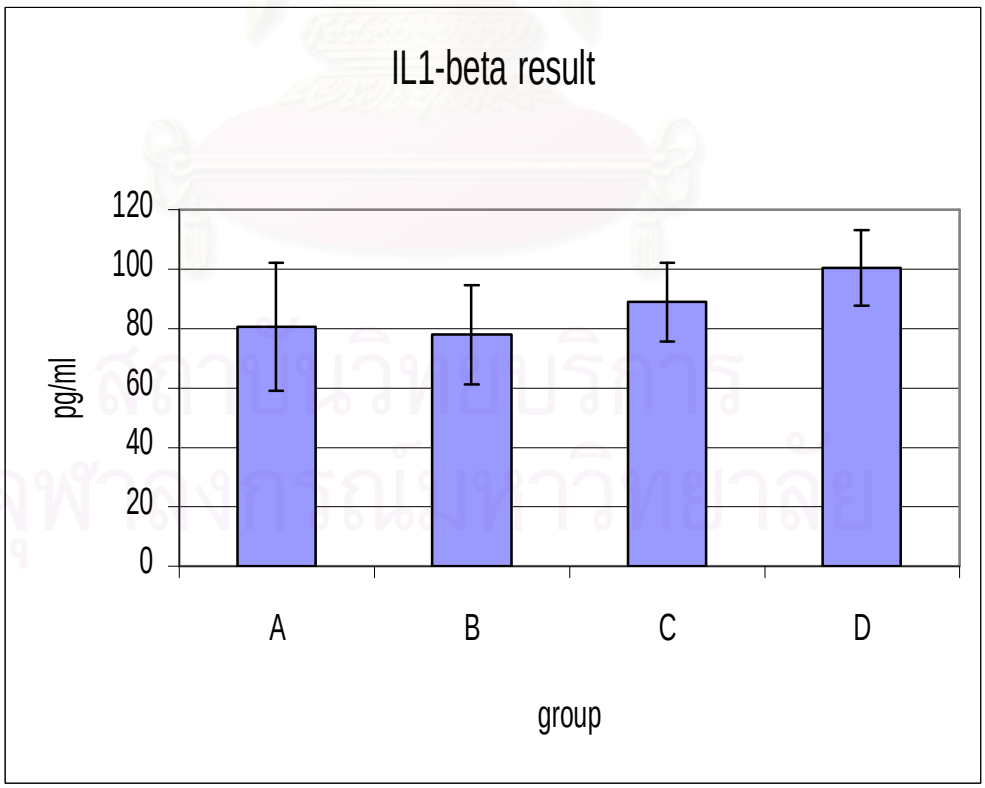


Figure 4.2 Comparison of the mean ± SD of the IL-1β levels.

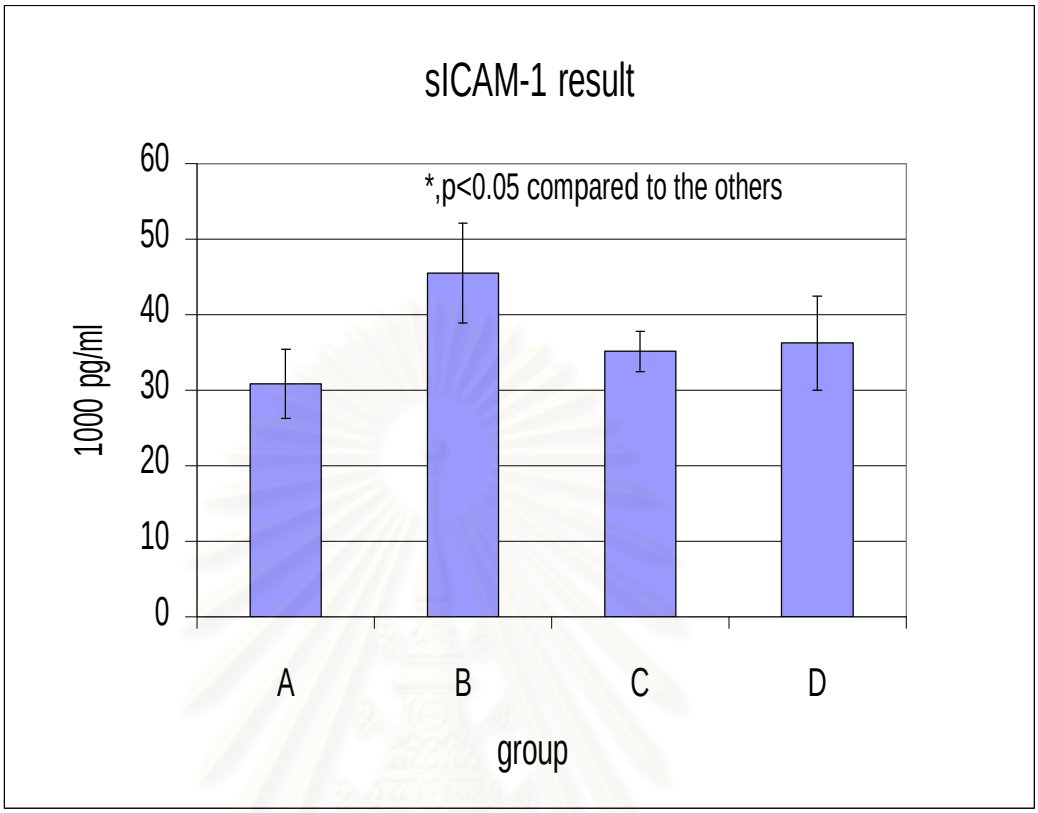


Figure 4.3 Comparison of the mean ± SD of the sICAM-1 levels.

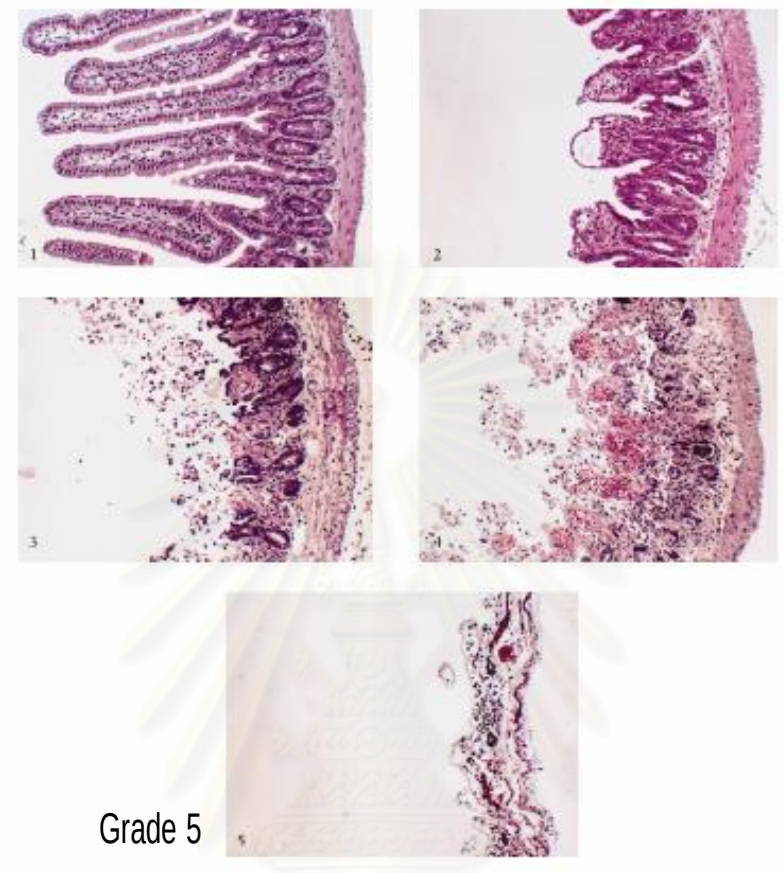
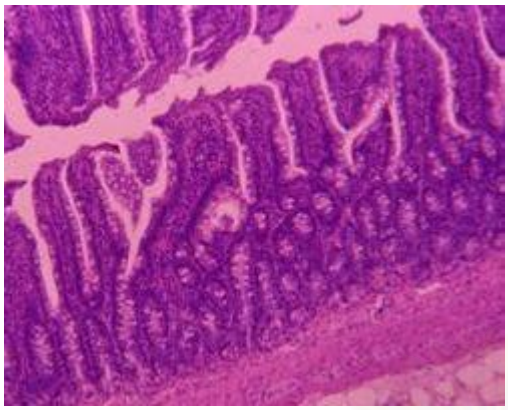
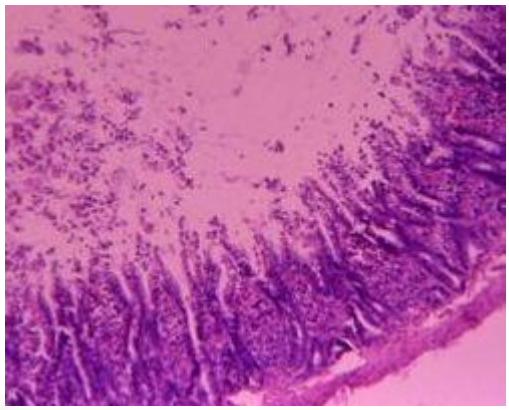


Figure 4.4 Representative hematoxylin-eosin stained sections of small bowel showing histologic intestinal injury grading. (Original magnification .40, Nikon microscope).[12]
Grade 1, Normal mucosal villi;
grade 2, development of mucosal sloughing;
grade 3, epithelial layer lifting up in sheets, presence of a few denuded villous tips, and mild capillary congestion;
grade 4, exposed lamina propria, dilated capillaries, and evidence of hemorrhage;
grade 5, digestion and disintegration of the lamina propria.



1



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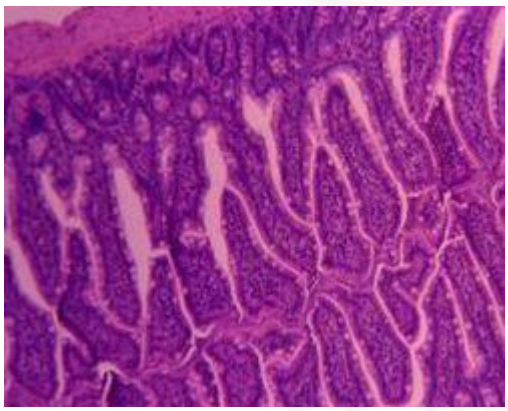

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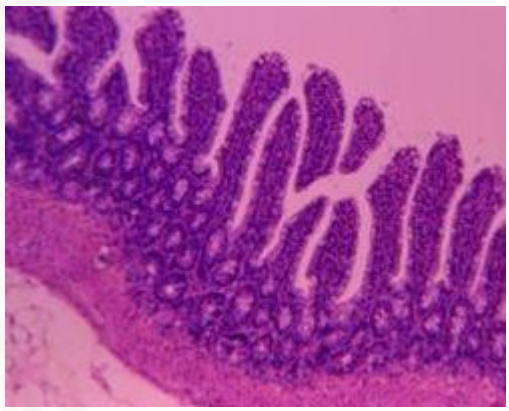

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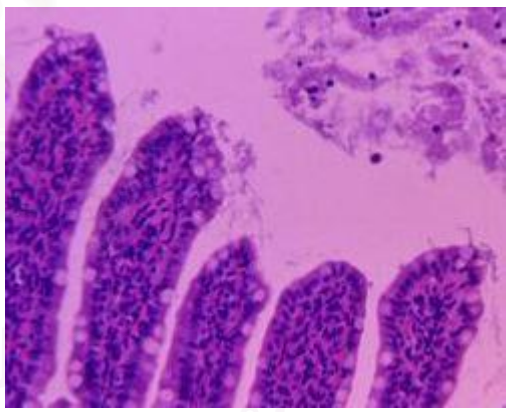

5

Figure 4.5 Representative stained section of terminal ileum in each group
(1) Group A
(2) Group B
(3) Group C
(4) Group D
(5) Tips of villi in Group D

Score	Group A	Group B	Group C	Group D
Grade 1	8	0	8	0
Grade 2	0	1	0	6
Grade 3	0	6	0	2
Grade 4	0	1	0	0
Grade 5	0	0	0	0

Table 4.3 The histological scores

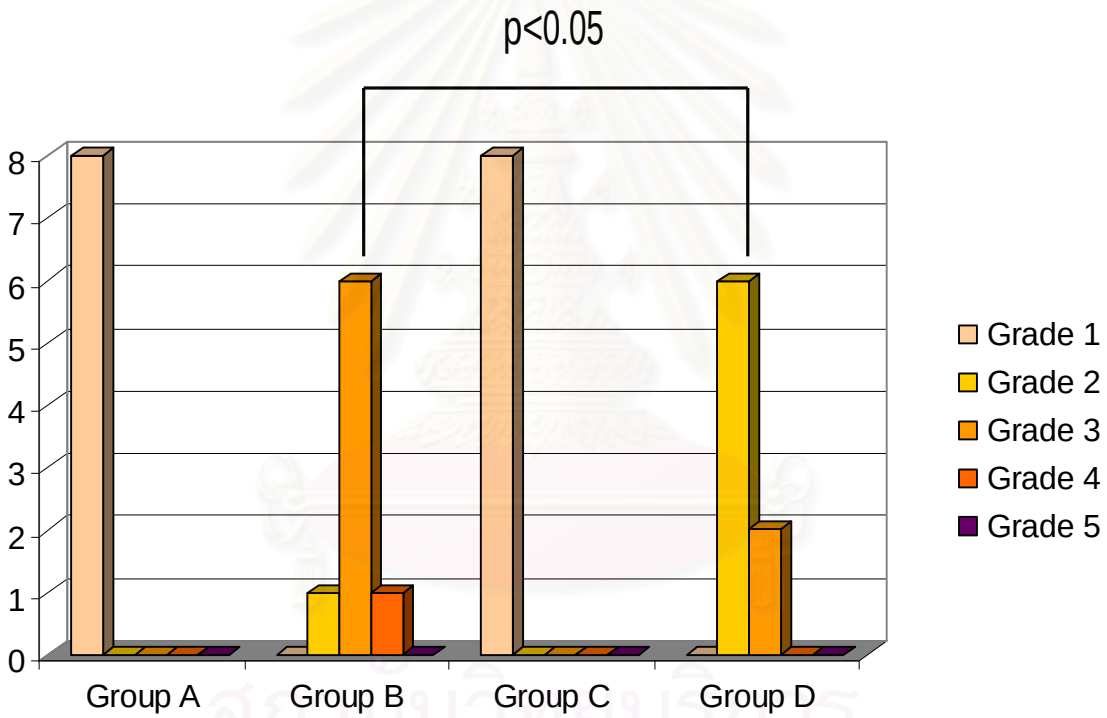

Figure 4.6 The histological scores

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Discussion

This study proposed two hypotheses. The first hypothesis was that whole-body-moderate hypothermia reduces serum inflammatory mediators following intestinal IR. This could be proved by measuring serum inflammatory mediators, which was collected from animals in the experiment. ELISA techniques were performed to determine the levels of three mediators involved in SIRS including TNF-α, IL-1β, and sICAM-1. The second hypothesis was that moderate hypothermia ameliorates the effects of intestinal ischemia-reperfusion injury. This could be proved by histological evaluation. Paraffin sections of terminal ileum were analyzed and histological injury was graded under a microscope.

TNF-α is the first cytokine in inflammatory process. Intestinal IR at normothermia (Group B) induced significant increases in serum TNF-α levels ($p<0.05$) compared with sham-operated animals (Group A). This result has been suggested that there is a systemic inflammatory response following intestinal IR and confirm the study by Lee-Wei Chen et al.[78] Intestinal IR at moderate hypothermia (Group D) had no significant difference in serum TNF-α levels compared with intestinal IR at northermia (Group B), although intestinal IR at hypothermia (Group D) resulted in significant decreases in histological injury ($p<0.05$) compared with intestinal IR at normothermia (Group B). This result suggested that moderate hypothermia did not effect the serum TNF-α levels after intestinal IR. Generally, TNF-α trigger inflammatory process, and inflammation also stimulate TNF-α production. This is probably due to the lasting inflammation following intestinal IR in Group D that stimulates TNF-α production.

There was no significant difference in serum IL-1β levels, a pro-inflammatory cytokine, in all experimental groups. There are several possible explanations. Firstly,

serum IL-1β may be not related to intestinal IR. Secondly, intestinal IR may bypass the activation of IL-1. Serum IL-1β levels did not change may cause by the differential time response. The study by Yamamoto et al. demonstrated that tissue IL-1β levels were significant increase at 120 min after reperfusion.[79] Finally, this study investigates intestinal IR but blood samples are collected from heart, not intestine. IL-1β, which are produced from intestine, may be diluted at the heart. Therefore, serum IL-1β levels are not a beneficial marker in this animal model.

ICAM-1 is an adhesion molecule that has an important role in leukocyte transmigration during inflammatory process. Intestinal IR at normothermia (Group B) caused an elevation of serum sICAM-1 levels and significant histological injury. Moderate hypothermia significantly attenuated the elevation of serum sICAM-1 and histological injury. These results suggest that moderate hypothermia is likely to decline the tissue injury at the leukocyte transmigration process. The mechanisms by which moderate hypothermia attenuated intestinal IR injury in this models is unlikely to be TNF-α de-activation but rather the other mechanisms that resulted in the decrease in ICAM-1 expression. In addition, there are no significant difference between the serum levels of TNF-α, IL-1β, and sICAM-1 in Group A and Group C (sham at hypothermia). This result suggested that moderate hypothermia did not decrease or increase the expression of TNF-α, IL-1β, and sICAM-1.

From the results of histological score, all of intestinal tissues in Group A are histologically normal, whereas intestinal tissues in Group B have significant musocal injury. These indicated that intestinal IR induced intestinal injury. Induction of moderate hypothermia without intestinal IR (Group C) did not affect intestinal histology. Comparison of histological score of Group B and Group D showed that Group D has less injury. These suggest that, histologically, moderate hypothermia attenuated tissue injury following intestinal IR.

5.2 Benefit

The small intestine may be accidentally or intentionally ischemic during surgery resulting in postoperative complications. It has been previously shown that moderate hypothermia (between 30◦C and 32◦C) has beneficial effects on liver function,[64] intestinal histology,[65] and prevents pulmonary neutrophil infiltration[80] subsequent to intestinal IR. The results of serum sICAM-1 levels from this study, together with previous studies showing the benefits of moderate hypothermia to the intestine, liver, lungs, and heart[81] supporting the potential clinical utility of moderate hypothermia in amelioration of multisystem organ failure caused by intestinal IR. However, clinical use has produced undesirable side effects such as lung complications, and some experimental models have demonstrated acute pancreatitis and inhibition of phagocytic activities of macrophages during the period of hypothermia.[82] Therefore, more studies on animals are needed to acquire further understandings of its mechanism before conducting a clinical trial of this model.

5.3 Future Work

This animal model was induced to moderate hypothermia at the beginning of the experiment, before ischemia, because of maximum effect. This model can be investigated as the prevention in the surgery, such as the surgery for aortic aneurysm that the SMA is sometimes clamped. The next step of investigation will induce moderate hypothermia during reperfusion period as the post-ischemic therapy.

The continuation of moderate hypothermia studies will provide the basis knowledge for setting up a clinical trial, and the development of further molecular techniques. Although this experiment made in small animals, such as rats, may not be directly applicable to larger animal models or clinical situations, the results will lead to further understanding of pathophysiology of intestinal IR, and to future possibilities for the clinical application.

5.4 Conclusion

This experimental study clearly demonstrated that moderate hypothermia attenuated serum sICAM-1 elevation and relieved histological injury of intestinal tissue following intestinal IR, although the attenuation did not appear in serum TNF-α levels. It is likely that one of the mechanisms by which moderate hypothermia attenuates the injury is at ICAM-1 signaling. This study provides more understanding in how moderate hypothermia affects intestinal IR. Further investigations are needed to decide whether induction of moderate hypothermia is a useful therapeutic means for intestinal IR.

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APPENDICES

APPENDIX A

Respiratory Rate (Times per minute)

Time (minutes)	A1	A2	A3	A4	A5	A6	A7	A8
0	80	84	100	80	84	80	68	64
15	84	84	80	80	96	84	68	76
30	76	80	80	76	96	80	68	64
45	80	80	80	72	72	80	72	68
60	80	76	80	76	80	84	76	64
75	84	80	76	76	84	84	76	60
90	76	84	76	80	84	84	72	64
105	80	84	80	80	80	80	72	60
120	80	76	80	80	80	80	72	64
Time (minutes)	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**
0	120	80	80	76	84	64	64	80
15	96	90	84	76	80	76	72	68
30	100	100	84	84	80	80	76	80
45	92	100	84	80	80	72	84	76
60	92	84	84	84	80	74	84	88
75	100	96	88	84	80	80	84	96
90	96	96	100	84	90	80	80	84
105	100	84	100	84	80	80	80	84
120	100	84	96	84	80	80	80	80
Time (minutes)	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**
0	52	60	64	56	52	56	64	60
15	80	60	64	56	48	56	64	64
30	80	72	68	56	52	52	64	64
45	80	64	68	56	56	52	60	68
60	80	64	64	56	52	56	56	64
75	60	60	60	52	48	56	52	60
90	60	60	60	52	52	56	52	56
105	64	56	56	56	52	60	52	56
120	64	56	56	56	52	56	52	56
Time (minutes)	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**
0	60	64	60	60	52	52	52	60
15	64	64	60	60	52	52	52	60
30	60	80	68	60	52	52	64	56
45	64	72	76	56	56	52	64	56
60	64	68	72	56	56	48	60	60
75	60	64	72	56	60	48	60	56
90	64	60	68	56	56	48	64	48
105	64	60	68	52	60	52	60	44
120	64	60	72	52	60	52	60	48

APPENDIX B

Temperature (Degrees Celsius)

Time (minutes)	A1	A2	A3	A4	A5	A6	A7	A8
0	37.4	36.6	36.5	36.5	37.0	36.6	36.2	36.0
15	36.0	36.0	36.0	36.6	36.9	37.0	36.4	36.2
30	36.1	36.0	36.1	36.8	36.9	37.2	37.0	36.2
45	36.0	36.1	36.2	37.1	36.9	36.7	36.5	37.2
60	36.0	36.3	36.0	36.7	36.7	37.0	36.5	36.9
75	36.2	36.5	36.1	36.6	37.1	37.4	36.7	36.4
90	36.6	36.6	36.0	36.5	37.0	37.5	36.5	36.4
105	36.5	36.5	36.3	36.7	36.6	37.2	36.7	36.4
120	36.7	36.5	36.5	36.7	36.5	37.1	36.7	36.6
Time (minutes)	B1	B2	B3	B4	B5	B6	B7	B8
0	36.0	36.7	36.7	36.1	36.1	36.0	36.0	36.8
15	36.1	36.4	36.6	36.0	36.0	36.0	36.1	36.9
30	36.2	36.5	36.5	36.1	36.2	36.6	36.3	36.7
45	36.3	36.4	36.3	36.0	36.5	36.8	36.6	36.9
60	36.4	36.1	36.8	36.0	36.7	37.0	37.0	36.7
75	36.4	36.5	37.2	36.2	37.0	37.2	37.0	37.0
90	36.4	36.4	37.3	36.3	37.1	37.1	36.2	36.7
105	36.5	36.3	37.4	36.5	36.9	37.1	36.5	37.0
120	36.7	36.3	37.4	36.5	36.9	37.1	36.7	36.9
Time (minutes)	C1	C2	C3	C4	C5	C6	C7	C8
0	32.2	32.2	32.2	32.2	32.0	32.2	32.4	33.0
15	32.5	32.2	32.1	32.3	32.0	32.1	32.3	32.3
30	32.0	32.5	32.8	32.0	32.1	32.1	32.2	32.0
45	32.3	32.0	32.2	32.5	32.5	32.5	32.2	32.2
60	32.3	32.6	32.3	32.4	32.7	32.0	32.1	32.5
75	32.5	32.0	32.0	32.0	32.2	33.0	32.2	32.1
90	32.1	32.5	32.0	32.2	32.3	32.1	32.3	32.2
105	32.6	32.0	32.2	32.4	32.5	32.4	32.0	32.0
120	32.0	32.0	32.6	32.3	32.2	32.0	32.3	32.0
Time (minutes)	D1	D2	D3	D4	D5	D6	D7	D8
0	32.1	32.0	32.0	32.0	32.0	32.5	33.0	32.9
15	32.5	32.0	32.2	32.4	32.0	32.4	32.1	32.1
30	32.2	32.6	32.1	32.2	32.1	32.5	32.3	32.1
45	32.1	32.4	32.5	32.2	32.3	32.5	32.3	32.0
60	32.3	32.3	32.2	32.0	32.0	32.1	32.3	32.5
75	32.0	32.5	32.1	32.7	32.5	32.7	32.0	32.4
90	32.5	32.0	32.6	32.3	32.0	32.3	32.4	32.3
105	32.1	32.9	32.0	32.0	32.3	32.3	32.3	32.1
120	32.7	32.6	32.4	32.2	32.4	32.2	32.3	32.3

APPENDIX C

Solutions

1) 10% Buffered Neutral Formalin (pH 7.0)

Formaldehyde, 37%-40%	100.0	ml
Distilled water	900.0	ml
Sodium phosphate monobasic ($NaH_2PO_4H_2O$)	4.0	gm
Sodium phosphate dibasic (Na_2HPO_4) anhydrous	6.5	gm

2) Harris's Hematoxylin and Eosin

Harris's Hematoxylin		
Hematoxylin	5.0	gm
Alcohol, 100% ethyl	50.0	ml
Potassium or ammonium, alum	100.0	gm
Distilled water	1000.0	ml
Mercuric oxide, red	2.5	gm
Eosin Stock Solution		
EosinY, water soluble	1.0	gm
Distilled water	100.0	ml
Phloxine Stock Solution		
Phloxine B	1.0	gm
Distilled water	100.0	ml
Eosin-Phloxine Working Solution		
Eosin Stock Solution	100.0	ml
Phloxine Stock Solution	10.0	ml
Alcohol, 95% ethyl	780.0	ml
Acetic acid, glacial	4.0	ml

3) Blueing Agent

2% aqueous sodium bicarbonate or saturated lithium carbonate

APPENDIX D

Raw Data

Animals	TNF (pg/ml)	IL-1 (pg/ml)	ICAM-1 (pg/ml)	Grading
A1	25.86	85.55	27549.11	1
A2	22.98	73.44	27771.15	1
A3	29.97	55.27	36592.10	1
A4	25.04	68.39	26943.56	1
A5	22.98	67.38	27448.19	1
A6	23.39	70.41	28962.08	1
A7	24.22	105.73	38328.04	1
A8	23.80	118.85	33261.54	1
B1	41.08	77.47	40144.71	2
B2	33.68	76.46	40709.90	3
B3	48.48	59.31	56272.72	3
B4	78.50	67.38	43394.53	4
B5	84.26	70.41	46563.62	3
B6	125.80	70.41	37883.96	3
B7	30.80	90.59	44968.98	3
B8	43.96	112.80	54294.57	3
C1	39.02	72.43	39680.45	1
C2	26.68	89.58	35542.47	1
C3	32.85	71.42	33160.61	1
C4	35.32	80.50	37702.29	1
C5	30.38	90.59	36814.14	1
C6	22.16	96.65	33665.24	1
C7	25.45	105.73	32676.17	1
C8	29.15	104.72	32030.24	1
D1	63.29	86.56	43394.53	2
D2	92.90	104.72	43212.87	3
D3	110.99	87.56	35300.25	2
D4	34.50	93.62	29042.82	2
D5	31.21	109.77	35986.55	3
D6	51.77	124.91	42788.98	2
D7	40.67	97.66	29305.23	2
D8	54.65	98.67	30879.68	2

Biography

Miss Nuchanan Leawhiran was born on 13th November 1979 in Bangkok. I graduated from Faculty of Science, Chulalongkorn University with a Bachelor degree of science in Biology in 2001. I have enrolled at Chulalongkorn University in graduate programme for the Master's degree of Science in Medical Science, Faculty of medicine in 2002.