

**EFFECTS OF SELENIUM AND VITAMIN E ON GASTRIC MUCOSAL
DAMAGE, ACID SECRETION AND HAEMATO-BIOCHEMICAL CHANGES
INDUCED BY WATER-IMMERSION RESTRAINT STRESS IN WISTAR RATS**

BY

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Declaration

I declare that the work in this thesis, entitled 'Effects of selenium and vitamin e on gastric mucosal damage, acid secretion and haemato-biochemical changes induced by water-immersion restraint stress in Wistar rats' has been carried out by me in the Department of Human Physiology. The information obtained from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

YakubuSADAU

Signature

Date

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Dedication

This work is dedicated to my father, MallamSadau (Dauda) Ibrahim and my mother, Malama Fatima Usman.

Abstract

A stress response is a natural reaction by the body against potentially harmful stimuli to enhance the chance for survival. Serious stress can induce organ injury or contribute to diseases, such as gastric ulcers. The aim of the present study was to determine the effects of selenium and vitamin E on gastric mucosal damage, secretions and haemato-biochemical changes due to water-immersion restraint stress (WRS) in Wistar rats. Seventy (70) Male Wistar rats (200-220 gram) were divided in to two main groups, 35 rats each for ulcer and acid secretions studies. Group 1 and 2 were sub-divided each into 5 sub-groups of 7 rats: (I) passive control (non-stress rats) (II) active control (WRS plus distilled water) (III) WRS plus selenium (IV) WRS plus vitamin E (V) WRS plus selenium plus vitamin E. The WRS procedure last for 3.5 hours. Gastric tissues were taken out and investigated macroscopically and histologically to determine mucosal damage. Blood samples were collected through cardiac puncture for haematological and biochemical analyses. Gastric secretion was collected after additional 3 hours of pyloric ligation. The result demonstrated that acute WRS significantly ($P < 0.001$) increased gastric ulcer and gastric secretion parameters as well as MDA concentration, activities of plasma ALT and AST, plasma Zn concentration, and haemolysis of erythrocytes in different concentration of NaCl. However pre-treatment with selenium and vitamin E individually or in combination significantly lowered the recorded increases, but the decrease were considerable in selenium and vitamin E co-administration. Exposure to WRS for 3.5 hours leads to significant ($P < 0.001$) decreases in the total plasma protein concentration, activities of CAT, SOD and GSH, Mg concentration, number of neutrophils. Pre-treatment with selenium or vitamin E singly or in combination resulted in significant ($P < 0.001$) upsurge the observed decrease. This study found no significant ($P > 0.05$) changes in PCV, RBC, Hb and MCV on exposure to WRS. Pre-

treatment with vitamin E or selenium singly or combine significantly ($P < 0.05$) increased some of the values of haematological parameters (PCV, RBC, WBC, Hb, and lymphocytes). It was thus concluded that WRS exposure causes significant alteration in macroscopic, histological structure of the gastric tissue, biochemical, haematological, lipid peroxidative parameters and shift in serum antioxidant balance. However pre-treatment with selenium or vitamin E singly or in combination ameliorate some of the recorded adverse effects of WRS. It was noted that co-administration of selenium and vitamin E have a synergistic effects in restoration of WRS-induced changes.

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List of Abbreviations

| | |
|-----------------------|-------------------------------------|
| AAS | Atomic absorption spectrophotometry |
| AC | Active control |
| ACTH | Adrenocorticotrophic hormone |
| Ach | Acetylcholine |
| AGML | Acute gastric mucosal lesion |
| ALT | Alanine aminotransferase |
| ANS | Autonomic nervous system |
| AMP | Adenosine monophosphate |
| AST | Aspartate aminotransferase |
| ASPP | Apoptosis stimulator |
| ATP | Adenosine triphosphate |
| bFGF | Basic fibroblast growth factor |
| CAT | Catalase |
| CCK | Cholecystokinin |
| COX | Cyclooxygenase |
| CGRP | Calcitonin gene-related peptides |
| Cl⁻ | Chloride anion |
| CRF | Corticotrophin releasing factor |
| CRS | Cold restraint stress |

| | |
|------------------------------------|---------------------------------|
| Cu | Copper |
| DNA | Deoxyribonucleic acid |
| DUs | Duodenal ulcers |
| ECL | Enterochromaffin-like cell |
| EGF | Epidermal growth factor |
| EGF-R | Epidermal growth factorReceptor |
| EOF | Erythrocyte osmotic fragility |
| EPO | Erythropoietin |
| FPP | Fermented papaya preparation |
| GI | Gastrointestinal |
| GRP | Gastrin-releasing peptide |
| GSH | Glutathione |
| GPx | Glutathione peroxidase |
| Gus | Gastric ulcers |
| H⁺ | Hydrogen ion |
| HCl | Hydrochloric acid |
| HCO₃⁻ | Bicarbonate anion |
| Hb | Haemoglobin |
| HDC | Histidine decarboxylase |
| HNE | 4-Hydroxyl-2-n |
| H₀ | Null hypothesis |
| HOCl | Hypochlororous acid |
| H₂O₂ | Hydrogen peroxide |

| | |
|--|---|
| HPA | Hypothalamic-pituitary-adrenal axis |
| <i>H.pylori</i> | <i>Helicobacter pylori</i> |
| IASPP | Apoptosis inhibitor |
| IL-1β | Interleukin-1 beta |
| LDL | Low-density lipoprotein |
| MAPKS | Mitogen-activated protein kinase |
| MCH | Mean corpuscular haemoglobin |
| MCV | Mean corpuscular volume |
| MCHC | Mean corpuscular haemoglobinconcentration |
| MDA | Malondialdehyde |
| ml | Millilitre |
| Mg | Magnesium |
| mmHg | Millimetre of mercury |
| mRNA | Messenger ribonucleic acid |
| Mn | Manganese |
| MPO | Myloperoxidase |
| MUC | Mucin glycoprotein |
| NaCl | Sodium chloride |
| Na₂SeO₃. 5 H₂O | Sodiumselenite pentahydrate |
| NADPH | Nicotinamide adenine dinucleotide |
| NaOH | Sodium hydroxide |
| NH₃ | Ammonia |
| NH₂Cl | Monocholaramine |

| | |
|----------------------------------|---|
| NO₂ | Nitrogen dioxide |
| NO | Nitricoxide |
| NSAIDs | Non-steroidal anti- inflammatory drugs |
| NPSH | Non-protein sulphhydryl |
| O₂ | Oxygen |
| O₂⁻ | Superoxide radical |
| PACAP | Pituitary adenylatecyclase-activity peptide |
| PC | Passive control |
| PCV | Packed cell volume |
| PDGF | Platelet derived growth factor |
| PGE | Prostaglandin E |
| PGI₂ | Prostacyclin |
| PGS | Prostaglandins |
| pH | Index of hydrogen ion concentratio |
| PVE | Palm vitamin E |
| PLP | Lipo protein lipase |
| PLTP | Phospho lipid transfer protein |
| PUFA | Poly unsaturated fatty acid |
| PUD | Peptic ulcer diseases |
| PPI'S | Proton pump inhibitors |
| RBC | Red blood cell |
| ROS | Reactive oxygen species |
| RS | Restraint stress |

| | |
|-------------------------------|------------------------------------|
| RNS | Reactive nitrogen species |
| SAM | Sympatho-adrenal medullary |
| Se | Selenium |
| SOD | Superoxide dismutase |
| S.E.M. | Standard error of mean |
| TFF | Trefoil factor |
| α-TF | Alpha-Tocopherol |
| TrxR | Thioredoxinreductase |
| VC | Vitamin C |
| VE | Vitamin E |
| VEGF | Vascular endothelial growth factor |
| VIP | Vasoactive intestinal peptide |
| WBC | White blood cell |
| WRS | Water immersion restraint stress |
| Zn | Zinc |

CHAPTER ONE

1.0 Introduction

Stress is an aversive stimulus, which disturbs physiological homeostasis (Anil *et al.*, 2010). It is a condition of highly-individualized response of an individual to external and internal challenges, which may be controlled with or without difficulties. A stress response is a natural reaction by the body against potentially harmful stimuli to enhance the chance for survival (Nayanatara *et al.*, 2012). It induces the strain upon both emotional and physical endurance, which has been considered the basic factor in the aetiology of a number of diseases (Sheldon *et al.*, 2012). Serious stress can induce organ injury or contribute to diseases, such as gastric ulcers, hypertension, diabetes mellitus and cancer.

Stress affects psychological and physiological balances which can lead to various pathological changes. One known pathological stress-induced condition is the formation of gastric lesion, and studies have shown that its pathogenesis is multi-factorial. It includes factors which disrupt the gastric mucosal integrity such as changes in gastric acid, mucus and bicarbonate secretions, inhibition of gastric mucosal prostaglandin synthesis (Nur *et al.*, 2012), reduction in gastric mucosal blood flow, changes in stress hormones (Ibrahim *et al.*, 2010) and gastric motility (Jun *et al.*, 2009). Psychogenic factors, such as stress, play a major role in the pathogenesis of gastric ulcers in man. Gastro-intestinal erosion is one of the consistent findings in man and in experimental animals subjected to different types of stress (Shawon and Gautam, 2012). It has been known for many years that patients with severe burns, trauma or other serious diseases develop severe intestinal bleeding or perforation caused by ulcers. Endoscopy has revealed that severe physiological stress induces lesions, ranging from erosion to complicated ulcers (Shawon and Gautam, 2012). Clinically, the term stress ulcer encompasses both upper gastrointestinal hemorrhages and lesions as a consequence of trauma, including burns, intracranial injuries and septic shock. In normal daily life, individuals encounter various types of stress. Accumulation of

daily life stress (chronic stress) often causes gastro-intestinal symptoms and functional gastro-intestinal diseases. Although some adaptational action may occur in chronic stress, the proximate mechanism underlying chronic stress remains unknown. Acute stress (single) delays gastric emptying and alters upper gastro-intestinal motility (Jun *et al.*, 2009).

Water-immersion restraint stress (WRS) mimics the clinical acute gastric ulceration caused by trauma, surgery or sepsis (Dur-Zong *et al.*, 2013), and has been widely accepted for studying stress ulceration (Ochi *et al.*, 2008). The animals immobilised for times in adjustable restraint stress (RS). A combination of RS with cold environment usually at 8°C is called cold restraint stress (CRS). Water-immersion restraint stress (WRS) is a variant of RS, The restraint method has therefore, been modified with the restraint animals subjected to additional water immersion (Johan and Mary, 2001). The WRS method involved prolonged starvation for 36 hours; both models involve elements of physical in addition to psychological stress, shown to induce changes in gastro-intestinal function. The model involves central nervous system in to play and the lesions produced by this method are located in the glandular portion region of the stomach and penetrate the muscularis mucosa and as such may be called ulcers. Thus stress-related animal experiments appear to be a very good mimic of human condition and have allowed studies in to pathogenic mechanism as well as useful therapeutic interventions (Shawon and Gautam, 2012). Body restraint, when combined with water immersion, is used as one of the methodologies of induction of reliable gastric ulceration in a very short time (Landeira-Fernandez, 2000). Physiological studies have shown that stress from any source may influence the endocrine, haemopoietic and immune systems (Ebulunlomo *et al.*, 2012). Cytokines and cortisol play an important role in the communication between these systems. The first report of the use of restraint as a stress factor was published by Selye in 1936. While Hanson and Brodie (1960) and Bonfils *et al.* (1966) described methods of studying the effects of anti-ulcer drugs on immobilisation stress in rats (Shawon and Gautam, 2012).

The autonomic nervous system is one of the main components involved in stress mechanism, exerting a profound influence on heart rate and digestion (Yuan-Fang *et al.*, 2005). However, the precise role of each component of the autonomic nervous system in the gastric ulcer after stress exposure remains unclear (Yuan-Fang *et al.*, 2005). Arawaka *et al.* (1997) reported that excessive peripheral sympathetic activity plays an important role in the WRS model. Shichijo *et al.* (1991) suggested the presence of a sympathetic hypofunction and parasympathetic hyperfunction in the stomach, contributing to the gastric lesion in the spontaneously-hypertensive rats, subjected to WRS. Vagal activity has been shown to play an important role in the protection of gastric ulcer to WRS (Brzozowski *et al.*, 2004). Although controversial, it is generally believed that sympatho-vagal imbalance plays an important role in the gastric lesion, induced in the rat by WRS model (Hashiguchi *et al.*, 1993); and that it stimulates numerous pathways, leading to increased production of reactive oxygen species (ROS) (Brzozowski *et al.*, 2004). The term ROS have one or more unpaired electrons which makes them very unstable and they move through the blood stream, taking electrons from other cells or giving away unpaired ones. Thus, ROS cause cell damage, linked to a host of diseases and cancer. According to Nayanatara *et al.* (2012), chronic restraint stress causes a significant alteration in physiological, biochemical and lipid peroxidative parameters and, consequently a shift in oxidant and antioxidant balance. Restraint is one of the best explored models, which combines both emotional (escape reactions) and physiological (muscle work) stress (Brzozowski *et al.*, 2004). Stress exposure activates the sympatho-neural system, resulting in release of catecholamines, which play an important role in the regulation of cardiac function, both in health and disease (Blumenthal *et al.*, 2000).

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the ignition or propagation of oxidizing chain reactions. They also act as

oxidizing inhibitors (Mansoor and Mahmood, 2009). Selenium (Se) is an essential trace element and its low status in human has been linked to increased risk of various diseases, such as cancer and heart diseases (Ujang, 2008). Selenium prevents-reperfusion induced gastri mucosal lesion, intraluminal bleeding and depletion of non-protein sulphhydryl (NPSH) level in the rat stomach, therefore play an antioxidant role to protect tissue against oxidative stress (Abu Taib *et al.*, 1997). Selenium beneficial effect is, however, expressed in a very narrow dosage range (Ostodalova, 2012). The high and low doses of selenium are connected with pathological manifestations. The toxicity depends on the chemical form of selenium, state of organism, interactions with heavy metals and on the stage of ontogenetic development. Whereas one dose of sodium selenite (20 $\mu\text{mol/kg}$ body weight) is lethal in adult rats, suckling rats are entirely resistant. Selenium is a trace element that is essential at small amounts but can be very toxic at larger amount. Humans and animals require selenium for the function of a number of selenium-dependent enzymes also known as selenoproteins. During selenoproteins synthesis it is incorporated into a very specific location in the amino acids sequence in order to form a functional protein (Siham *et al.*, 2008). At least two types of selenoproteins are necessary for each animal cell, the first form is the family of GSH-peroxidase and the second form is the family of deiodinase. GSH-peroxidase are the most power antioxidant enzymes which depend the cell against oxidative damage and thus oxidative stress-related diseases and disorders (Gabryel and Maleck, 2006).

Vitamin E is known to possess many biological properties including antioxidant activity to modulate protein function and gene expression (Dan *et al.*, 2010). Vitamin E (α -tocopherol) is a lipid-soluble antioxidant and a well-accepted first line defence mechanism against lipid

peroxidation. It functions as a chain-breaking antioxidant for lipid peroxidation in cell membranes and as a scavenger of ROS such as superoxide anion, hydrogen peroxide and single oxygen (Ibrahim *et al.*, 2010). Vitamin E and selenium are micronutrients that share a common biological function in the animal body. Selenium is a component of glutathione peroxidase enzyme which destroys free radicals in the cytoplasm, whereas vitamin E is a non-enzyme scavenger of free radicals (Shinde *et al.*, 2007). This antioxidant activity of vitamin E in preventing lipid peroxidation may be one of the mechanisms by which vitamin E enhances immunity (Shinde *et al.*, 2007). Vitamin E causes a reduction of glucocorticoids, which are known to be immunosuppressive. It also alters arachidonic acid metabolism and subsequent synthesis of prostaglandin, thromboxanes and leukotrienes.

Under stress conditions, increased levels of these compounds by endogenous synthesis or exogenous entry may adversely affect immune cell function similarly; selenium deficiency results in immunosuppression, reduced resistance to infection, neutrophil function, antibody production, proliferation of T and B cells in response to mitogens and cytodestruction by T lymphocytes and NK cells (Kiremidjian and Stotzky, 1987). Samanta *et al.* (2006) in crossbred calves and Rajeesh (2006) in buffalo calves reported that supplementation of vitamin E improved the immune status of the animals. During oxidative stress, an increase in the exogenous supply of antioxidants improves the capacity of the tissue to cope with high antioxidant demands (Ambaliet *al.*, 2010b). The results of the present study may show that oxidative stress, occurring in gastric mucosal damage, may be ameliorated or prevented by the administration of selenium and vitamin E.

1.1 Statement of the Research Problem

Several pharmacologic therapies have been studied for the prevention of stress-induced gastric ulcers and bleeding, including proton pump inhibitors (PPIs), histamine-2 receptor antagonist, sulcrafate and enteral nutrition. Despite decades of research significant controversy continues to surround standardization of prophylactic therapy, particularly because of in appropriate use and cost (Mark *et al.*, 2014). Many synthetic antioxidant agents have been developed to remediate oxidative stress, but factors such as lack of availability and side effects remain a major setback in combating oxidative (Zhang *et al.*, 2011).

1.2 Justification of the Study

There is paucity of information on the effect of selenium and vitamin E on gastric mucosal damage, acid secretion and haemato-biochemical changes in rats exposed to water-immersion restraint stress. Knowledge obtained from this study may be used to develop new, more effective strategies to prevent and manage stress-related gastric disorders. Stress ulcer is a highly prevalent clinical complication, and full understanding of the mechanism of stress ulcer may increase our knowledge of the prevention and treatment of stress-related organ injury (Yuan-Fang *et al.*, 2005).

1.3 Aim and Objectives

1.3.1 Aim

The aim of the study was to determine the effect of selenium and vitamin E on gastric mucosal damage, secretions and blood biochemical changes due to WRS in Wistar rats.

1.3.2 Objectives

The objectives of the study are:

- i) To investigate the effects of selenium and vitamin E on gastric mucosal damage and acid secretion.
- ii) To evaluate changes in oxidative stress biomarkers, liver injury and haematological parameters in Wistar rats, subjected to WRS.
- iii) To determine the mineral changes induced by restraint stress in Wistar rats.

1.4 Research Hypothesis

Selenium and vitamin E do not ameliorate the deleterious effects of water immersion restraint stress on gastric mucosal damage, secretions and haemato-biochemical changes in Wistar rats.

CHAPTER TWO

2.0 Literature Review

2.1 Introduction

The stomach plays a pivotal role in the digestion of food. The vertebrate stomach performs a variety of functions, including serving as a reservoir for food, exposing ingested food to acid secreted by the parietal cells and pepsin secreted by the chief cells. It also provides a barrier that prevents microorganisms from entering the intestine (Duan *et al.*, 2006). In addition, the stomach is exceedingly rich in the following peptide hormone producing cells: enterochromaffin-like cells (*ECL*) (histamine), D cells (somatostatin), A- like cells (ghrelin and obestatin), D₁/p cells (unknown product), and EC cells (serotonin) and G cells (gastrin). The diverse physiological functions of the stomach depend on an intact gastric mucosal integrity. The stomach wall consists of a mucous layer, mucosa, sub-mucosa, *muscularis* and serosa. The

mucous layer protects the mucosal surface from harmful components in the lumen (HCl and pepsin) capable of damaging the epithelial barrier (Duan *et al.*, 2006).

The major gastric secretions are HCl, pepsin, intrinsic factor, mucus and bicarbonate ion (Sheree- Lehman, 2014). The cells of the gastric glands secrete about 2500 mL of gastric juice daily in man. This juice, in addition to the major previous secretions, also contains Na, K, Mg, sulphate, gelatinase and enzymes (Shereen-Lehman, 2014). Multiple chemical, neural and hormonal factors participate in the regulation of gastric acid secretion. Gastric juice is corrosively acidic and its protein-digesting enzymes can digest the stomach itself. The stomach is protected by the mucosal barrier. Anything that breaches the gel-like mucosal barrier producers can induce inflammation of the underlying layers of the stomach wall. Conditions such as gastritis and persistent damage to the underlying tissues can promote gastric ulcer (Shereen-Lehman, 2014). With exception of rare cases, this organ can resist to a large variety of noxious factors, including hydrochloric acid, refluxed bile salts and alcohol, with a wide range of temperatures and osmolality. This high resistance to injuries depends on a number of physiological responses elicited by the mucosal lining of the stomach against potentially harmful luminal agents, as well as the ability of the organ to rapidly repair the mucosal damage when it occurs (Laine *et al.*, 2008). Nevertheless, when the protective mechanisms such as mucin, bicarbonate and prostaglandins are overwhelmed by injurious factors like *Helicobacter pylori*, non-steroidal anti-inflammatory drugs (NSAIDs) and high gastric acid, a gastric mucosal lesion may develop. Major detrimental effects on gastric mucosa are exerted by NSAIDs, which exert gastric injuring effects and delay healing of ulcer lesion through a variety of local and systemic mechanisms (Dalia and Mai, 2011).

Various factors that are implicated play a pivotal role in the pathogenesis of ulceration like sedentary life style, alcohol intake, spicy food, drugs and various bacterial infections. Moreover,

several endogenous substances are involved in the production of gastro-intestinal lesions in animals. They include some bacterial infections, various drugs and chemicals, gastric secretion, lipid metabolites, neuropeptides, inflammatory mediators and ROS (Kumar *et al.*, 2012). Oxidative stress has emerged as one of the major pathogenic factors in progression of gastric ulcer that directly impairs the cellular functions and promotes damage to cellular organelles, including the mitochondria, lysosomes and nucleus (Kumar *et al.*, 2012).

2.2 Prevalence of Stress-related Gastrointestinal Disorder

Depending on patient disease and severity of illness, stress ulceration and related mucosal erosion and sub-epithelial haemorrhage were demonstrated in 70-100% critically-ill patients in U.S.A. These lesions are generally superficial and asymptomatic, but can extend into the *submucosa* and *muscularis propria* and erode larger vessel causing overt and clinically significant bleeding (Mark *et al.*, 2014). Earlier studies suggested that overt gastro-intestinal bleeding occurred frequently, and in some studies up to 25% of critically ill patient, it is accepted condition with prevalence between 0.6 and 4% of patients (Mark *et al.*, 2014). Lanre (2009) investigated the prevalence of job stress among primary school teachers in South West, Nigeria with ulcer symptoms of 21.8%.

The true prevalence rate of peptic ulcer (PUD) in Nigeria populace is not certain, although over three decades ago Nigeria was listed as an area of high PUD prevalence (Ndubaba and Adeyemi, 2008). With perforation being the most frequent indication for surgery, more recent studies begin to show similar prevalence rates for duodenal ulcers and gastric ulcers in both Southern and Northern Nigeria, and this is attributed to improved diagnostic facilities (Ndubaba and Adeyemi, 2008). Complications of PUD vary in frequency geographically. In the United

States, haemorrhage is the most common complication of PUD (73 percent), followed by perforation (9 percent) and obstruction (3 percent). The mortality rate from complication of PUD is more than 10 times that of acute appendicitis or acute cholecystitis. Perforation has the highest mortality rate, followed by obstruction and haemorrhage.

By contrast, a 13-year review of all surgical procedures for peptic ulcer complications at a Nigeria hospital found that obstruction was the most common complication (56 percent), followed by perforation (30 percent) and bleeding (10 percent) (Irabor, 2005). Some regional factors that may account for the differences include the rate of NSAID use, the prevalence of *H. pylori* infection, and the distribution and extent of gastritis (Sadice *et al.*, 2009). The control of PUD represents a major triumph for modern pharmacology. Proton pump inhibitors are considered superior for acid suppression in most clinically significant acid peptic diseases. Recently, a global market sale of PPIs, only of esomeprazole was 8.4 billion dollars (Shawon and Gautam, 2012). Currently, there are two main approaches for the treatment of PUD. The first deals with the reduction of gastric acid secretion and the second with re-enforcement of gastric mucosal protection. (Shawon and Gautam, 2012).

2.3 Antioxidants

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the ignition or propagation of oxidizing chain reactions. They also act as oxidizing inhibitors (Mansoor and Mahmood, 2009). At any point of time, one antioxidant molecule can react with single free radical, and is capable to neutralize free radical(s) by donating one of its own electrons, ending the carbon “stealing” reaction. Antioxidants prevent cell and tissue damage as they act as scavengers. A variety of components act against free radicals to neutralize them from both endogenous and exogenous origin (Sujogya, 2012). These include endogenous enzymatic and non-enzymatic antioxidants. The mammalian cells reduce the effect of lipid peroxidation

via the utilisation of enzymatic and non-enzymatic antioxidants which scavenge for free radicals in the system. Oxidative stress results when the endogenous antioxidants are overwhelmed by the rate and extent of free radical generation, therefore, during oxidative stress an increase in the exogenous supply of antioxidants improves the capacity of the tissue to cope with high antioxidant demand (Ambaliet *al.*, 2010b).

2.3.1 Antioxidant enzymes

Cells, tissue and body fluids are equipped with powerful defence systems that help counteract oxidative challenge. To maintain a steady-state of metabolites and functional integrity in the aerobic environment, antioxidant defence is organized at three principal levels of protection, prevention, interception and repairs (Klimczack *et al.*, 2007). The biological mechanism of defence against oxidative stress includes antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Edem *et al.*, 2012). The SOD plays a major role as first line of the antioxidant defence system by catalysing the dismutation of superoxide radical (O_2^-), a highly potent ROS, into a less reactive species, hydrogen peroxide (H_2O_2) (Edem *et al.*, 2012). CAT is an ubiquitous enzyme present in cells of aerobic organisms. It converts two molecule of hydrogen peroxide (H_2O_2) to molecular oxygen (O_2) and two molecules of water (H_2O). Glutathione peroxidase (GP_x) also inactivates, by degradation the hydrogen peroxide (H_2O_2), formed from superoxide radical anion (O_2^-) by SOD into water. This is accompanied by the conversion of glutathione from reduced form (GSH) to oxidized form (GSSG) (Kwiecien *et al.*, 2004).

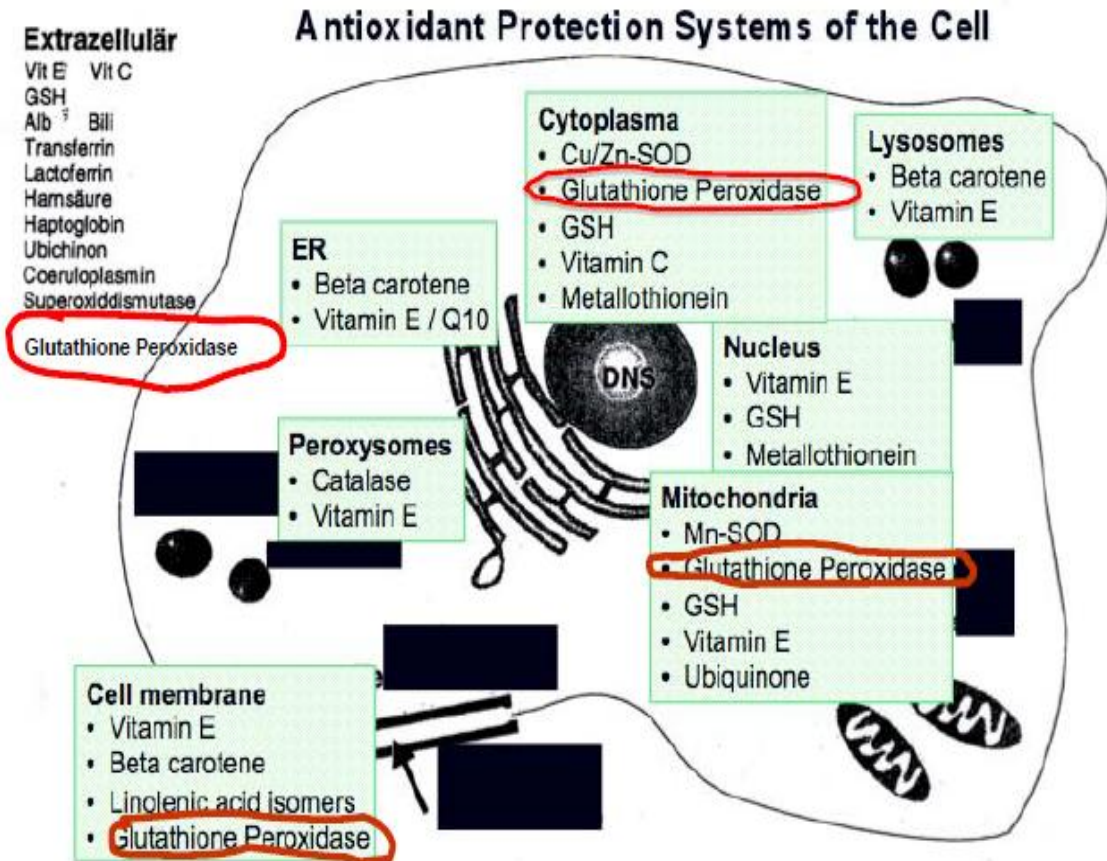
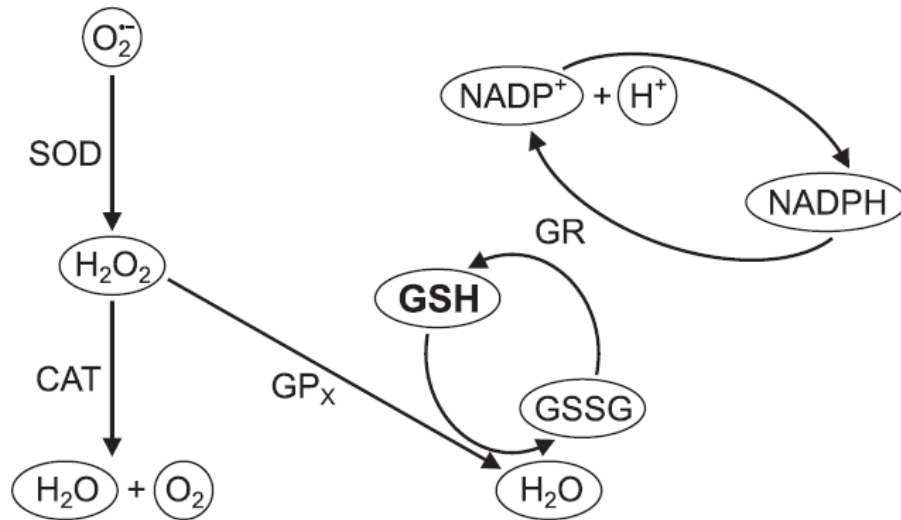


Figure 2.1: Anti-oxidative Cellular Mechanisms (Adapted from Trace Trial, 2007).



| | |
|---------------------------------|---|
| SOD - superoxide dismutase | GSSG - oxidized glutathione |
| CAT - catalase | $NADP^+$ - nicotinamide adenine dinucleotide phosphate |
| GP_x - glutathione peroxidase | $NADPH$ - reduced nicotinamide adenine dinucleotide phosphate |
| GR - glutathione reductase | |
| GSH - glutathione | |

Figure 2.2: Transformation pathways of superoxide radical anion (O_2^-) in the body and role of glutathione metabolism in neutralization of reactive oxygen species (Adapted from Kwiecien *et al.*, 2004).

2.3.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants are divided into metabolic and nutrient antioxidants. Metabolic antioxidants are the endogenous antioxidants, which are produced by metabolic activity in the body, like lipoid acid, glutathione, L-arginine, co-enzyme Q₁₀, melatonin, uric acid, bilirubin and metal-chelating proteins. While nutrient antioxidants belong to exogenous antioxidants, which cannot be produced in the body, but are provided through diet or supplement, viz. trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids (Sujogya, 2012).

Vitamin E (VE) and vitamin C (VC) are the non-enzymatic antioxidants that exist within normal cells; and they can be supplied through diet. Comparative study of the protective effect of VE and VC against gastric mucosal lesion induced by WRS in rats, either 0.05 or 0.5 mmol/kg of VE or 0.5 or 1.5 mmol/kg of VC was orally administered to rats with six hours of WRS, just before the onset of the stress. Both doses of pre-administered VE prevented gastric mucosal lesion development and attenuated all the changes in gastric mucosal components and enzymes studied. Only the higher dose of pre-administered VC suppressed the changes in all the studied parameters that is mucus concentration, NO synthase activity and increased in lipid peroxidation concentration and activities of myeloperoxidase and xanthine oxidase. This suggests that orally-administered VE protects against WRS-induced gastric mucosal lesion in rats more effectively than orally-administered VC (Ohta *et al.*, 2010). Vitamins are ideal antioxidants to increase

tissue protection from oxidative stress due to their easy, effective and safe dietary administration in a large range of concentrations (Sushma *et al.*, 2009). Vitamin E and selenium are micronutrients that share a common biological function in the animal body. Selenium is a component of glutathione peroxidase enzyme which destroys free radicals in the cytoplasm, whereas vitamin E is a non-enzyme scavenger of free radicals (Shinde *et al.*, 2007). That functions as a specific lipid soluble antioxidant in cell membranes. Vitamin E reacts with peroxide radicals produced from polyunsaturated fatty acids in membrane phospholipids or lipoproteins to yield a stable lipid hydroperoxide.

2.4 Biological Activity of Selenium

2.4.1 Selenium as antioxidant

Selenium (Se) is an essential trace element and its low status in humans has been linked to increased risk of various diseases, such as cancer and heart diseases. Foods are major natural source of Se and its levels generally depend on soil Se level. Since its discovery as an important component of antioxidant enzymes such as *glutathione peroxidase* (GPx), thioredoxin reductase (TrxR) and iodothyronine deiodinase (IDD), there has been an increased interest in the study of other Se-containing proteins (selenoproteins) or enzymes (seleno-enzymes) (Ujang, 2008). There are at least 30 selenoproteins that have been identified in mammals, and it has been estimated that humans have about 25 selenoproteins which are still not fully understood, even though they have been conserved throughout evolution because of their unique physico-chemical properties (Ujang, 2008). Due to their antioxidant activity there has been a tremendous interest in the study of selenium and its compounds involved in chemo-prevention of heart diseases and enhancement of immunity. Selenium prevents reperfusion, induced gastric mucosal lesion, intraluminal bleeding and depletion of non-protein sulphhydryl level in the rat stomach. Therefore the trace element plays an antioxidant role in protecting the tissue against oxidative stress (Abu Taib *et al.*, 1997). Selenium functions as a component of at least 25 different

selenoproteins (Andrieu, 2008). In these proteins, sulphur is replaced with Se, which allows the proteins to donate hydrogen and take part in reduction reactions. Selenoproteins include the enzyme iodothyronine deiodinase which is important in regulating metabolism and thioredoxin reductase. The enzymes are important components of antioxidant and immune system (Andrieu, 2008). Se is incorporated in the amino acid *cystein* to form selenocystein; and when selenocystein is added to polypeptide chain, a selenoprotein is formed. When selenocystein is specifically incorporated in the active site of an enzyme, it is called seleno-enzyme. Examples of seleno-enzymes are glutathione peroxidase, GSHPx-1, GSHPx-2, GSHPx-3 and GSHPx-4, Thioredoxin, and iodothyronin-5-deiodinase (Weiss, 2005).

Selenium is a trace element that is essential at small amounts, but can be toxic at larger amount. Humans and animals require selenium for the function of a number of selenium dependent enzymes, also known as selenoproteins. During selenoproteins synthesis, it is incorporated into a very specific location in the amino acid sequence in order to form a functional protein (Siham and Nabila, 2008). At least two types of selenoproteins are necessary for each animal cell, the first form is the family of GSH-peroxidase and the second form is the family of deiodinases. GSH-peroxidases are the most powerful antioxidant enzymes, which defend the cell against oxidative damage and thus oxidative stress-related diseases and disorders such as cardiovascular disease, malignancies, bacterial or viral diseases, muscle dystrophy, arthropathy, arterial plaques and others (Gabryel and Malecki, 2006). GSH-PX has many other regulatory functions, such as regulation of biosynthesis of prostaglandins, prostacycline, leukotrienes, and thromboxane, while deiodinases regulate the metabolism of biologically active triiodothyronine and thus is implicated in thyroid hormone regulation of the whole organism (Siham and Nabila, 2008).

2.4.2 Nutritional Source of selenium

Selenium can be present in inorganic form, a metallic form (Se^0) and oxyanions such as selenite [$\text{SeO}(\text{OH})_2$], selenite [$\text{SeO}_2(\text{OH})_2$] and also in organic form as seleno-amino acids such as Secys and selenomethionine (Semet) (Weiss, 2005).

2.4.3 Animal response to organic selenium

Mechanisms of absorption and metabolism of inorganic and organic Se differ substantially. Inorganic Se is recognized by the digestive tissue and is absorbed and converted to selenoprotein (Weiss, 2005). In contrast, organic Se in the form of Se-met is not recognized as Se-containing by mammalian cells (Behne and Kyriakopoulos, 2001). As a consequence, Se-met is absorbed and metabolised relative to methionine needs. If Se-met is broken down within the cell, Se is released and recognized by the cell as mineral and processed according to the animal's need for Se. However, if the cell does not break-down Se-met, it will be incorporated in to a wide variety of proteins that do not require Se (Behne and Kyriakopoulos, 2001). Organic selenium is more digestible than inorganic Se. However, in ruminants whether inorganic selenite or selenite is formed, selenite is the primary compound available for absorption, because the reducing conditions of the rumen convert the majority of selenite to selenite (Weiss, 2005).

2.4.4 Metabolism of selenium

Selenium is a trace bio-element essential for the normal function of the body, but this metalloid is quite unique in its metabolism, compared with typical essential element metals such as copper and zinc (Kazuo, 2005). It belongs to the same group on the periodic table as oxygen, sulphur and tellurium; that is group, 16. It is present at around 10mg/60kg bodyweight (Kazuo, 2005). Selenium is similar to sulphur in chemical property and has to be discriminated biologically from abundant sulphur during its metabolism in the body. At the time, selenium is known to be a

highly toxic element, with a narrow adequate range between deficiency and excessive dose, being of the range 0.1-1.0 μ g/g diet or ml drinking water) in experimental animals. Selenium is essential for the body because it forms the active centre for seleno-enzymes that carry out redox reaction, such as glutathione peroxidase (GPx), thioredoxin reductase, thyroid hormone deiodinase (Suzuki and Ogra, 2002).

2.4.5 Excretion of Selenium

Once selenium is taken up by the body, it is mostly excreted in the urine. Therefore, the amount of selenium present in urine depends on the dose in the nutritional range. However, excessive selenium is excreted not only in urine, but also in breath (Kazuo, 2005). In either case, selenium is excreted after being methylated stepwise. Urinary metabolites are known to be monomethylated selenium is exhaled in the form of dimethylselenide (Kazuo, 2005). Once selenium compounds are recognised as selenium species, they are transformed to the common intermediate metabolite selenide and then utilized for the synthesis of selenoproteins or excreted after being methylated stepwise (Kazuo, 2005).

2.4.6 Gastro-protective effect of selenium

Gastric cancer results from chronic superficial gastritis, atrophic gastritis, intestinal metaplasia, and heterotypic hyperplasia. Previous studies showed that selenium (Se) supplementation or selenium deficiency causes changes in the cellular ultrastructure (Yan-ping *et al.*, 2005). Selenium is an essential trace element in mammals. The results of laboratory investigations and cohort studies suggest that selenium exhibits a bivalent effect in cancer, either increasing or decreasing the risk of cancer (Yan-ping *et al.*, 2005). Yan-ping *et al.* (2005) found that selenium supplement can lead to the expansion of secretory canaliculus, increase in the number of endocrine cells in gastric mucosa, alterations in the mitochondria and changes in the number

and shape of pinosomes. Furthermore, our results also indicate that selenium supplement as sodium selenite can alter the ultrastructure of gastric mucosa. Jeong-Hwan *et al.* (2012) also found that selenium markedly attenuated ethanol-induced lipid peroxidation in gastric mucosa and increased activities of radical scavenging enzymes such as SOD, CAT and GSH. Surajit and Sudipta (2014) discovered appreciable beneficial effects of selenium and vitamin E co-administration against arsenic-induced changes in biochemical and histopathological parameters. Both selenium and vitamin E significantly reduced the basal gastric acid secretion when given individually, using Shay's ligation model (Ahmad and Mohammad, 1996). Selenium and vitamin E protect gastric mucosa against the lesion produced by hypothermic restraint stress and chemical (Ahmad and Mohammad, 1996).

2.4.7 Effect of selenium on immune and endocrine systems

Selenium deficiency has been linked to many health problems in young animals such as increased neonatal mortality, decreased sucking reflex, weakness, higher occurrence of infectious diseases and white muscle disease (Pechova *et al.*, 2012). Selenium deficiency may also result in immune and endocrine disorders, especially thyroid dysfunction, as Se is essential for thyroid function and thyroid homeostasis (Kohrle *et al.* 2005). Selenium is also able to modify the immune response in patients with autoimmune thyroiditis. Low sperm production and poor sperm quality are consistent features of Se-deficient animals. The pivotal link between Se, sperm quality and male fertility is GPX4 since the enzyme is essential to allow the production of the correct architecture of the mid-piece of spermatozoa (Geoffery and John, 2005). Selenium also has insulin mimetic properties, an effect that is probably brought about by stimulating the tyrosine kinases involved in the insulin signalling cascade. Furthermore, in the diabetic rat, Se not only restores glycaemic control but it also prevents or alleviates the adverse effects that diabetes has on cardiac, renal and platelet function (Geoffery and John, 2005). Adequate supplies of iodine and thyroid hormones are needed for the normal

development of the central nervous system (CNS). Thus iodine deficiency during the development of the CNS can cause a wide range of damaging effects (Mitchell *et al.*, 1997). Thyroxin (T_4) produced in the thyroid gland is biologically inactive without further metabolism to 3, 3, 5'-tri-iodothyronine (T_3). Up 80% of circulating T_3 is produced by the activity of selenium-containing enzyme type I iodothyronine diiodinase (ID-I). Thus thyroid hormone metabolism may be impaired by both selenium and iodine deficiencies (Mitchell *et al.*, 1997). Selenium deficiency result in immune suppressive, reduced resistance to infection, neutrophil function, antibody production, proliferation of T and B cells in response to mitogens and cyto destruction by T lymphocytes and NK cells (Kiremidjian and Stoczky, 1987).Mudagal (2005) found that supplementation of 0.3 ppm Se in buffalo calves significantly improved their humoral immune response, as compared to supplemented animals. Song *et al.* (2006) reported that supplementation of different levels of Se in the diet of laying hens significantly improved their lipopolysaccharide (LPS) stimulation index, concanavalin A (Con A) stimulation index and peroxidase enzyme activity. Lin and Chang,

(2006) noticed enhance immune response in cocreels when their diet was added with 20mg / Kg of vitamin E.

2.4.8. Effect of selenium on cardiovascular system

Low levels of selenium can contribute to heart failure, and being deficient in selenium seems to make atherosclerosis worse. Studies have shown that. Selenium supplements do neither seem to have any effect on the progression of heart disease, nor do they protect against heart attack (Latheef *et al.*, 2014). Selenium, supplementation (1 ppm) has been shown to increase LDL receptor activity and m-RNA expression (Dhingra and

Bansal, 2006). Selenium concentrations were found to be inversely associated with the coronary heart disease and dilated cardiomyopathy in observational studies and the evidence from a few randomized studies is still inconclusive (Flores-Meto *et al.*, 2006). Latheef *et al.* (2014). Showed that 1mg of selenium supplementation elevated serum lipid profile in experimental rabbits. Lower tissue Se status has been linked to an increased risk of several aging-related diseases, including cardiovascular diseases (Rijin *et al.*, 2014). Several animal studies have also demonstrated the cardiovascular benefits of Se, whereby dietary Se supplementation prior to ischemia-reperfusion injury resulted in improved cardiac functional recovery, reduced incidence of reperfusion arrhythmias, and preservation of ventricular ultra-structure (Venados *et al.*, 2004). Supplementation with selenium-enriched yeast may ameliorate, at a transcriptional level, the effects of aging on cardiac function in mitochondrial DNA mutator mice. If similar effects hold true in relation to human heart, the health implications could be of major benefit from the perspective of geriatric health and well-being (Rijin *et al.*, 2014).

.2.4.9 Effect of selenium on liver and kidney

Siham and Nabila. (2008) found that selenium and garlic ameliorated kidney and liver damage induced by HgCl₂ injection. Either selenium or garlic when given with

HgCl₂ induced a decrease in the elevated serum level of both blood urea nitrogen (BUN) and creatinine as compared with the HgCl₂ group, while no marked alteration was observed in serum levels of both BUN and creatinine in rats given selenium or garlic alone as compared with saline control group. The elevated serum level of

BUN and creatinine is an indicator of kidney damage as blood urea nitrogen is derived from normal metabolism of protein and is excreted in the urine. Elevated BUN usually indicates glomerular damage while creatinine is a metabolite of creatine and is excreted completely in the urine *via* glomerular filtration; an elevation of its level in the blood is, thus, an indication of impaired kidney function. Rats injected with HgCl₂ showed elevation of serum level of both ALT and AST. However selenium treatment in rat injected with HgCl₂ decreased the elevated serum level of ALT and AST (Siham and Nabila, 2008).

2.4.10 Effect of selenium on the central nervous system

Selenium is a powerful preventive and therapeutic substance to excitotoxic brain damage distinct from known antioxidative substances (Savaskan *et al.*, 2008). Excitotoxic conditions were induced in neurons by treatment with an excess of glutamate, the most abundant excitatory neurotransmitter in the brain. Glutamate treatment reduced cell survival by 80%. This glutamate-induced cell death could be prevented by simultaneous application of selenite in a concentration-dependent manner (Savaskan *et al.*, 2008).

2.4.11 Effect of selenium on growth

Selenium deficiency causes decrease in plasma 3, 5, 3'-triiodothyronine(T3) concentration and this can cause reduced growth in birds and selenium supplementation increased it (Boostani *et al.*, 2014). In the oxidative stress, selenium pool of body maybe depleted for antioxidant activity and deficiency in selenium occur for conversion of T4 to T3. Pechova *et al.* (2012) found that supplementation of mothers with Se both in organic and an inorganic form was sufficient to prevent Se deficiency in kids at the time of weaning.

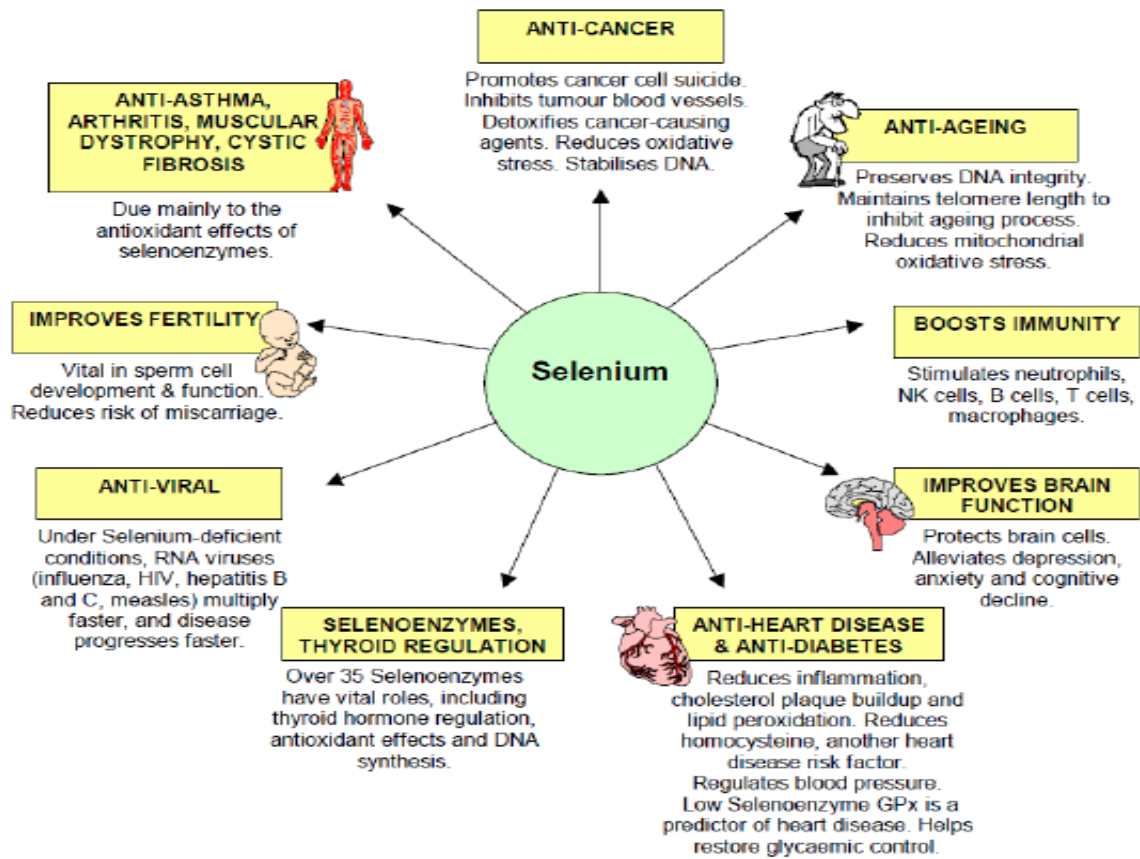


Figure 2.3: Roles of selenium in the body (Adapted from Trace Trial, 2007).

2.5 Biological Activities of Vitamin E

Vitamin E is a group of eight antioxidant lipophilic molecules, four of which are tocopherols and four of which are tocotrienols. It is mostly found in green vegetables, grains, nuts and various vegetable oils, as well as in eggs and milk. Although it is commonly known for its antioxidant properties, the first biological role attributed to VE was its necessity for foetal survival (Dan *et al.*, 2010). VE is known to possess many biological properties including antioxidant activity to modulate protein function and gene expression (Ibrahim *et al.*, 2010). Samanta *et al.* (2006) in cross bred calves and Rajesh (2006) in buffalo calves reported that supplementation of vitamin E improved the immune status of the animals.

2.5.1 Role of vitamin E in cellular antioxidant defence

Vitamin E (α -tocopherol) is a lipid-soluble antioxidant and a well-accepted first line defence mechanism against lipid peroxidation. It functions as a chain-breaking antioxidant for lipid peroxidation in cell membranes and as a scavenger of ROS such as superoxide anion, hydrogen peroxide and singlet oxygen (Ibrahim *et al.*, 2010). Yoshikawa *et al.* (1991) reported a decrease in gastric mucosal VE level and an increase in gastric mucosal lipid peroxidation in ischaemia-reperfusion-induced gastric mucosal injury and severity of the injury was enhanced in VE-deficient rats. According to Yoshikawa *et al.* (1991), in nitric oxide-depleted rats, VE played an important protective role against ischaemia-reperfusion-induced gastric mucosal injury. The authors suggested that the gastro-protective effect of VE was not only due to its antioxidant action, but also inhibitory action on neutrophil infiltration into the gastric mucosa. Ibrahim *et al.* (2010) concluded that dietary supplementation of palm VE or α -tocopherol was able to reduce

gastric lesion significantly in comparison to the stressed control rats. The WRS increased plasma ACTH and corticosterone significantly. Palm VE treatment reduced the parameters significantly compared to the stressed control. Therefore, supplementation with either palm VE reduces the formation of gastric lesions, probably by inhibiting the elevation of ACTH and corticosterone levels, induced by stress. VE scavenges peroxy radical intermediate in lipid peroxidation and is responsible for protecting poly-unsaturated fatty acids present in cell membrane, and low-density lipoprotein (LDL) against lipid peroxidation (Sujogya, 2012). Vitamin E pre-treatment has been reported to be beneficial in preventing formaldehyde-induced tissue damage in rats (Gulec *et al.*, 2006).

The preventive effect of VE on cypermethrin or endotoxin-induced oxidative stress in rat tissue is suggestive of its antioxidant activity (Kheir-Eldin *et al.*, 2000). Vitamin E has been shown to protect the liver from injury via inhibiting the oxidative damage (Sushma *et al.*, 2009). Its supplementation leads to significant decrease in MDA concentration (Ashok and Sushil, 2005). Tocotrienol caused a reduction in the gastric acid concentration in rats not exposed to stress, which suggests that it may have an anti-secretory effect and also showed that a single large dose (300mg/kg) of alpha-tocopherol caused a reduction in gastric acid secretion in non-stressed rats. Based on this finding it was proposed that that one of the protective effects by vitamin E against gastric injury could be through its anti-secretory function (Nur *et al.*, 2005). The antioxidant activity of vitamin E in preventing lipid peroxidation may be one of the mechanism by which vitamin E enhances immunity (Shinde *et al.*, 2007). Vitamin E causes a reduction of glucocorticoids, which are known to be immune suppressive. It also alters arachidonic acid metabolism and subsequent synthesis of prostaglandins, thromboxanes and leukotrienes. Under stress condition, increased level of these compounds by endogenous synthesis or exogenous entry may adversely affect immune cell function (Shinde *et al.*, 2007).

2.5.2 Absorption and metabolism of vitamin E

Vitamin E as a lipophilic molecule is absorbed in the gut via micelles and then incorporated into chylomicrons (Dan *et al.*, 2010). When it reaches the circulation, VE is transferred to other lipoprotein by the action of phospholipid transfer protein, and to cells by the action of phospholipid transfer protein and lipoprotein lipase (Dan *et al.*, 2010).

2.6 Trace Elements

The role of minerals in enzyme functions has been studied extensively in nutrition and biochemistry. Magnesium, for example, is a co-factor for glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, two pentose-cycle enzymes catalysing the production of NADPH from NADP⁺, thus the deficiency of dietary magnesium reduces glutathione reductase activity and result in radical-induced protein oxidation indicated by the generation of protein carbonyl and marked lesions in tissues (Rock *et al.*, 1995). Iron promotes ROS production, lipid peroxidation and oxidative stress (Dabbagh *et al.*, 1994). Iron is the most abundant trace element in the body, and iron occurs mostly bound to proteins. Free iron concentrations are particularly low because Fe³⁺ is not water soluble, and Fe²⁺ participates in the generation of free radicals. Thus, an increase in extracellular iron concentration can result from dietary protein deficiency, dietary iron loading, low concentration of iron-binding proteins or cell injury. Copper, zinc and manganese are indispensable metals for the activities of Cu, Zn-SOD and Mn-SOD, respectively. Therefore, dietary deficiencies of these minerals markedly decrease tissue Cu, Zn-SOD and Mn-SOD activities and result in peroxidative damage and mitochondrial dysfunction (Yun-Zhong *et al.*, 2002).

Copper and Mn function as component of metallo-enzymes that take part in reduction reactions. These metallo-enzymes are involved in multiple physiological processes including respiration,

carbohydrate and lipid metabolism, antioxidant activities and collagen formation (Andrieu, 2008). One of the copper-containing enzymes, ceruloplasmin binds up to 95% of circulating copper, regulates iron availability, takes part in oxidation-reduction reactions, and may regulate immune function (Healy and Tipton, 2007). Both Cu and Mn are important for keratin formation, and are components of SOD (Tanya, 2009). Zinc is widely distributed throughout the body as component of metallo-enzyme and metallo-proteins (Tanya, 2009). Zinc finger proteins play an integral role in regulating gene expression, consequently impacting a wide variety of body functions, including cell division, growth hormone production, appetite control and immune function (Tanya, 2009). Zinc has a co-active role in SOD. It is important to note that the concentration of trace elements changes under different infectious or inflammation. The ion changes reflect changes in cation binding plasma proteins, and, more importantly, alterations in cellular uptake mechanisms (Mohd *et al.*, 2013).

These ionic changes help in preventing infection or disease. In dairy cattle, plasma Fe and Zn concentrations decrease during the acute phase response to immunological challenges, where plasma Cu concentration may increase (Mohd *et al.*, 2013). Zinc sulphate and zinc chloride exert dose-dependent in ulcer index in three models, of gastric ulceration pyloric ligation, aspirin induced-ulcer and WRS (Sonali *et al.*, 2012).

2.7 Haematological Parameters

Blood is specialized bodily fluid that delivers necessary substances to the body cells such as nutrients, oxygen and transport of waste products away from those of same cells. Blood is the most important body, fluid that governs vital functions of the body like respiration, circulation, excretion, osmotic balance and the transport of metabolic substance. Circulation of the blood within the cardiovascular system is essential for transportation of gases, nutrients, minerals, metabolic products and hormones between organs (Savithri *et al.*, 2010). Blood parameters are probably the more rapid and detectable variations under stress and are fuel in assessing the

health condition (Savithri *et al.*, 2010). The haematological parameters are important because they have proven to be valuable indicators of diseases or stress in animals (Ebulunlomo *et al.*, 2012).

2.7.1 Stress exposure and haematological response

Physiological studies have shown that stress from any source has influence on the endocrine, haemopoietic and immune systems (Ebulunlomo *et al.*, 2012). Cytokines and cortisol play an important role in the communication between these systems. Previous studies have shown that stress increase erythrocyte, neutrophil and platelet counts, but decrease the number of lymphocytes, eosinophil's and monocytes (Ebulunlomo *et al.*, 2012). The magnitude of stress-induced changes is significantly reduced in adrenalectomised animals. It is suggested that endocrine factors, released during stress modulate leucocyte trafficking and distribution of leucocytes between the blood and other immune compartments. The activation of sympathetic nervous system may also have a role to play (Bamidele *et al.*, 2010). Lymphocytes and monocytes express receptors for several stress hormones, including norepinephrine and epinephrine, thus stressful event could alter immune function. Immobilisation stress induces distinct changes in concentration of erythrocytes (Artemy *et al.*, 2013). Recently, it has been shown that erythropoietin production and erythrocyte differentiation are regulated by ROS especially H_2O_2 , which are involved in redox-sensitive signaling pathways through down-regulation of transcription factors. This means that ROS generation can suppress erythropoietin synthesis, whereas antioxidants can stimulate its synthesis (Anand *et al.*, 2010). Antioxidants such as β -carotene, desferrioxamine, teapolyphenol, VE, VC, reduced glutathione, N-acetylcystine and α -lipoic acid have been shown to modulate haematological parameters (RBC count, WBC count, MCH, MCHC and MCV) and erythropoietin gene expression under normoxia or hypoxic conditions (Anand *et al.*, 2010). Studies on N-acetylcystine and α -lipoic acid show a direct relationship between oxidative stress and erythropoiesis

and that some antioxidants could modulate haematological parameters (Zembron *et al.*, 2009). For example, there is a significant reduction in platelet count in non- smokers and in smokers after VE supplementation. An inverse relationship between platelet count and dietary vitamin levels has also been reported (Anand *et al.*, 2010). According to Anand *et al.* (2010), administration of taurine significantly increased neutrophil count and decreased lymphocyte count, without a significant change in total leucocyte count.

Stress-related behavioural activities and haematological response may be triggered for a long period by an exposure to psychological, emotional stressor (Ogundeji *et al.*, 2013). According to Minka and Ayo (2008), psychological stress acting singly or in combination with other factors, may induce adverse haematological effects due to increases ROS activity. Ogundeji *et al.* (2013), found no significant differences in the haematological parameters of rats, treated with lycopene (an antioxidant), and subjected to psychological stress

2.8 Erythrocyte Osmotic Fragility

Erythrocytes are frequently used to evaluate oxidative stress. This is because their membrane is rich in polyunsaturated fatty acids, a primary target for reaction, involving ROS, and is very susceptible to lipid peroxidation (Alhassan *et al.*, 2010). Peroxidation results in the loss of membrane fluidity and cellular lysis. Increased haemolysis indicated by fragiligram shifting to the right side has been used as an indirect way of quantifying oxidative stress in animals during stress (Adenkola and Ayo, 2009). Increased lipoperoxidative is indicated by increase in MDA concentration, resulting from an increase in erythrocyte osmotic fragility in rats, exposed to chlorpyrifos (Ambali *et al.*, 2010a). ROS can adversely affect the proteins, resulting in modification of activity of enzymes. Thus, damage to the membrane transport proteins may produce disturbed cellular ionic homeostasis, leading to alteration in intracellular calcium and potassium and a series of changes in the cells. ROS can directly affect the conformation and activities of all sulphhydryl-containing molecules by oxidation of their thiol moiety (Ambali *et*

al., 2010a). Zinc is an antioxidant that ameliorates the increased erythrocyte osmotic fragility, induced by chronic chlorpyrifos exposure (Ambali *et al.*, 2010a).

According to Olorunshola *et al.* (2011), who investigated the effect of two-and-half hours of road transport on osmotic fragility test on humans during harmattan season, there was a significant decrease in percentage haemolysis recorded at 0.5% NaCl concentration after transportation in subjects administered with VC. The result suggested that erythrocyte osmotic fragility could be used as a biomarker of stress. VE, VC and carnitine may be of benefit in overcoming oxidative stress and haemolysis under situations such as intermittent hypobaric hypoxia (HH) and hypobaric therapy (Asha *et al.*, 2007). Co-administration of VC and VE has been shown to ameliorate chlorpyrifos-induced erythrocyte fragility (Ambali *et al.*, 2010a). This, apparently, resulted from the antioxidant properties of the vitamins as observed by reduced lipoperoxidative damage to the erythrocyte membranes (Ambali *et al.*, 2010a).

2.9 Aetiologies of Gastric Ulcer Development

Despite its robust and multi-faceted nature, many factors directly related to impairment of mucosal defence can alter the epithelial barrier and encourage the formation of mucosal injury, the most important of which are acid secretion, bacteria and their products, NSAIDs, alcohol, ROS, as well as different chemical compounds. Their effects on the gastric barrier represent important mechanism of the pathogenesis of gastric ulcers, chronic gastritis and other gastric diseases, which are frequently generated through an imbalance between mucosal aggressive/injurious (acid, pepsin, stress and *Helicobacter pylori*) and defensive mucosal factors (mucin, prostaglandins, bicarbonate, NO and growth factors) (Tulassay and Herszenyi, 2010).

2.9.1 Oxidative stress

Increase oxidative stress generally describes a condition in which cellular antioxidant defences are inadequate to completely inactivate the ROS (Stocker and Keany, 2004). Over-production of ROS frequently either by excessive stimulation of NADPH oxidase by cytokines or by the mitochondrial electron transport chain and xanthine-oxidase results in oxidative stresses (Valko *et al.*, 2007). The ROS, such as superoxide anions, hydrogen peroxide and hydroxyl radicals, are involved in the aetiology and pathophysiology of several human diseases, including neurodegenerative disorders, viral infections, inflammation, autoimmune pathologies in digestive disturbances such as gastro-intestinal inflammation and gastric ulcer (Repetto and Llesuy, 2002). During gastric oxidative stress, the imbalances of aggressive and defensive factors in the stomach play a pivotal role in gastric haemorrhage and ulcer formation (Hung, 2005). Over-production of ROS is one of the major pathogenic factors that directly result in oxidative damage, including lipid peroxidation, protein oxidation and DNA damage, including cell death. Additionally, the agents act as second messenger to activate diverse redox-sensitive signaling transduction cascades including mitogen-activated protein kinase (MaPKs) and downstream transcription factors such as NF-KB and AP-1, which regulate the expression of several pro-inflammatory genes and, thereby, lead to the elaboration of chemical and humoral mediators of tissue inflammation and injury (Ali and Harty, 2009).

This is frequently evidenced by pro-ulcerative factors in the stomach and gut such as *H. pylori*, use of NSAIDs, ethanol, smoking, psychological stress, corticosteroid use and loss of sleep while defensive factors involve glutathione (GSH), an important endogenous sulfhydryl compound and mucus biosynthesis (Olaleye *et al.*, 2007). In diseased state oxidative stress of the stomach may occur, and this results in an elevation of mucosal lipid peroxide, generated from the reaction of oxy-radicals and cellular polyunsaturated fatty acid. The GSH may prevent this aggressive action that can damage gastric mucosal cells. Malondialdehyde (MDA) is an end product of peroxidation of polyunsaturated fatty acids and related esters within cell membrane. The measurement of MDA is a suitable index of oxidative tissue damage. On the other hand,

sulfhydryl compound such as GSH are involved in the maintenance of gastric integrity, particularly when ROS are implicated in the pathophysiology of tissue injury (Blandizzi *et al.*, 2005). Lipid free radicals and MDA appear in the blood and gastric juice as a result of ROS-initiated chain reaction or indirect mechanisms that suppress the antioxidant capacity of both blood and gastric wall to scavenge ROS (Tuorkey and Abdul-Aziz, 2011).

Numerous studies have demonstrated a decrease in GSH level in inflammatory and ulcerated gastric mucosa. The protective effect of GSH on gastric damage induced by ethanol; non-steroidal anti-inflammatory drugs or lipopolysaccharide has been well documented. A large body of research evidence in both animals and human studies has demonstrated the adverse effect of psychological stress on the gastro-intestinal tract. For instance, in accordance to Shinki *et al.* (2012), susceptibility to gastric lesions increased in rats by social stressors as premature separation of the rat pup from its mother. Chang (2008) showed that stressors can be acute or chronic and range from daily hassle to life-threatening situations like natural disasters and violence that trigger the “fight or flight” over-time, recurrent stress which result in an increased demand on physiological systems. Thus, several terms have been used to describe stress-related mucosal damage in critically ill patients, including stress ulcers, stress gastritis, stress erosions, hemorrhagic gastritis, erosive gastritis and stress-related mucosal diseases (Ali and Harty, 2009). Stimulation of gastric acid secretion has historically been considered a mechanism by which physiological stress increase, susceptibility to gastro-duodenal ulceration. It is also known to modify gastric blood flow, which plays an important role in the gastric mucosal barrier and affect some possible mediators, such as cytokines, corticotropin-releasing hormone and thyrotropin releasing hormone. Furthermore, stress may exert different effects on gastric motility including delayed gastric emptying which could increase the net acid load delivered to the duodenum and enhancing the risk of duodenal ulcer. Psychological stress may also promote the growth of *H. pylori* in the duodenum, if it increases duodenal acid load.

This is because *H. pylori* inhibitory effects of bile may reverse by acid (Shinki *et al.* 2012). A variety of factors produce damage of gastric mucosa, including systemic events such as thermal stress or local application of various irritants that are commonly named breakers of gastric mucosal barrier (Kwiecien *et al.*, 2002). This mucosal barrier is composed by epithelial cell with tight junctions and superimposed layer of mucus. The aim of this barrier is to protect the mucosa against damage of deeper structures by hydrogen ions (H⁺) and other noxious substances, originating from the gastric lumen (Kwiecien *et al.*, 2002).

2.9.1.1 Lipid peroxidation

Lipid peroxidation is the introduction of a functional group, containing two catenated oxygen atoms, O-O, to unsaturated fatty acids in a free-radical reaction (Eqbal *et al.*, 2011). Attacks are initiated by the formation of a carbon-centred radical by the abstraction of a hydrogen atom at one of the double bonds of the lipids. Lipid peroxidation is one of the major causes of quality deterioration during the storage of fats oils or other lipid-rich foods (Eqbal *et al.*, 2011). It is broadly defined as oxidative deterioration of polyunsaturated fatty acids which are fatty acid containing more than two carbon-carbon double bond (Maneesh and Jayalekshmi, 2006). Lipid when reacted with ROS can undergo the highly-damaging chain reaction of lipid peroxidation, leading to both direct and indirect effects (Devasagayam *et al.*, 2004).

Peroxidation of lipids is a binding process, connected with the formation of aldehydes, MDA (Niedworok and Bielaszka, 2007). It is the end-product of lipid peroxidation and a good marker of ROS-mediated damage and oxidative stress (Atip *et al.*, 2010). It has been utilised as suitable biomarker of lipid peroxidation.

2.9.1.2 Biomarkers of Oxidative Damage

The WRS is known to provoke acute inflammation and injury of gastric mucosa. The interleukin-1 beta (IL-1B) and tumor necrosis factor-alpha (TNF α) are the major pro-inflammatory cytokines, playing important role in the development of acute inflammation, mediated by neutrophil infiltration of gastric mucosa (Kwiecien *et al.*, 2004). Neutrophils produce superoxide radical anion, which belongs to the group of ROS. Superoxide radical anion reacts with cellular membrane lipids, leading to the formation of lipid peroxides, metabolised to MDA and 4-hydroxynonenal (4-HNE) as biomarkers of lipid peroxidation. The localisation and effects of oxidative stress, as well as information regarding the nature of the ROS may be gleaned from the analysis of discrete biomarkers of oxidative damage, isolated from tissues and biological fluids. Biomarkers are defined as characteristics that can be objectively measured and evaluated as indicators of normal biological process, pathogenic process or pharmacological response to a therapeutic intervention (Finkel and Holbrook, 2000). To function as suitable biomarkers of oxidation modification in relation to disease, it is critical that such oxidation are stable, can accumulate to a detectable concentration, reflect specific oxidation pathways and correlate with severity of diseases so that they can be used as diagnostic tools. (Halliwell and Whiteman, 2004). Products of lipid peroxidation such as α , β -unsaturated reactive aldehydes include MDA, 4-hydroxyl -2- nonenal (4-HNE), 2-propenal (acrolein) and isoprostane. All the products can be measured in plasma as indirect index of oxidative stress (Halliwell and Whiteman, 2004). Compared with ROS the aldehydes are relatively stable and can diffuse within or escape from cell and attack targets far from the site of the original event. The aldehydes therefore, not only end-products and remnants of lipid peroxidation process, but may also act as second cytotoxic messenger (Klaunig and Komendulis, 2004).

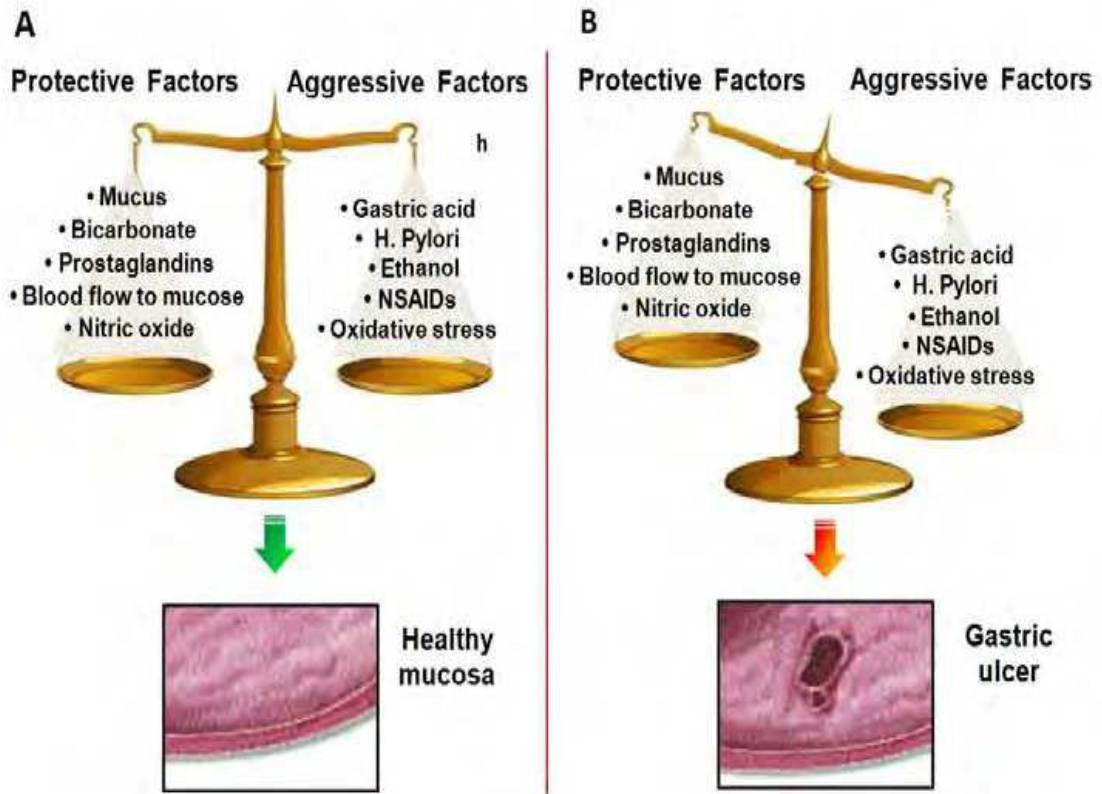


Figure 2.4: Mechanism of ulcer: The imbalance between gastro-toxic/aggressive agents and protective factors. Adapted from (Tulassay and Herzenyi, 2010).

2.9.1.3 What are Free Radicals?

Free radicals are defined as molecules having an unpaired electron in the outer orbit (Yun-Zhong *et al.*, 2002). They are generally unstable and very reactive. Oxygen free radicals are superoxide, hydroxyl, peroxy (RO_2^*), alkoxy (RO^*), and hydroperoxyl (HO_2^*) radicals. Nitric oxide and nitrogen dioxide ($^*\text{NO}_2$) are two nitrogen free radicals. Oxygen and nitrogen free radicals can be converted to other non-radical species, such as hydrogen peroxide, hypochlorous acid (HOCl), hypobromous acid (HOBr) and peroxynitrite (ONOO^*). ROS, RNS and reactive chlorine species are produced in animals and human under physiological and pathological condition (Evans and Halliwal, 2001). Thus, the ROS and reactive nitrogen species (RNS) include radical and non-radical species (Klaunig and Komendulis, 2004).

2.9.1.4 Production of Free Radicals

Oxygen is required for the generation of all ROS, RNS and reactive chlorine species (Andrey *et al.*, 2007). The physiological generation of free radicals can occur as a by-product of biological reactions in mitochondria, peroxisomes, cytochrome P_{450} . In resting cells, superoxide anion is produced at 1-2% of total daily oxygen consumption during electron transfer and oxidative phosphorylation for ATP generation by mitochondria (Finkel and Holbrook, 2000). Mitochondrial ROS are recognized as regulators of mitochondrial functions including electron transfer chain enzymes and mitochondrial membrane potential (Finkel and Holbrook, 2000).

2.9.1.5 Biological Role of Free Radicals

There are “two faces” of ROS in biology in that they serve as signaling and regulatory molecules at physiologic levels but highly deleterious and cytotoxic oxidants at pathologic levels (Evans and Halliwal, 2001). The beneficial effects of ROS may play an important role in

the origin of life and biological evolution of organisms. For example, oxygen radicals exert critical actions such as signal transduction, gene transcription and regulation of soluble guanylate cyclase activity in cells (Andrey *et al.*, 2007). Also NO is one of the most widespread signaling molecules, participating in virtually every cellular and organ function in the body (Evans and Halliwal, 2001). Physiologic levels of NO produced by endothelial cells are essential for regulating the relaxation and proliferation of vascular smooth muscle cells, leucocyte adhesion, platelet aggregation, angiogenesis, thrombosis, vascular tone and hemodynamics (Andrey *et al.*, 2007). In addition, NO produced by neurones serves as neurotransmitter while NO generated by activated macrophages is an important mediator of the immune response (Valko *et al.*, 2007). However as oxidants and inhibitors of enzymes containing an iron-sulfur centre, ROS and other reactive species cause the oxidation of biomolecules (protein, amino acid, lipid, and DNA), which leads to cell injury and death (Valko *et al.*, 2007).

2.4.1.6 Mechanism of Stress Gastric Injury

Stress ulcer is a highly prevalent clinical complication. Fully understanding the mechanism of stress ulcer will increase the knowledge for the prevention and treatment of stress-related organ injury (Nie *et al.*, 2003). Both psychological and physiological responses occur during WRS, and are involved in the pathogenesis of gastric ulceration.

The stomach is one of the main targets of stress. Stress-induced gastric ulceration is a typical example of stress-associated organ injuries (Anil *et al.*, 2010). It is an easy and convenient method to induce both psychological and physical stress. The psychological responses include anxiety, depression, helplessness, fear and threat of drowning. The physiological responses are neuro-hormonal and immunological activations, involving corticotrophin-releasing factor. These two systems interact during stressful challenges (Robles and Carroll, 2011). Stress affects psychological and physiological balances which can lead to various pathological changes. One

known pathological stress-induced condition is the formation of gastric lesion, and studies have shown that its pathogenesis is multi-factorial. It includes factors which disrupt the gastric mucosal integrity such as changes in gastric acid, mucus and bicarbonate secretions, inhibition of gastric mucosal prostaglandin synthesis (Nur *et al.*, 2012), reduction in gastric mucosal blood flow, changes in stress hormones (Ibrahim *et al.*, 2010) and gastric motility (Jun *et al.*, 2009). Psychogenic factors, such as stress, play a major role in the pathogenesis of gastric ulcers in man. Gastro-intestinal erosion is one of the consistent findings in man and in experimental animals subjected to different types of stress (Shawon and Gautam, 2012). It has been known for many years that patients with severe burns, trauma or other serious diseases develop severe intestinal bleeding or perforation caused by ulcers. Endoscopy has revealed that severe physiological stress induces lesions, ranging from erosion to complicated ulcers (Shawon and Gautam, 2012). Clinically, the term stress ulcer encompasses both upper gastrointestinal haemorrhages and lesions as a consequence of trauma, including burns, intracranial injuries and septic shock.

Role of autonomic nervous system in stress ulcer: It is known that the autonomic nervous system (ANS) is one of the main components of stress, which exerts a profound influence on heart rate and digestion. However, the precise role of each component of the ANS in gastric ulcer after stress exposure remains unclear as conflicting mechanistic explanations have been provided (Arawaka *et al.*, 1997). Arawaka *et al.* (1997) reported that excessive peripheral sympathetic activity plays an important role in the WRS model. However, Shichijo *et al.* (1991) suggested the presence of a sympathetic hypofunction and parasympathetic hyperfunction in the stomach, contributing to the gastric lesion in the spontaneously hypertensive rats, subjected to WRS. A recent report showed that vagal activity played an important role in the protection of gastric ulcer to WRS (Brzozowski *et al.*, 2004). According to Yuan-Fang *et al.* (2005), WRS induced serious gastric mucosal lesion, and parasympathetic over-activity is the predominant autonomic response in the WRS model. Bilateral cervical vagotomy prevented the gastric mucosal lesion

induced by WRS, and this is a causal relationship between parasympathetic over activity and gastric mucosal lesion in rat WRS model. Although controversial, it is generally believed that sympathovagal imbalance plays an important part in the gastric lesion, induced in the rat by WRS model (Hashiguchi *et al.*, 1993) and that it stimulates numerous pathways, leading to increased ROS production.

Effects of reactive oxygen species on gastric mucosa: Excessive production of ROS, loss of antioxidant defence or both. Major consequences of oxidative stress are damage to nucleic acid bases, lipids and proteins, which can severely compromise cell health and viability or induce a variety of cellular responses through generation of secondary reactive species. This ultimately leads to cell death by necrosis or apoptosis. Oxidative damage of any of these biomolecules, if unchecked, can theoretically contribute to development of diseases (Stocker and Keaney, 2004).

Changes in stress hormones: During stress, the other mechanism involved is the activation of the hypothalamic pituitary–adrenal axis (HPA) and sympatho–adrenal–medullary (SAM) systems, causing the release of corticosterone together with noradrenalin and adrenalin (Nur, *et al.*, 2012). Furthermore, the elevation in catecholamines level may generate ROS, which may be cytotoxic and mediate tissue damage by injuring cellular membranes and releasing intracellular components. It has been demonstrated that stress induces activation of the hypothalamus–pituitary–adrenal axis. When exposed to stress, the first system to respond is the ANS, which sends a message to the hypothalamus. The hypothalamus, in turn, release corticotropin releasing factor (CRF), which is picked up by the nearby pituitary. This neuropeptide stimulates the pituitary to release adrenocorticotrophic hormone (ACTH) to the blood stream. The ACTH stimulates the production and release of glucocorticoids (cortisterone in rats, cortisol in humans) from the adrenal glands. Corticosterone stimulates the release of glucose from body stores which provides energy to fight off the danger or to turn away from it (Nur, *et al.*, 2012). Once

the threat is over, the mediators return to base-line level due to negative feed-back on the HPA axis (Ibrahim *et al.*, 2010). The plasma corticosterone level is the most striking hormonal change found in stress, and is used as a sensitive index of stress (Ibrahim *et al.*, 2010). Increase in catecholamines level during stress causes vasoconstriction and reduction in mucosal blood flow due to impaired gastric microcirculation, which results in ischaemia. The changes can ultimately result in formation of gastric lesion (Nur *et al.*, 2012).

Xanthine-xanthine oxidase and neutrophil infiltration in the gastric mucosa in restraint rats: The mechanism of acute gastric mucosal lesion is assumed to relate to ischaemia or hypoxaemia, which can induce increased generation of ROS through the xanthine-xanthine oxidase system and infiltrated neutrophils in the gastric mucosa. Xanthine oxidase produces superoxide anions and H₂O₂ by catalysing the oxidation of hypoxanthine or xanthine using O₂ as the electron acceptor. High levels of xanthine oxidase activity and high serum uric acid (xanthine oxidase-derived metabolite) concentration have been found in rats exposed to WRS (Shinki *et al.*, 2012). Inflammatory response is also known to be an important pathway in the development of acute gastric mucosal lesion. NADPH oxidase is present in the membranes of neutrophils, which is the major source of superoxide radical anion. Neutrophils play a crucial role in WRS-induced mucosal injury, including ROS generation. Myeloperoxidase activity is considered as index of neutrophil infiltration in gastric mucosa (Shinki *et al.*, 2012). Its activity in gastric mucosa significantly increased in WRS rats, but administration of fermented papaya preparation, an antioxidant, reduced the activity.

2.9.2 *Helicobacter pylori*

Helicobacter pylori are common human pathogens and public health problem, associated with the pathogen of gastritis and peptic ulcers. With a prevalence of up to 90% in developing population, this microorganism is the second most common pathogen for human beings. It is a

non-sporulating, Gram negative micro-aerophilic bacilli, spiral-shaped, having one to six polar-sheathed flagellae, emerging from one of its rounded ends and a smooth surface (Monica and Armelle 2013). This pathogen multiplies with great efficiency in the hostile environment within the stomach, but survives poorly in the lumen. It is mainly found under the mucous layer and in close proximity or even attached to gastric superficial epithelial cells, without substantial invasion of host tissue (Monica and Armelle 2013). *H.pylori* induces chronic gastritis of varying severity in infected subjects, which in around 10-15% progress to peptic ulcer, while in 1-2 % of subjects ultimately result in *MALT* lymphoma or gastric adenocarcinoma. The initial response to infection is an interaction of the host epithelial cells with the bacteria. However, the pathogenetic mechanisms of chronic infection with and gastric ulcer are yet to be fully determined (Calvino-Fernandez and Parra-Cid, 2010).

A characteristic feature of *H. pylori* is the synthesis of urease, which was its first virulence factor studied. This enzyme may explain the extra-ordinary ability of the bacteria to colonize the gastric mucosa and survive in an acid environment (Monica and Armelle, 2013). Since the ecologic niches of the bacteria are rich in urea, the pathogen catalyses urea hydrolysis with the formation of ammonium (NH_3), carbon dioxide and hydroxyl ions. By this mechanism, *H. pylori* neutralize the surrounding gastric acid and protects itself from the strong acidity of the stomach (Monica and Armelle, 2013). On the other hand, although the neutralisation of gastric acid benefits the bacteria, metabolites from urease activity are toxic to gastric epithelial cells. The formed ammonium reacts with OCl^- produce by activated neutrophils to form highly-toxic monochloramine (NH_2Cl) in the stomach. A hallmark of *H.pylori* urease has been shown to significantly decrease this toxicity, suggesting that ammonia is at least partially responsible for the cytotoxicity, found in association with this bacterium. Moreover, hydroxide ions are also considered toxic to gastric epithelial cells (Handa *et al.*, 2010). Beside urease activity, further important virulence factors from *H. pylori* are their spiral shape and the motility of their flagella, which render them resistant to peristaltic flushing of the gastric contents and enable

them to persist in the mucous layer. Additionally, this pathogen produces other enzymes, including catalase, oxidase, protease and phospholipase, and it synthesizes specific adhesion proteins that enable them to adhere to mucous and epithelial cells (Handa *et al.*, 2010).

2.9.3 Non-steroidal anti-inflammatory drugs

Another important factor directly related to gastric injury initiated by impairment in mucosal defence is the prominent non-steroidal anti-inflammatory drugs (NSAID_s) use. As the prevalence of *H. pylori* infection has declined because of continued efforts to eradicate the organism, the prevalence of NSAID_s-induced ulcers has risen and is acquiring greater clinical importance. Studies show that NSAID_s are among the most commonly used drugs in the world. In United States, approximately 70 million prescriptions are written each year While in Europe these medications represent more than 7.7 % of all prescriptions (Graumlich, 2001). The use of NSAID_s is more frequent among women and it increases with age, as does the incidence of rheumatic diseases. In fact, more than 90 % of prescriptions for NSAID_s are made to patients, aged > 65 years. The major problem with the use of these drugs is that they induce predictable gastric mucosal injury, including complication in both upper and lower gastro-intestinal tract (Laine *et al.*, 2008).

The major mechanism via which NSAID_s cause ulcers and gastro-intestinal complication is thought to be by inhibition of COX, the key enzyme involved in the biosynthesis of *prostaglandins* (PGS). There are two well-identified isoforms of COX: COX1 and COX2 (Laine *et al.*, 2008). COX1 isoform is expressed in most tissues producing *prostaglandins* that play an essential protective role in the stomach by stimulating the synthesis and secretion of mucus and bicarbonate, and increasing mucosal blood flow and promoting epithelial proliferation. So, the

COX-1-mediated PG synthesis is mainly responsible for maintaining gastric mucosal integrity at the base-line. On the other hand, COX-2 has little or no expression in most tissues, but is rapidly induced in response to inflammatory stimuli. Therefore, this isoform is the primary target for anti-inflammatory drugs. In this context, the traditional NSAIDs, non selective inhibitors of both COX-1 and COX-2 such as indomethacin or ibuprofen, cause damage the stomach with a marked decrease in the gastric mucosal PGE₂ content. This effect occurs via COX1 isoform inhibition, creating a gastric environment that is more susceptible to topical attack by endogenous and exogenous factors (James, 2013).

Moreover, the inhibition of the COX1 blocks platelet production of thromboxane, which increases bleeding when active gastro-intestinal bleeding site is present (Lanas and Scheiman, 2007). This contention is further supported by the fact that COX-2 selective inhibitors, which do not inhibit COX-1 at therapeutic doses, do not affect the PGS production and do not produce gross gastric damage in experimental model (Laine *et al.*, 2008). Therefore, the development of NSAIDs which selectively inhibit COX-2 (Coxibs), while having little or no effect on COX-1 should result in effective pain relief, with reduced adverse gastro-intestinal effects. Data from large gastrointestinal outcome studies reveal that Coxibs significantly decrease with five Coxibs (Celecoxib, Valdecoxib, Rofecoxib, Lumiracoxib, Etoricoxib), when the drugs were tested against any of the most commonly used NSAIDs (diclofenac, naproxen or ibuprofen) (Rostom *et al.*, 2007). Large randomized controlled outcome trials demonstrate a considerable reduction in upper gastro-intestinal complication and overall clinical events with coxibs, compared to traditional NSAIDs (Silverstein *et al.*, 2000).

It has been recognized that prostaglandins derived from COX-2 can be generated at ulcer margins and appear to play an important role in ulcer healing through triggering the cell proliferation, promotion of angiogenesis and restoration of mucosal integrity (Laine, 2008).

These observations indicate that, in contrast to the initial concept, COX-2 plays an important role in gastric mucosal defence. Accordingly, experimental studies have reported that inhibition of both COX-1 and COX-2 was required for NSAIDs-induced gastric injury (Tanaka *et al.*, 2001). Indomethacin and similar NSAIDs, which inhibit both isoform of the COX enzyme, produce more severe damage in gastric tissue, even gastro-intestinal bleeding than more selective drugs (Delaney *et al.*, 2007). Therefore, indomethacin becomes one of the first choice drugs to produce an experimental ulcer model (Suleyman *et al.*, 2004).

In addition, studies have shown evidence that NSAIDs may induce tissue and cell injury by mechanisms independent of prostaglandin inhibition, which include the inhibition of phosphorylating enzymes (kinase), inhibition of oxidative phosphorylation in mitochondria and activation of apoptosis (Husain *et al.*, 2001). The mechanisms, in combination with those related to micro-vessels occlusion and subsequent hyper-production of ROS. Such agents are then able to induce oxidative injury that seems to play a prominent role in the development of mucosal ulceration, caused by NSAIDs (Blandizzi *et al.*, 2005). Furthermore, with the decrease in arachidonic acid metabolism via the COX pathway in NSAID users, arachidonic acid metabolism may be shifted to the alternative 5-lipoxygenase pathway, with a resultant increase in leukotriene production. In this way, a potential role for leukotriene in NSAIDs-induced gastric injury also has been postulated. This is because, licofelone, an inhibitor of COX-1, COX-2 and 5-lipoxygenase, do not increase gastric mucosal injury (Bias *et al.*, 2004). In this sense, since NSAIDs and even coxib therapy delay the healing of active peptic ulcers, the best approach to prevent mucosal injury is to avoid the use of NSAIDs or replace it with an agent less toxic to the gastro-duodenal mucosa (Bias *et al.*, 2004).

2.9.4 Gastric acid secretion

When pharmacologic means were developed such as histamine-2 blocker drugs such as cimetidine, which effectively eliminate acid, many patients treated with the drugs found that their ulcer disease was healed. The observations validated the dictum “no acid no ulcer” (Gustafson and Welling, 2010). As a result, the quantitative measurement of gastric acid secretion, for the most part, has become obsolete. Although there are multiple processes involved in the development of gastric lesion, the presence of acid hyper-secretion continues to be a necessary condition for ulcer production and for a variety of common gastro-intestinal disorders, this is because medical therapy for the illness involves both removing the injurious agent (NSAIDs and *H. pylori*) and inhibiting acid secretion (Schubert and Peura, 2008). Parietal cells secrete hydrochloric acid at a concentration of approximately 160 mmol/L or pH 0.8. Acid facilitates the digestion of proteins and absorption of calcium, iron and vitamin B₁₂. It is also the first line of mucosal defence to avoid microorganisms’ colonisation, thus preventing the bacterial overgrowth and consequent enteric infection by *H. pylori*. However, when level of acid and pepsin overwhelm mucosal defence mechanism, common and potential serious acid-related clinical conditions occur, including gastro-oesophageal reflux diseases, Barrett’s oesophagus, where the usual squamous mucosal lining becomes replaced by columnar epithelial cells of putative specific aspect, peptic ulcer diseases (Schubert and Peura, 2008).

Acid is thought to gain access to the lumen by means of channels in the mucus layer created by the relatively high intra-glandular hydrostatic pressure, generated during secretion (approximately 17 mmHg) (Johansson *et al.*, 2001). Thus, luminal acid interferes with the process of restitution, resulting in the conversion of superficial injury to deeper mucosal lesion. It is inactivated by the acid-labile growth factors, important for maintenance of mucosal integrity and repair of superficial injury. A large amount of studies show that the rate of acid secretion by the human stomach changes little with ageing, unless there is co-existing diseases of the oxyntic mucosa, such as atrophic gastritis, infection with *H. pylori* or both (Schubert and Peura, 2008). To prevent acid-induced mucosal damage, gastric acid must be precisely regulated through a

highly-coordinated interaction of neural, hormonal and paracrine pathways (Schubert and Peura, 2008).

In this sense, the principal stimulant of acid secretion include gastrin, histamine, gastrin-releasing peptide, orexin, ghrelin and glucocorticoids, while the main inhibitor is somatostatin, released from oxyntic and pyloric D. cells (paracrine). Gastrin, released from antral G. cells to the blood stream during meal stimulates acid secretion primarily by releasing histamine from histamine-secreting enterochromaffin-like cells. The GRP, released from antral-nerve fibres in response to proteins, stimulates gastrin secretion. Ghrelin and orexin appear to stimulate acid secretion, although their physiological actions in the stomach are not known. Glucocorticoids stimulate acid secretion, acting via phosphoinositide-3-kinase, serum-inducible kinase and glucocorticoid-inducible kinase (Schubert and Peura, 2008). In addition, acetylcholine, released from postganglionic enteric neurones (neuronal stimulation), acts directly stimulating parietal cell acid secretion, and indirectly, by eliminating the inhibitory paracrine influence of somatostatin on parietal and enterochromaffin-like cells (Schubert and Peura, 2008). Acid secretion by the parietal cells involves intracellular elevation of calcium, cyclic-AMP or both, followed by a cascade that triggers the translocation of the proton pump, $H^+ K^+$ -ATPase, from cytoplasmic tubule vesicle to the apical plasma membrane. This pump is an integral membrane protein that transports hydronium ions from the cytoplasm to the caniculus of the parietal cells in exchange for potassium. Most of the adult population chronically infected with *H. pylori* produce less than normal amounts of acid, probably due to increased apoptosis via secreted mediators (such as IL-1b) and inhibition of the $H^+ K^+$ -ATPase activity (Schubert and Peura, 2008).

This condition may cause further reduction of acid production and eventually, atrophy of the stomach cancer (Seubaum and Michetti, 2002). Thus, continued progress in understanding of gastric acid

secretion may enhance the development of new, more effective strategies to prevent and manage gastric disorders.

2.9.4.1 Regulation of gastric acid secretion

The acid secretion is stimulated or inhibited by endocrine, paracrine and neurocrine signals via at least three messenger pathways; Gastrin-histamine, CCK-somatostatin and acetylcholine (Duan *et al.*, 2006). Gastric acid (H^+) secretion results from the interplay of stimulatory and inhibitory neuro-hormonal mechanisms, activated by the ingestion of food and the presence of nutrients in the upper gastro-intestinal lumen (Konturek, 2003).

2.9.4.2 The gastrin–histamine pathway

Circulating gastrin acts on the CCK_2 receptors of the ECL cells, resulting in increased HDC mRNA expression and accelerated release and synthesis of histamine; which, in turn, stimulate acid secretion by activating the histamine H_2 receptors of the parietal cells (Duan *et al.*, 2006). Acid secretions were impaired in gastrin knockout mice and even more so in CCK_2 -receptor knock-out mice (Cheng *et al.*, 2004). The greater impairment of acid secretion in the CCK_2 -receptor knock-out mice could be due to the loss of typical ECL cells and their replacement by histamine-free like cells, displaying an ultra-structural distinct from that of the ECL cells (Cheng *et al.*, 2004). HDC knock-out mice had little or no *de novo* histamine synthesis in the mucosa, resulting in severely impaired acid secretion and a failure to respond to gastrin (Cheng *et al.*, 2004). H_2 -receptor knock-out mice showed a complete lack of acid response to both histamine and gastrin (Kobayashi *et al.*, 2000).

Histamine: Histamine is a chemical messenger that mediates a wide range of cellular responses, including allergic and inflammatory reactions, gastric acid secretion and possibly

neurotransmission in parts of the brain. Additionally, it is secreted by mast cells as a result of allergic reactions or trauma. Pharmacologically, histamine produces vasodilation and increase in permeability of blood vessel wall that may contribute to gastric haemorrhage (Hung and Wang, 2004). Histamine in the stomach is synthesized by histidine decarboxylase (HDC), stored in enterochromaffin-like (ECL) cells and released in response to gastrin, acting on CCK₂ receptors on the ECL cells. Most histamine in the body is stored in mast cells and basophils, although some is found also in eosinophils and platelets. In the gastric mucosa, histamine occurs mainly in ECL cells and mast cells (Hakanson *et al.*, 2001). The role of mast cell histamine probably reflects the pathophysiologic role of mast cells in immune reaction (Hakanson *et al.*, 2001). The ECL cells, which produce a peptide hormone of unknown identity, are located mainly in the basal part of oxyntic mucosa. They are rich in histidine decarboxylase and are actively producing and releasing histamine. In experimental animals increased mucosal histamine has been reported to elicit gastric secretion and mucosal lesion (Yuxin *et al.*, 2011). Histamine may cause increase in gastric secretion with gastric mucosal permeability to electrolytes and render the stomach more susceptible to acid-induced damage (Yuxin *et al.*, 2011). The role of histamine in the secretion of acid from acid producing-parietal cells is widely reported (Norlen *et al.*, 2000); and histamine has been shown to activate histamine-2 receptors on the acid producing parietal cells to stimulate acid production. The over-production of acid inhibits through low antral pH gastrin released from G cells, thus preventing the stimulatory effect of gastrin on enterochromaffin-like (ECL) cells and further histamine release (Yuan *et al.*, 2006). This inhibitory control is mediated via the release of somatostatin from D- cells situated in close proximity to the G cells (Norlen *et al.*, 2000).

2.4.4.3 CCK–somatostatin pathways

The CCK mobilises somatostatin from D cells by acting on CCK₁ receptors in both the antral and oxyntic mucosa, thereby inhibiting the gastrin-histamine pathway (G cells and ECL cells)

and the activity of the parietal cells by an effect of somatostatin on sst₂ receptors (Allen *et al.*, 2002). In gastrin and CCK double-knock-out mice, little or no histamine was mobilised from the ECL cells to stimulate the H₂ receptors. At the same time, there was no circulating CCK to stimulate gastric mucosal D cells to release somatostatin. Despite the lack of gastrin and CCK, the parietal cells were capable of producing gastric acid in response to vagal stimulation (pylorus ligation) (Chenget *et al.*, 2004). Thus, the net-acid output may be determined by the balance between stimulating signals from gastrin-histamine pathway on one hand and inhibiting signals from the CCK-somatostatin pathway on the other.

2.4.4.4 Neural pathway involving acetylcholine and neuropeptides

Central mechanism controls the sympathetic and parasympathetic inputs to myenteric and sub-mucosal ganglia in the stomach wall; command neurones in these ganglia control nerve signaling to the parietal cells. Thus, parietal cell function is regulated not only by circulating hormones such as gastrin and CCK or paracrine messengers like histamine and somatostatin, but also by neurotransmitters from enteric neurones like acetylcholine, catecholamine and neuropeptides, including pituitary adenylate cyclase-activating peptide (PACAP), VIP and galanin. (Duan *et al.*, 2006). Acetylcholine is known to stimulate acid secretion. M₃- receptor knock-out mice have an impaired parietal function as evidenced by elevated intra-gastric pH, reduced acid output in response to pylorus ligation, reduced proportion of secreting parietal cells (Aihara *et al.*, 2003).

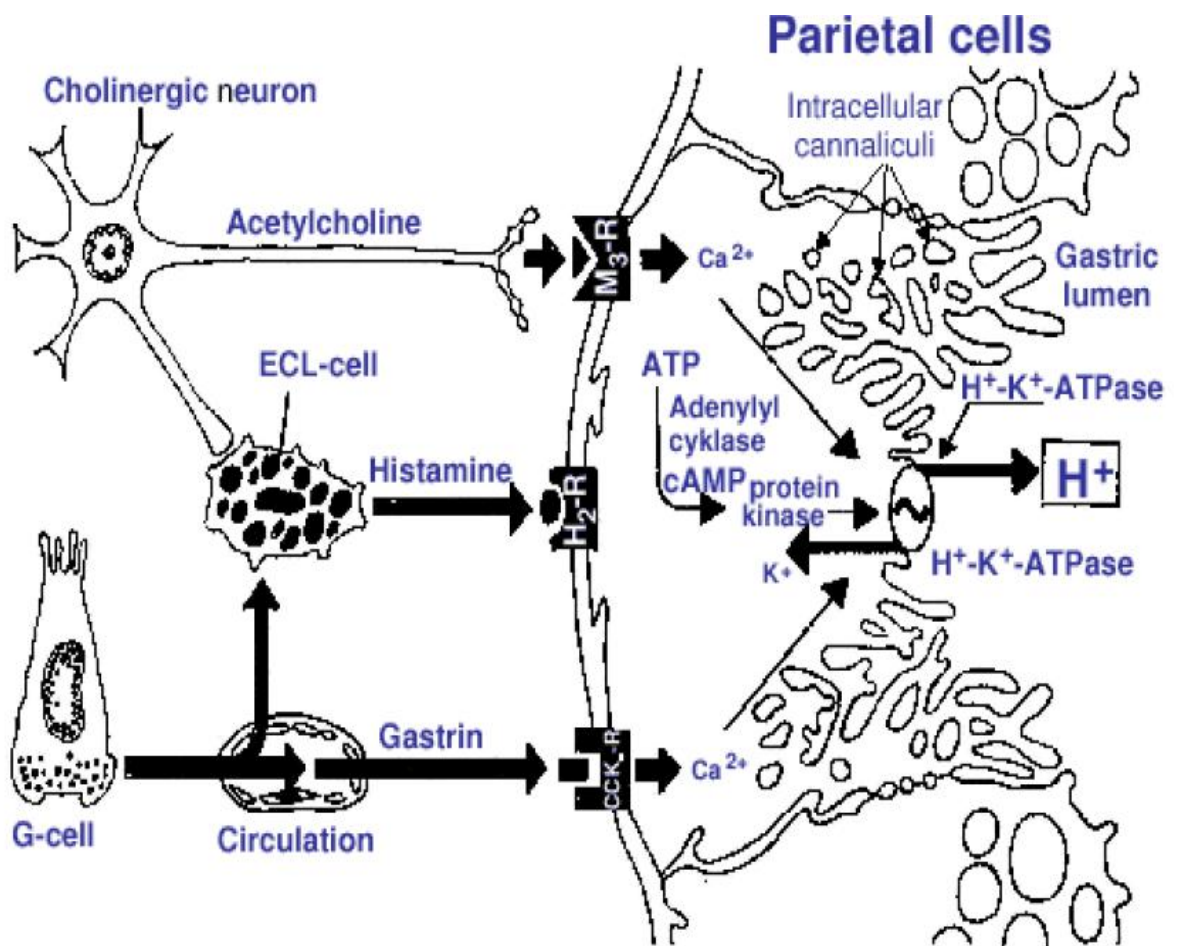


Figure 2.5: Control of gastric acid secretion. Adapted from El-Yassin (2012).

Secretion of hydrochloric acid by parietal cell

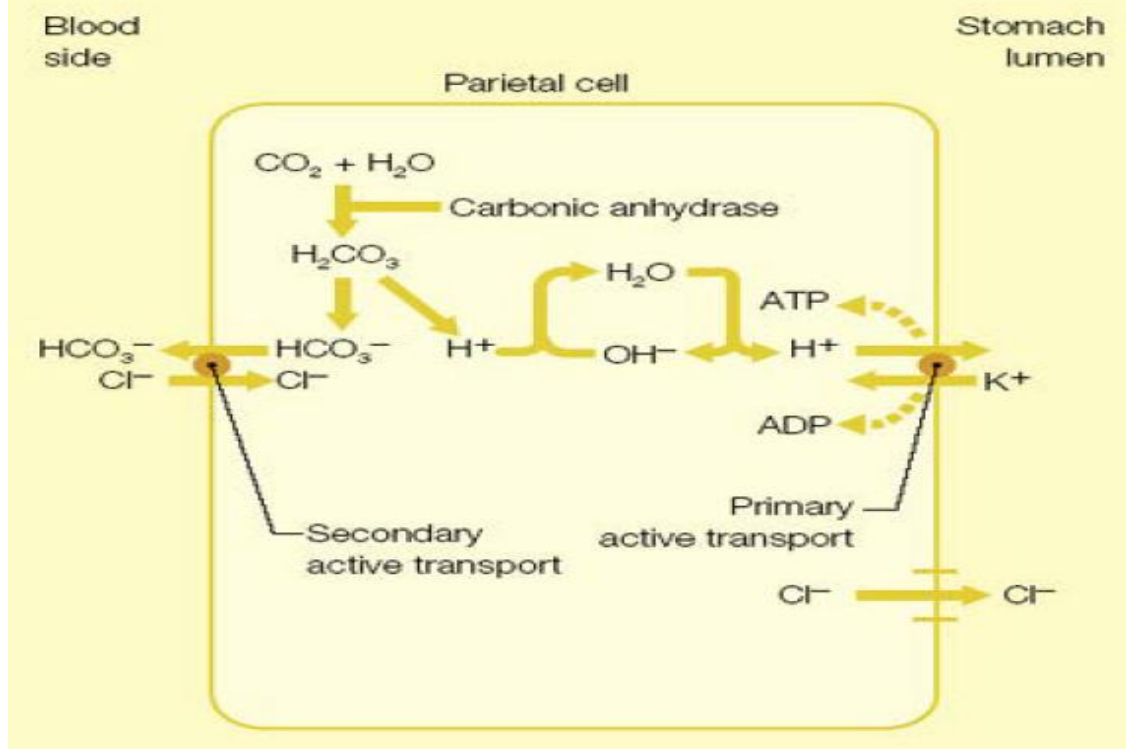


Figure 2.6: Mechanism of gastric acid secretion in parietal cells. Adapted from El-Yassin (2012).

2.9.5 Alcohol

The gastro-intestinal tract plays a particularly important role among the various organ systems that mediate the effect of alcohol on the human body and health. Alcohol absorption into the blood stream occurs through the gastro-intestinal and its direct contact with the mucosa can induce numerous metabolic and functional changes. The alteration may lead to marked mucosal damage, which can result in a broad spectrum of acute and chronic diseases such as gastro-intestinal bleeding and ulcers (Lin *et al.*, 2011). Pathogenesis of ethanol-induced gastric lesion is complex. Alcohol may interact directly with the gastric mucosa, or it may act through a more general mechanism affecting the release of hormones and the regulation of nerve functions involved in acid secretion (Lin *et al.*, 2011).

Intra-gastric application of absolute ethanol has long been used as a reproducible method to induce gastric mucosal lesion in experimental animals (Arafa and Sayed-Ahmed, 2003). The effects of acute administration of absolute ethanol-induced gastric mucosal lesion and erosion are similar to those occurring in gastric ulcer (Repetto and Llesuy, 2002). Thus, alcoholic gastritis leads to the impairment of the integrity of gastric mucosal barrier, contributing to acid reflux in the subluminal layer of the mucosa and sub-mucosa (Oh *et al.*, 2005). Oxidative stress and depletion of anti-oxidants have been considered a crucial step in alcohol-induced mucosal damage, so they have been widely investigated in a number of studies (Arafa and Saye-Ahmed, 2003). Ethanol treatment induces intracellular oxidative stress and produces mitochondrial permeability transition and mitochondrial depolarisation, which precede cell death in gastric mucosal cells. Thus, considering that ethanol is involved in the formation of oxidative stress generated extracellularly and intracellularly, cytoprotective role of anti-oxidants in the prevention and healing of gastric lesion has also been widely investigated (Silva *et al.*, 2009). The widening web of epigenetic regulatory mechanisms also encompasses ethanol-induced changes in the gastro-intestinal hepatic system. Alcohol modifies several epigenetic parameters in the gastro-intestinal tract and liver. The major pathways affected include DNA methylation, different site-specific modification in histone proteins and micro-RNAs. Ethanol metabolism cell-signaling cascades and oxidative stress have implicated these responses (Shivendra and Robert, 2013).

2.9.6 Incidence of apoptosis

Apoptosis was initially defined by Kerr *et al.* (1972), who suggested that cells dying in this process go through defined morphological changes that include chromatin condensation, cytoplasmic and nuclear blebbing and eventual cellular demise, without loss of membrane integrity. Caspase-3 has been shown to be a key component, involved in the underlying mechanism of apoptosis and relies on action of the initiator caspases, including caspase-8 and

caspase-9 for its action (Kamal *et al.*, 2013). The investigations include also the intra-mucosal DNA fragmentation as a gold standard for the process previously involved in diagnosis of gastric ulceration (Xia *et al.*, 1999). Under normal physiological conditions, the balance between gastric epithelial cell proliferation and death is of great importance in maintaining gastric mucosal integrity. The balance between cell apoptosis and cell proliferation has important role to keep the gastric mucosa healthy (Kalia *et al.*, 2000). Disturbance of this balance could result in either cell loss, leading to mucosal damage and ulcer formation or cell accumulation leading to cancer development (Meng *et al.*, 2013). There was a higher rate of *H.pylori* infection, and increased expression of apoptosis inhibitor IASPP and decreased expression of apoptosis stimulator ASPP2 in gastric cancer or precancerous tissue. This suggests that *H. pylori* may cause gastric cancer by up- regulating IASPP and down-regulating ASPP2 (Meng *et al.*, 2013).

2.10 Composition of Gastric Juice

The extracellular fluid that is secreted into the stomach is called gastric juice. It is the mixture of secretions from different gastric glands that drain into the lumen. It is also a secretion from the surface cells that produce primarily mucus and bicarbonate to protect the stomach from digesting itself as well as trefoil peptides substances that stabilise the mucus bicarbonate layer (Shereen-Lehman, 2013). Gastric juice contains 99.5% water and 0.5% solids. The solids are organic and inorganic substances. The organic substances are the enzymes, mucus and intrinsic factors, while the inorganic substances include hydrochloric acid cations like sodium, calcium and potassium and the anions include chloride, phosphate and sulfate. Secretion of gastric juice is regulated, through specific neural and hormonal pathways, by the act of eating and by the presence of food in the stomach and intestine (Shereen-Lehman, 2014).

2.11 Stress and Gastric Secretion

According to Shichijo *et al.* (1993), restraint stress alone induces moderate sympathetic hyperactivity, while sympathetic hyperactivity in the stomach prevents WRS-induced gastric injury formation, mainly via the inhibition of gastric acid secretion as observed in stroke-prone spontaneously-hypertensive rats. Recent studies showed that ghrelin level increases, following chronic stress in rats and mice (Lutteret *et al.*, 2008; Ochi *et al.*, 2008). Ghrelin regulates the motility of the gastric motility, gastric acid secretion and defence (Bulbul *et al.*, 2011). An intravenous administration of rat ghrelin dose dependently increases both gastric acid secretion and gastric motility actions, blocked by the treatment with either atropine or bilateral cervical vagotomy, but not by histamine H₂-receptor antagonist famotidine. Thus suggesting that ghrelin may play a physiological role in vagal control of gastric function in rats (Torsello *et al.*, 2000).

Yuan-Fang *et al.* (2005) reported that rat gastric output increases remarkably by WRS, and this increase lasted while the stress was present. The stress-induced increase in acid output correlated well with the severity of mucosal lesions. Bilateral cervical vagotomy prevented the gastric mucosal lesion in the WRS model, which suggests that WRS induces gastric mucosal lesion is basically a digestive ulcer, resulting from parasympathetic over-activity and related increased in acid output at the basis of reduced mucosal resistance. Corticotropin-releasing factor (CRF), a stress-related neuropeptide, acts in the brain to influence the gastro-intestinal tract. Gastric emptying and acid secretion are greatly attenuated when CRF is exogenously applied to the central nervous system (Junet *et al.*, 2009). Parasympathetic over-activity is the predominant autonomic response to WRS, and is most probably the leading mechanism of WRS-induced gastric mucosal lesion in rat (Yuan-Fang *et al.*, 2005).

2.12 Stress and Gastric Blood Flow

The maintenance of the relatively high pH microenvironment is dependent on undisturbed mucosal blood flow. If blood flow to the stomach is interrupted, then the pH within the mucoid

caps drops precipitously and hemorrhagic lesions form. This is the case either with mechanical occlusion of the gastric arterial supply or by administration of a vasoconstrictor such as endothelin (Wallace and Mcknight, 1993). Acid is permitted to diffuse deeper to the mucosa, causing extensive necrosis and hemorrhage. Prostaglandins play a significant role in the maintenance of mucosal blood flow during critical period of epithelial repair (Lei *et al.*, 2013). The gastric mucosa can be exposed to high concentration of acid without significant epithelial injury. Part of the reason for this is that the mucosal vasculature responds very quickly to the presence of acid in the superficial mucosa, so as to buffer, dilute and remove the acid (John, 2008). According to Yuan-Fanget *al.*(2005), the gastric mucosae in rats subjected to RS alone remained intact, with no apparent lesion both in gross and histological inspections. However five hours of WRS were associated with profound gastric mucosal lesion that was evident grossly and in histological sections in all WRS indicating clearly serious mucosal hemorrhage. Yuan-Fanget *al.* (2005) reported an increase in gastric acid output by WRS and no corresponding increase in gastric mucosal blood flow.

2.13 Mechanisms of Gastric Mucosal Defence

Mucosal defence is used to describe the various factors and components that permit the gastric mucosa to remain intact. The defence occurs despite frequent exposure of the gastric mucosa to substances with a wide range of temperature, pH and osmolarity and detergent or cytotoxic actions and bacterial products. The substances are capable of causing local and systemic inflammatory reactions (Laine *et al.*, 2008). The gastric mucosa maintains its structure and function despite continuous exposure to noxious factors, including 0.1mol/HCl and pepsin that are capable of digesting tissue under normal conditions. Mucosal integrity is maintained by defence mechanisms which include pre-epithelial factors (mucus-bicarbonate-phospholipid barrier) and epithelial barrier, comprising surface epithelial cells, connected by tight junction and generating bicarbonate ions, The defence mechanism of the epithelial barrier also include

themucus, phospholipids, trefoil peptides, prostaglandins and heat-shock proteins. Other components of the defence mechanism are continuous cell renewal accomplished by proliferation of progenitor cells regulated by growth factors, prostaglandin E₂ and continuous blood flow through mucosal microvessels and endothelial barrier, sensory innervation, and generation of prostaglandins and nitric oxide (Andrzej *et al.*, 2014).

2.13.1 Local mechanisms of gastric mucosal defence

The stomach is lined by complex epithelium that forms a selective barrier between the external environments (lumen). The body of the stomach is folded into several branching, tubular gastric glands deeply embedded into the *muscularis mucosa*. The diverse range of functions performed by gastric epithelial cells is maintained in the face of hostile luminal environment that can contain up to 150 mm of HCl and aggressive protease, capable of digesting the tissue and many noxious pathogens (Dimelina and Varro, 2007). Despite continuous exposure to the injurious factors, under normal conditions the defence mechanisms prevent local damage and maintain structural and functional mucosal integrity (Tulassy and Herszenyi, 2010).

2.13.1.1 Mucus–bicarbonate–phospholipid barrier

The first line of gastric mucosal defence is the mucosal-bicarbonate-phospholipid barrier (Andrzej *et al.*, 2014). The surface of gastric mucosa is covered by a layer formed by mucus gel, bicarbonate anions and surfactant phospholipids. This unstirred layer is capable of retaining the bicarbonate ions, secreted by the surface epithelial cells and maintaining a micro environment with a pH near 7 at the mucus–mucosa interface. The mucus layer prevents the penetration of pepsin, thus avoiding the proteolytic digestion of epithelium (Allen and Flemstrong, 2005). In addition, the luminal surface of mucus gel is covered by a film of surfactant phospholipids, which confers hydrophobic properties to the mucus layer (Lichtenberger, 1999). The mucus gel

is secreted by surface epithelial cells and is formed by large amount of water about 95%, and various mucinglycoproteins, including MUC2, MUC5AC, MUC5B and MUC6, the production of which may vary in different regions of the gastric mucosa (Ho, 2004, Allen and Flemstrom, 2005). Gel forming mucin units polymerize in to large mucin multimers, which are essential for gel formation. The mucus gel is secreted along with low-molecular weight trefoil factor family (TFF) peptides, which play a relevant role in the formation of the mucus layer (Newton, 2000). For instance, TFF₂ increases the viscosity of gastric mucin and stabilizes the network. The secretion of bicarbonate into the mucus gel layer is essential to maintain a pH gradient at the epithelial surface, which represents a first line of defence against gastric acid (Allen and Flemstrom, 2005). Bicarbonate secretion from the apical membrane of surface epithelial cells is mediated by a Cl⁻/HCO₃⁻ anion exchanger. It is stimulated by various factors, including prostaglandins (via EP1 receptors), luminal acid, corticotrophin releasing factor, melatonin, uroguanylin and orexin A (Allen and Flemstrom, 2005).

The mucus–bicarbonate barrier is the only system, which segregates the epithelium from the gastric lumen. Therefore, when this protective barrier breaks down during pathological events or upon detrimental actions by injuring agents, a second line of protective mechanisms comes into play. They include intracellular acid neutralisation, rapid epithelial repair and maintenance of mucosal blood flow. Moreover, if some ROS are generated in surface epithelium containing mucus, intracellular mucus can scavenge them, acting as antioxidant, and thus, reducing mucosal damage-mediated by ROS (Allen and Flemstrom, 2005). Even when cells containing mucus are damaged by ROS, extracellular mucus may be released to the gastric tissue to prevent additional damage by scavenging them (Seno *et al.*, 1995). The efficacy of protective properties of the mucus barrier depends not only on the gel structure, but also on the amount of thickness of the layer covering the mucosal surface (Allen and Flemstrom, 2005). The thickness of this layer is the result of a dynamic balance between its secretion and its erosion mechanically by shear forces of the digestive process and by proteolytic degradation, particularly from luminal

pepsin in the stomach. The mucus bicarbonate is the only pre epithelial barrier between epithelium and lumen. When it is overwhelmed or broken down in different disease conditions, the next series of protective mechanism come into play, including epithelial repair and maintenance, and distribution of mucosal blood flow (Tulassay and Herszenyi, 2010).

2.3.1.2 *Epithelial cells*

The continuous layer of surface epithelial cells represents the next line of mucosal defence. This epithelial tissue is responsible for the production of mucus, bicarbonate and other components of the gastric mucosal barrier. These cells are hydrophobic in nature, being able to repel acid and water-soluble injuring agents, owing to the presence of phospholipids on tight junctions, forming a continuous barrier, which prevents back diffusion of acid and pepsin (Allen and Flemstrom, 2005). Another relevant protective factor, available in the epithelial cells, is represented by heat-shock proteins, which are activated in response to stress, including temperature increments, oxidative stress and cytotoxic agents. These proteins can prevent protein denaturation and protect cells against injury (Tanaka *et al.*, 2007). Cathelicidin and beta-defensin are cationic peptides, which play relevant role in the innate defensive system at the mucosal surface, preventing bacterial colonisation (Taupin and Podolsky, 2003). In addition, TFF secreted by the epithelial cells regulate the re-epithelisation process and exert mucosal (Taupin and Podolsky, 2003). Consistent with the notion that there are several layers of mucosal defence (with degree of redundancy of function), reducing experimentally the effectiveness of the mucus-bicarbonate layer on the epithelial surface does not usually result in epithelial damage (Malin *et al.*, 2013). This may be in part related to inherent ability of gastric epithelial cells to remain intact and functional, when continuously exposed to high concentration of acid (Malin *et al.*, 2013).

2.13.2 **Mucosal cell renewal**

The integrity of gastric epithelium is maintained by a continuous process of cell renewal, ensured by mucosal progenitor cells. These cells are subjected to a continuous, well-coordinated and controlled proliferation, which ensures the replacement of damaged or aged cells on the epithelial surface. The process of complete epithelial renewal takes about 3-7 days, while the overall glandular cell replacement requires months. However, the restitution of surface epithelium after damage occurs very quickly within few minutes and results by migration of preserved cells located in the neck area of gastric glands (Laine et al., 2008). The process of turn-over of cells is regulated by growth factors; in particular a marked expression of epidermal growth factor (EGF-R) has been detected in gastric progenitor cells. Such a receptor can be activated by mitogenic growth factor, such as transforming growth factor alpha (TGF- α) and insulin-like growth factor-1 (IGF-1) (Millani and Calabro, 2001). Ulcer healing is a complex process that depends on regeneration of mucosal glandular structure and migration of epithelial cells to cover ulcer crater (Tulassy and Herszenyi, 2010). Results from animal studies are in agreement with clinical impression regarding the importance of rapid spontaneous healing, and suggest the involvement of the following growth factors: Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). Growth factors, such as EGF, PDGF and bFGF, activate epithelial cell migration and proliferation, and accelerate ulcer healing *in vivo*. However, the growth of granulation tissue and formation of new micro-vessels through angiogenesis is stimulated by VEGF. Thus, in order to understand the ulcer healing activity of the test compound, alterations in the level of protein expression of the factors are observed by Western Blot analysis (Shawon and Gautam, 2012).

In addition, PGE₂ and gastrin are able to trans-activate the EGF-R and promote the activation of mitogen-activated protein kinase (MAPK) pathway, with consequent stimulation of cell proliferation (Millani and Calabro, 2001). Notably, the presence of EGF has not been detected in the normal mucosa, although it is contained in the gastric juice, as a product of salivary and

oesophageal glands and can stimulate mucosal cell in case of injury (Millani and Calabro, 2001).

2.13.3 Modulation of inflammatory response during gastric ulcer healing

In chronic gastric ulcer, there is disruption of the mucosal integrity, leading to a local defect or exaction due to active inflammation. Thus, healing of chronic ulceration not only requires cell migration, proliferation and angiogenesis, but also amelioration of active inflammation. Inflammation of the mucosal layer is a feature, which is usually associated with ulceration of gastric tissue. Interleukin-1 beta (IL - 1 β) and tumor necrosis factor-alpha (TNF- α) are the major pro inflammatory cytokines and IL-10, the anti-inflammatory cytokine that plays an important role in inflammatory response accompanied with chronic ulceration of gastric mucosa (Millani and Calabro, 2001). Furthermore, cyclooxygenase (COX-2) is an important rate-limiting enzyme, involved in inflammatory process due to its role in the production of prostaglandin from arachidonic acid. Thus, effects of the test compounds are observed on the gene expression of these inflammatory mediators by reverse transcriptase polymerase chain reaction (RT-PCR) (Andrzej *et al.*, 2014).

2.13.4 Mucosal blood flow

Mucosal blood flow is essential to deliver oxygen and nutrients and to remove toxic metabolites from gastric mucosa. Arteries embedded in the *muscularis mucosae* branch into capillaries, which then enter the *lamina propria* and travel towards the proximity of glandular epithelial cells. Endothelial cells, lining these micro-vessels produce NO and prostacyclin (PGI₂), which act as potent vasodilators. Thus, the endothelial cells protect the gastric mucosa against damage and counteract the detrimental effects of various vasoconstrictors, including leukotriene C₄, thromboxane A₂ and endothelin. In addition, NO and PGI₂ maintain the viability of

endothelial cells and inhibit platelet and leucocyte adhesion to the micro-vasculature thus preventing the occurrence of micro-ischemic phenomena (Laine *et al.*, 2008).

When the gastric mucosa is exposed to irritants or acid back-diffusion, a massive and rapid increase in mucosal blood flow occurs. This process allows removal and dilution of back diffusing acid or noxious agents. The increase in blood flow is regarded as pivotal mechanism for preventing gastric mucosal cell injury, and its decrease results in the development of tissue necrosis. The increase in mucosal blood flow is mediated by NO release. There is experimental evidence demonstrating that NO protects the gastric mucosa against injury, induced by ethanol or endothelin 1, while the inhibition of NO synthase enhances mucosal injury (Holzer, 2006). It is clear that in an *in vivo* setting, vascular perfusion is crucial in providing a "back-up" level of mucosal defence during the critical period after injury has occurred, and the basement membrane is exposed to luminal contents. The mucus released from damaged epithelial cells and plasma exuding from the mucosal vasculature coalesce to form a protective layer over the denuded region that has been termed the "mucoïd cap" (Wallace, 1990). Even in the presence of very high level of hydrochloric acid in the stomach that is $\text{pH} < 1$. The pH within the mucoïd cap can be maintained close to neutrality (Wallace, 1990).

2.13.5 Prostaglandins

Prostaglandins (PG_S) are also synthesized by gastric mucosal epithelial cells from arachidonate metabolism through the action of *cyclooxygenase* (COX). The ability of exogenous PG_S to attenuate or even completely prevent mucosal damage caused by corrosive substances such as absolute ethanol, concentrated bile or hyper-osmolar solutions has been termed cytoprotection (Farhadi *et al.*, 2003). Particularly, prostaglandin E_2 and prostacyclin have long been known to have cytoprotective "effects" on the gastro-intestinal epithelium and, therefore, they are crucial for the maintenance of the gastric integrity. Although the precise mechanisms of

cytoprotective action of prostaglandins remain unknown, it appears to result from a complex ability to stimulate mucosal mucus and bicarbonate secretion. The stimulated mucus and bicarbonate increase, mucosal blood flow and sulfhydryl compounds particularly, in the stomach, to limit back diffusion of acid to the epithelium (Kato *et al.*, 2005). Prostaglandins inhibit mast cell activation as well as leucocyte and platelet adhesion to the vascular endothelium (Brzozowski *et al.*, 1995). The beneficial action exerted by PGE₂ is mediated by activation of EP₁ receptors, mediating the most important protective effects of prostaglandins through an increase in gastric motility (Kato *et al.*, 2005). Other EP receptor subtypes are also involved in the protective actions of PGE₂. EP₃ receptors inhibit the gastric acid secretion, while EP₄ receptors stimulate the secretion of mucus (Kato *et al.*, 2005).

2.13.6 Neuro-hormonal mechanism of gastric mucosal defence

In addition to local mucosal protective factors, gastric mucosal defence is also regulated, at least, in part, by the central nervous system and hormonal factors (Peskar, 2001). Gastric mucosa and mucosal vessel are innervated by primary afferent sensory neurons and nerves forming a dense plexus at the mucosal base. Afferent neurones constitute an emergency system that is requested, when the gastric mucosa is endangered by noxious agents. Thus, activation of the nerves in the presence of gastric acid promotes the release of neurotransmitters such as substance P and calcitonin gene-related peptides (CGRP), which relax the smooth muscle surrounding the arterioles. The activation results in an elevation of mucosal blood flow increase in mucus gel and surface cell intracellular pH in the stomach. This mucosal protective action occurs most likely through vasodilatation of sub-mucosal vessel, mediated by NO generation. In this sense, interference with any aspect of the sensory innervation impairs the hyperaemic response and, therefore, diminishes resistance of the gastric mucosa to injury (Roeland *et al.*, 2011).

Mediators such as NO, CGRP as well as some hormones, including gastrin and cholecystokinin (CCK), ghrelin, leptin and gastrin-releasing peptide (GRP) have also been found to protect gastric mucosa against the damage, induced by corrosive substances. This protective action has also been attributed in part to the release of PGS because it could be abolished by pre-treatment with indomethacin (a non-selective inhibitor of COX 1 and 2) and restored by the addition of exogenous PGE₂ (Farhadi *et al.*, 2003). Glucocorticoids have been shown to support the mechanism of protection at gastric level. These hormones are involved in the response to stress and represent potent gastro-protective factors against injury. Consistent with this contention, glucocorticoid antagonists enhanced the severity of stress-induced erosion, further supporting a protective role of the hormones during stress (Filaretova, 2001). The mechanism through which glucocorticoids exert their protective effects include the maintenance of glucose homeostasis, the increase in mucosal blood flow and mucus secretion and the attenuation of both enhanced gastric motility and micro-vascular permeability (Filaretova, 2001).

CHAPTER THREE

3.0 Materials and Method

3.1 Materials

3.1.1 Experimental animals

Seventy (70) male Wistar rats were purchased from the Department of Pharmacology, Ahmadu Bello University, Zaria and whose body weights range from 200-220 g were used for this study. The animals were housed at ambient room temperature (26°C), and exposed to 12 hrs light. They were given free access to food and drinking water. Forty eight hours before the induction of stress, animals were deprived of food to allow for complete gastric emptying, but they were allowed access to water *ad libitum*. This procedure has been proved to be non-ulcerogenic and sufficient for absolute emptying of the stomach (Magaji *et al.*, 2007). During fasting, rats were housed each in separate cages with a wide- raised, mesh bottom to prevent coprophagy. (Shu *et al.*, 2012). This study was conducted in accordance with internationally accepted principles for laboratory animal use and care.

3.2 Methods

The rats were divided in to two main groups of 35 rats each, for ulcer and acid secretions studies. Group 1 and 2 were further sub-divided into 5 sub-groups of 7 rats:

Group I: Passive control animals (unstressed control)

Group II: Animals received distilled water as the vehicle at the dose of 5 mL/kg bodyweight orally for 3 days plus WRS (active control).

Group III: WRS plus Selenium pentahydrate at the dose of 100 µg/kg body weight orally for 3 days (Jeong-Hwan *et al.*, 2012).

Group IV: WRS plus VE at the dose of 250mg/kg body weight (Vivian *et al.*, 2011)

Orally for 3 days

Group V: WRS plus Selenium pentahydrate plus VE for 3 days.

3.2.3 Induction of water immersion restraint stress

The rats were first anaesthetized with chloroform inhalation. Thereafter, they were kept conscious during the experiment, and no additional anaesthetic agent was applied. The four limbs of each rat were bound on a wooden board (25 cm x 19 cm) with upper limbs anchored at horizontal position and the lower limbs extended downwards as reported by Shu *et al.* (2012). According to Anil *et al.* (2010), rats were immobilised by taping all the four limbs to the board after placing them on their backs. In WRS group, the animals were restrained after awakening(after anaesthesia) and [anchored plate]were immersed to the level of xiphoid process vertically (head up)in water bath, thermostatically-controlled at $23 \pm 0.5^{\circ}\text{C}$ (Shu *et al.*, 2012). The stress procedure lasted for 3.5 hours. At the end of this experimental procedure, the rats were released from the plate and anaesthetized by chloroform inhalation.

3.2.4 Collection of blood samples

Blood samples were collected through cardiac puncture (Ebunlomo *et al.*, 2012) and kept in two different containers. The first container was plain bottle without anticoagulant and contained the blood samples, used for biochemical analysis, (biomarkers of oxidative stress, activities of antioxidant enzymes, concentration of minerals and total protein).The second container contained blood samples used for haematological parameters, including: PCV, RBC, WBC and differential leucocyte counts.



Plate I: Water-immersion restraint stress procedure (Snapped by the candidate, 02/3/2015).

3.2.5 Evaluation of gastric mucosal lesion

After the stress procedure, animals were released from the plate and sacrificed under chloroform anaesthesia (Shu *et al.*, 2012). The stomach was then harvested and opened along lesser curvature. The severity of mucosal lesions was grossly inspected and photographed.

Gastric tissue was fixed in 10% formalin, dehydrated and embedded in paraffin wax. Paraffin section of 5 µm was cut and stained with haematoxylin and eosin. Histological changes were examined under a light microscope. Ulcer index was determined as follows: Lesion size in millimeters was determined by measuring each lesion at its greatest diameter with a transparent millimeter scale rule (Magaji *et al.*, 2007). Five petechiae lesions were equal to 1mm lesion. The total length in each group of rats were averaged and expressed as lesion index (Wong *et al.*, 2002). Preventive index (%) = ulcer index of control- ulcer index of treated / ulcer index of control x 100.

3.2.6 Measurement of gastric acid secretion

To avoid interrupting the development and observation of gastric erosion, additional rats were used to measure gastric secretion parameters, which included: volume, titratable acidity and total acid output. Collection of gastric secretion was performed according to the method of Reymond *et al.* (1995), the rats were anaesthetized using light chloroform. An abdominal incision of about 2.5 cm from below the xiphisternum was carried out, and the stomach was brought into view by gentle traction on the omentum. Then, pyloric ligation was done using a curved needle attached to a silk thread size 0 or 1. The needle was passed along the upper border of pylorus behind its posterior surface, avoiding the gastro-duodenal artery, and passed out on its lower border, where it crosses the omentum. The ligature was tied in order to close the pylorus, without crushing its wall. The abdomen was closed by silk sutures, and rats were placed in the same cage and received neither food nor water. After three hours, the rats were sacrificed, and the abdomen was re-opened by removal of the silk sutures.

Measurement of gastric acidity was carried out following the method described by Shay *et al.* (1954). The junction between the stomach and the oesophagus and the duodenum and pylorus was secured before the stomach was isolated. Then 3ml of distilled water was introduced into

the stomach and the organ was carefully shaken. The gastric juice was collected and centrifuged at 1000 x g for 10 minutes. The supernatant was taken and diluted 10 times; following this, a few drops of phenolphthalein was added to the solution. Titration was done using 0.01 N NaOH solutions until the colour of the test solution changed to light pink, indicating pH 7.0. The volume of NaOH needed for titration was used in the calculation to derive the hydrogen ion concentration (Shay *et al.*, 1954).

3.2.6.1 Analysis of gastric juice

Volume of the gastric juice: -After the centrifugation, the supernatant was measured as the volume of the gastric juice (Magaji *et al.*, 2007).

Titritable acidity: A given volume of the gastric juice was titrated against 0.01N NaOH using an endpoint of pH 7.0 with phenolphthalein as an indicator (Magaji *et al.*, 2007). It was calculated as milliequivalent per liter (mEq/L) which was equal to the number of milliliters (mL) of 0.01N NaOH, required to neutralize 100 mL of the gastric juice (Zhongzhi *et al.*, 2011).

Titritable acidity = Volume of 0.01N NaOH which neutralised 1mL of gastric juice x 100

10

Acid output: This was calculated as $\mu\text{Eq/h}$ by multiplying the volume of gastric secretion (mL/h) of an animal by the titritable acidity (acid concentration) (mEq/L) of the gastric secretion in the animal (Magaji *et al.*, 2007).

3.2.7 Assessment of lipid peroxidation

Lipid peroxidation as evidenced by the formation of Thiobarbituric acid-reactive substances (TBARS) was measured by the modified method of Niehaus and Samuel (1968) and as described by Akanji *et al.* (2009).

To 150 μL (0.15 mL) of serum were treated with 2 mL of (1:1:1 ratio) of Thiobarbituric Acid, Trichloroacetic Acid, and Hydrochloric Acid (TBA-TCA-HCL), reagent (Thiobarbituric Acid 0.37%, 15% Trichloroacetic Acid and 0.25N Hydrochloric Acid) and place in water bath for 1 hour at 90°C. The mixture was cooled and centrifuged at 1000 x g for 5 minutes at 4°C. The absorbance of the pink supernatant 2.0 mL was measured against reference blank using spectrophotometer at 535nm.

Calculations: The MDA was calculated using the molar extinction coefficient of $1.56 \times 10^5 \text{cm}^{-1} \text{M}^{-1}$. MDA concentration = $\text{absorbance} / 1.56 \times 10^5 \text{cm}^{-1} \text{M}^{-1} \times 1$

3.2.8 Assay of reduced glutathione concentration

Reduced glutathione (GSH) concentration measurement was done according to Ellman (1959) and as described by Rajagopalan *et al.* (2004). 0.2M phosphate buffer: 8.40g of NaH_2PO_4 and 9.94 of Na_2HPO_4 were dissolved in distilled water and made up to 1000 mL mark in volumetric flask. The buffer was adjusted to pH 8.

To 150 μL of serum, 1.5 ml of 10% TCA was added and centrifuged at 1500g for 5 minutes. The supernatant (1mL) was treated with 0.5 mL of Ellman's reagent (19.8 mg of 5, 5'- dithiobis (nitro benzoic acid) (DNTB) in 100 mL of 0.1% sodium nitrates), and 3 mL of phosphate buffer (0.2 M, pH 8). The absorbance was read at 412nm.

3.2.9 Determination of superoxide dismutase activity

Superoxide Dismutase (SOD) activity was determined by the method described by Fridovich (1989). The ability of SOD to inhibit auto-oxidation of adrenaline at pH 10.2 forms the basis of this assay. 0.1 mL of microsome was diluted in 0.9 mL of distilled water to make 1:10 dilution of microsome. An aliquot mixture of 20 mL of the diluted microsome was added to 2.5 mL of 0.05M carbonate buffer. The reaction was started with addition of 0.3 mL of 0.3 mM adrenaline and 0.20 mL of distilled water. Absorbance was measured 30 seconds up to 150 seconds at 480nm. Increase in absorbance per minute = $(A_5 - A_1) / 2.5$. Percentage inhibition = $100 - \text{Increase in absorbance of substrate} / \text{Increase in absorbance of blank} \times 100$. 1 unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the oxidation of adrenaline to adrenochrome in 1 minute.

3.2.10 Determination of catalase activity

Catalase activity was determined using the method described by Sinha (1972). The method is based on the reduction of dichromate in acetic acid to chromic acetate, when heated in the presence of hydrogen peroxide with formation of perchromic acid as unstable intermediate. Chromic acetate so produced was measured colorimetrically using spectrophotometer at 570nm. This is because dichromate has no absorbance at 570 nm and does not interfere with the determination of catalase activity. Distilled water (0.9 mL) was added to 0.1 mL of microsome (serum) and mixed thoroughly. Phosphate buffer (2.5 mL) was put in a small conical flask, 0.5 mL of microsome was added, and 2.0 mL of H₂O₂ added. The stop-watch was started. The reaction mixture was thoroughly mixed and was stopped after every 60 seconds for 3 minutes with 1 mL dichromate/acetic acid solution. It was heated in water bath for 10 minutes at 80°C. Absorbance was read at 570 nm. Standard curve was obtained using absorbance obtained at various H₂O₂. The quantity of H₂O₂ consumed was obtained from the graph of the catalase standard curve, it determined the catalase activity.

3.2.11 Estimation of protein concentration

The protein concentration of various samples was determined using the Biuret method as described by Edem *et al.* (2012). The principle of the test was based on the formation of coloured complex between proteins and cupric ions in alkaline solution. The result was expressed in mg/mL.

3.2.12 Determination of serum alanine aminotransferase and aspartate aminotransferase

The measurements of the activities of both enzymes were carried out by spectrophotometric determination of their absorbance using analytical grade reagents kits (Randox Laboratories Limited, Crumlin, and County Antrim, United Kingdom). The evaluation was done as described by Friday *et al.* (2010). AST catalyses the reversible transfer of an amino group from aspartate to α -ketoglutarate, forming glutamate and oxaloacetate. The oxaloacetate produced was reduced to malate by malate dehydrogenase and NADH. The rate of decrease in concentration of NADH was measured spectrophotometrically, and was proportional to the catalytic concentration of AST present in the sample. The ALT also catalysed the reversible transfer of an amino group from alanine to α -ketoglutarate, forming glutamate and pyruvate. The pyruvate produced was reduced to lactate by lactate dehydrogenase and NADH. The rate of decrease in the concentration of NADH was measured spectrophotometrically, and was proportional to the catalytic concentration of ALT present in the sample (Friday *et al.*, 2010).

3.2.13 Determination of haematological parameters

Red blood cell (RBC) count was carried out using the method of Akinnuga *et al.* (2011). Blood sample was diluted to 1:200 with Hayem's fluid, which was used to preserve and then count the corpuscles with a Neubauer counting chamber under a light microscope. The counting of total

white blood cells (WBCs) was done according to the method of Akinnuga *et al.* (2011), using a diluting fluid (Turk's fluid) in a ratio of 1:20. Haemoglobin (Hb) concentration of the blood and pack cell volume (PCV) were estimated by the macrohaematocrit method. Erythrocytic indices of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were calculated from values of RBC, PCV and Hb as follows (Saleh *et al.*, 2010).

$$\text{MCV (fl)} = \text{PCV (\%)} \times 10/\text{RBC count.}$$

$$\text{MCH (pg)} = \text{Hb (g/dL)} \times 10/\text{RBC count.}$$

$$\text{MCHC (g/dL)} = \text{Hb (g/dL)} \times 100/\text{PCV (\%)}$$

3.2.14 Evaluation of erythrocyte osmotic fragility

The erythrocyte osmotic fragility test was determined using the method of Faulkner and King (1970) as described by Olayinka *et al.* (2011). 10 test tubes containing 0.1% to 0.9% concentration of sodium chloride. Each tube contained 5 mL of the corresponding sodium chloride and they were arranged serially in a rack of 10 tubes. A pipette (1 mL) was used to transfer 0.02 mL of blood into each of the 10 test tubes. The contents of the test tubes were gently mixed by inverting each test tube five times and allowing it to stand at room temperature (26°C) for 30 minutes. Thereafter the content of the test tubes was centrifuged at 2000 x g for 10 minutes. The supernatant was then transferred in a glass cuvette and the absorbance of the supernatant was measured at a wavelength 540nm using a spectrophotometer. The percentage haemolysis was calculated according to Faulkner and King (1970) as follows:

$$\text{Percentage haemolysis (\%)} = \frac{\text{Optical density of the test}}{\text{Optical density of the standard (Distilled water)}} \times 100$$

Erythrocyte osmotic fragility curve was obtained by plotting percentage haemolysis against the saline concentration.

3.2.15 Assessment of mineral changes

Atomic absorption spectrophotometry (AAS) is the most effective method for trace mineral element analyses in plasma (Bowers and Rains, 1988). Diluted plasma was aspirated directly into the AAS flame (AAS: Factory name Varian, model number: AA240FS), used to determine magnesium, zinc and copper.

3.2.16 Statistical analyses

Results were computed for mean values \pm S.E.M. Statistical comparisons between variables were carried out using one way analysis of variance, and Tukey's post-*hoc test* was used to compare the differences between the means. Values of $P < 0.05$ were considered significant

CHAPTER FOUR

4.0 Result

4.1 Ulcer and Preventive Indices

The mean (\pm SEM) ulcer index as well as ulcer inhibition capacity of selenium and vitamin E in the pre-treated rats are shown in Figures 4.1, 4.2 and appendix I. WRS induced ulcers as shown by the mean increase of ulcer index of active control (27.08 ± 0.08 mm) compared with that of the passive control (0.00 ± 0.00 mm) ($P < 0.001$) and the mean values of the pre-treated groups, 14.35 ± 3.20 mm, 13.22 ± 2.50 mm and 7.10 ± 1.50 mm for selenium, vitamin E, and selenium

plus vitamin E groups were compared with active controls ($P < 0.001$). Selenium inhibited ulcer by 47.01%, vitamin E inhibited ulcer by 50.89 %, and the combination of selenium and vitamin E inhibited ulcer by 73.78 %. The macroscopic and histopathological features of gastric mucosa are shown on plates, i, ii, iii, iv, v, vi, vii, viii, ix, x

4.2 Gastric Juice Volume, Titratable Acidity and Acid Output

The WRS induced gastric ulcer with a significant ($P < 0.001$) increase in titratable acidity as the mean value of active control (78.71 ± 1.38 mEq / L) was compared with that of the passive control (36.14 ± 0.77 mEq / L). The mean values of selenium, vitamin E and selenium plus vitamin E groups were (36.43 ± 0.53 mEq / L), (35.14 ± 1.50 mEq / L) and (34.29 ± 7.33 mEq / L) respectively. There were significant decreases ($P < 0.001$) in the titratable acidity as the mean value of the active control was compared with mean values of the pre-treated groups as shown in figure 4.3 and appendix II. There was a significant increased ($P < 0.001$) in gastric acid output when the mean value of the active control (127.06 ± 2.75 μ Eq / L) was compared with the values of the passive control (78.91 ± 2.73 μ Eq / L). The increased in the gastric acid output was significantly ($P < 0.001$) lowered when the mean values of the pre-treated groups (107.67 ± 3.87 μ Eq / L, 83.60 ± 4.18 μ Eq / L, 41.60 ± 2.68 μ Eq / L) for selenium, vitamin E and selenium plus vitamin E co-administered group (figure 4.4 and appendix II). Gastric juice volume was significantly ($P < 0.001$) higher in the active control (4.81 ± 0.09 mL / 3 hours) was compared with the value in passive control (2.19 ± 0.06 mL / 3 hours). Pre-treatment with selenium and/or vitamin E significantly ($P < 0.001$) decreased the gastric juice volume as the mean values in pre-treated groups (2.96 ± 0.11 mL / 3 hours, 2.37 ± 0.16 mL / 3 hours, and 1.53 ± 0.08 mL / 3 hours for selenium, vitamin E and selenium plus vitamin E) were compared with the mean value of the active control as shown in appendix II.

4.3 Protein Concentration

Protein concentration was lower in the active (12.17 ± 1.85 mg / ml) control, and was significantly ($P < 0.001$) different, when compared with the passive control (60.00 ± 2.15 mg / ml). The protein concentrations in all the pre-treated groups (52.24 ± 9.15 mg / ml, 53.37 ± 9.18 mg / ml, and 62.46 ± 3.75 mg / ml for selenium, vitamin E, and selenium plus vitamin E groups) were higher and significantly different ($P < 0.001$) than that of the active control (Fig 4.5 and Appendix III)

4.4 Malondialdehyde Concentration

Figure 4.6 and appendix IV, shows the mean \pm SEM of malondialdehyde (MDA) concentration in rats. There was a significant ($P < 0.001$) increase in the MDA concentration of the active controls (3.36 ± 0.19 nanomol / ml) compared with the passive controls (1.69 ± 0.21 nanomol / ml). There was a significant ($P < 0.001$) decrease in the MDA concentration in all the selenium and vitamin E pre-treated groups, when the mean values (1.07 ± 0.18 nanomol / ml, 1.17 ± 0.28 nanomol / ml, and 0.78 ± 0.13 nanomol / ml for selenium, vitamin E and selenium plus vitamin E pre-treated groups) were compared with the active control. This decrease was more pronounced in the group co-administered with selenium and vitamin E.

4.5 Antioxidant Enzymes

The activities of the antioxidant enzymes, catalase, SOD and reduced glutathione (GSH) are shown in Table 4.1. There was a significant ($P < 0.001$) decrease in the mean activities of catalase, SOD and GSH in the active control groups (13.43 ± 0.82 , 0.81 ± 0.04 and 5.84 ± 0.28) were compared with the mean values of passive controls (32.00 ± 1.99 , 1.84 ± 0.13 , and 8.83 ± 0.33). In all the selenium and vitamin E pre-treated groups, there was a significant ($P < 0.001$) increase in the mean activity of catalase, SOD, and GSH when the mean values of the pre-treated groups were compared with the active controls. The increase was more marked in the vitamin E

group, followed by the selenium group, while the least increase in the activities was obtained in the group co-administered with vitamin E and selenium.

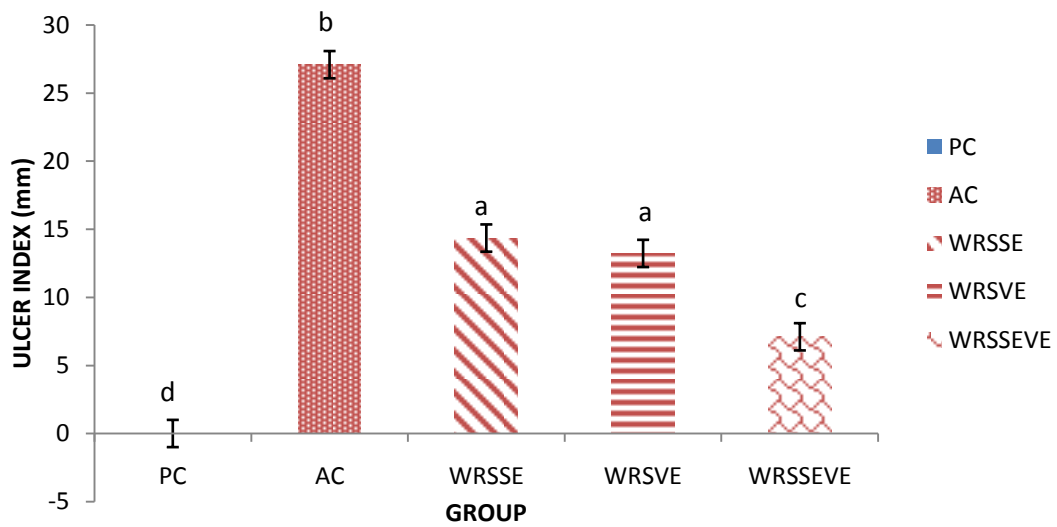


Figure4.1:Effects of selenium and vitamin E on ulcer index in rats subjected to water-immersion restraint stress.

a, b, c, d = mean with different superscript letters are significantly different at $P < 0.001$ respectively compared with active control (n= 7).

PC = Passive control (Non stress animals). AC = Active control (WRS + Distilled water). WRSSE = (WRS + selenium). WRSVE = (WRS + Vitamin E). WRSSEVE = (WRS + Selenium + Vitamin E). WRS = water immersion restraint stress.

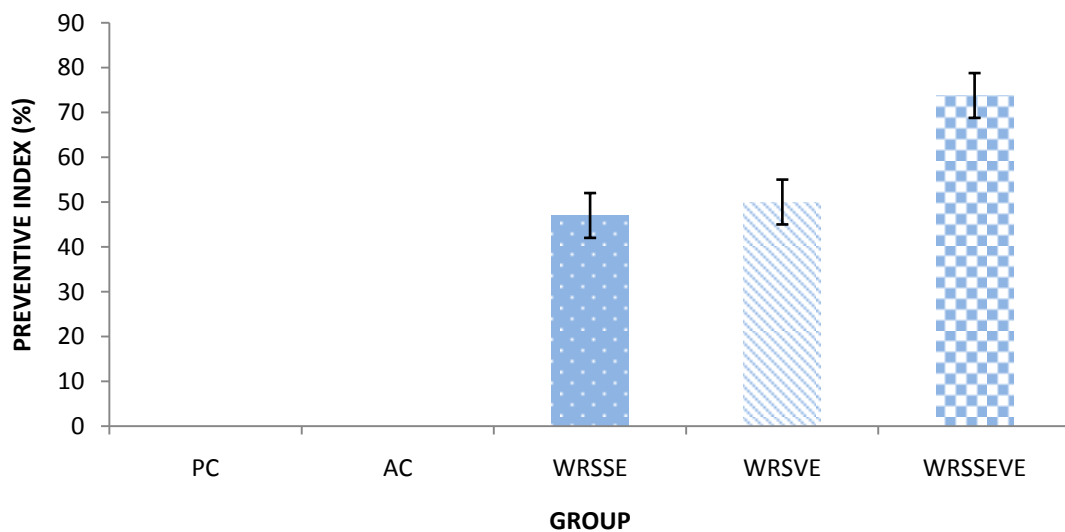


Figure 4.2: Effects of selenium and vitamin E on preventive index in rats subjected to water immersion restraint stress (n = 7).

PC = Passive control (Non stress animals). AC = Active control (WRS + Distilled water). WRSSE = (WRS + Selenium). WRSVE = (WRS + Vitamin E). WRSSEVE = (WRS + Selenium + Vitamin E). WRS = water immersion restraint stress.



Plate ii: Macroscopic picture of gastric mucosa in passive control (non-stressed rats)



Plate iii: Macroscopic picture of gastric mucosa in active control (stress rat plus distilled water)



Plate iv: Macroscopic picture of gastric mucosa in stressed rats pre-treated with selenium (WRS plus Se).



Plate v: Macroscopic picture of gastric mucosa in stressed rats pre-treated with vitamin E (WRS plus VE).

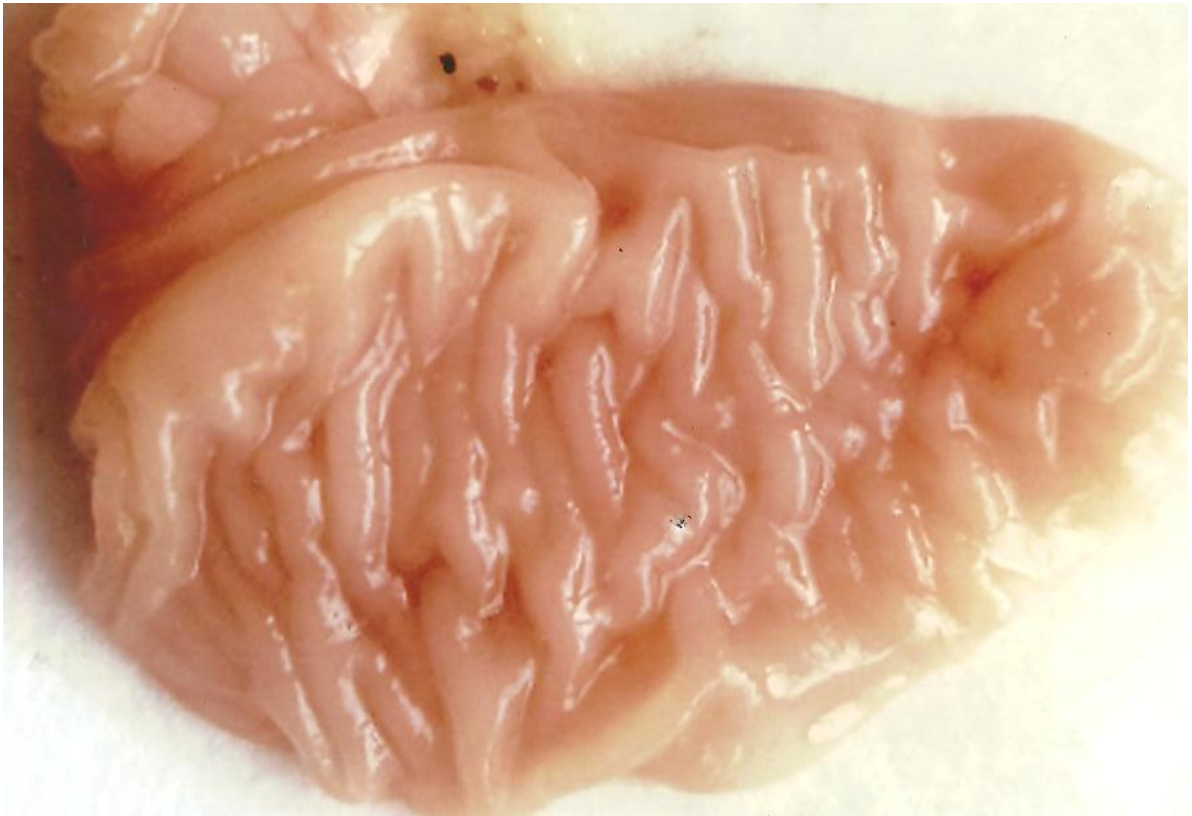


Plate vi: Macroscopic picture of gastric mucosa in stressed rats pre-treated with selenium and vitamin E (WRS plus Se plus VE).

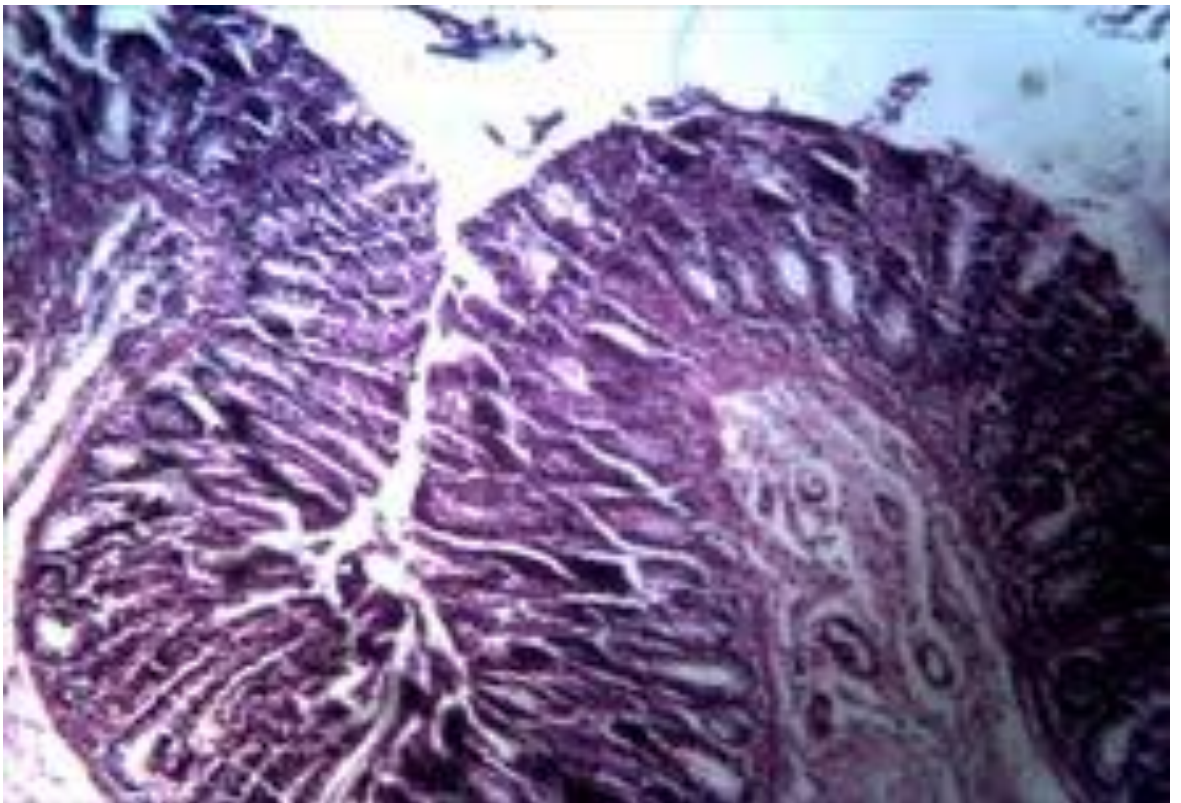


Plate vii: Photomicrograph of histological features of gastric tissue of non-stressed rats (passive control) (H&E Mag x100)

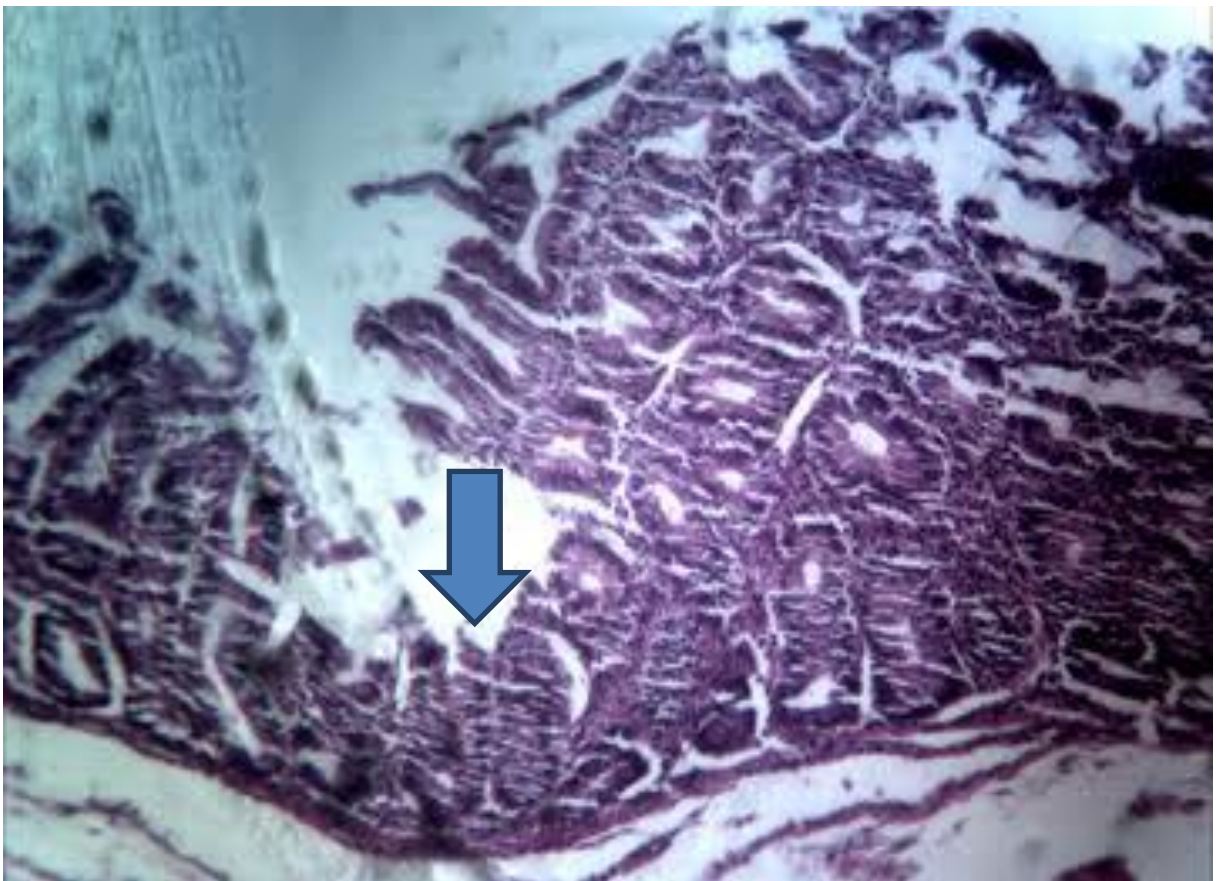


Plate viii: Photomicrograph of histological features of gastric tissue of rats subjected to water-immersion restraint stress (active control) (H&E Mag x100)

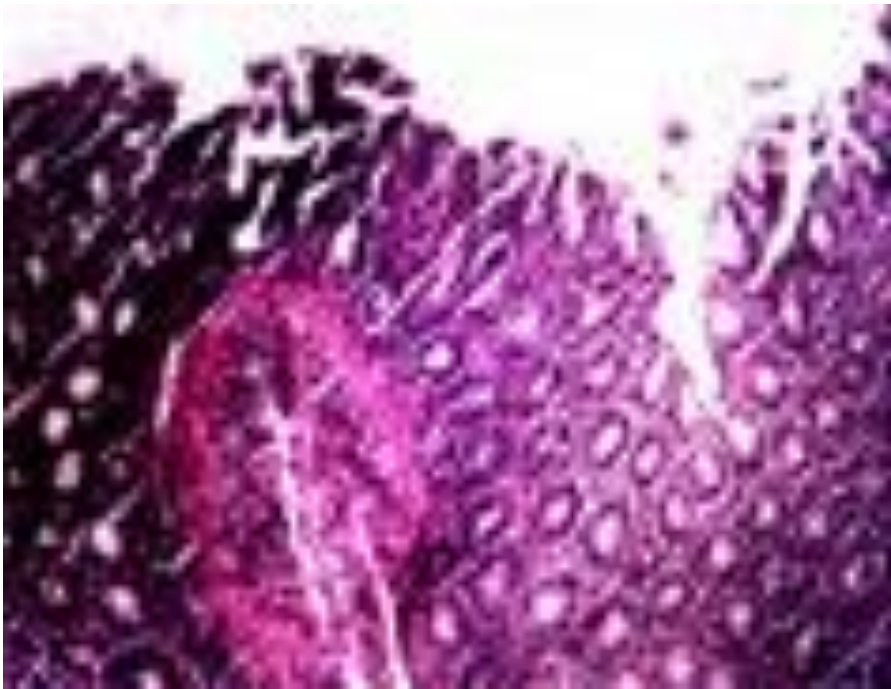


Plate ix: Photomicrograph of histological features of gastric tissue of rats subjected to WRS and modulatory role of selenium (WRS plus Se) (H&E Mag x100).

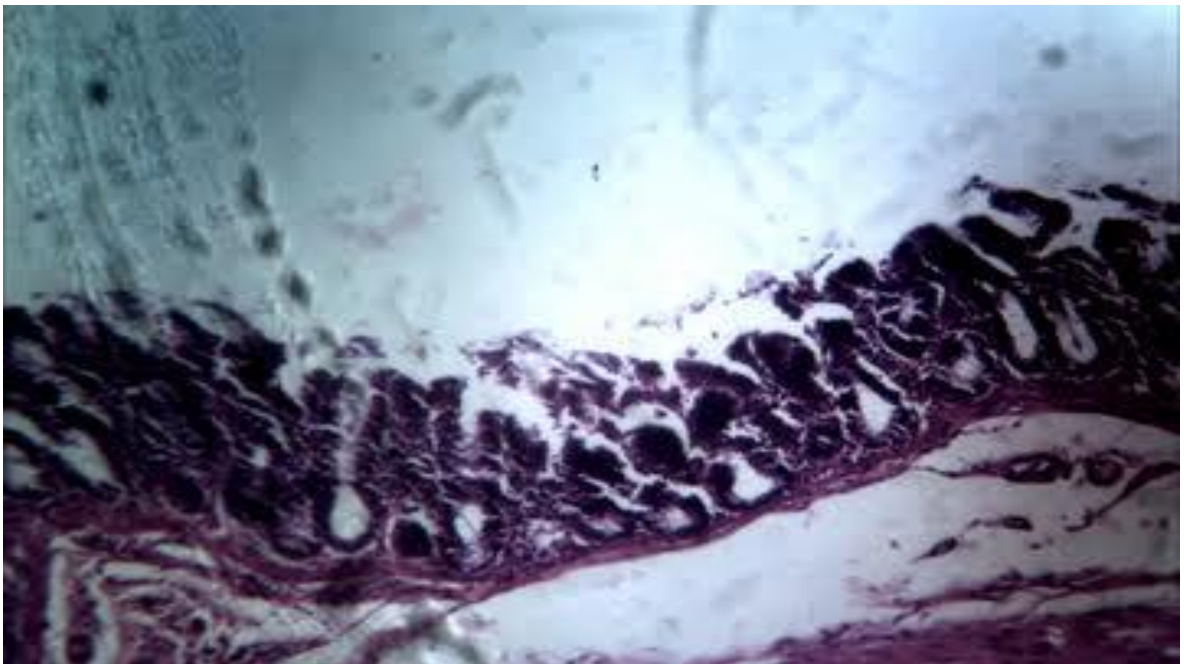


Plate x: Photomicrograph of histological features of gastric tissue of rats subjected to WRS and modulatory role of VE (WRS plus VE) (H&E Mag x100).

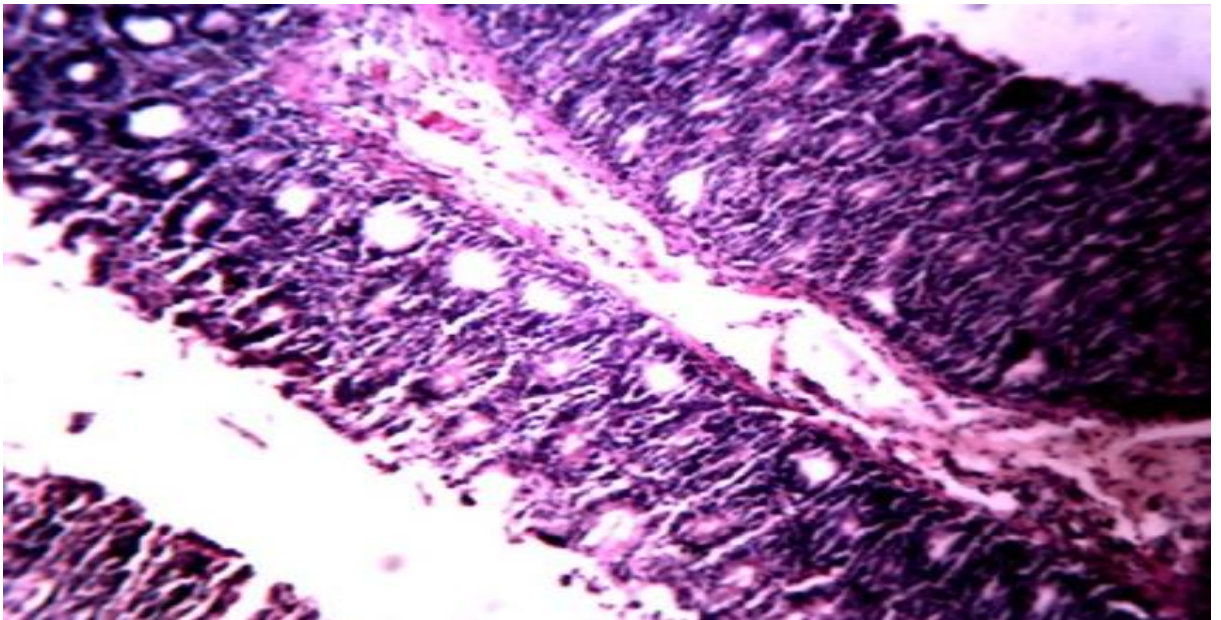


Plate xi: Photomicrograph of histological features of gastric tissue of rats subjected to WRS and modulatory role of selenium and VE(WRS plus Se plus VE) (H&E Mag x100)

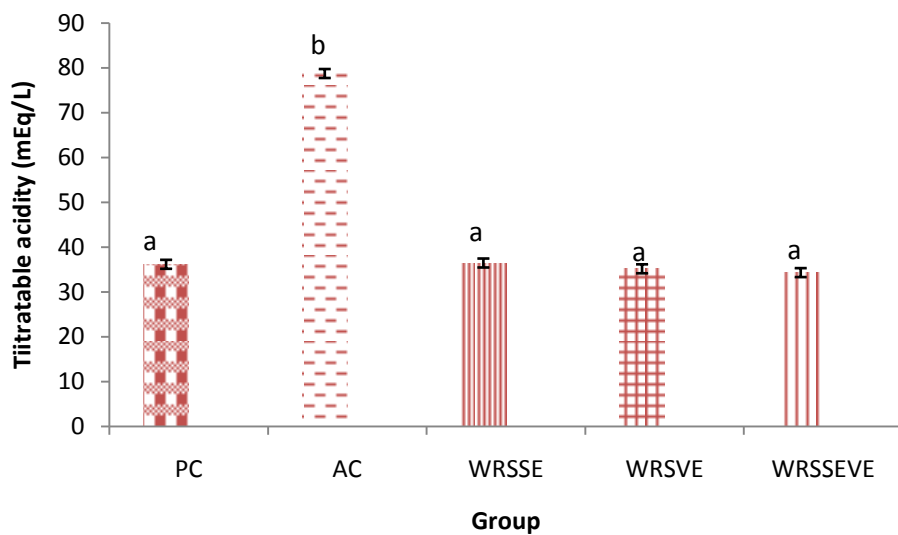


Figure 4.3: Effects of selenium and vitamin E on gastric juice titratable acidity in rats subjected to water-immersion restraint stress.

a, b = means with different superscript letters are significantly ($P < 0.001$) different compared with active control ($n = 7$).

PC = Passive control (Non-stressed animals). AC = Active control (WRS + Distilled water). WRSSE = (WRS + Selenium). WRSVE = (WRS + Vitamin E). WRSSEVE = (WRS + Selenium + Vitamin E). WRS = water- immersion restraint stress.

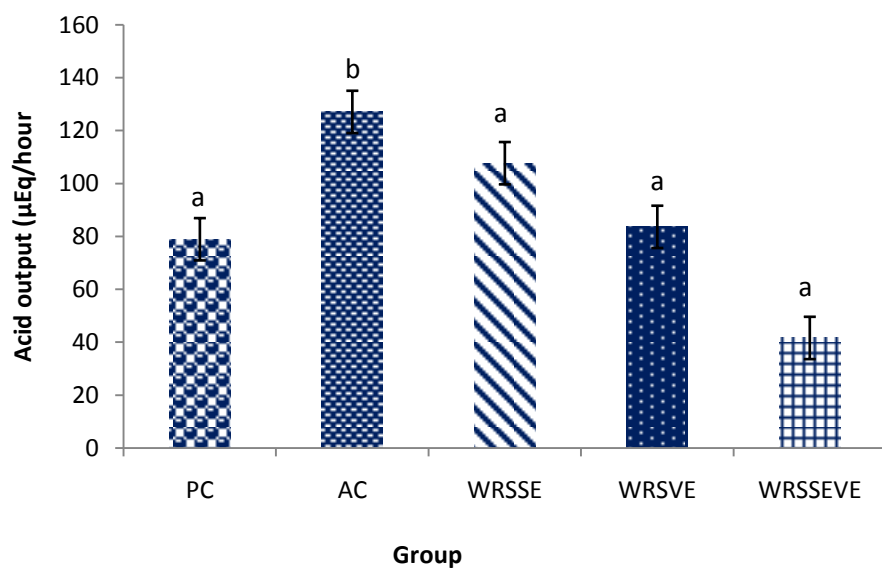


Figure 4.4: Effects of selenium and vitamin E on gastric acid output in rats subjected to water-immersion restraint stress.

a, b = means with different superscript letters are significantly ($P < 0.001$) different compared with active control ($n = 7$).

PC = Passive control (Non stress animals). AC = Active control (WRS + Distilled water). WRSSE = (WRS + Selenium). WRSVE = (WRS + Vitamin E). WRSSEVE = (WRS + Selenium + Vitamin E). WRS = water- immersion restraint stress.

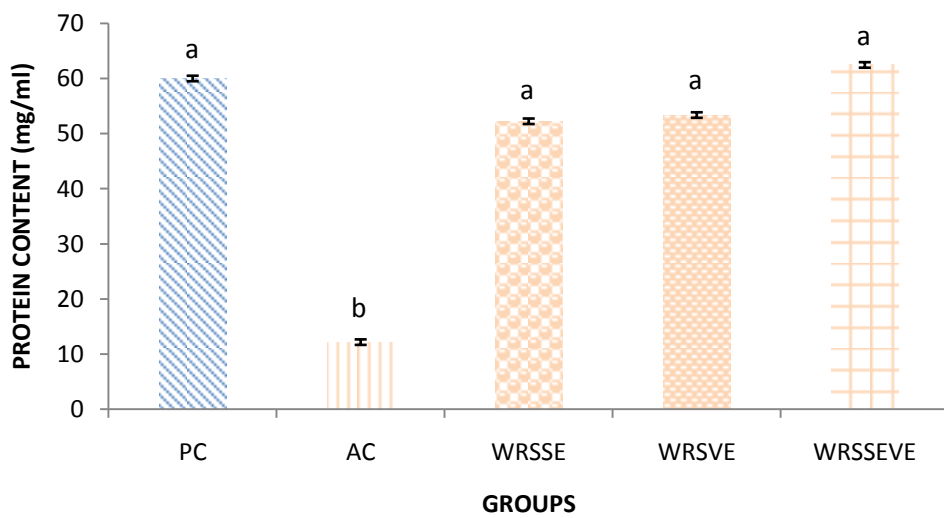


Figure 4.5: Effects of selenium and vitamin E on protein concentration in rats subjected to water-immersion restraint stress.

a, b = means with different superscript letters are significantly ($P < 0.001$) different ($n = 7$).

PC = Passive control (Non stress animals). AC = Active control (WRS + Distilled water). WRSSE = (WRS + Selenium). WRSVE = (WRS + Vitamin E). WRSSEVE = (WRS + Selenium + Vitamin E). WRS = water- immersion restraint stress.

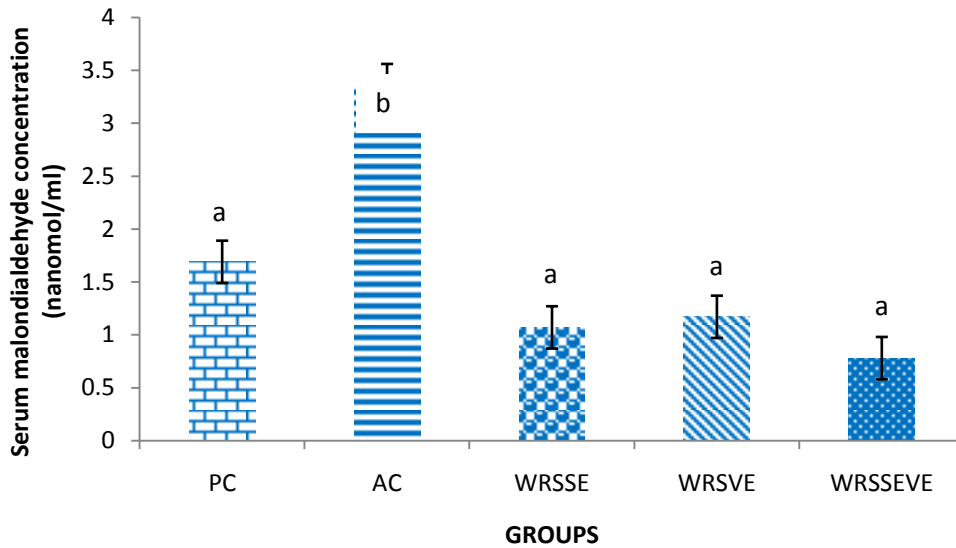


Figure 4.6: Effects of selenium and vitamin E on serum Malondialdehyde concentration in rats subjected to water-immersion restraint stress.

a, b = means with different superscript letters are significantly ($P < 0.001$) different compared with active control ($n = 7$).

PC = Passive control (Non-stressed animals). AC = Active control (WRS + Distilled water). WRSSE = (WRS + Selenium). WRSVE = (WRS + Vitamin E). WRSSEVE = (WRS + Selenium + Vitamin E). WRS = water- immersion restraint stress.

Table 4.1: Effect of selenium and vitamin E on the activities of antioxidant enzymes in rats subjected to water-immersion restraint stress (mean \pm SEM, n = 7)

| Group | Catalase(μ moles of H ₂ O ₂ consumed/mgprotein) | SOD(U/mg protein) | GSH(μ g of GSH/mg protein) |
|-----------------|--|------------------------------|---------------------------------|
| Passive control | 32.00 \pm 1.99 ^a | 1.84 \pm 0.13 ^a | 8.83 \pm 0.33 ^a |
| Active control | 13.43 \pm 0.82 ^b | 0.81 \pm 0.04 ^b | 5.84 \pm 0.28 ^b |
| WRS+ Se | 28.30 \pm 1.38 ^a | 1.53 \pm 0.08 ^a | 7.53 \pm 0.46 ^a |
| WRS + VE | 30.97 \pm 1.93 ^a | 1.46 \pm 0.13 ^a | 8.51 \pm 0.30 ^a |
| WRS + Se +VE | 23.33 \pm 2.33 ^a | 1.65 \pm 0.13 ^a | 8.26 \pm 0.19 ^a |

** = P < 0.001 significant differences between active and passive controls and also active control compared with vitamin E and selenium groups

Pass. Cont. = Passive control (Non-stressed animals). Act. Cont. = Active control (WRS + Distilled water). WRS + Se = (WRS + Selenium). WRS + VE = (WRS + Vitamin E). WRS + Se + VE = (WRS + Selenium + Vitamin E). WRS = water-immersion restraint stress.

4.6 Activities of Liver Enzymes

Figures 4.7 and 4.8 show the result of hepatoprotective effect of selenium and vitamin E in rats, subjected to WRS. The mean \pm SEM of serum alanine aminotransferase (ALT) (185.50 ± 39.84 U/L) and aspartate aminotransferase (AST) (347.43 ± 82.36 U/L) activities in active controls were higher and significantly different ($P < 0.001$) when compared with mean values of passive control (79.5 ± 6.48 U/L and 63.43 ± 4.11 U/L). A significant ($P < 0.001$) decrease in the mean activities of ALT (69.68 ± 11.22 , 71.61 ± 13.56 , 59.99 ± 13.16 U/L for selenium, vitamin E and selenium plus vitamin E groups respectively) and AST (75.85 ± 5.54 , 78.33 ± 7.28 , 70.10 ± 6.46 U/L for selenium, vitamin E and selenium plus vitamin E groups respectively) were recorded in all the selenium and vitamin E pre-treated groups when the mean values of activities were compared with that of the active control. There was a considerable decrease in the activities of ALT and AST in the selenium and vitamin E co-administered group.

4.7 Trace Mineral Concentration

The mean values of serum concentrations of Cu, Zn and Mg in rats subjected to WRS are shown in Table 4.2. No significant ($P > 0.05$) difference recorded in the mean values of Cu concentration in active control (0.054 ± 0.01 ppm) when compared with that (0.046 ± 0.03 ppm) of passive control. Similarly there was no significant difference ($P > 0.05$) in Cu concentrations in all the selenium and vitamin E pre-treated groups when compared with the mean of the active control (0.043 ± 0.01 ppm, 0.096 ± 0.07 ppm, 3.252 ± 0.01 ppm for selenium, vitamin E and selenium plus vitamin E groups). The mean value of Zn concentration in active control ($0.515 \pm$

0.33 ppm) was significantly higher ($P < 0.05$), when compared with that of the passive controls (0.215 ± 0.06 ppm). Pre-treatment with selenium or vitamin E did not alter Zn concentration significantly ($P > 0.05$), when the mean concentration (0.287 ± 0.04 ppm, 0.292 ± 0.12 , 0.430 ± 0.04 ppm for selenium, vitamin E and selenium plus vitamin E groups respectively) of the pre-treated groups were compared with that of the active control. There was a significant ($P < 0.05$) decrease in the concentration of Mg, when the mean concentration of the active controls (1.950 ± 0.44 ppm) was compared with that of the passive controls (3.546 ± 0.15 ppm). Pre-treatment with selenium and vitamin E before induction of WRS resulted in a significant ($P < 0.05$) increase in the serum Mg concentration, when the mean concentrations (3.043 ± 0.15 ppm, 2.822 ± 0.21 ppm, 3.043 ± 0.15 ppm for selenium, vitamin E, and selenium plus vitamin E groups respectively) compared with that of the active control. Pre-treatment with either selenium or vitamin E increased Zn concentration greater than any other pre-treatment.

4.8 Haematological Parameters

Table 4.3 shows the mean values of the haematological parameters obtained in the various groups. PCV, RBC, Hb, WBC, MCV, MCH, and MCHC were relatively higher in active control (47.14 ± 0.67 %) PCV, ($4.80 \pm 0.14 \times 10^{12} / L$) RBC, (15.90 ± 0.29 g / dl) Hb, ($7.18 \pm 0.44 \times 10^9 / L$) WBC, compared with the corresponding values in passive controls, although differences were not significant ($P > 0.05$). The values of PCV, RBC in all groups pre-treated with selenium and vitamin E were higher ($P < 0.001$) compared with that of the active controls, The WBC counts in pre-treated groups were significantly ($P < 0.001$) higher in WRS + Se group, when compared with the count recorded in active controls.

The mean values of MCV, MCH and MCHC did not show any significant ($P > 0.05$) difference between the pre-treated groups and those of the active controls. The neutrophil, lymphocyte counts were significantly ($P < 0.05$) higher when the active controls was compared with that of

the passive controls. There was no significant difference between the mean monocyte counts recorded of active and passive controls. The mean counts of neutrophils, lymphocytes in all the pre-treated groups were higher ($P < 0.05$), when compared with that of the active controls. There was no significant difference in the mean count of monocytes in the pre-treated groups, when compared with that of the active control.

4.9 Erythrocyte Osmotic Fragility Test

The mean percentages of haemolysis at different concentrations of NaCl were significantly ($P < 0.001$) higher in active controls compared with the corresponding percentages in passive controls (Fig 4.9). A significant decrease ($P < 0.001$) in percentage haemolysis was recorded in selenium and vitamin E pre-treated groups, when compared with those of the active controls, and the decrease was higher in selenium and vitamin E pre-treated group. (Figure 4.10).

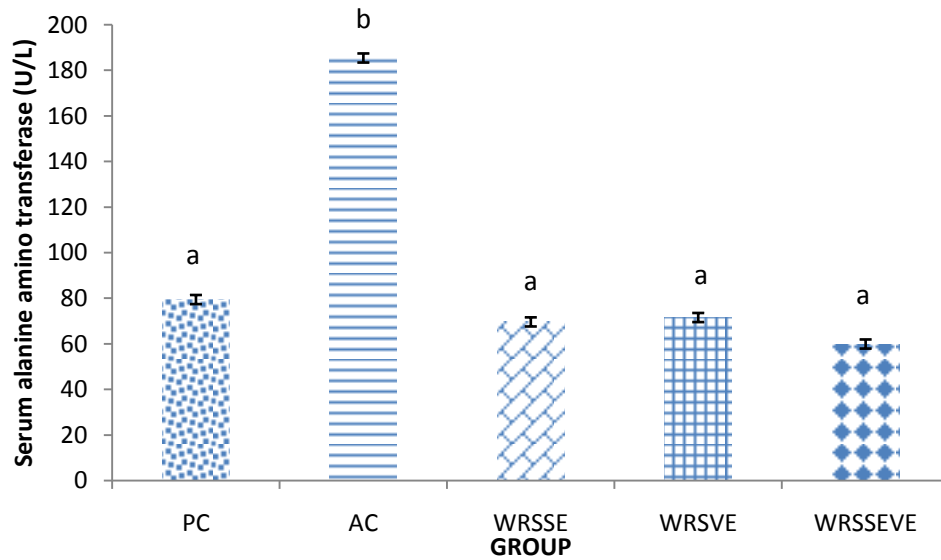


Figure 4.7: Effects of selenium and vitamin E on serum alanine amino transferase activity in rats subjected to water-immersion restraint stress.

a, b = means with different superscript letters are significantly ($P < 0.001$) different compared with active control ($n = 7$).

PC = Passive control (Non-stressed animals). AC = Active control (WRS + Distilled water). WRSSE = (WRS + Selenium). WRSVE = (WRS + Vitamin E). WRSSEVE = (WRS + Selenium + Vitamin E). WRS = water-immersion restraint stress.

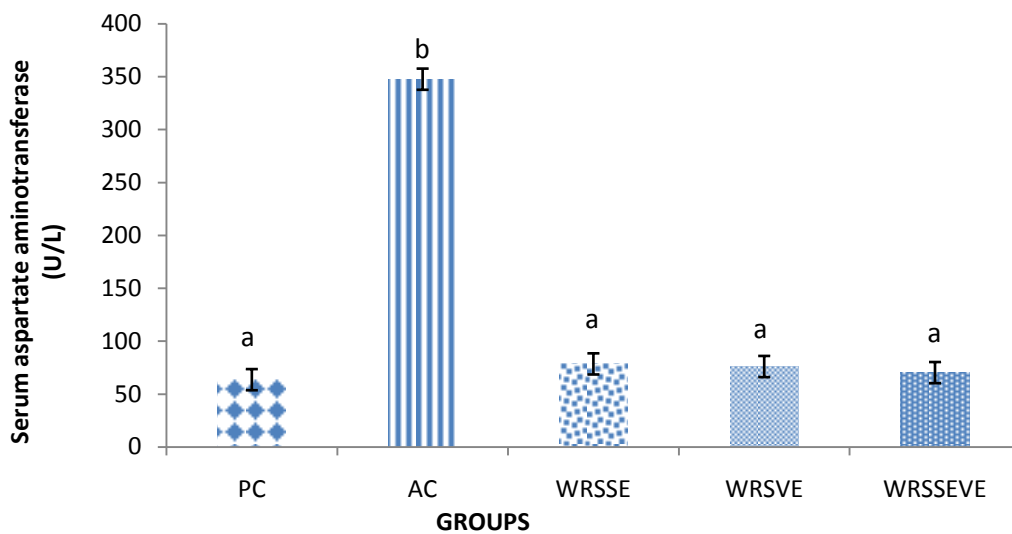


Fig 4.8: Effects of selenium and vitamin E on serum aspartate transferase activity in rats subjected to water immersion restraint stress.

a, b = means with different superscript letters are significantly ($P < 0.001$) different compared with active control ($n = 7$).

PC = Passive control (Non-stressed animals). AC = Active control (WRS + Distilled water). WRSSE = (WRS + Selenium). WRSVE = (WRS + Vitamin E). WRSSEVE = (WRS + Selenium + Vitamin E). WRS = water- immersion restraint stress.

Table 4.2: Effects of selenium and vitamin E on the concentration of copper, zinc and magnesium in rats subjected to water immersion restraint stress (mean \pm SEM, n = 7)

| Group | Cu (ppm) | Zn (ppm) | Mg (ppm) |
|---------------|------------------|-------------------|-------------------|
| Pass. cont. | 0.046 \pm 0.03 | 0.215 \pm 0.06 | 3.546 \pm 0.15* |
| Act. Cont. | 0.054 \pm 0.01 | 0.515 \pm 0.33* | 1.950 \pm 0.44 |
| WRS + Se | 0.043 \pm 0.01 | 0.287 \pm 0.04 | 3.043 \pm 0.15* |
| WRS + VE | 0.096 \pm 0.07 | 0.292 \pm 0.12 | 2.822 \pm 0.21* |
| WRS + Se + VE | 3.252 \pm 0.01 | 0.430 \pm 0.04 | 3.043 \pm 0.15* |

* = P < 0.05 significant differences between active and passive controls and active control compared with selenium and vitamin E groups

Pass. Cont. = Passive control (Non-stressed animals). Act. Cont. = Active control (WRS + Distilled water). WRS + Se = (WRS + Selenium). WRS + VE = (WRS + Vitamin E). WRS + Se + VE = (WRS + Selenium + Vitamin E). WRS = water-immersion restraint stress.

Table 4.3: Effect of selenium and vitamin E on some haematological parameters in rats subjected to water immersion restraint stress(mean \pm SEM, n=7)

| Parameter | Passive Control | Active Control | WRS +Se | WRS+VE | WRS+Se+VE |
|-----------------------------|--------------------|---------------------|---------------------|---------------------|---------------------|
| PCV (%) | 47.14 \pm 0.67 | 48.14 \pm 0.51 | 55.29 \pm 0.89** | 56.86 \pm 0.96** | 55.29 \pm 1.36** |
| Hb (g/dL) | 15.90 \pm 0.29 | 16.04 \pm 0.17 | 18.29 \pm 0.29** | 18.90 \pm 0.32** | 18.86 \pm 0.44** |
| RBC (x 10 ¹² /L) | 4.80 \pm 0.14 | 4.59 \pm 0.09 | 5.06 \pm 0.10** | 5.28 \pm 0.09** | 5.37 \pm 0.11** |
| WBC (x10 ⁹ /L) | 7.18 \pm 0.44 | 7.94 \pm 0.54 | 6.09 \pm 0.36** | 6.46 \pm 0.33 | 6.43 \pm 0.33* |
| MCV (fL) | 9.88 \pm 0.36 | 10.46 \pm 0.25 | 10.35 \pm 0.44 | 10.51 \pm 0.43 | 10.35 \pm 0.32 |
| MCH (pg) | 33.01 \pm 1.19 | 34.94 \pm 0.79 | 33.40 \pm 1.26 | 36.56 \pm 0.68 | 34.16 \pm 1.08 |
| MCHC (g/dL) | 33.49 \pm 0.36 | 33.34 \pm 0.43 | 32.98 \pm 0.44 | 34.98 \pm 0.43 | 32.58 \pm 0.32 |
| Neutrophils (x 1000/L) | 245.59 \pm 15.46 | 222.29 \pm 27.31* | 243.79 \pm 3.86* | 244.03 \pm 2.59* | 245.30 \pm 3.84* |
| Lymphocytes (x 1000/L) | 443.94 \pm 27.63 | 487.36 \pm 38.66 | 520.53 \pm 32.31* | 553.41 \pm 32.03* | 555.85 \pm 34.18* |
| Monocytes (x 1000/L) | 31.16 \pm 1.89 | 39.67 \pm 2.71 | 35.62 \pm 3.00 | 36.21 \pm 3.08 | 36.80 \pm 2.70 |

* = P < 0.05 Significant Differences between active and passive controls and also active control compared with selenium and vitamin E groups. Pass. Cont. = Passive control

(Non-stressed animals). Act. Cont. = Active control (WRS + Distilled water). WRS + Se = (WRS + Selenium). WRS + VE = (WRS + Vitamin E). WRS + Se + VE = (WRS + Selenium + Vitamin E). WRS = water-immersion restraint stress

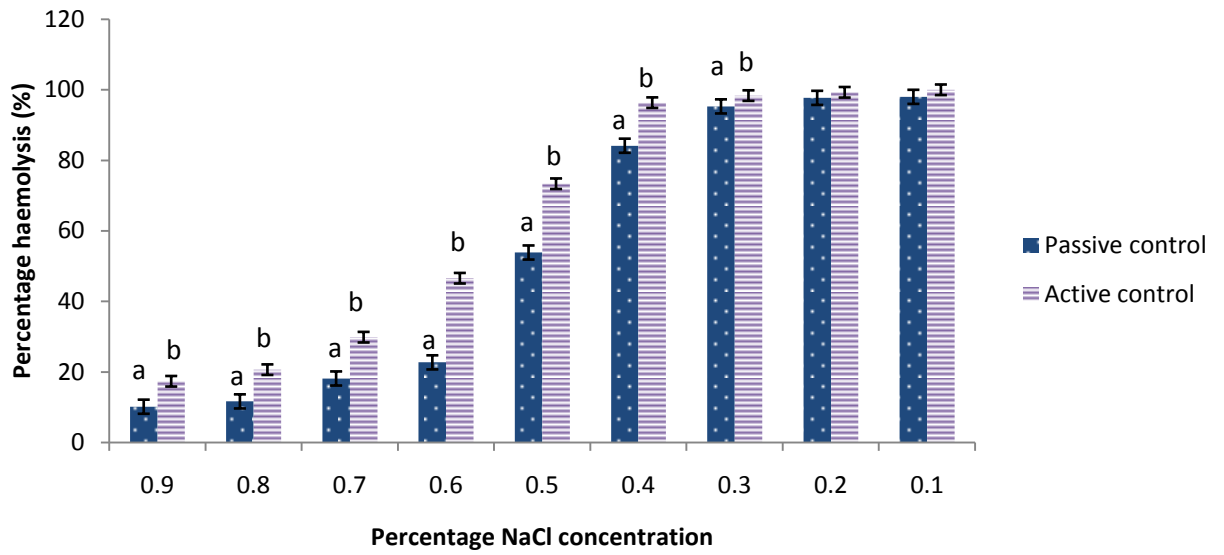


Figure 4.9: Effects of water-immersion restraint stress on erythrocyte osmotic fragility in Wistar rats

a, b = Means with different superscript letters are significantly ($P < 0.001$) different ($n = 7$).

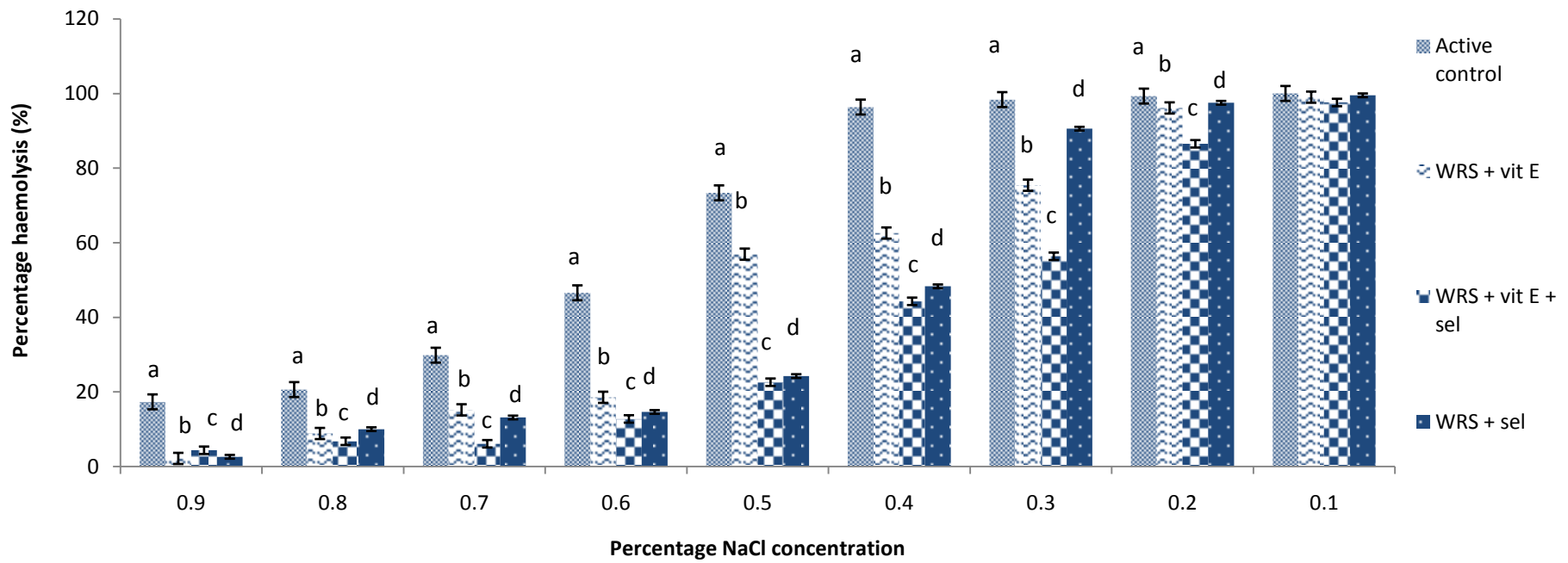


Figure 4.10: Effects of selenium and vitamin E on erythrocyte osmotic fragility in rats subjected to water immersion restraint stress

a,b, c,d = Values with no common super script letters are significantly ($P < 0.001$) different

Pass. Cont. = Passive control (Non stress animals). Act. Cont. = Active control (WRS + Distilled water). WRS + vit E = (WRS + vitamin E). WRS + vit E + sel = (WRS + vitamin E + selenium). WRS + sel = (WRS + selenium). WRS = water immersion restraint stress

CHAPTER FIVE

5.0 Discussion

The effects of selenium and vitamin E on gastric mucosal damage, gastric secretion and haemato-biochemical changes induced by water immersion restraint stress (WRS) in Wistar rats were investigated. WRS-induced gastric lesion in Wistar rats. Significant difference in the gastric ulcer parameter (ulcer index) when the values obtained in active controls were compared with those of passive control. The result agreed with the report of Anil *et al.* (2010) that the stomach is one of the main targets of stress and that stress-induced gastric ulceration is a typical example of stress-associated organ injuries. However administration of selenium and vitamin E individually or in combination decreased significantly the studied gastric ulcer parameters. This is because selenium and vitamin E apparently inhibited the oxidation of molecules and, therefore, prevent tissue damage as they act as scavengers (Mansoor and Mahmood, 2009). The above findings were in accordance, with the report of Ibrahim *et al.* (2010), who concluded that supplementation with palm vitamin E or α -tocopherol reduce gastric lesion significantly. According to Abu Taib *et al.* (1997) selenium prevents reperfusion-induced gastric mucosal lesion, intraluminal bleeding and depletion of non-protein sulphhydryl level in the rat stomach, thus playing potent antioxidant role to protect tissue against oxidative damage.

This study found that selenium or vitamin E inhibited ulcer, but greatest inhibition was achieved by co-administration of selenium and vitamin E. The result showed that the co-administration of the two antioxidants was more potent in the ulcer inhibition than their individual administration. Therefore selenium and/or plays an antioxidant role in protecting the tissue against oxidative stress. The macroscopic findings of the opened stomach in this study indicated haemorrhagic gastric ulcers with clotted blood were more apparent in the active control than in the passive control group. WRS-induced gastric ulceration was inhibited by selenium and vitamin E administration and ulcer formation was completely inhibited by the co-administration

of selenium and vitamin E. Histopathological findings of the gastric mucosa obtained in this study, passive control rats showed an intact cellular architecture while active control showed ulcer combined with distorted gastric glands, a damage mucosal epithelium and cellular debris were also found. Selenium and vitamin E pre-treated group's show the protection against these histopathological changes, induced by WRS in rats, resulted in the maintenance of glandular organisation and the structure of the *muscularis mucosa*. Yan-ping *et al.* (2005) found that selenium supplement can lead to the expansion of secretory canaliculus, increase in the number of endocrine cells in gastric mucosa, alterations in the mitochondria and changes in the number and shape of pinosomes. Ahmad and Mohammad, (1996) found that Selenium and vitamin E protect gastric mucosa against the lesion produced by hypothermic restraint stress and chemical. According to Yoshikwa *et al.* (1991), in nitric oxide-depleted rats, vitamin E played an importance protective role against ischaemia-reperfusion-induced gastric mucosal injury. The authors suggested that the gastro-protective effect of VE was not only due to its antioxidant action, but also inhibitory action on neutrophil infiltration into the gastric mucosa. Ibrahim *et al.* (2010) concluded that dietary supplementation of palm VE or α -tocopherol was able to reduce gastric lesion significantly. Vitamin E causes a reduction of glucocorticoids, which are known to be immune suppressive. It also alters arachidonic acid metabolism and subsequent synthesis of prostaglandins, thromboxanes and leukotrienes. Under stress condition, increased level of these compounds by endogenous synthesis or exogenous entry may adversely affect immune cell function (Shinde *et al.*, 2007). The present study demonstrated that exposure of the rats to WRS resulted in significant increase in gastric secretion parameters (gastric juice volume, titrable acidity and acid output) in the active controls compared to the passive controls. However, pre-treatment with selenium and vitamin E showed a significant decreased in gastric secretion parameters. The result corroborated with the report of Yuan-Fang *et al.* (2005) that gastric acid output increased remarkably in rats, exposed to WRS, and the increase lasted while the stress was present. Thus, the stress-induced increase in acid output obtained in the present study correlated well with severity of mucosal lesion. Nur *et al.* (2005) suggested that vitamin E

may have an anti-secretory effect and also showed that a single large dose (300mg/kg) of alpha-tocopherol caused a reduction in gastric acid secretion in non-stressed rats. Based on this finding it was proposed that that one of the protective effects by vitamin E against gastric injury could be through its anti-secretory function. The result of the present study reported an increase in the volume, titratable acidity and acid output in the active control, but selenium and vitamin E pre-treatment lower significantly the recorded increases. This effect may contribute to gastric mucosal protection activity of selenium and vitamin E.

The results of the study demonstrated that WRS significantly reduced total protein concentration. The mean protein concentration of the active control was significantly lower compared with that of the passive control. The total protein concentration significantly increased in selenium and/or vitamin E, pre-treated animals. The concomitant administration of selenium and vitamin E showed a greater enhancement of total protein concentration. The finding agreed with the report of Abdulaziz *et al.* (2011), who demonstrated that treatment with α -tocopherol and selenium in combination or individually increased total protein concentration, which were not significantly different from that of the normal control in stressed rats. The results showed that ROS, apparently, leading to lipid peroxidation. The increase was reduced by antioxidants in the plasma of rats administered with selenium or vitamin E. The MDA is a product of lipid peroxidation that is an indicator of ROS to tissues (Naime *et al.*, 2001). It is also a basic indicator of tissue damage in the heart, lungs, small intestine and stomach (Jeong-Hwan *et al.*, 2012). In the present study the MDA concentration increased significantly in the active controls, with respect to the passive controls. Thus, the, selenium and vitamin E pre-treatments reduced the concentration of the MDA significantly in comparison to the active control. The results of the present study clearly revealed that selenium and vitamin E inhibited the formation of gastric mucosal lesion in rats subjected to WRS through prevention of lipid peroxidation. Co-administration of selenium and vitamin E produced considerable decrease in the MDA concentration, compared to selenium or vitamin E alone in the present study. Jeong-Hwan *et al.*

(2012) reported that pre-treatment with 100 µg/ kg selenium for 3 days showed a significant decrease in MDA concentration in ethanol-induced gastric mucosal lesion in rats. Naime *et al.* (2001) investigated the effects of selenium, which is a co-factor of GSH-Px, on gastric mucosal injury induced by cold restraint stress and found that selenium has protective effect on gastric mucosa and reduced lipid peroxidation-induced damage, significantly as evidenced by decreased in gastric tissue MDA concentration. The result of the present study agreed with the findings of Naime *et al.* (2001) who showed that combination of selenium and vitamin E resulted in significant decrease in gastric mucosal damage and lipid peroxidation-induced MDA levels in rats subjected to cold restraint stress. Vitamin E scavenges peroxy radical intermediate in lipid peroxidation and is responsible for protecting poly-unsaturated fatty acids present in cell membrane, and low-density lipoprotein (LDL) against lipid peroxidation (Sujogya, 2012).

The enzymatic antioxidant system CAT, SOD and GSH counteracted the ROS and reduced the oxidative stress (Ramasundaram *et al.*, 2007). Then SOD accelerates the conversion of superoxide radical to hydrogen peroxide, while CAT or GSH converts hydrogen peroxide to water. The present study also investigated the effect of selenium and vitamin E on the activities of radical scavenging enzymes, such as CAT, SOD and GSH in the blood serum. The WRS significantly decreased the activities of CAT, SOD and GSH, which was recorded when the activities of the enzymes in active controls were compared with those of passive controls. However, selenium and vitamin E pre-treatment increased significantly the activities of the enzymes, when their activities in pre-treated groups were compared with those of the active controls. The findings of the present study were in agreement with the result of Gulgun *et al.* (2011) that vitamin E inhibits oxidant damage in gastric mucosa in acetic acid-induced ulcerative colitis in rats. Jeong-Hwan *et al.* (2012) also found that selenium markedly attenuated ethanol-induced lipid peroxidation in gastric mucosa and increased activities of radical scavenging enzymes such as SOD, CAT and GSH. Surajit and Sudipta (2014) discovered appreciable beneficial effects of selenium and vitamin E co-administration against arsenic-induced changes in biochemical and histopathological parameters, indicating that

supplementation of vitamin E along with micronutrient selenium exert anti-oxidative effects against arsenic-induced cardio- toxicity.

The liver is an important organ for metabolism and detoxification. It contains considerable amount of polyunsaturated fatty acids, which are prone to damage by ROS. Liver enzymes (ALT and AST) are released into the blood, whenever the liver cells are damaged, and the activities of the liver enzymes in the plasma increase (Ebunlomo *et al.*, 2012). In the present study, elevated activities of ALT and AST demonstrated liver damage in the rats, exposed to WRS. There were significant increases in both the ALT and AST activities in the active control in comparison to the passive control. This increased of activities might be due to alteration in the cell membrane permeability, which permits the enzymes to leak from the cell with intact membrane due to the effect of stress on the liver cells. The enzymes escape into the blood circulation and so the ALT and AST activities increase. The results of the above findings are in agreement with the work of Ebunlomo *et al.* (2012). However, supplementation with vitamin E and selenium 3 days before WRS induction decreased significantly the activities of ALT and AST, when compared with the active controls. The pre-treatment with selenium and vitamin E thus, alleviated the increased serum ALT and AST activities to near normal, either individually or when co-administered. The result agrees with the findings of Tareek *et al.* (2012), who showed that this could be attributed to the antioxidant activities of selenium and vitamin E as well as the metal-chelating efficacy of selenium. Siham and Nabila, (2008) found that rats injected with HgCl₂ showed elevation of serum level of both ALT and AST. However selenium treatment in rats injected with HgCl₂ decreased the elevated serum level of ALT and AST.

Trace elements such as Cu and Zn are required in small amount, usually less than 100 mg/kg dry matter (Yatoo *et al.*, 2013), and are present in very minute quantities in animal serum. Copper and zinc function as component of metallo-enzymes that take part in reduction reactions, these metallo-enzymes are involved in multiple physiological processes including respiration, carbohydrate and lipid metabolism, antioxidant activities and collagen formation (Andrieu, 2008). Pre-treatment with selenium and vitamin E had no effects on both Cu and Zn concentrations. The result of the present study also showed a significant decrease in serum Mg concentration in active control compared with that of passive control. However, pre-treatment with selenium and vitamin E significantly elevated serum Mg concentration when the active control was compared with the pre-treated groups. The above findings were in accordance with the result obtained by Huang *et al.* (2012) and Antonyuk *et al.* (2009) that trace elements act as co-factors of enzymes like SOD, GPx and CAT. The elements contribute in antioxidant functions as well as playing vital roles in many physio-biochemical processes, like protein, hormone and enzyme syntheses.

The changes in haematological parameters in Wistar rats, subjected to WRS and the effects of selenium and vitamin E in attenuating some of the adverse effects were one of the focal points of the present study. Physiological studies have shown that stress from any source can influence the endocrine, haemopoietic and immune systems (Ebunlomo *et al.*, 2012). Dhabhar *et al.* (1995) found that stress increases RBC, neutrophils and platelets counts, whereas lymphocyte, eosinophils and monocyte decrease in number, and suggested further that endocrine factors released during stress modulate leucocyte trafficking resulting in distribution of leucocytes between the bloods other immune compartments. The result of the present study showed that there were no significant changes in PCV, Hb, RBC, WBC, MCV, MCH, MCHC, Lymphocytes and monocytes in response to WRS for 3.5 hours in the active controls in comparison to passive

controls. The reason for this result might be that WRS for 3.5 hours did not exert considerable effects on blood-forming organs, such that there was no inhibition of erythropoiesis and consequently, haemosynthesis. The MCV, MCH and MCHC remain normal because there was no significant destruction in RBC (size and shape) and decrease in haemoglobin content in the active controls. A significant decrease in neutrophil count was observed when the active controls were compared with the passive controls. This finding may be because neutrophils are involved in phagocytosis, during which some of the neutrophils have ruptured. The present study was in agreement with findings of Ebunlomo *et al.* (2012), who also found no significant increase in the values of PCV, RBC, Hb, and MCV. Although the present study contrasts the significant increase in Hb and MCV values reported by Maes *et al.* (1998), number of some of the haematological values (PCV, RBC, WBC, Hb, neutrophils and lymphocytes) showed significant increases in selenium and vitamin E pre-treated groups. The findings suggest that selenium and vitamin E as antioxidants increased the immune function of the rats.

The result of the erythrocyte osmotic fragility showed that 3.5 hours of WRS increased haemolysis in the rats. The percentage haemolysis was significantly highest in the active control group, when compared with passive controls. The increased erythrocyte osmotic fragility may be attributed to effects of stress, to cause increased ROS generation in animals (Olayinka *et al.*, 2011). The erythrocytes are highly susceptible to oxidative stress apparently because of high amount of poly-unsaturated fatty contents in their membrane (Nazifi *et al.*, 2009). The present study showed that pre-treatment with selenium and vitamin E decreased the percentage haemolysis in the pre-treated groups, compared to the active controls. This result agreed with the findings of Alhassan *et al.* (2010), who suggested that the beneficial effects of administration of antioxidant vitamins in ameliorating the adverse effects of stress including the susceptibility to haemolysis in heat-stressed rats

CHAPTER SIX

6.0 Summary, Conclusion and Recommendation

6.1 Summary

i. Water-immersion restraint stress (WRS) mimics the clinical acute gastric ulceration caused by trauma, surgery or sepsis. It affects psychological and physiological balance, which can lead to various pathological changes; the psychological responses include anxiety, depression, helplessness, fear and threat of drowning, while the physiological responses are neuro-hormonal and immunological activation. Psychogenic factors such as stress play a major role in the pathogenesis of gastric ulcers in man. Thus, stress-related animal experiment appears to be a very good mimic of human condition and have allowed studies in to pathogenic mechanisms as well as useful therapeutic interventions.

ii. Acute WRS for 3.5 hours carried out in this study was found to cause significant alterations in physiological, biochemical, haematological and lipid peroxidative parameters and, consequently, a shift in oxidant and antioxidant balance.

iii. Antioxidant or free radical scavengers, vitamin E and the mineral selenium destroyed ROS by preventing the oxidation process. Exposure to WRS increased significantly lipid peroxidative parameter, activities of plasma-biomarkers of liver injury enzymes (ALT and AST), plasma zinc concentration, and haemolysis in different concentrations of NaCl, increased gastric ulcer and secretion parameters. Pre-treatment with selenium and vitamin E individually or co-administered significantly lowered the recorded increases.

6.2 Conclusions

Water-immersion restraint stress causes significant increases in the concentration of serum lipid peroxidative parameter, plasma-biomarkers of liver injury, plasma Zn, and significant increases in haemolysis, gastric ulcer and secretion parameters. Furthermore, exposure to WRS significantly lowered the concentrations of the serum proteins, antioxidant enzymes, Mg and number of neutrophils. Pre-treatment with selenium or vitamin E singly and/or in combination ameliorated some of the recorded adverse effects of WRS. Consumption of diet containing selenium or vitamin E could help to ameliorate the oxidation effects caused by stress.

6.3 Recommendations

It is recommended that:

- (i) More investigation needs to be performed to determine role of selenium and vitamin E on ulcer healing, by observing gene expression of certain growth factors.
- (ii) There is need to investigate physiological doses of selenium and/or vitamin E or their long-term effects on mutagenicity and genotoxicity.
- (iii) There is need to investigate, if H₂-receptors are involved in increased gastric acid secretion following exposure to WRS.

6.4 Contributions to knowledge

(i) Selenium and vitamin E concurrent administration achieved better gastroprotective effect in WRS than singly, considering the result of ulcer inhibition (73.78%) and highest decrease in lipid peroxidative and gastric acid secretion parameters.

(ii) There was a synergistic effect between selenium and vitamin E in ameliorating the adverse effects of WRS.

(iii) Selenium and vitamin E increased RBC counts in WRS rats.

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APPENDICES

APPENDIX I

Effect of selenium and vitamin E on ulcer indices in rats subjected to water-immersion restraint stress (Mean \pm SEM, n = 7)

| Group | Ulcer index (mm) | Preventive index (%) |
|-----------------|------------------------------|----------------------|
| Passive control | 0.00 \pm 0.00 ^b | ----- |
| Active control | 27.08 \pm 0.80 | ----- |

| | | |
|---------------|---------------------------|-------|
| WRS+ Se | 14.35 ± 3.20 ^a | 47.01 |
| WRS + VE | 13.22± 2.50 ^a | 50.89 |
| WRS + Se + VE | 7.10 ± 1. 50 ^b | 73.78 |

a and b = mean with different superscript letters are significantly different at $P < 0.05$ and $P < 0.001$ respectively compared with active control.

Pass. Cont. = Passive control (Non-stressed animals). Act. Cont. = Active control (WRS + Distilled water). WRS + Se = (WRS + Selenium). WRS + VE = (WRS + Vitamin E). WRS + Se + VE = (WRS + Selenium + Vitamin E). WRS = water-immersion restraint stress.

Appendix II

Effect of selenium and vitamin E on gastric acid secretion and output in rats subjected to water immersion restraint stress (Mean ± SEM, n = 7)

| Group | Gastric juice volume (ml/3hours) | Titrateable acidity (mEq/L) | Acid output (μEq/hour) |
|---------------|---|--|--|
| Pass. Control | 2.19 \pm 0.06 ^a | 36.14 \pm 0.77 ^a | 78.91 \pm 2.726 ^a |
| Act. Control | 4.81 \pm 0.09 | 78.71 \pm 1.38 | 127.06 \pm 2.75 |
| WRS + Se | 2.96 \pm 0.11 ^a | 36.43 \pm 0.53 ^a | 107.67 \pm 3.87 ^a |
| WRS+ VE | 2.37 \pm 0.16 ^a | 35.14 \pm 1.50 ^a | 83.60 \pm 4.18 ^a |
| WRS + Se + VE | 1.53 \pm 0.08 ^a | 34.29 \pm 7.33 ^a | 41.60 \pm 2.68 ^a |

a = means with same superscript letters are significantly ($P < 0.001$) different compared with active control.

Pass. Cont. = Passive control (Non-stressed animals). Act. Cont. = Active control (WRS + Distilled water). WRS + Se = (WRS + Selenium). WRS + VE = (WRS + Vitamin E). WRS + Se + VE = (WRS + Selenium + Vitamin E). WRS = water-immersion restraint stress.

Appendix III

Effect of selenium and vitamin E on protein concentration in rats subjected to water immersion restraint stress (Mean \pm SEM, n = 7)

| Group | Protein content (mg/ml) |
|---------------|-------------------------------|
| Pass. control | 60.00 \pm 2.15 ^a |
| Act. Control | 12.17 \pm 1.85 |
| WRS + Se | 52.24 \pm 9.15 ^a |
| WRS + VE | 53.37 \pm 9.18 ^a |
| WRS + Se+ VE | 62.46 \pm 3.75 ^a |

a = means with same superscript letters are significantly ($P < 0.001$) different compared with active control.

Pass. Cont. = Passive control (Non-stressed animals). Act. Cont. = Active control (WRS + Distilled water). WRS + Se = (WRS + Selenium). WRS + VE = (WRS + Vitamin E). WRS + Se + VE = (WRS + Selenium + Vitamin E). WRS = water-immersion restraint stress.

Appendix IV

Effect of selenium and vitamin E on lipid peroxidation parameter (MDA) in rats subjected to water immersion restraint stress (Mean \pm SEM, n = 7)

| Group | MDA(nanomol/ml) |
|--------------|------------------------------|
| Pass control | 1.69 \pm 0.21 ^a |
| Act. Control | 3.36 \pm 0.19 |
| WRS + Se | 1.07 \pm 0.18 ^a |
| WRS + VE | 1.17 \pm 0.28 ^a |
| WRS + Se+ VE | 0.78 \pm 0.13 ^a |

a = means with same superscript letters are significantly (P < 0.001) different compared with active control.

Pass. Cont. = Passive control (Non-stressed animals). Act. Cont. = Active control (WRS + Distilled water). WRS + Se = (WRS + Selenium). WRS + VE = (WRS + Vitamin E). WRS + Se + VE = (WRS + Selenium + Vitamin E). WRS = water-immersion restraint stress.

Appendix V

Effect of selenium and vitamin E on ALT and AST activities in rats subjected water- immersion restraint stress. (Mean \pm SEM, n= 7)

| Group | ALT (U/L) | AST (U/L) |
|--------------|--------------------------------|-------------------------------|
| Pass. Cont. | 79.5 \pm 6.48 ^a | 63.43 \pm 4.11 ^a |
| Act. Cont. | 185.50 \pm 39.84 | 347.43 \pm 82.36 |
| WRS + Se | 69.68 \pm 11.22 ^a | 75.85 \pm 5.54 ^a |
| WRS + VE | 71.61 \pm 13.56 ^a | 78.33 \pm 7.28 ^a |
| WRS + Se+ VE | 59.99 \pm 13.16 ^a | 70.10 \pm 6.46 ^a |

a = means with same superscript letters are significantly (P < 0.001) different compared with active control.

Pass. Cont. = Passive control (Non-stressed animals). Act. Cont. = Active control (WRS + Distilled water). WRS + Se = (WRS + Selenium). WRS + VE = (WRS + Vitamin E). WRS + Se + VE = (WRS + Selenium + Vitamin E). WRS = water-immersion restraint stress. ALT = alanine aminotransferase. AST = aspartate aminotransferase

Appendix VI

Effect of selenium and vitamin E on erythrocytes osmotic fragility in rats subjected to water-immersion restraint stress (Mean \pm SEM, n= 7)

| GROUP | NaCl Concentration (%) | | | | | | | | |
|-------------|------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|-----------------------|---------------------|
| | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 |
| Pas cont | 98.00 \pm 0.00 | 97.71 \pm 0.29 | 95.29 \pm 0.89 | 84.14 \pm 2.01 | 53.86 \pm 2.01 | 22.71 \pm 0.79 | 18.14 \pm 0.91 | 11.64 \pm .6 | 10.14 \pm 0.83 |
| Act cont | 100.00 \pm 0.0* | 99.29 \pm 0.10 | 98.35 \pm 0.13 | 96.35 \pm 0.1* | 73.36 \pm 1.9* | 46.57 \pm 1.6* | 29.85 \pm 0.65* | 220.64 \pm 0.38* | 17.35 \pm 0.* |
| WRS+VE | 99.00 \pm 0.25 | 96.11 \pm 0.67* | 75.41 \pm 2.01* | 62.59 \pm 0.97* | 56.93 \pm 1.12* | 18.57 \pm 0.23* | 15.21 \pm 0.43* | 8.87 \pm 1.19* | 2.19 \pm 0.27* |
| WRS+V | 97.57 \pm | 86.50 \pm | 56.36 \pm | 44.29 \pm | 22.61 \pm | 12.77 \pm | 6.11 \pm | 6.79 \pm | 4.37 \pm |
| E+Se | 0.25* | 0.44* | 1.89* | 0.29* | 0.93* | 0.57* | 1.28* | 0.72* | 0.88* |
| WRS + Se | 99.50 \pm 0.01 | 97.50 \pm 0.27* | 90.56 \pm 1.49* | 48.31 \pm 4.21* | 24.26 \pm 4.21* | 14.64 \pm 0.49* | 13.16 \pm 0.33* | 10.03 \pm 2.17* | 2.63 \pm 0.31* |

* = Significant Differences between active control and passive control also active control compared with vitamin E and selenium groups at P < 0.05

Pass. Cont. = Passive control (Non-stressed animals). Act. Cont. = Active control (WRS + Distilled water). WRS + Se = (WRS + Selenium). WRS + VE = (WRS + Vitamin E). WRS + Se + VE = (WRS + Selenium + Vitamin E). WRS = water-immersion restraint stress. ALT = alanine aminotransferase. AST = aspartate aminotransferase