EFFECT OF ZINC SUPPLEMENTATION ON THE MORPHOLOGY AND PERMEABILITY OF THE SMALL INTESTINE MUCOSA SECONDARY TO CHRONIC MILD STRESS IN RATS

by

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
APC	Antigen-presenting cells
CD	Crohn's disease
CRF	Corticotrophin releasing factor
CRH	Corticotrophin-releasing hormone
CRS	Cold restraint stress
DTH	Delayed type hypersensitivity
EDTA	Ethylene diamine tetraacetic acid
FITC	Fluorescein isothiocyanate dextran
fMLP	N-formylmethionylleucyl-phenylalanine
G.A.S.	General adaptation syndrome
GALT	Gut-associated lymphoid tissues
GI	Gastrointestinal
GLP	Glucagon-like peptide
GLUT	Glucose transporter
H&E	Hematoxylin and eosin
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
KLF	Kruppel-like factor

MLN	Mesenteric lymph nodes
MMC	Mucosal mast cells
MT	Metallothionein
MTX	Methotrexate
MUC	Mucins
MYC	Myelocytomatosis
NK	Natural killer
PAS	Periodic acid schiff
PG	Prostaglandins
PKC	Protein kinase C
PML	Polymorphonuclear leukocyte
ROS	Reactive oxygen species
RS	Restraint stress
SOD	Superoxide dismutase
Tff	Trefoil factor
TGF	Transforming growth factor
Th	T helper
TNF	Tumor necrosis factor
UC	Ulcerative colitis
WAS	Water avoidance stress
WIRS	Water immersion restraint stress

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KESAN PENAMBAHAN ZINK KE ATAS TATABENTUK DAN KETELUSAN MUKOSA USUS KECIL SEKUNDER KEPADA TEKANAN SEDERHANA KRONIK TERHADAP TIKUS-TIKUS

ABSTRAK

Tekanan psiko-fisiologi telah menarik secara meluas penyelidik-penyelidik untuk mengkaji kesan-kesan tekanan pada sistem fisiologi. Walaubagaimanapun, tiada kajian berkenaan peranan faedah Zn dalam tekanan-mencetuskan perubahan fisiologi telah mapan. Oleh itu, kajian ini meneliti kesan penambahan zink pada parameter-parameter fisiologi, tatabentuk, ketelusan dan infiltrasi sel radang mukosa usus kecil sekunder kepada tekanan sederhana kronik terhadap tikus-tikus. 40 ekor tikus-tikus Sprague Dawley jantan berumur 6 minggu dengan berat badan badan mean 149.69 ± 3.92 g yang dibahagikan ke dalam 4 kumpulan (n=10 bagi setiap kumpulan); kumpulan kawalan (C), kawalan dengan penambahan zink (CZ), tekanan (S) dan tekanan dengan penambahan zink (SZ). Tekanan pengelakan air untuk 1 jam diberi setiap hari untuk 10 hari berterusan. Penambahan berat badan, ambilan makanan dan air dan bilangan ketulan najis selepas setiap sesi tekanan telah didokumentasikan sepanjang kajian. Pada hari ke-11, selepas satu malam berpuasa, tikus-tikus dibius dengan diethyl-ether dan solusi fluorescein isothiocyanate dextran disuntik ke dalam gelungan usus kecil. Selepas satu jam, tikus-tikus dibius lagi dan darah dikumpulkan dari abdominal aorta untuk menyukat ketelusan usus. Tikus-tikus telah dikorbankan dan ileum dan jejunum telah diambil untuk penilaian tatabentuk dan ujian sel radang. Perbezaan statistik telah dibuat dengan one-way analysis of variance diikuti dengan ujian Post Hoc Tukey. Sesi-sesi tekanan harian di dalam kajian ini telah dengan signifikasinya mengurangkan penambahan berat badan kumpulan S jika dibandingkan dengan kumpulan C, walaupun tiada perbezaan signifikasi dilihat telah di dalam kumpulan yang sama perbandingan ambilan

makanan dan air. Walaubagaimanapun, kumpulan SZ telah dengan signifikasinya meningkatkan penambahan berat badan dan ambilan makanan jika dibandingkan dengan kumpulan S. Bilangan ketulan najis telah dengan signifikasinya tinggi di dalam kumpulan S jika dibandingkan dengan kumpulan C dan kumpulan SZ jika dibandingkan dengan kumpulan CZ. Kumpulan S telah dengan signifikasinya pendek kedalaman crypt di dalam ileum, rendah ketinggian villus di dalam jejunum, rendah bilangan goblet sel di dalam crypts ileum dan jejunum dan rendah bilangan goblet sel di dalam villus jejunum jika dibandingkan dengan kumpulan C. Kumpulan SZ telah dengan signifikasinya panjang kedalaman crypt, tinggi ketinggian villus dan tinggi bilangan goblet sel di dalam crypt dan villus ileum dan jejunum jika dibandingkan dengan kumpulan S. Kumpulan S telah dengan signifikasinya tinggi ketelusan FITC di dalam usus kecil dan infiltrasi sel radang di dalam ileum dan jejunum jika dibandingkan dengan kumpulan C. Walaubagaimanapun, kumpulan SZ telah dengan signifikasinya rendah ketelusan FITC di dalam usus kecil dan infiltrasi sel radang di dalam ileum dan jejunum jika dibandingkan dengan kumpulan S. Secara kesimpulannya, tekanan sederhana kronik mengkompromikan tatabentuk mukosa usus kecil, ketelusan dan infiltrasi sel radang yang mana telah berkurangan oleh penambahan zink. Penemuan ini mengusulkan bahawa zink berkemungkinan terlibat di dalam pencegahan ketidakfungsian halangan usus kecil sekunder kepada tekanan sederhana kronik dan barangkali menjadi nutrisi harapan untuk penyakit berkaitan dengan ketidakfungsian halangan usus.

EFFECT OF ZINC SUPPLEMENTATION ON THE MORPHOLOGY AND PERMEABILITY OF THE SMALL INTESTINE MUCOSA SECONDARY TO CHRONIC MILD STRESS IN RATS

ABSTRACT

Psycho-physiological stress has widely attracted researchers to investigate the effects of stress on physiological system. However, no study about the beneficial role of zinc in stress-induced physiological alterations has been established. Hence, this study examines the effect of zinc supplementation on the physiological parameters, morphology, permeability and inflammatory cell infiltration of the small intestine mucosa secondary to chronic mild stress in rats. 40 male Sprague Dawley rats aged 6 weeks with a mean body weight of 149.69 ± 3.92 g were divided into 4 groups (n=10 for each group); control (C), control with zinc supplement (CZ), stress (S) and stress with zinc supplement (SZ) groups. Water avoidance stress for 1 hour was given daily for 10 consecutive days. Body weight gain, food and water intake and number of stool pellet after every stress session were documented throughout the study. On the 11th day, after overnight fasting, rats were anesthetized with diethyl-ether and fluorescein isothiocyanate dextran solution was injected into small intestinal loop. One hour later, rats were re-anesthetized and blood was collected from the abdominal aorta to measure the intestinal permeability. Rats were sacrificed and ileum and jejunum were taken for histological evaluation and inflammatory cell assay. Statistical comparisons were made by one-way analysis of variance, followed by Post Hoc Tukey test. Daily stress sessions in this study had significantly reduced the body weight gain of S group as compared to C group, although no significant difference was seen in the same groups' comparison of food and water intake. However, SZ group had significantly increased the body weight gain and food intake as compared to S group. The number of stool pellet was significantly higher in S

group as compared to C group and SZ group as compared to CZ group. S group had significantly shorter crypt depth in ileum, lower villus height in jejunum, lower number of goblet cell in crypt of ileum and jejunum and lower number of goblet cell in villus of jejunum as compared to the C group. SZ group had significantly longer crypt depth, higher villus height and higher number of goblet cell in crypt and villus of ileum and jejunum as compared to the S group. S group had significantly higher permeability of FITC in small intestine and inflammatory cell infiltration in ileum and jejunum as compared to C group. Yet, SZ group had significantly lower permeability of FITC in small intestine and inflammatory cell infiltration in ileum and jejunum as compared to S group. In conclusion, chronic mild stress compromised the small intestinal mucosal morphology, permeability and inflammatory cell infiltration which were attenuated by zinc supplementation. These findings suggest that zinc might be involved in the prevention of small intestinal barrier dysfunction secondary to chronic mild stress and possibly be a promising nutrient for diseases related to intestinal barrier dysfunction.

CHAPTER ONE
INTRODUCTION

1.1 Intestine

1.1.1 Background and discoveries of intestine

The wall of the gastrointestinal (GI) tract is formed by four distinct functional layers, which are the mucosa, submucosa, muscularis and serosa or fibrosa (Figure 1.1).

The mucosa is the inner most layer of the cavity of GI tract and consists of three layers, which are the epithelial lining, lamina propria and muscularis mucosa. Cells of the epithelial lining are joined together by tight junctions that create a barrier, restricting uptake of luminal material (Bhagavan, 2002). The type of cells varies among different parts of the GI tract. The inner surface of mouth, tongue, pharynx and esophagus are lined by stratified squamous epithelial cells, whereas, the stomach, small intestine and large intestine are lined by columnar epithelial cells. The lamina propria lies just below the epithelial lining and is formed by connective tissues that contain fibroblasts and various immunocytes such as mast cells, macrophages, neutrophils, lymphocytes and eosinophils. These cells react nonspecifically to certain bacterial products or specifically to foreign protein antigens (Steven, 2005). Therefore, their numbers increase during inflammation. The muscularis mucosa which is the outermost layer of the mucosa consists of a thin layer of smooth muscle fibers. This layer is absent in both the mouth and pharynx but is present from the esophagus downwards (Lichtenstein, Rustgi, & Wu, 2004).

The submucosa is situated below the mucosa. This layer is present from the esophagus downwards but is absent in the mouth and pharynx. The submucosa is made of loose collagen fibers, elastic fibers, reticular fibers and a few connective tissue cells. Blood vessels, lymphatic vessels and nerve plexuses are present in this layer. The submucosal plexus (Meissner's nerve plexus) is present in between this layer and the muscularis mucosa. It is responsible for the regulation of the secretory function of the GI tract and the constriction of blood vessels (Badylak et al., 2002).

The muscularis layer of the GI tract is a muscular layer made of two types of muscles; skeletal and smooth muscles. The muscularis of lips, cheeks and wall of pharynx consists of only skeletal muscle fibers but the esophagus has both skeletal and smooth muscle fibers. The wall of the stomach and intestine is formed by smooth muscle fibers. The muscle fibers of stomach are arranged in three layers which are inner oblique layer, middle circular layer and outer longitudinal layer, whereas, smooth muscle fibers of the intestine are arranged in two layers which are inner circular layer and outer longitudinal layer. The myenteric plexus (Auerbach's nerve plexus) is present between the circular and longitudinal muscle fibers to regulate the movements of GI tract (Sembulingam & Sembulingam, 2003).

The outermost layer of the wall of GI tract is the serosa or fibrosa. The serosa is formed by connective tissue and mesoepithelial cells. It covers the stomach, small intestine and large intestine, whereas, the fibrosa is formed by connective tissue which covers the pharynx and esophagus (Gourtsoyiannis, 2002).

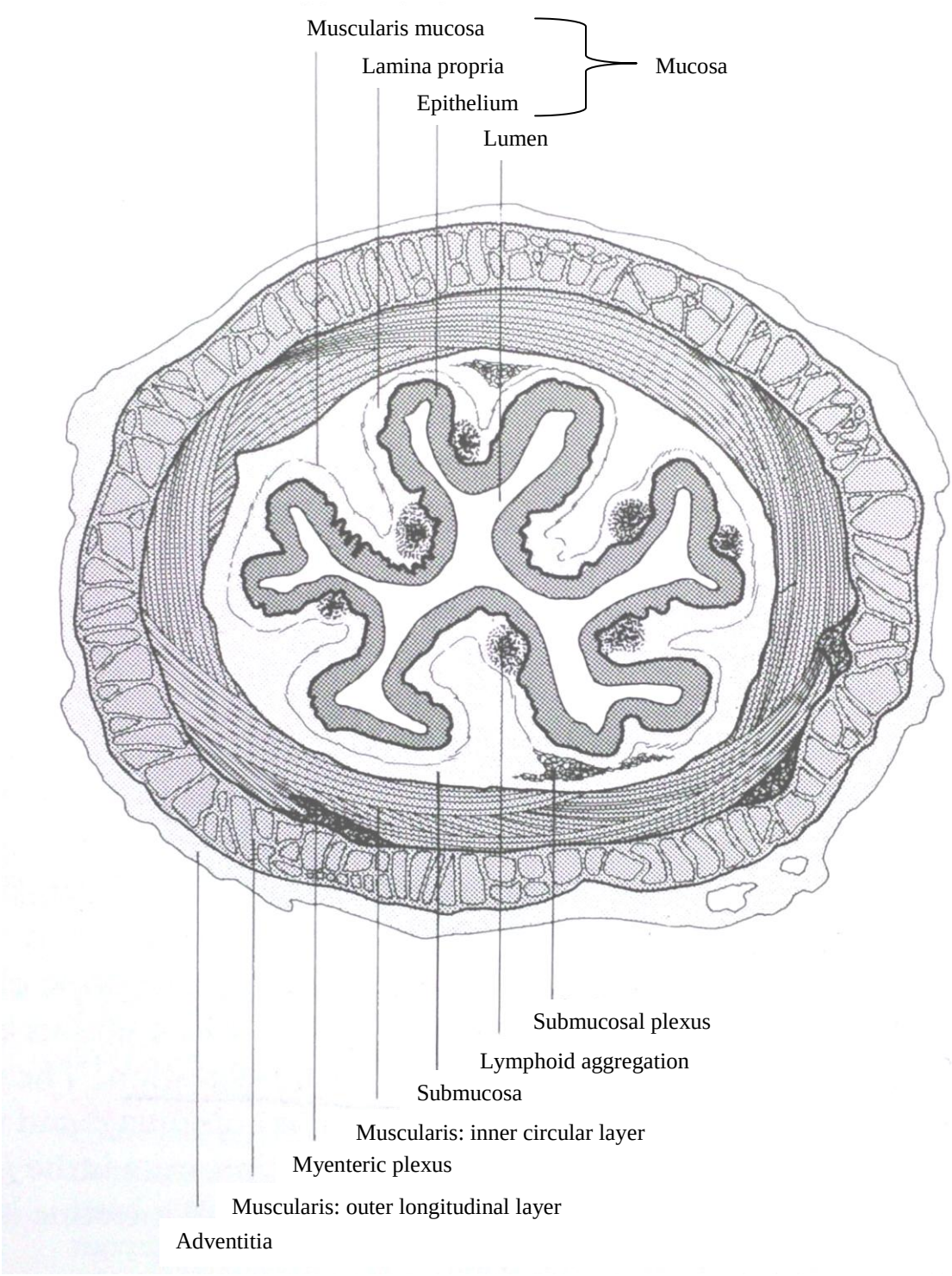

Figure 1.1: Structure of intestinal wall (Adapted from Young et al., 2006)

1.1.2 Small intestine

The small intestine is the part of the GI tract extending between the pyloric sphincter of the stomach and ileocecal valve which opens into the large intestine. It is described as small intestine as it has a small diameter compared to large intestine. The small intestine consists of three parts: proximal (duodenum) part, middle (jejunum) part and distal (ileum) part (Herlinger, Maglinte, & Birnbaum, 2001). The functional importance of the small intestine is absorption as this is where most of the absorption of digested food products takes place. In a recent finding, biopsy of the duodenum and upper jejunum shows a 'saw tooth' appearance of the villi, which is reported to be lacking in the ileum. It also shows the more proximal part of the small intestine, the fewer goblet cells in the villus epithelium (Prasad, 2007).

The mucous membrane of the small intestine is covered by minute projections called villi. The height of the villi is about 1 mm and the diameter is less than 1 mm (Marieb & Hoehn, 2009). The villi are lined by columnar cells which are called enterocytes. They give rise to a hair like projections called microvilli. The villi and microvilli increase the surface area of the mucous membrane by many folds. Within each villus there is a central channel called a lacteal. This channel opens into the lymphatic and blood vessels (Tortora & Grabowski, 2003).

The crypts of Lieberkuhn or intestinal glands are simple tubular glands of intestine. The intestinal glands do not penetrate the muscularis mucosa of the intestinal wall but open into the lumen of intestine between the villi (Seeley, Stephens, & Tate, 2008). Intestinal glands are lined by columnar cells. The lining of each gland is continuous with the epithelial lining of the villi (Figure 1.2). The

epithelial cells lining the intestinal glands undergo division by mitosis more rapidly than other body tissues (Duff & Ettarh, 2002). The newly formed cells push the older cells upward over the lining of villi. The cells which move to the villi are called enterocytes. These cells secrete enzymes for digestion and absorption as well as transport substances from the intestinal lumen to the circulatory system. Old enterocytes are continuously shed into the lumen every 3 to 5 days (Söderholm et al., 1999) along with enzymes. There are 3 types of cells interposed between columnar cells of the glands; argentaffin or enterochromaffin cells which secrete cholecystokinin and secretin hormones, goblet cells which secrete mucus, and Paneth cells which secrete cytokines called defensins (Sembulingam & Sembulingam, 2003).

In addition to intestinal glands, the first part of the duodenum contains mucus gland which is called Brunner's gland. This gland penetrates the muscularis mucosa and extends up to the submucosa coat of the intestinal wall. It opens into the lumen of intestine directly and secretes mucus and traces of enzymes (Young et al., 2006).

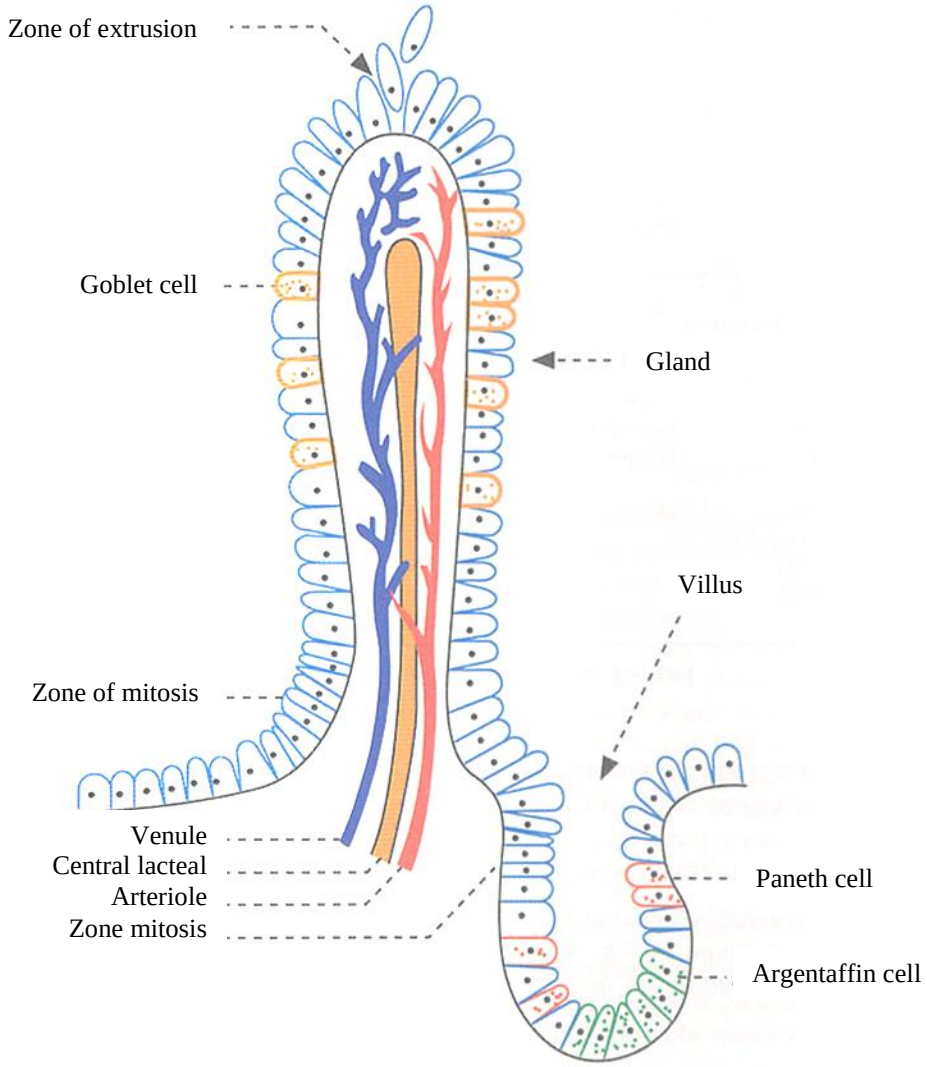

Figure 1.2: Intestinal gland and villus (Adapted from Sembulingam & Sembulingam, 2003)

Small intestinal crypts are the residence of stem cells which provide a continuous replenishment of villi epithelial cells that die and are discarded into the lumen (Johnson & Barrett, 2006). Protection of these stem cells is important for long term maintenance of the intestinal epithelium. Location of Paneth cells adjacent to stem cells suggests that they play a significant role in defending epithelial cell renewal through its antimicrobial function (Garabedian et al., 1997).

Nevertheless, decreased crypt cell proliferation may result in a disruption of mucosal structure and function. Duff and Ettarh (2002) have demonstrated that deficiency of zinc (Zn) caused lower rate of crypt cell division in rats' jejunum. However, no direct proof has been reported to indicate whether the decreased mucosal cell proliferation is involved with decrease crypt cell division and migration to the villus tip. Moreover, Simmen et al. (2007) found that Kruppel-like factor 9 (KLF-9) controls elaboration of intestinal smooth muscle, molecular mediators of crypt cell proliferation, villus cell migration and Paneth and goblet cell differentiation. A more recent finding showed insulin-like growth factor-2 (IGF-2) is essential in increased crypt fission and accelerated the adaptation of crypt number associated with increased insulin-like growth factor-1 (IGF-1) and accelerated the adaptation of mucosal mass of jejunum followed by ileocecal resection (Garrison et al., 2008). All these findings demonstrated the importance of the crypt in maintaining and sustaining intestinal morphology.

Many studies have emphasized the role of goblet cells for epithelial lining preservation. Goblet cells are distributed throughout the crypts and villi which produces epithelial-protective mucins 2 (MUC2) and trefoil factor-3 (Tff3). These

two products have been demonstrated to be involved in post injury repair of the small intestine (Beck et al., 2004). Aside from this, Helmrath et al. (2007) found that mRNA C-myelocytomatosis (C-MYC) is involved with goblet cells proliferation while mRNA Tff3 and MUC2 are associated with goblet cells differentiation. In the current finding, Zheng et al. (2008) discovered that goblet cells differentiation is controlled by Kruppel-like factor 4 (KLF-4) gene expressions which are inhibited by the notch signaling pathway.

Apart from that, it is essential to understand the factors that are involved in the changes of intestinal morphology. 2 weeks of total parenteral nutrition has been noticed to cause intestinal atrophy and low quantity of mucus gel (Sakamotoa et al., 2000). Hemorrhagic shock also has been reported to damage gut morphology of rats by causing injury and atrophy of the intestinal mucosa which was followed by increased intestinal permeability (Chang et al., 2005). Besides, intestinal allergic reaction in the rats by horseradish peroxidase (HRP) has caused alteration of the architecture of jejunal mucosa with swelling of the villi due to edema and detachment of the lining epithelium from the basement membrane by blebs-like materials as well as disruption of the lining epithelium detached from the basement membrane (Yang et al., 2001). In addition, acute diarrhea with *Escherichia coli* infection also altered intestinal morphology with diminution of the villus length and crypt depth in piglets (Hornich et al., 2010). Despite all the factors, research related to stress induced intestinal morphological changes is very limited. Nevertheless, there has been a study involving water avoidance stress which led to the degeneration of the epithelial and glandular cells of the rat ileum (Ersoya et al., 2008).

It is important to maintain healthy intestinal morphology to protect intestinal mucosal barrier as well as to prevent higher permeability of the intestine. Intestinal barrier protects the body from different antigens present in the intestinal lumen. This barrier consists of enterocytes which are joined at the apical poles by tight junctions to form a physical barrier. It is also dynamically active and regulated by neuroendocrine and immunological factors (Soderholm et al., 1999). Under normal circumstances, tight junctions are impermeable to proteins (Di Leo et al., 2002) but permeable to ions (Cameron & Perdue, 2005; Zareie et al., 2006; Keita et al., 2007) and small molecules. However, under certain conditions such as cholinergic stimulation with carbachol, HRP has been reported to penetrate the tight junctions (Bijlsma et al., 1996). Carbachol stimulated Ca^{2+} release from intracelllular stores which activated protein kinase C (PKC) in epithelial cell culture (Luo, Lindeman, & Chase, 1992). Activation of PKC has been connected with loosening of tight junctions (Stenson et al., 1993). This process results in decreased resistance of tight junctions on epithelial monolayers associated with F-actin rearrangements and phosphorylation of myosin light chain in response to T cell activation or bacterial attachment (Spitz et al., 1995; McKay, Croitoru, & Perdue, 1996). The intestinal mucosal barrier might also be compromised in several conditions such as nutritional deprivation (Rodriguez, Darmon & Chappuis, 1996) during prolonged total parenteral nutrition (Khan et al., 1999), immune deficiency states (Madsen et al., 1999) and stress (Saunders et al., 2002).

1.1.3 Mucosal immunity of intestine

Approximately 25% of the intestinal mucosa and submucosa weights are contributed by gut-associated lymphoid tissues (GALT) which comprises the largest

extra thymic site of lymphocytes in humans (McBurney, 1993). GALT cells respond to gut pathogens through three stages. Initially, antigens are processed for recognition by lymphocytes. Then, a cascade of specialized immune responses to antigens is initiated by regulating the migration of immune mediators from periphery to the infected area. Finally, cytotoxic activities are participated to limit pathogens establishment and survival (Van der Hulst et al., 1998). Beside these specific immunological responses, intestinal mucosa provides nonspecific barrier function. Formation of tight junctions and secretion of mucus prevent the entry of pathogenic antigens, meanwhile, rapid mucosal cell turnover enables the repair of epithelial or lymphoid cells damaged during pathogenic infections (Welsh et al., 1998).

The intestinal mucosal immune system comprises of two separate anatomical and functional compartments. The specialized local inductive sites are made of Peyer's patches, isolated lymphoid follicles and mesenteric lymph nodes and these are where intestinal antigens are first recognized (Bogen, Weinber, & Abbas, 1991; Laissue et al., 1993; Regoli et al., 1994). The other site is diffuse effector sites consists of intraepithelium and lamina propria and this is the site where the outcome of an effective immune response eliminates the infectious agents (Beagley & Elson, 1992).

Antigens from the intestinal luminal foods and pathogens are transported across epithelial barrier through specialized epithelial microfold cells or intraepithelial lymphocytes to organized lymphoid tissues within mucosa. Then, the antigens are processed and presented by antigen-presenting cells (APC), for instance dendritic cells, B cells, macrophages and other intestinal epithelial cells. Afterwards,

naive T lymphocytes interact with antigen-primed APC in aggregated Peyer's patches and single lymphoid follicles and further differentiate in the germinal centers of lymphoid follicles. Following this, the antigen-specific T and B cells leave the epithelial barrier to travel in the mesenteric lymph nodes (MLN). Then, they are moved into the mucosa and the peripheral blood stream with either intestinal-derived or locally activated immune cells. The lymphocytes migrate to systemic lymphoid tissues (spleen and peripheral lymph nodes) from blood stream, where the lymphocytes proliferate and mature into effector lymphocytes (secrete cytokines and mediate T cell-dependent humoral immunity) or memory cells (rapidly respond to the infection on the secondary encounter) (Nagler, 2001). Peripheral lymphocytes preferentially leave the blood stream and move into the intestinal lamina propria and intraepithelium (Mennechet et al., 2002; Kamada et al., 2010) by expressing adhesion receptors recognized through specific endothelial molecules lining the gut mucosal lymphoid tissues (Smith & Weis, 1996).

GALT protects the intestine against pathogens by continuous movement of lymphocytes from gut lymphoid tissues to and back the blood stream. It enables the delivery of the pathogenic antigens to peripheral sites by disseminating immune response widely and promotes gut to receive lymphocytes from blood stream of effector sites within the intestinal epithelium. It also secretes cytokines that regulate the suitability, level and phenotypic expression of immune responses (Mosmann & Sad, 1996; Finkelman et al., 1997).

Depending on the type of antigen stimulus, undifferentiated T helper (Th) cells transform into either Th1 or Th2 cells (Figure 1.3). Th1 is usually stimulated by

virus, bacteria and protozoan infections. It is known as cell-mediated immunity, involving phagocytosis in functional immunity and is characterized by elevated levels of Th1 cytokines [for example interferon (IFN)-γ, interleukin (IL)-2, IL-12] and effectors (for example macrophages, natural killer cells, neutrophils) (Salmi & Jalkanen, 1991; Kagnoff, 1996). Meanwhile, Th2 responds to intestinal extracellular parasites (nematode) infection. It depends on the Th2 cytokines (for example IL-4, IL-5, IL-10) production which mediate antibody-dependent (humoral immunity) effector responses (Moran et al., 2006). These cytokines are delivered into GALT which attract progenitors of B cell, mucosal mast cells (MMC) and eosinophils by chemo-taxis to the mucosal epithelium where they are proliferated and matured (Wershil et al., 2002). Th2 cytokines also promote mucosal mastocytosis, proliferation of IgE-secreting B cells in situ, transport of systemic IgE into the intestinal lumen of nematode-infected mice and IgE-mediated MMC degranulation and release of cytotoxic inflammatory substances (Ramaswamy, Negrao-Correa, & Bell, 1996; Wastling et al., 1997).

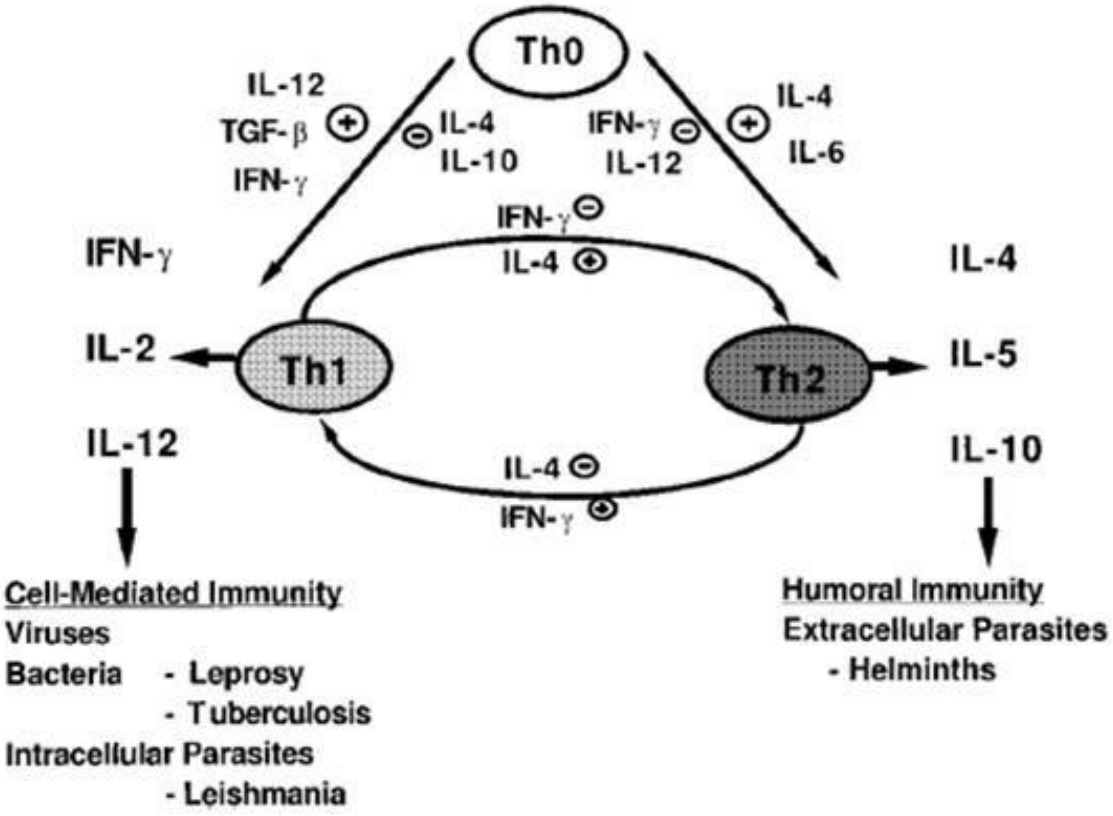

Figure 1.3: Differentiation and cross regulation of T helper (Th) cells in relation to the cytokine milieu and infectious agent (Adapted from Mosmann & Sad, 1996).

Note: (TGF) Transforming growth factor, (+) Stimulatory effect, (-) Inhibitory effect.

1.1.4 Intestinal diseases of barrier integrity disorder

The GI barrier has immunological and non-immunological components. The non-immunological components are essential for the GI protective barrier which prevent absorption of damaging substances from the external environment (Farhadi et al., 2003). This defensive functions of the GI mucosa is described as permeability, which is highly selective and permits the absorption of nutrients, water and electrolytes from lumen but prevents the passsage of proinflammatory molecules into the mucosa. In contrast, loss of GI barrier integrity allows the penetration of normally excluded luminal substances into the mucosa and leads to the initiation or continuation of inflammatory processes and mucosal damage (Hollander, 1998; Banan et al., 1999). Hyperpermeability of this barrier is believed to contribute in the pathogenesis of several GI disorders including inflammatory bowel disease (IBD) and celiac disease (Keshavarzian et al., 1999).

IBD is a group of pathologic conditions of the GI tract namely Crohn's disease and ulcerative colitis (Scaldaferri & Fiocchi, 2007). They are associated with intestinal and extra-intestinal clinical symptoms including weight loss, diarrhea with blood and mucus, fever, gastric dysmotility and shortening of the colon (Fiocchi, 1998; Hendrickson, Gokhale, & Cho, 2002). The pathogenesis of IBD is associated with an increase in inflammatory mediators consisting of reactive oxygen species (ROS) such as nitric oxide (NO) (Kolios, Valatas, & Ward, 2004; Araki, Sugihara, & Hattori, 2006) and prostaglandins (PG) (Singer et al., 1998; Bonner, 2002) as well as an incline of inflammatory cytokines like tumor necrosis factor (TNF)-α, IL-1 and IL-6 (Rojas-Cartagena, Flores, & Appleyard, 2005; Kwon et al., 2005; Mudter & Neurath, 2007). Moreover, general feature of IBD in inflammatory mediators such as

cytokine interplay with intestine may provide a key puzzle for the etiology of IBD (Kolios, Valatas, & Ward, 2004; Araki, Sugihara, & Hattori, 2006; Mudter & Neurath, 2007; Brown & Mayer, 2007).

Crohn's disease (CD) has been independently described by Lesniowski, a Polish surgeon in 1904 and by Burrill Bernard Crohn, an American gastroenterologist in 1932 as the disease named after. Crohn with his colleagues described a series of patients with inflammation of the terminal ileum, commonly affected by the disease (Loftus, Schoenfeld, & Sandborn, 2002). CD is a chronic, periodic and inflammatory condition of the GI tract, identified by transmural inflammation of the entire wall of infected area and skip lesions of inflammatory area with normal lining in between (Podolsky, 2002; Shanahan, 2002). Histomorphological features of CD are aphthous ulcers of the mucosa, mural abscesses, suppurative fistulas, macrophage and epithelioid cell granulomas (Zumla & James, 1996). The common GI tract symptoms of this disease are abdominal pain, diarrhea with blood and mucus, constipation, vomiting and weight loss (Hanauer, 1996; Podolsky, 2002; Mueller et al., 2002; Fix et al., 2004) while external GI tract symptoms are skin rashes, arthritis and inflammation of the eye (Danese et al., 2005; Bernstein, 2006). Although the pathogenesis of CD is still unknown but the interacting components of genetic susceptibility factors, immune-mediated tissue injury and infectious agents in ample discoveries have suggested the etiology of the disease (Quirke, 2001; Shanahan, 2002; Podolsky, 2002; Darfeuille-Michaud et al., 2004). Genetic factors, associated with autoimmune disease have been invoked for this riddle. The condition occurs as the immune system contributes to damage the GI tract by causing inflammation (Ogura et al., 2001; Cobrin & Abreu, 2005). Besides

this, the role of luminal bacteria has also been suggested in the etiology of CD. It is supported by observations that CD is clinically deviated when luminal bacterial concentrations are decreased (Rutgeerts et al., 1995).

Another disorder of GI tract within the IBD is ulcerative colitis (UC). UC is a form of chronic inflammation and ulceration of the colon and rectum lining (Leighton et al., 2006). It is an intermittent disease with periods of exacerbated symptoms (Podolsky, 2002). The main symptom in an extensive severe cases is bloody diarrhea and faeces containing mucus. However, fever, general malaise, abdominal pain and tenderness might also be seen as the general symptoms of the disease (Shivananda et al., 1996; Kornbluth & Sachar, 2004). Although the pathogenesis of UC is still unknown, animal models and clinical observations suggest that luminal bacteria induce chronic inflammation in this disease (Schultsz et al., 1999; Campieri & Gionchetti, 2001; Farrell & LaMont, 2002; Farrell & Peppercorn, 2002). Alterations of immune responses toward commensal intestinal microflora also play a key role to the development and maintenance of these conditions (Duchmann et al., 1995; Linskens et al., 2001). Besides, UC can be induced in knockout or transgenic mice with genetic susceptibilities to inflammatory disease only when their colons are populated with normal commensal bacteria but not in germ-free mice (Sadlack, Merz, & Schorle, 1993; Taurog et al., 1994). Furthermore, human studies have suggested that intestinal bacterial populations in patients with UC may alter towards a more proinflammatory phenotype and can be a root of the pathogenesis of the disease (Hartley et al., 1992; Matsuda et al., 2000; Campieri & Gionchetti, 2001; Macfarlane et al., 2004). Hence, eradicating bacterial

antigens and adjuvants that regularly cause the pathogenic immune response may be considered as an approach for UC therapy currently (Sartor, 2004).

Apart from CD and UC, intestinal hyperpermeability is also believed to be involved in the pathogenesis of celiac disease (Vogelsang, Schwarzenhofer, & Oberhuber, 1998). This disease is an autoimmune disorder that affects GI tract in genetically predisposed individual after introduction of gluten containing food (Ciclitira, 2001; Green & Cellier, 2007). It is identified as chronic inflammation of the small intestinal mucosa that results in atrophy of intestinal villi and deficient absorption of nutrients (Dewar & Ciclitira, 2005; Elson et al., 2005). There is a genetic predisposition to celiac disease that attribute to the specific genetic markers known as human leukocyte antigen HLA–DQ2 and HLA–DQ8 present in the affected individuals (Kagnoff, 2005). Dietary glutens (wheat, rye, barley and gliadin) are suggested to interact with these HLA molecules which activate an abnormal mucosal immune response and stimulate tissue damage. Most affected individuals experience remission after excluding gluten from their diets (Kemppainen et al., 2009). Celiac disease also involves the adaptive immune response and the innate immune response which is characterized by the presence of anti-gluten and anti-transglutaminase 2 antibodies as well as lymphocyte infiltration in the intestinal epithelial membrane and the lamina propria (Briani, Samaroo, & Alaedini, 2008). Under normal conditions, the immune system tolerates all the proteins introduced into the GI tract with the diet (Briani, Samaroo, & Alaedini, 2008). Immune tolerance to harmless proteins in intestinal tissues is daily guaranteed by several regulatory T cell subsets which are important components of the homeostasis of the immune system since impaired regulatory T-cell activity may cause celiac disease as

well as other autoimmune diseases (Lan, Mackay, & Gershwin, 2007). Besides, several compelling evidences suggest that T regulatory cells not only prevent autoimmunity or assure immune tolerance (Van Driel & Ang, 2008) but are also involved in controlling virtually all kinds of immune response, including the one against malignant tumors (Gallimore & Godkin, 2008).

1.2 Stress

1.2.1 Background and discoveries of stress

The term 'stress' was first used by Austro-Canadian endocrinologist Hans Selye in 1936 (Selye, 1936). He discovered the effects of stress when he infused ovarian hormones into the glandular system of laboratory rats. The hormones stimulated the outer tissues of the adrenal glands of rats which caused atrophy of the thymus gland, produced ulcers and eventually death (Selye, 1952). He finally determined that these effects could be produced by administering virtually any toxic substance, physical injury or environmental stress. Selye (1952) was able to extend his theory in humans indicating that stress may induce breakdown of the hormonal system and lead to conditions like heart disease as well as high blood pressure which he termed “diseases of adaptation”.

Selye (1978) has described stress as a state where physical or psychological stimuli disrupt the body homeostasis and the body responses nonspecifically to that demand whether it is caused by or the results of pleasant or unpleasant conditions. In recent literature, stress has been elucidated as a condition that results when a person’s transaction with the environment leads the individual to perceive a discrepancy whether real or not between the demands of a situation and the resources

of the person's biological, psychological or social systems (Winfield & Richards, 2004). During stress, our body tries to maintain homeostasis by various ways. In prolonged stress, capacity of the body to maintain the homeostasis could be exhausted leading to various illnesses of many parts of the body (Mehic-Basara & Mehic, 2002; Natelson, 2004; Violanti et al., 2006).

Within the general concept of stress, Hans Selye differentiated between distress (from the Latin dis = bad, as in dissonance, disagreement), and eustress (from the Greek eu = good, as in euphonia, euphoria). During both distress and eustress the body undergoes virtually the same nonspecific responses to the various positive or negative stimuli acting upon it. However, the fact that eustress causes much less damage than distress explicitly demonstrates "how the body can take it" and determines ultimately whether the body can adapt successfully to changes (Selye, 1974).

The human body responds to stressors by activating the nervous system and specific hormones. Corticotrophin-releasing hormone (CRH) from hypothalamus signals the pituitary gland to produce adrenocorticotrophic hormone (ACTH) which signals adrenal glands to produce more of the hormones adrenaline and cortisol and release them into the bloodstream (Gonzalez-Heydrich et al., 2001) (Figure 1.4). These hormones speed up the heart rate, breathing rate, blood pressure and metabolism. Blood vessels open wider to let more blood flow to large muscle groups, putting our muscles on alert while pupils dilate to improve the vision. Furthermore, the liver releases some of its stored glucose to increase the body's energy and sweat is produced to cool the body (Sapolsky, Romero, & Munck, 2000). All of these

physical changes prepare a person to react quickly and effectively to handle the pressure of the moment. This natural reaction is known as the stress response and if it is working properly, the body's stress response enhances a person's ability to perform well under pressure. However, the stress response can also cause health hazards, when it overreacts or fails to turn off and reset itself properly (Martini & Nath, 2008).

In general, the hormonal system aids body to adapt towards the environmental changes or stimuli. Selye (1946) has described a physiological response to physical and physiological stimuli as a stressor. This condition sometimes could cause diseases especially if the state of stress is prolonged or intense. Then, he elaborated on the process that the body under stress goes through three stages of the "general adaptation syndrome" (G.A.S.). The first stage is the alarm reaction which is characterized by the changes of hormones due to environmental alterations. If the stressor (stress-producing agent) is so severe that prolong exposure is incompatible with life, a person or animal will die within a few hours during this stage. Otherwise, the second stage which is adaptation of resistance will arise since no organism can be maintained continuously in a state of alarm. This adaptive stage is characterized by the vanishing or diminishing of the initial symptoms since the body has achieved optimal adaptation. After further prolonged exposure to the stressor, this acquired adaptation is lost and a third stage of exhaustion is entered, unless a person or animal receives emergency aid from outside sources. It illustrates apparently that the adaptability of an organism is finite.

Recently, chronic stress has been reported to compromise intestinal barrier function in animal models. Chronic water avoidance stress induced bacterial adhesion and penetration into epithelial cell surface which caused overreaction of the body with possible damaging inflammatory responses (Zareie et al., 2006). Meanwhile, Levenstein et al. (2000) have ascertained psychosocial factors such as stress result in physiological diseases which have lead to many clinical and experimental interests (Morrow & Garrick, 1997; Rao et al., 1998). Moreover, it is speculated that psychological stress could alter the function of small and large intestine (Saunders, Hanssen, & Perdue, 1997; Saunders et al., 2002; Soderholm et al., 2002). Alteration of GI function under stress associates with functional dyspepsia and irritable bowel syndrome (IBS) where psychosomaticist described it as classical psychosomatic disorders (Mayer et al., 2001).

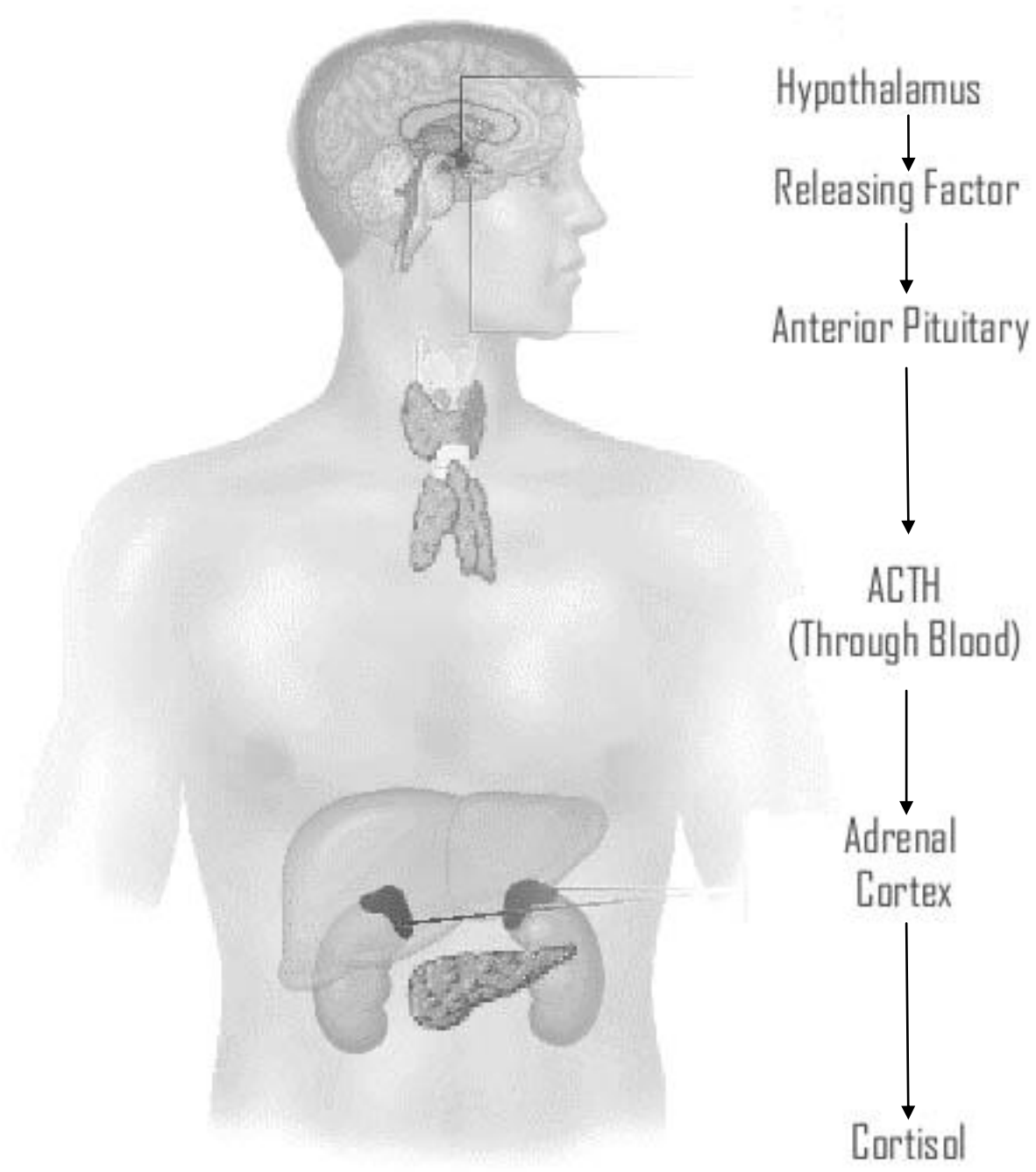

Figure 1.4: Hypothalamic-pituitary-adrenal axis (Adapted from Goodell, 2008)

1.2.2 Human and animal stress studies

Stress has been studied for more than a century and investigators still cannot agree on a satisfactory definition of this concept. Generally, researchers simply classify stressor as circumstances that most people find tension and pressure psychologically. Elliot and Eisdorfer (1982) characterized stressors into five categories. These categories have the advantage of distinguishing among stressors on two essential dimensions which are duration and course, for instance, continuous versus discrete. The first category is acute time-limited stressors. It involves laboratory challenges such as a public speaking or mental arithmetic. The second category is brief naturalistic stressors. It involves a person confronting a real-life short-term challenge for example academic examinations. The third category is related to stressful event sequences. It is a focal event, such as the loss of a spouse or a major natural catastrophe which gives rise to a series of related challenges. They usually do not know exactly when these challenges will settle but they know it will stop at some point in the future. The fourth category is chronic stressors. It usually pervades a person's life, forcing a person to restructure his or her identity or social roles. Chronic stressors also has stable characteristic that a person either know or not know when the challenge will end or can be certain that it will never end. Examples of chronic stressors are suffering traumatic injury that causes physical disability or being a refugee forced out of a nation because of a war. Fifth category is distant stressors. It is a traumatic experience that occurred in the distant past, yet has potential to continue interfering immune physiological functions because of their long lasting cognitive and emotional sequelae. Examples of distant stressors include being sexually assaulted during childhood, have witnessed the death of a fellow soldier during combat and have been a prisoner of war (Baum, Cohen, & Hall, 1993).

Stress in human studies has been thought to be a protective reaction from environmental insults. It results by alterations of hypothalamic-pituitary-adrenal axis function where it stimulates the elevation of glucocorticoid hormone. This hormone activates glyconeogenesis to supply a readily available source of energy for the adaptive reactions necessary to promote acute coping with stressful challenges (Sapolsky, Romero, & Munck, 2000). Nevertheless, the response could be detrimental if it exceeds an individual's adaptive capability to a variety of deleterious consequences (Korte, 2001). In human, chronic psychological stress has been speculated to be the etiology of many diseases including neurological damage, psychopathology and even premature deaths (Mc Ewen & Stellar, 1993). It also has been reported that glucocorticoids are responsible for the thymicolymphatic involution, eosinopenia and lymphopenia in acute stress (Berczi, 2001). Apart from that, the effects of acute stress on intestinal secretion have been demonstrated using intestinal perfusion technique in human jejunum. By this technique, Barclay and Turnberg (1987) have discovered that psychological stress induced by dichotomous listening reduced mean net water absorption and reversed net sodium ion (Na^+) and chloride ion (Cl^-) from absorption to secretion. They have concluded that stress induced ion secretion could be mediated by the parasympathetic nervous system since the effect of stress has been inhibited by atropine. Psychological stress has also been shown to influence the clinical course of chronic intestinal disorders such as CD, UC (Levenstein et al., 2000) and IBS (Mayer et al., 2001). By extending this model, Santos et al. (1998) have found that jejunal water secretion induced by cold pain stress has been linked with luminal release of mast cell mediators, tryptase and histamine. They proposed that during stress, central nervous system has signalled the intestinal mucosa to release mast cells.

In animal studies, GI abnormalities have been reported to be associated with acute and chronic psychological stress. These conditions have been described as increased secretory state, altered colonic motility, increased epithelial permeability to small and large probes, damaged mitochondria in epithelial cells, altered epithelial/bacterial interactions and increased inflammatory infiltration (Saunders, Hanssen, & Perdue, 1997; Saunders et al., 2002). These manifestations could be the consequence of excess level of glucocorticoids which inhibits intestinal epithelial cells proliferation (Senguptaa & Sharma, 1993; Ersoy et al., 2008).

Some models of stress have been developed to examine the possibility of life stressors promoting diseases. These models expose the animals to stressors including in both the elements of psychological and physical stress. An increase in the psychological element and a decrease in the physical element during stress session has been a trend in selecting a model which better imitate the experience of ongoing environmental or life stress in human (Soderholm & Perdue, 2001).

The acute stress model is generally used in animal research to study intestinal functions. Rats are exposed to single and relatively short exposure of restraint or immobilization stress (Castagliuolo et al., 1996a; Saunders et al., 1994). In restraint stress (RS), the animals are immobilized from 30 minutes to 4 hours in adjustable restraint equipment or wrap restraint (gentle wrapping of the upper and lower limbs) (Castagliuolo et al., 1996b; Castagliuolo et al., 1998). Besides that, in cold restraint stress (CRS), animals are restrained under a cold environment (usually at 8°C) (Saunders et al., 1994). Another method is water immersion restraint stress (WIRS) where the restrained animals are positioned upright in 20°C water to the level of the