EFFECTS OF CHLOROQUINE, ARTHEMETER-LUMEFANTRINE AND ARTESUNATE ON EXPERIMENTAL COLITIS IN RATS

BY

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CERTIFICATION

I certify that **Miss Ebunlomo, Ayobami Omolara** carried out this work titled "**Effects of Chloroquine, Arthemeter-lumefantrine and Artesunate on Experimental Colitis in Rats"** under my supervision in the Department of Physiology, College of Medicine, University of Ibadan, Ibadan, Nigeria.

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DEDICATION

This work is dedicated to the pillar of my life. The only God qualified to be called “I AM”. I love you with every piece of my being. Also to my loving and exceptional parents Hon and Mrs Kolawole Ebunlomo. I will live to ever remember your reassuring words and prayers. I love you.

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All honour, glory and adoration be unto him who sits upon the throne of my life and that of the universe, Most High God indeed you reign and rule in the affairs of men.

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ABSTRACT

Ulcerative colitis is a chronic inflammatory bowel disease characterised by inflammation, ulceration and bleeding of the colonic mucosa. The etiology is still unknown. Studies have shown that some anti-malarial drugs interfere with gastric mucosal integrity, leading to the development of gastric ulceration. However the effect of this class of drugs on the integrity of the colon is not known. This study was designed to investigate the effects of chloroquine, artesunate and artemether-lumefantrine on acute ulcerative colitis in rats.

Eighty male albino rats (160–180 g) were randomly assigned to four groups of twenty animals each viz: control (distilled water), chloroquine phosphate (8 mg/kg), artemether-lumefantrine combination (8 mg/kg) and artesunate (2 mg/kg) p.o. Five days post-treatment, colitis was induced by intrarectal administration of 6% acetic acid (1mL/200 g). Colonic mucosal injury was assessed using a macroscopic diarrhoea score scale. Lipid peroxidation in the colon was determined by measuring the levels of Thiobarbituric Acid Reactive Substances using spectrophotometry. Histological findings were derived from paraffin sections of the colonic tissue stained with haematoxylin and eosin and scored according to the degree of neutrophil infiltration and tissue damage. The readings were taken over the period of 12 days post-induction in order to monitor the healing rate. Data were analysed using Student's t test at p= 0.05.

The diarrhoea score in the control group was 2.4 ± 0.1 and 1.1 ± 0.3 for days 3 and 12 post-induction respectively. These scores were significantly increased by chloroquine (3.1 ± 0.3 and 2.0 ± 0.0) and decreased by artesunate (1.6 ± 0.3 and 0.8 ± 0.2) respectively. Artemether-lumefantrine combination did not affect the diarrhoea score significantly. Colonic lipid peroxidation in the artesunate treated rats was significantly lower than the control, chloroquine

and artemether-lumefantrine values throughout the healing period (3.1 ± 0.1 and 2.4 ± 0.1µmol/mL, 4.4 ± 0.1 and 3.61 ± 0.2 µmol/mL, 4.8 ± 0.1 and 4.4 ± 0.3 µmol/mL and 4.7 ± 0.2 and 3.8 ± 0.2 µmol/mL at days 3 and 12 for all the groups respectively), suggesting an interference with colon oxidative system. High rate of colonic injury and inflammatory cell infiltration were observed in the untreated colitis-induced animals (Infiltration score = 4.0 ± 0.2 on day 12). Treatment with chloroquine and artemether-lumefantrine did not significantly change the infiltration score when compared with the control values. However, artesunate reduced inflammatory cell infiltration (Infiltration score of 1.4 ± 0.4) with less severe ulceration at the same reference period.

Chloroquine and artemether-lumefantrine delayed healing of acetic acid-induced colitis, while artesunate accelerated the process. The use of chloroquine and artemeter-lumefantrine for malaria treatment in colitis patients may not be advisable.

Keywords: Ulcerative colitis, Chloroquine, Artesunate, Artemether-lumefantrine

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CHAPTER ONE

1.0 INTRODUCTION

Ulcerative colitis (colitis ulcerosa) is an idiopathic inflammatory bowel disease (IBD) with diffuse, recurrent inflammation of the colon and rectum, which is predominantly characterised by cycles of acute inflammation, ulceration and bleeding of the colonic mucosa. Inflammation is a protective attempt by organisms to remove injurious stimuli and to initiate healing process. It is a short term process, usually appearing within a few minutes or hours and ceasing upon the removal of the injurious stimulus (Cotran, 1998). However, in certain instances, the inflammatory response fails to shut down and becomes chronic leading to a host of diseases.

Inflammatory bowel disease (IBD) is an umbrella term used to describe ulcerative colitis and Crohn's disease.IBD may lead to serious gastrointestinal and extraintestinal complications, involving the hepatobiliary, cardiovascular and neural systems. There is evidence for an intense local immune response associated with recruitment of lymphocytes and macrophages followed by release of soluble cytokines and other inflammatory mediators. Subsequent activation of these cells causes a self-augmenting cycle of cytokine production, cell recruitment and inflammation (Shanahan, 2002).

Ulcerative colitis is an intermittent disease with periods of exacerbated symptoms and periods that are relatively symptom free. Although the symptoms of ulcerative colitis can sometimes diminish on its own, the disease usually requires treatment to go into remission (Hanauer, 1996). Ulcerative colitis affects female more than males and its clinical presentation depends on the extent of the disease process. It is classified by the extent of involvement depending on how far from the rectum up the colon the disease extends. Ulcerative colitis patients can also be characterized by the severity of their disease. There is significantly increased

risk of colorectal cancer in patients with ulcerative colitis (Kornbluth and Sachar, 2004).The typical symptom of ulcerative colitis is constant diarrhoea mixed with blood. Bowel movements are frequent but small in volume as a result of rectal inflammation. The disease is usually accompanied with different degrees of abdominal pain from mild discomfort to severely painful cramps (Hanauer, 1996).

The cause of ulcerative colitis is still unknown though several probably interrelated causes have been suggested (Hanauer, 1996). Many hypotheses have been raised for environmental contributions to the pathogenesis of ulcerative colitis, these include diet. As the colon is exposed to many dietary substances which may encourage inflammation, dietary factors have been hypothesized to play a role in the pathogenesis of both ulcerative colitis and Crohn's disease. There have been few studies to investigate such association. Diet and other lifestyle factors typical for the western countries, such as low intake of fruits and vegetables, sedentary life style, obesity and probably high intake of cooked meat and sugar have been implicated in colon inflammation.

Chloroquine was until recently the most widely used antimalarial drug. It was the original prototype from which most other methods of treatment are derived. It is the least expensive and best tested of all available drugs. Though the emergence of drug resistant parasitic strains is rapidly decreasing its effectiveness, it is still used for the treatment of rheumatoid arthritis, lupus erythematous and various inflammatory conditions (Onigbogi *et al.*,2000). Chloroquine has been found to possess serious adverse effects which include hypotension (Abiose *et al.*,1997), toxicological consequences on retina, antifertility activities including reduction in sperm motility and viability (Adeeko and Dada 1998; Salman and Ajayi, 2007), reduction in Leydig cell population and testosterone concentration in rats (Ebong, 1999). It also interferes with blood

biochemistry (Obi *et al.*,2003) and induces oxidative stress in animals and man (Toler and Steven, 2004). Nonetheless emergence and spread of plasmodium parasite resistance against chloroquine and other major antimalarial drugs has brought the urgency to develop a new generation of safe and effective drugs against malaria (Chattopadhyay *et al.*, 2007).

The problem of the development of malaria resistance has led to the administration of combination therapy which is the simultaneous use of two or more blood schizonticidal drugs with independent modes of action and different biochemical targets in the parasite (White, 2004). This current practice of combination therapy offers several advantages such as reduced risk of treatment failure, reduced risk of developing resistance, enhanced convenience and reduced side effects.

Due to the common antimalarial resistance, most malaria endemic countries are switching antimalarial drug policy to artemisinin - based combination therapies (ACT).Artemisinin is a chinese herb (qinghaosu) that has been used in the treatment of fevers for over one s years (Dondorp *et al.*,2010). Artemisinin based combinations are known to improve cure rates, reduce development of resistance and they might decrease transmission of drug resistant parasites (Yeung *et al.*, 2008). At present, artemisinin is strictly controlled under WHO guidelines as it has proven to be effective against all forms of multi-drug resistant Plasmodium falciparum. It also has very good therapeutic effects on patients infected with Plasmodium viva, also effective in cerebral malaria and very helpful for drug resistant malaria (Bharel *et al.*, 1996; Gulati *et al.*, 1996).

Semi synthetic artemisinin derivatives (eg artesunate, artemether) are easier to use than the parent compound because they are converted rapidly once in the body to the active compound dihydroartemisinin. Artesunate used primarily as treatment for malaria is unique

among anti-malaria drugs in killing the young intra-erythrocytic malaria thereby preventing their development to more pathological mature stages. This results in rapid clinical and parasitological responses to treatment and life-saving benefit in severe malaria (Targett *et al.,* 2001; White 2008). Artesunate is also known to be efficacious at reducing egg production in Schistosoma haematobium infection (Boulangier *et al.,* 2007). Artesunate even though believed to lack adverse effect at usual therapeutic dose may reduce spermatogenesis under prolonged use (White 2008; Jewo *et al.,* 2008). The combination artemether/ lumefantrine is an artemisinin –based combination therapy (ACT) indicated for the treatment of acute uncomplicated plasmodium falciparum malaria. This combination is an effective and well tolerated malaria treatment providing cure rates of up to 97% even in areas of multi drug resistance (Makanga *et al.,* 2006). This drug combination can cause anaphylactic reactions. It frequently causes headache, dizziness, anorexia, sleep disorders, palpitation and unspecific reactions such as gastrointestinal disorders (Bakshi *et al.,* 2000). Other side effects also associated with artemisinin use include nausea, vomiting, abnormal bleeding, itching etc.

Some of the effects associated with antimalarial drugs on the gastrointestinal tract have been documented. Chloroquine has been shown to be a weak stimulant of gastric acid secretion in rats (Etimita *et al.*,2005) and also worsens gastric lesion generated by indomethacin and acidified ethanol (Ajeigbe *et al.*,2008a). Artemisinin on the other hand significantly decrease gastric lesion induced by indomethacin (Ajeigbe *et al.,* 2008b). Unlike the stomach, very few studies are available on the effects of antimalarial drugs on colon inflammation. In one of the studies, chloroquine and hydrochloroquine were shown to be beneficial in the treatment of ulcerative colitis (Goenka *et al.,* 1996). This contradicts the findings of ulcer-promoting activity

of chloroquine on the stomach (Etimita *et al.*, 2005; Ajeigbe *et al.*, 2008a). No mechanism has been proposed for the effect of these drugs on experimental colitis.

AIM AND OBJECTIVES

The present work is aimed at investigating:

(1) the effects of chloroquine, artesunate and Lonart^{R} (antimalarial combination therapy of artemether and lumefantrine) on the formation and healing of experimental colitis.

- To assess colitis using stool constituency
- To determine colonic protein
- To measure rate of lipid peroxidation
- To conduct histopathological studies on tissues obtained from the colon.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 THE COLON: The colon is a muscular tube composed of lymphatic tissues, blood vessels, connective tissues, and specialised muscles. It is the last part of the digestive system in most vertebrates. It is approximately 4.5 feet long (140 cm and 170 cm) and 2.5 inches wide.The colon has no villi (multiple, minute projections of the intestinal mucous layer which serve to absorb fluids and nutrients) as compared to the small intestine and produces no digestive enzymes. The only secretion of importance in the colon is the mucus which acts as a lubricant for the transport of intestinal contents. The main function of the colon is absorption of water, Na^+, and other minerals from the gut contents following nutrient absorption during passage through the small intestine. By removal of about 90% of the fluid, it converts the 1000 -2000 ml of isotonic chyme that enters it each day from the ileum to about 200 – 250 ml of semisolid feces (Ganong, 2005). The diameter of the colon is greater than that of the small intestine. The fibers of its external muscular layer are collected into three longitudinal bands, the teniae coli.Because these bands are shorter than the rest of the colon, the wall of the colon forms outpouchings (haustral) between the teniae.

The colon has the largest reservoir of macrophages in the body. Colonic macrophages do not proliferate but are recruited from the blood monocytes (Smythies *et al.,* 2006). In normal colon, tissue macrophage does not function as antigen presenting cell (APC) but demonstrates high phagocytic and bactericidal activity (Kanai and Watanabe, 2004). Consequently, they exhibit low expression of clutter of differentiation (CD) 14, 80, 86 and respond poorly to chemotatic agents (Smythies *et al.,* 2006).

2.1.1 Anatomy of the Colon

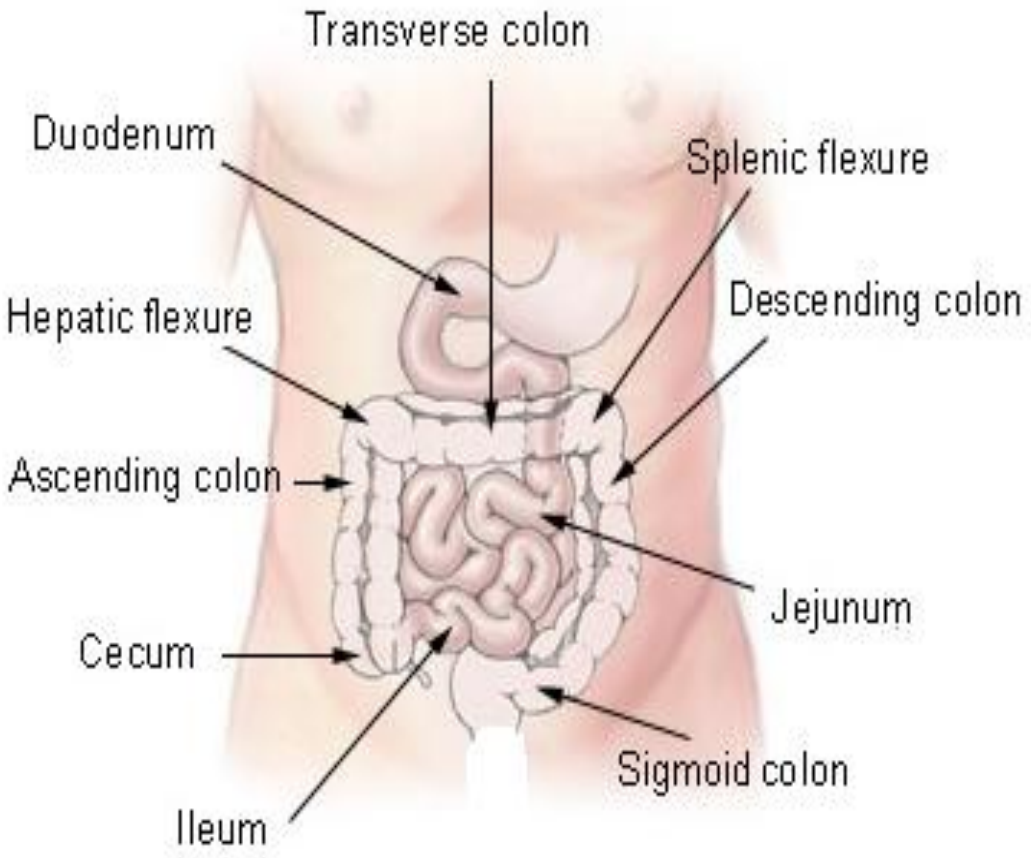


Figure 1: The gastrointestinal tract of man showing structures including the colon.

(Virtual Dictionary Online)

www.web-books.com

In mammals, the colon consists of four sections:

1. The ascending colon,

2. The transverse colon,

3. The descending colon, and

4. The sigmoid colon

The proximal colon usually refers to the ascending colon and transverse colon.

1. The Ascending Colon: - This is on the right side of the abdomen; it's about 25 cm long in humans. It is the part of the colon from the cecum to the hepatic flexure (the turn of the colon by the liver). It is secondarily retroperitoneal in most humans. In ruminant grazing animals, the cecum empties into the spiral colon. Anteriorly it is related to the coils of small intestine, the right edge of the greater omentum, and the anterior abdominal wall. Posteriorly, it is related to the iliacus, the iliolumbar ligament, the quadratus lumborum, the transverse abdominis, the diaphragm at the tip of the last rib; the lateral cutaneous, ilioinguinal, and iliohypogastric nerves; the iliac branches of the iliolumbar vessels, the fourth lumbar artery, and the right kidney. The ascending colon is supplied by parasympathetic fibers of the vagus nerve. Arterial supply of the ascending colon comes from the ileocolic artery and right colic artery, both branches of the Superior Mesenteric Artery (SMA). While the ileocolic artery is almost always present, the right colic artery may be absent in 5–15% of individuals (Kapoor, 2011).

2. The Transverse Colon: - The transverse colon is the part of the colon from the hepatic flexure to the splenic flexure (the turn of the colon by the spleen). The transverse colon hangs off the stomach, attached to it by a wide band of tissue called the greater omentum. On the posterior side, the transverse colon is connected to the posterior abdominal wall by a mesentery known as

the transverse mesocolon. The transverse colon is encased in peritoneum, and is therefore mobile (unlike the parts of the colon immediately before and after it). Cancers form more frequently further along the large intestine as the contents become more solid (water is removed) in order to form feces. The proximal two-third of the transverse colon is perfused by the middle colic artery, a branch of Superior Mesentry Artery, while the latter third is supplied by branches of the Inferior Mesenteric Artery (IMA). The watershed area between these two blood supplies, which represents the embryologic division between the midgut and hindgut, is an area sensitive to ischemia (Kapoor, 2011).

3. The Descending Colon: - The descending colon is the part of the colon from the splenic flexure to the beginning of the sigmoid colon. The function of the descending colon in the digestive system is to store food that will be emptied into the rectum, absorb fluid and also secrete ions. It is retroperitoneal in two-thirds of humans. In the other third, it has a (usually short) mesentery. The arterial supply comes via the left colic artery (Kapoor, 2011)

4. Sigmoid Colon: -The sigmoid colon is the part of the large intestine after the descending colon and before the rectum. The name sigmoid means S-shaped (see sigmoid). The walls of the sigmoid colon are muscular, and contract to increase the pressure inside the colon, causing the stool to move into the rectum. The sigmoid colon is supplied with blood from several branches (usually between 2 and 6) of the sigmoid arteries, a branch of the Inferior Mesenteric Artery (IMA). The IMA terminates as the superior rectal artery. Sigmoidoscopy is a common diagnostic technique used to examine the sigmoid colon**.**

One variation on the normal anatomy of the colon occurs when extra loops form, resulting in a longer than normal organ. This condition, referred to as redundant colon, typically has no direct major health consequences, though rarely volvulus occurs resulting in obstruction

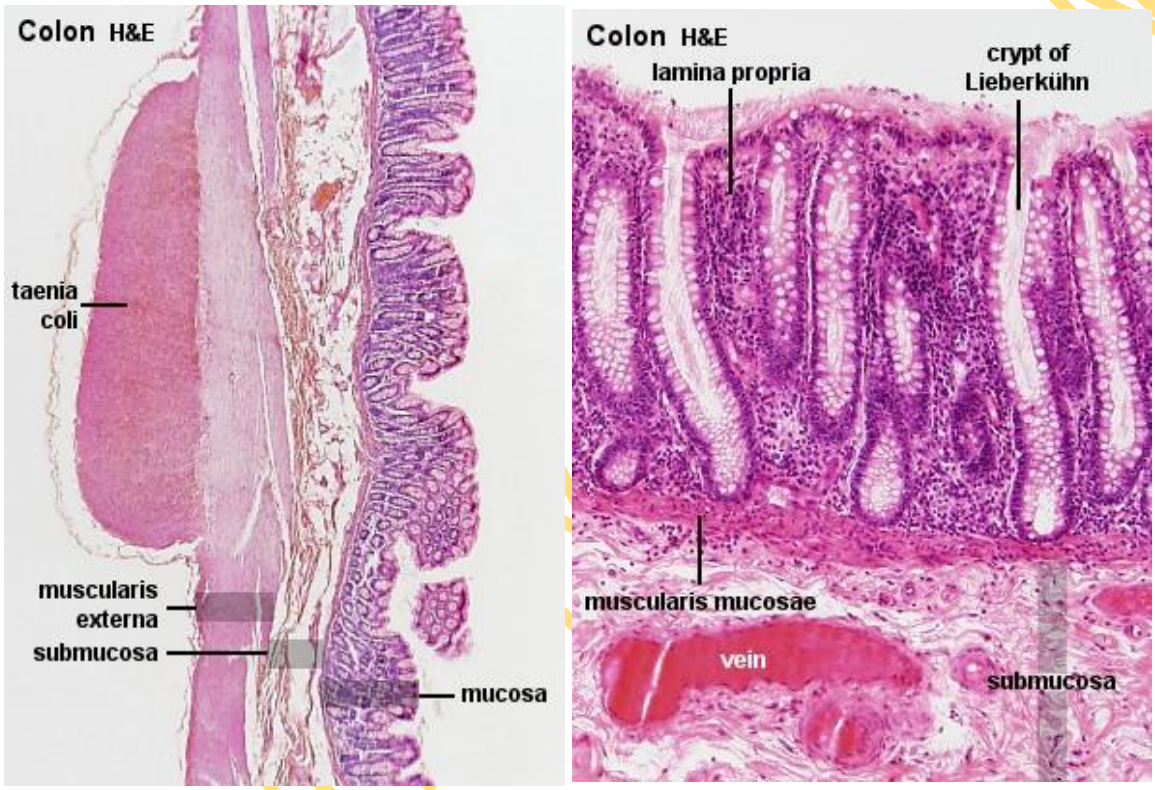
and requiring immediate medical attention. A significant indirect health consequence is that use of a standard adult colonoscope is difficult and in some cases impossible when a redundant colon is present, though specialised variants of the instrument (including the paediatrics variant) are useful in overcoming this problem (Lichtenstein *et al.*, 1998).

2.1.2Histological Features of the Colon

The colon and the rectum share a common mucosal descriptionwith many straight crypts but no villi, and with the epithelium consisting of many goblets cells intersped among absorptive cells. The mucosa of the colon has a simple columnar epithelium shaped into straight tubular crypts.

In cellular composition, the epithelium of the colon resembles that of the small intestine, but with a higher proportion of goblet cells interspersed among the absorptive cells. Although the absorptive cells remain more numerous, the colon epithelium sometimes appears to consist mostly of goblets. The surface of the mucosa is relatively smooth as there are no plicae circulares or intestinal villi. Crypts of Lieberkühn are present and usually longer and straighter than those of the small intestine. Goblet cells account for more of the epithelial cells than in the small intestine. There is only little lamina propria squeezed between the glands. The crypt epithelium also includes stem cells which replenish the epithelium every few days, enteroendocrine cells, and (in the caecum and proximal colon) paneth cells. The crypts are separated by conspicuous lamina propria, whose composition is similar to that of small intestine, loose connective tissue infiltrated by many white blood cells, with capillaries and thin strands of smooth muscle. The muscularis mucosa of the colon forms a thin layer beneath the deep ends of the crypts. The submucosa of the lower tract is relatively unspecialised, however, considerable amounts of fat may be found in the submucosa. The appearance of the muscularis externa is different from that of the small intestine. The inner circular layer of muscle forms the usual sheath around the large

intestine, but the outer longitudinal muscle layer forms three flattened strands, the taenia coli. Only a thin layer of longitudinal muscle surrounds the inner circular muscle layer between the taenia coli. The adventitia forms small pouches (appendices epiploicae) filled with fatty tissue along the large intestine.



Histology of the Colon

Fig 2: Virtual Slidebox of Histology (USA) Gastrointestinal tract, Human colon (Kumar *et al.*, 2007)

2.1.3. Functions of the Colon.

The large intestine is mainly responsible for storing waste, reclaiming water, maintaining the water balance, absorbing some vitamins, such as vitamin K, and providing a location for flora-aided fermentation. By the time the chyme has reached this tube, most nutrients and 90% of the water has been absorbed by the body. At this point some electrolytes like sodium, magnesium, and chloride are left as well as indigestible parts of ingested food (e.g., a large part of ingested amylose, protein which has been shielded from digestion before, and dietary fiber, which is largely indigestible carbohydrate in either soluble or insoluble form). As the chyme moves through the large intestine, most of the remaining water is removed, while the chyme is mixed with mucus and bacteria (known as gut flora), and becomes faeces. The ascending colon receives fecal material as a liquid. The muscles of the colon then move the watery waste material forward and slowly absorb all the excess water. The stools get to become semi solid as they move along into the descending colon. The bacteria break down some of the fiber for their own nourishment and create acetate, propionate, and butyrate as waste products, which in turn are used by the cell lining of the colon for nourishment. No protein is made available (Terry and Meyer, 1996). In humans, perhaps 10% of the undigested carbohydrate thus becomes available; in other animals, including other apes and primates, who have proportionally larger colons, more is made available, thus permitting a higher portion of plant material in the diet. This is an example of a symbiotic relationship and provides about one hundred calories a day to the body. The large intestine produces no digestive enzymes,chemical digestion is completed in the small intestine before the chyme reaches the large intestine. The pH in the colon varies between 5.5 and 7 (McNeil, 1984).

2.1.4. Immunobiology of Colon

The environment of the colon is constantly in a state of controlled inflammation. This control is believed to be maintained by a phenomenon called oral tolerance although this process is completely not understood (Kraus and Mayer, 2005).

The polarised single mucus- covered epithelial cell layer of the gut is the physical barrier against the outside world and its integrity is maintained by tight junction proteins (Rescigno *et al.,* 2005). It is here that the first line of defence against pathogens is mounted (Baumgart and Dignass, 2002). The mucus covering the epithelium contains around 500 species of commensal microbes (Swidsinki *et al.*,2002). These microbes affect the expression of different genes, the absorption of nutrients, xenobiotics metabolism, angiogenesis and post natal intestinal maturation (Hooper *et al.,* 2001). This is also the place where the first recognition and processing of the luminal antigens take place (Hershberg, 2004). This is possible because the epithelial cell expresses pattern recognition receptors (PRRs) i.e toll like receptor (TLR) which through nuclear factor kappa B (NFkB) activation can increase the production of inflammatory cytokines (Rakoff-Nahoun *et al.,* 2002). When no pathogen is present in the gut, TLR interacts with the commensals thereby contributing to the intestinal homeostasis and maintenance of the epithelial barrier (Giradin *et al.,* 2003).

In the sub-epithelial space, many types of immune cells are gathered i.e T cell, B cells, granulocytes, mast cells, Natural killer (NK) cells, macrophages and dendric cells (DC). In the healthy gut, immature DC opens tight junction between the epithelial cells and extend dendrites through this opening out into the lumen to sample antigens from microbes (Niess *et al.,* 2005). This can induce unresponsiveness probably by stimulating naive T cell differentiation into the regulatory cluster of differentiation 4 (CD4) + T cells such as Th3cells rather than Th 1 or Th2

cells. The DCs are the key cells in control against pathogens and tolerance towards commensal since they express the entire spectrum of TLR and nucleotide oligomerisation domain (NODs) (Iwasaki and Medzhitov, 2004). When DC senses danger, they mature, acquire an activated phenotype and induce immunity. This process can involve remodelling of the cell cytoskeleton and activation of TLR (West *et al.*, 2004).

2.1.5 The Gut Flora

Gut flora consists of microorganisms that live in the digestive tracts of animals and this is the largest reservoir of human flora. The human body, consisting of about 100 trillioncells, carries about ten times as many microorganisms in the intestines (Björkstén *et al.*, 2001; Guarner and Malagelada, 2003 ; Steinhoff, 2005 ; Sears, 2005). The metabolic activities performed by these bacteria resemble those of an organ, leading some to liken gut bacteria to a forgotten organ (O'Hara and Shanahan, 2006). It is estimated that these gut flora have around 100 times as many genes in aggregate as there are in the human genome (Junjie, 2009).

Bacteria make up most of the flora in the colon and up to sixty percent 60% of the dry mass of feces (Guarner and Malagelada, 2003). Somewhere between 300 and 1000 different species live in the gut, with most estimates at about 500 (Gibson, 2004; Steinhoff, 2005; O'Hara and Shanahan, 2006). However, it is probable that 99% of the bacteria come from about 30 or 40 species (Beaugerie and Petit, 2004). Fungi and protozoa also make up a part of the gut flora, but little is known about their activities.

Research suggests that the relationship between gut flora and humans is not merely commensal (a non-harmful coexistence), but rather a symbiotic relationship (Sears, 2005). Though people can survive without gut flora (Steinhoff, 2005), the microorganisms perform a host of useful functions, such as fermenting unused energy substrates, training the immune

system, preventing growth of harmful pathogenic bacteria,regulating the development of the gut, producing vitamins for the host such as biotin and vitamin K and producing hormones to direct the host to store fats (Guarner and Malagelada, 2003). However, in certain conditions, some species are thought to be capable of causing disease by producing infection or increasing cancer risk for the host. Over 99% of the bacteria in the gut are anaerobes (Vedantam and Hecht, 2003), but in the cecum, aerobic bacteria reach high densities (Guarner and Malagelada, 2003). Not all the species in the gut have been identified because most cannot be cultured and identification is difficult (Shanahan, 2002). Populations of species vary widely among different individuals but stay fairly constant within an individual over time, even though some alterations may occur with changes in lifestyle, diet and age (Guarner and Malagelada, 2003; O'Hara and Shanahan,2006). An effort to describe the microflora of the gut and other body locations better has been initiated by Tap *et al* (2009), scientists highlighted the existence of a small number of species shared by all individuals constituting the human intestinal microbiota phylogenetic core (Tap *et al.*,2009).

Most bacteria belong to the genera *Bacteroides*, *Clostridium*, *Fusobacterium*, *Eubacterium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, and *Bifidobacterium* (Vedantam and Hecht, 2003; Beaugerie and Petit, 2004). Other genera, such as *Escherichia* and *Lactobacillus*, are present to a lesser extent (Guarner and Malagelada, 2003). Species from the genus *Bacteroides* alone constitute about 30% of all bacteria in the gut, suggesting that this genus is especially important in the functioning of the host (Sears, 2005).

2.1.6. Some Common Pathologies of the Colon

1. **Angiodysplasia**: -Angiodysplasia is a small vascular malformation of the gut. It is a common cause of otherwise unexplained gastrointestinal bleeding and anemia. Lesions are often multiple,

and frequently involve the cecum or ascending colon, although they can occur at other places. (Warkentin *et al.,* 2003)

2. **Appendicitis**: - This is a condition characterized by inflammation of the appendix. It is classified as a medical emergency and many cases require removal of the inflamed appendix, either by laparotomy or laparoscopy. Untreated, mortality is high, mainly because of the risk of rupture leading to peritonitis and shock (Hobler, 1998).

3. **Constipation**: - Constipation refers to bowel movements that are infrequent and/or hard to pass (Chatoor and Emmanuel, 2009) Constipation is a common cause of painful defecation. Severe constipation includes obstipation (failure to pass stools or gas) and fecal impaction. Constipation is a symptom with many causes.

4. **Diarrhoea: -** This is the condition of having three or more loose or liquid bowel movements per day. The most common causesof diarrhoea are cholera toxin that stimulates the secretion of anions, especially chloride ions. Therefore, to maintain a charge balance in the lumen, sodium is carried with it, along with water. In this type of diarrhoea intestinal fluid secretion is isotonic with plasma even during fasting. It continues even when there is no oral food intake. Other causes of diarrhoea are bacterial infections, viral infections, parasitic infections, or autoimmune problems such as inflammatory bowel diseases.

5**. Colorectal cancer** :- Colorectal cancer, less formally known as bowel cancer, is a cancer characterised by neoplasia in the colon, rectum, or vermiform appendix. Colorectal cancers start in the lining of the bowel. If left untreated, it can grow into the muscle layers underneath then through the bowel wall. Most begin as a small growth on the bowel wall: a colorectal polyp or adenoma. These mushroom-shaped growths are usually benign, but some develop into cancer over time. Localised bowel cancer is usually diagnosed through colonoscopy. The symptoms of

colorectal cancer depend on the location of tumor in the bowel, and whether it has spread elsewhere in the body (metastasis). While no symptom is diagnostic of colorectal cancer, rectal bleeding or anemia are high risk features (Astin *et al.,* 2011).

6. **Hirschsprung's disease (aganglionosis)** :- Hirschsprung's disease (HD), or congenital aganglionic megacolon, involves an aganglionic section of bowel (the normal enteric nerves are absent) that starts at the anus and progresses proximally. The length of bowel that is affected varies but seldom stretches for more than about 30 cm. It arises when certain nerve cells in the gut (called ganglion cells) fail to develop and mature correctly. The result is a section of bowel that is essentially paralysed. Hirschsprung's disease is a congenital disorder of the colon in which certain nerve cells, known as ganglion cells, are absent, causing chronic constipation (Worman and Ganiats,1995). The lack of ganglion cells is in the myenteric plexus, which is responsible for moving food in the intestine.

7. **Ileus**: - Ileus is decreased motor activity of the gastrointestinal tract due to non-mechanical causes (Townsend *et al.,* 2004). In such sense, this does not include motility disorders that result from structural abnormalities, and, therefore, some mechanical obstructions are misnomers, such as gallstone ileus and meconium ileus, and are not true examples of ileus (Feldman *et al.,* 2006).

8. **Intussusception:** - An intussusception is a medical condition in which a part of the intestine has invaginated into another section of intestine, similar to the way in which the parts of a collapsible telescope slide into one another. This can often result in an obstruction. The part that prolapses into the other is called the *intussusceptum*, and the part that receives it is called the *intussuscipiens* (Andromanakos *et al.,* 2006).

9. **Irritable bowel syndrome**: - Irritable bowel syndrome (IBS or spastic colon) is a diagnosis of exclusion. It is a functional bowel disorder characterised by chronic abdominal pain, discomfort,

bloating, and alteration of bowel habits in the absence of any detectable organic cause. In some cases, the symptoms are relieved by bowel movements, Diarrhoea or constipation may predominate, or they may alternate. IBS may begin after an infection (post-infectious, IBS-PI), a stressful life event, or onset of maturity without any other medical indicators. The most common theory is that IBS is a disorder of the interaction between the brain and the gastrointestinal tract, although there may also be abnormalities in the gut flora or the immune system. IBS does not lead to more serious conditions in most patients (Minderhoud *et al.*, 2004).

10. **Inflammatory bowel disease (IBD)**: - refers to an inflammation of the colon and is often used to describe an inflammation of the large intestine (colon, caecum and rectum). Colitides may be acute and self-limited or chronic, i.e. persistent, and broadly fit into the category of digestive diseases. This disease is characterized by superficial infiltration of the bowel wall by inflammatory white blood cells, resulting in multiple mucosal ulceration and crypt abscesses. The lesions are contiguous, typically extending retrograde from the rectum, involving the descending, transverse, or the entire colon. Ulcerative colitis (UC) and Crohn's disease are major forms of inflammatory bowel disease (IBD) which affect millions of people worldwide and are characterized by chronic uncontrolled inflammation of intestinal mucosa. These two forms of IBD comprise a widespread health hazard in modern society (Baumgart and Sandborn, 2007; Xavier and Podolsky, 2007).

2.2. COLITIS

Colitis is a group of conditions which are inflammatory and auto immune, affecting the tissue that lines the gastrointestinal system mainly the large intestine. It is a digestive disease characterised by inflammation of the colon. It causes inflammation and sores called ulcers in the top layers of the lining of the large intestine. The inflammation makes the colon empty

frequently, causing diarrhoea. Ulcers form in places where the inflammation has killed colon lining cells. The ulcers bleed and produce pus and mucus (Shanahan, 2002).

2.2.1 Types of Colitis

Crohn's disease

Crohn's disease (also known as regional enteritis) is a chronic, episodic, inflammatory condition of the gastrointestinal tract characterised by transmural inflammation (affecting the entire wall of the involved bowel) and skip lesions (areas of inflammation with areas of normal lining in between). Crohn's disease is a type of inflammatory bowel disease (IBD) and can affect any part of the gastrointestinal tract from mouth to anus; as a result, the symptoms of Crohn's disease vary between affected individuals. The main gastrointestinal symptoms are abdominal pain, diarrhea (which may be bloody), and weight loss. Crohn's disease can also cause complications outside of the gastrointestinal tract such as skin rashes, arthritis, and inflammation of the eye (Hanauer1996).

Diversion colitis

Diversion colitis is an inflammation of the colon which can occur as a complication of ileostomy or colostomy, often occurring within the year following the surgery. It can also occur in a neovagina created by colovaginoplasty, sometimes several years after the original procedure. In many milder cases after ileostomy or colostomy, diversion colitis is left untreated and disappears naturally. If treatment is required, possible treatments include short-chain fatty acid irrigation, steroid enemas, and mesalazine (Geraghty and Talbot, 1991).

Ischemic colitis

Ischemic colitis is a medical condition in which inflammation and injury of the large intestine results from inadequate blood supply. Although uncommon in the general population, ischemic

colitis occurs with greater frequency in the elderly, and is the most common form of bowel ischemia (Higgins *et al.,* 2004).

Chemical colitis

Chemical colitis is a type of colitis caused by the introduction of harsh chemicals to the colon by an enema or other procedure. Chemical colitis can resemble ulcerative colitis, infectious colitis, and pseudomembranous colitis endoscopically. Prior to 1950, hydrogen peroxide enemas were commonly used for certain conditions. This practice will often result in chemical colitis. Soap enemas may also cause chemical colitis. Harsh chemicals, such as compounds used to clean colonoscopes, are sometimes accidentally introduced into the colon during colonoscopy or other procedures. This can also lead to chemical colitis (Harish *et al*., 2006).

2.2.2. Ulcerative colitis

Ulcerative colitis (Colitis ulcerosa, UC) is a type of colitis that includes characteristic ulcers, or open sores, in the colon.Ulcerative colitis (UC) is a condition that affects primarily the superficial layer of the colon mucosa, and histological analyses have shown ulceration of the mucosa, blunting and loss of crypts and inflammatory infiltrates (Strober *et al.*,2002). The immune pathogenesis of inflammatory bowel disease namely ulcerative colitis and Crohn's disease are associated with intestinal and extra-intestinal clinical manifestations of disease, including weight loss, diarrhoea accompanied by blood and/or mucus, fever, gastric dysmotility and shortening of the colon (Fiocchi, 2004; Hendrickson*et al.*,2002). The gastrointestinal epithelium forms a barrier that separates the finely regulated homeostasis of the body interstitium from the harsh environment of the intestinal lumen.

In colitis, the intestinal mucosal barrier is disrupted by inflammation and ulceration. In these circumstances, translocation of enteric bacteria and their products including reactive

oxygen species (ROS) such as nitric oxide (NO) through the bowel wall to extra-intestinal sterile sites may occur. The intestinal mucosal barrier plays a pivotal role in preventing microorganisms and bacterial toxins from entering an organism's bloodstream. However, the barrier becomes impaired during colitis. As a result, a large quantity of endotoxin can enter into systemic circulation through the impaired intestinal mucosa. In addition, oxygen free radicals (OFRs) and proinflammatory cytokines are induced, facilitating impairment of intestinal mucosal permeability (Simmonds *et al.*, 1992; Babbs, 1992). Oxidants alter cytoskeletal components (such as actin), resulting in disruption of the structural integrity of epithelial cells. In addition, proinflammatory cytokines, including tumor necrosis factor (TNF)-α and interferon (IFN)-γ, disrupt tight junctions, decrease the transendothelial electrical resistance (TEER), and regulate the expression of intestinal mucosal barrier-associated proteins. Therefore, restoring the impaired intestinal mucosa is beneficial for controlling and reducing the inflammation and immunologic reaction occurring in the intestinal mucosa of patients with IBD.

2.2.3. Aetiology of Colitis

Although ulcerative colitis has been known as a clinical entity since 1859, the aetiological mystery has not yet been completely revealed. However, the incorporation of new molecular biology techniques has yielded considerable progress in the understanding of the aetiology of ulcerative colitis. Among the pathological findings associated with ulcerative colitis are;

- An increase in certain inflammatory mediators such as prostaglandin, cytokines etc.
- Signs of oxidative stress.
- Abnormal glycosaminoglycan (GAG) content of the mucosa,

- A deranged colonic milieu: This is caused by change in bacterial profile, decreased oxidation of short chain fatty acids (SCFAs), increased sulfide production, and decreased methylation.
- Increased intestinal permeability.

While no one factor has been identified as the initial trigger for ulcerative colitis, pieces of the puzzle have been elucidated; fitting them together to create a complete picture remains to be accomplished (Sartor, 1995).

2.2.3.1 ENVIRONMENTAL FACTORS

Dietary Involvement: Several studies have examined dietary risk factors for the development of ulcerative colitis. An Italian study of 104 patients with ulcerative colitis and Crohn's disease found, using a dietary recall questionnaire, that total carbohydrate, refined sugar and starch intakes immediately prior to onset of the disease were significantly higher in both ulcerative colitis and Crohn's disease patients than in healthy controls. Total protein intake was significantly higher in ulcerative colitis but not Crohn's patients. A case-control Netherlands study of 43 recently-diagnosed (within the previous six months) ulcerative colitis patients and 43 age and gender matched controls examined dietary intakes for five years prior to the study using a cross-check dietary history method. Fat intake was determined by adipose tissue fatty acid composition. In this study, high intakes of vitamin B6 and mono- and polyunsaturated fats were associated with increased risk. No significant differences in composition of adipose tissue were noted. The connection between vitamin B6 and increased ulcerative colitis risk is baffling and may be an anomaly (Geerling *et al.*, 2000).

Non Steroidal Anti-inflammatory Drugs (NSAIDs) as a Causative Factor: Epidemiological data indicate that non-steroidal anti-inflammatory drugs can trigger exacerbations of ulcerative

colitis and even, occasionally, induce *de novo* disease. Possible mechanisms include decreased production of protective mucosal prostanoid and increased leukocyte adherence and migration. Although these effects were initially thought to be due to inhibition of cyclo-oxygenase-1 (COX-1), selective COX-2 inhibitors seem as potent as indomethacin in this context. Based on previous animal studies demonstrating ibuprofen inhibited short chain fatty acid (SCFA) oxidation in isolated mitochondria of mouse liver.

Roediger *et al*(1993) studied ibuprofen's effect on colonocytes from rats and humans and found that, at concentrations of 2.0-7.5 mmol/L, ibuprofen selectively inhibited oxidation of butyrate. This concentration may not occur at doses typically consumed. Other NSAIDs have been implicated in acute episodes and relapses of proctocolitis. Four cases were reported, involving flufenamic acid, mefenamic acid, naproxen, and ibuprofen.

2.2.3.2. THE ROLE OF INTESTINE'S BACTERIAL CONTENTS ON ULCERATIVE COLITIS

The human body consisting of about 100 trillion cells carries about ten times as many microorganisms in the intestine. They are usually referred to as gut flora (Sears 2005;Steinhoff 2005). Bacteria make up most of the flora in the colon and about 60% of dry mass of faeces. It has been estimated that between 300 and 1000 species of bacteria live in the gut. sThe metabolic activities performed by these bacteria resemble those of an organ, leading some to liken gut bacteria to a "forgotten" organ. It is estimated that these gut flora have around 100 times as many genes in aggregate as there are in the human genome (Junjie, 2009).

(a) Functions of Intestinal Bacterial

The intestinal microorganisms perform a host of useful functions. These include:

i. Carbohydrate fermentation and absorption

The body is unable to digest all carbohydrates; this is because the body cells lack the necessary enzymes for the digestion of these carbohydrates. Examples include certain starches, fiber, oligosaccharides and sugars. However, bacteria present in the gut contain enzymes that could help digest these carbohydrates so that the body can utilize them. Bacteria turn carbohydrates they ferment into short chain fatty acids (SCFAs). These materials can be used by host cells, providing a major source of useful energy and nutrients for humans They increase the gut's absorption of water, reduce counts of damaging bacteria, increase growth of human gut cells, and are also used for the growth of indigenous bacteria. SCFAs can be produce by two different type of fermentation; saccharolytic fermentation which also include the production of acetic acid, propionic acid, butyric acid, lactic acid and certain gases which are useful to the body. The second is proteolytic fermentation which is less favourable and breaks down proteins like enzymes, dead host and bacterial cells, and collagen and elastin found in food, and can produce toxins and carcinogens in addition to SCFAs (Beaugerie and Petit, 2004; Gibson, 2004).

Evidence also suggests that bacteria enhance the absorption and storage of lipids. Bacteria also produce and help the body absorb needed vitamins like vitamin K. In addition, the SCFAs they produce help the body absorb nutrients such as calcium, magnesium, and iron (Guarner *et al.*,2003; Sears, 2005). Short chain fatty acids (SCFAs) also help to increase growth of intestinal epithelial cells and control their proliferation and differentiation. Bacterial cells also alter intestinal growth by changing the expression of cell surface proteins such as sodium/glucose transporters. In addition, changes they make to cells may prevent injury to the gut mucosa from occurring (Keeley, 2004).

ii. Repression of pathogenic microbial growth:

Gut flora prevent species that would harm the host from colonising the gut; an activity termed the "barrier effect". Yeasts and harmful bacteria species such as *Clostridium difficile* (the overgrowth of which can cause pseudomembranous colitis) are unable to grow excessively due to competition from helpful gut flora species adhering to the mucosal lining of the intestine, thus animals without gut flora are infected very easily. The barrier effect protects humans from both invading species and species normally present in the gut at low numbers, whose growth is usually inhibited by the gut flora. Helpful bacteria prevent the growth of pathogenic species by competing for nutrition and attachment sites to the epithelium of the colon. Indigenous gut floras also produce bacteriocins which are proteinacious toxins that inhibit growth of similar bacterial strains, substances which kill harmful microbes and the levels of which can be regulated by enzymes produced by the host. Also importantly is that acids produce during fermentation helps to lower the pH, thus preventing the proliferation of harmful species of bacteria and facilitate that of helpful species (Guarner and Malagelada, 2003).

iii. Immunity

Bacteria play key role in promoting the early development of the gut's mucosal immune system both in terms of its physical components and function and continue to play a role later in life in its operation. The bacteria stimulate the lymphoid tissue associated with the gut mucosa to produce antibodies to pathogens. The immune system recognises and fights harmful bacteria, but leaves the helpful species alone, a tolerance developed in infancy (Shanahan, 2002).

As soon as an infant is born, bacteria begin colonising its digestive tract. The first bacteria to settle in are able to affect the immune response, making it more favorable to their own survival and less so to competing species; thus the first bacteria to colonize the gut are important in

determining the person's lifelong gut flora makeup. However, there is a shift at the time of weaning from predominantly facultative aerobic species such as *Streptococci* and *Escherichia coli* to mostly obligate anaerobic species (Guarner and Malagelada, 2003; Steinhoff, 2005).

Recent findings have shown that gut bacteria play a role in the expression of Toll-like receptors (TLRs) in the intestines, molecules that help the host repair damage due to injury. TLRs cause parts of the immune system to repair injury caused for example by radiation. TLRs are one of the two classes of pattern recognition receptors (PRR) that provide the intestine the ability to discriminate between the pathogenic and commensal bacteria. These PRRs identify the pathogens that have crossed the mucosal barriers and trigger a set of responses that take action against the pathogen which involve 3 main immunosensory cells; surface enterocytes, M cells and dendritic cells. The other class of pattern recognition receptors is known as the nucleotide-binding oligomerization domain/caspase recruitment domain isoforms (NOD/CARD) which are cytoplasmic proteins that recognize endogenous or microbial molecules or stress responses and forms oligomers that activate inflammatory caspases. This would result in the cleavage and activation of important inflammatory cytokines and/or activate NF-κB signaling pathway to induce the production of inflammatory molecules (O'Hara and Shanahan, 2006).

Microorganisms cover the surface of the large bowel mucosa and bacterial cell densities in adjacent luminal contents are around 1012 per gram. Most people tolerate this complex metabolically active and antigenic microflora, but in approximately two per 1000 of adults living in industrialised Western countries develop an intense inflammation in the mucosa, associated with bloody diarrhoea, urgency to defaecate and general malaise, that is not associated with known pathogens (Sears, 2005).

(b) Factors Modifying the Intestinal Bacterial Profile

- Western type of diet
- Use of antibiotics and chemotherapeutics
- Modern infant nutrition
- Public health measures
- High hygienic standards and sanitation

The gut bacteria have an essential role in the development of the gut immune system, as they stimulate the lymphocytes to clonal expansion and also prevent lymphocyte apoptosis. Selective bacterial stimulation may occur, with Gram-positive bacteria preferentially stimulating interleukin (IL)-12 productions whereas Gram-negative organisms induce IL4 production. Gram-negative bacteria and lipopolysaccharide are responsible for inducing oral tolerance (Sartor, 2004).Although standard cultivation techniques are capable of detecting up to 30% of total microflora, new techniques (including analysis of bacterial 16S ribosomal RNA, polymerase chain reaction, in situ hybridisation, flow cytometry and DNA microarray or chip analysis) have markedly increased the detection rate (Hessle *et al.,* 2000). The beneficial bacterial strains, such as bifidobacteria and lactobacilli, are generally absent from mucosa-associated bacterial flora in patients with active ulcerative colitis. On the other hand, an increased mucosal concentration of Gram-negative anaerobes, particularly *Escherichia coli*, *Fusobacterium varium* and bacteroides, along with a high frequency of *Peptostreptococcus* invasion, has been shown. Various authors have also shown severe bacterial invasions of the mucosa in most colonicspecimens from patients with ulcerative colitis, contrary to that in healthy controls (Ohkusa *et al.,* 2002).The high

bacterial mucosal invasion in patients with inflammatory bowel disease corresponds well with titres of immunoglobulin G to bacterial antigens. Some of these are used for distinguishing between ulcerative colitis (eg, anti-*Peptostreptococcus anaerobius* antibody) and Crohn's disease (eg, anti I2-from *Pseudomonas fluorescens* antibody or antibody to an outer membrane porin of *E coli*anti-OmpC). Nevertheless, these differences in bacterial mucosal concentrations between ulcerative colitis and Crohn's disease were not found by several investigators.

2.2.3.3. Signs and Symptoms of Colitis

Signs and symptoms of colitis include pain, tenderness in the abdomen, fever, swelling of the colon tissue, bleeding, erythema (redness) of the surface of the colon, rectal bleeding, and ulcerations of the colon. Common tests which reveal these signs include X-rays of the colon, testing the stool for blood and pus, sigmoidoscopy, and colonoscopy. Additional tests include stool cultures and blood tests, including blood chemistry tests. A high erythrocyte sedimentation rate (ESR) is one typical finding in acute exacerbations of colitis.

2.2.4. Inflammation

Inflammation is a pattern of response to injury, in which cells and exudates accumulate in irritated tissues and tend to protect them from further damage. Even though inflammation is considered as a harmful process that should be avoided, it is also a homeostatic response aimed at limiting entry of foreign materials to the body and of facilitating repair (Collins, 1999).

Inflammation can be classified as acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increase movement of plasma and leucocytes from the blood into the injured tissues. A cascade of biochemical event propagates and matures the inflammatory response, involving the local vascular system, the immune system and various

cells within injured tissues. The process of acute inflammation is initiated by the cells already present in all tissues, mainly resident macrophages, dendritic cells, histocytes, Kupffer cells and mastocytes. At the onset of an infection such as burns or other injury, these cells undergo activation and release inflammatory mediators responsible for the clinical signs of the inflammation. The mediator's molecules are also altered in the blood vessels to permit the migration of leucocytes, mainly neutrophils, outside the blood vessels (extravation) into the tissues. The neutrophil migrate along a chemotactic gradient created by the local cells to reach the site of the injury (Cotran and Collin, 1998).

Chronic inflammation is most appropriately defined in terms of the process, in which continuing inflammation and attempted tissue healing by repair occur simultaneously. It is characterised by inflammation and repair occurring concurrently, rather than consecutively (Jackson *et al.*, 1997).

Although it is often defined simply in terms of time course, with lesions of over 6 weeks' duration traditionally being regarded as chronic, any such definition is entirely arbitrary. At a microscopic level, chronic inflammation is sometimes defined in terms of the pattern of cellular response, although this is variable and not altogether reliable. The systemic effects of inflammation are more pronounced in chronic inflammatory diseases and may contribute significantly to the clinical consequences. These systemic effects are largely mediated by cytokines. Whereas the most prominent systemic effects of acute inflammation are fever and leucocytosis, chronic inflammation is usually associated with fatigue, sleepiness, weight loss and wasting (Cotran and Collin, 1998).

2.2.4.1 Inflammation and its role in disease

Inflammation is a protective attempt by an organism to remove injurious stimuli and to initiate healing process. Tissue injury can be caused by bacteria, trauma, chemicals, heat etc. When this occurs, multiple substances are released by the injured tissue which causes a dramatic change in the nature of the tissue leading to inflammation. Without inflammation wounds and infections would never heal and continuous destruction of tissue is a threat to the survival of the organism. However, on certain circumstances, the inflammatory response fails to shut down and becomes chronic, which could lead to a host of diseases. It is important to note that inflammation is not a synonym for infection. Even in cases when it is caused by infection, it is incorrect to use the terms as synonyms. Infection is caused by an outside agent, while inflammation is the body's response. Inflammatory response is closely intertwined with the process of repair. Repair begins during early phases of inflammation but reaches completion usually after the injurious influence has been neutralized. During repair, the injured tissue is replaced through regeneration of native parenchymal cell, by filling of the defect with fibrous tissue (scarring) or, most commonly, by a combination of these two processes. The inflammatory response consists of two main components, a vascular reaction and a cellular reaction (Cotran and Collins, 1998).

Vascular changes

Acute inflammation is characterised by marked vascular changes, including vasodilation, increased permeability, and the slowing of blood flow, which are induced by the actions of various inflammatory mediators. Vasodilation occurs first at the arteriole level, progressing to the capillary level, and brings about a net increase in the amount of blood present, causing the redness and heat of inflammation. Increased permeability of the vessels results in the movement of plasma into the tissues, with resultant stasis due to the increase in the concentration of the

cells within blood - a condition characterised by enlarged vessels packed with cells. Stasis allows leukocytes to marginate (move) along the endothelium, a process critical to their recruitment into the tissues (Williams, 2012).

Cellular response

Many tissues and cells are involved, including circulating cells, cellular and extracellular constituents of connective tissue. The circulating cells include neutrophils, monocytes, eosinophils, lymphocytes, basophils, and platelets. The connective tissue cells are the mast cells, which intimately surround blood vessels; the connective tissue fibroblasts; resident macrophages; and lymphocytes. The extracellular matrix consist of the structural fibrous proteins (collagen, elastin), adhesive glycoproteins (fibronectin, laminin, nonibrillar collagen, tenascin, and others), and proteoglycans (Esposito *et al*., 2003).

2.2.4.2 General Features of Inflammation

Inflammation is characterized by

- Vasodilation of the local blood vessels with consequent excess local blood flow which is the cause of heat and redness. Vasodilation is induced by the action of several mediators, notably histamine and nitric oxide, on vascular smooth muscle.
- Increased permeability of the microvasculature, with the out pouring of protein rich fluid into the extravascular tissue.
- Clotting of the fluid in the interstitial spaces because of excessive amounts of fibrinogen and other proteins leaking from the capillaries.
- Migration of large members of granulocytes and monocytes into the tissue.
- Swelling of the tissue cells.
- Phagocytosis.

2.2.5. Inflammatory mediators

In acute inflammation, the ordered process of cellular accumulation and activation is dependent upon the sequential release of chemical mediators of inflammation. Some of these are preformed and stored in the granules of platelets and mast cells while some, such as complement components, are generated by activation of plasma enzyme cascades but the majority is newly synthesized by cells of the tissue or by previously recruited inflammatory cells. Prominent among the latter group are relatively small protein molecules, collectively referred to as cytokines, which act as potent biological signals for cellular migration and activation (Wakefield and Lloyd, 1992).

In chronic inflammation, cytokines play critical roles in macrophage and T-cell recruitment, activation and local replication in the survival of inflammatory cells by inhibition of apoptosis of an immune response and in the induction of granulation tissue and fibrosis (Jackson *et al.,* 1997). Cytokines exert their effects by binding to cell membrane receptors on the same cell (autocrine action), adjacent cells (paracrine action) or remote cells (acting as a hormone). These mediators are characterised by redundancy (with different cytokines having overlapping effects) and pleiotropism (in which one particular cytokine has multiple effects). The families of cytokines include molecules referred to as chemokines, interleukins, interferons, colony-stimulating factors and growth factors, many of which are important in chronic inflammation. Chemokines regulate leucocyte migration by modifying the expression and affinity of adhesion molecules on the leucocyte surface (Wakefield and Llyod, 1992). During inflammation, circulating cells attach to the vascular endothelium and migrate between endothelial cells. When stimulated by cytokines, endothelial cells regulate the recruitment of leucocytes via sets of surface adhesion molecules that tether the two cells together. Activation of the endothelial cells

induces a variety of cytokines, as well as adhesion molecules. The ability of chemokines to attract and activate specific leucocytes subsets at sites of inflammation appears to be an important determinant of the nature of the inflammatory cellular infiltrate, as well as of the subsequent evolution of the inflammatory response. Acute inflammation is initially characterised by recruitment of neutrophils, in part mediated by the activity of the so-called chemokines such as interleukin 8. This is followed by T-cell and monocyte accumulation, believed to be mediated by other chemokines. Additional chemokines exhibit relative specificity for eosinophils or basophils, which are frequently associated with allergic disorders (Segal, 2005).

Proinflammatory cytokines such as interleukin 1, tumour necrosis factor α and interleukin 6, which are primarily produced by macrophages, play multiple roles in inflammation. These include:

(1) Activation of vascular endothelium, resulting in enhanced expression of leucocytes adhesion molecules;

(2) Induction of chemokine synthesis;

(3) Activation of the effectors cells of inflammation, notably neutrophils and macrophages;

(4) Induction of fever;

(5) Synthesis by the liver of 'acute-phase proteins' such as fibrinogen, amyloid A and C-reactive protein; and

(6) Subsequent induction of other systemic manifestations of chronic inflammation such as fever, night sweats, tiredness, anorexia and weight loss.

2.2.6. Oxidative Stress

Increasing attention has been focused recently on the role of free radicals, in both normal metabolism and defense against disease (Grisham and Granger,1988). Oxygen derived active

species are readily available in the gastrointestinal tract. Their major potential sources include stimulated leucocytes, xanthine oxidase, colonic bacteria, and epithelial lipooxygenase activity. Xanthine oxidase, which catalyses reduction of oxygen, yielding O_2 and H_2O_2, is activated by proteases released either from inflammatory cells or from dying epithelial cells. The potential pathogenicity of free radicals has been emphasized by recent work which suggests that reactive oxygen metabolites are not just one of a number of mediators and cytokines involved in the inflammatory process in inflammatory bowel disease but many have a pivotal role by initiating the expression of genes controlling many other aspects of the inflammatory, immune, and acute phase response, by activation of the transcription factor NF-kβ (Schreck *et al.*, 1991).

A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons, an unpaired electron being defined as one that is alone in an orbital (Babbs, 1990). Since electrons are more stable when paired together in orbitals, radicals are generally more reactive than non-radicals. Radicals may react with non-radicals in a number of ways: by donating the unpaired electron, or by abstracting an electron from, or combining with, another molecule. All of these reactions, however, result in the production of another radical and will usually result in a chain reaction (Valko *et al.*, 2007). These reactions may be terminated by the interaction with another radical or with one of the 'chain-breaking antioxidant molecules' (such as vitamin E), or by one of the enzymatic antioxidant defenses (for example superoxide dismutase, catalase, or glutathione peroxidase). An outline of reactive oxygen metabolite (ROM) chemistry in biological tissues is given in the figure below.

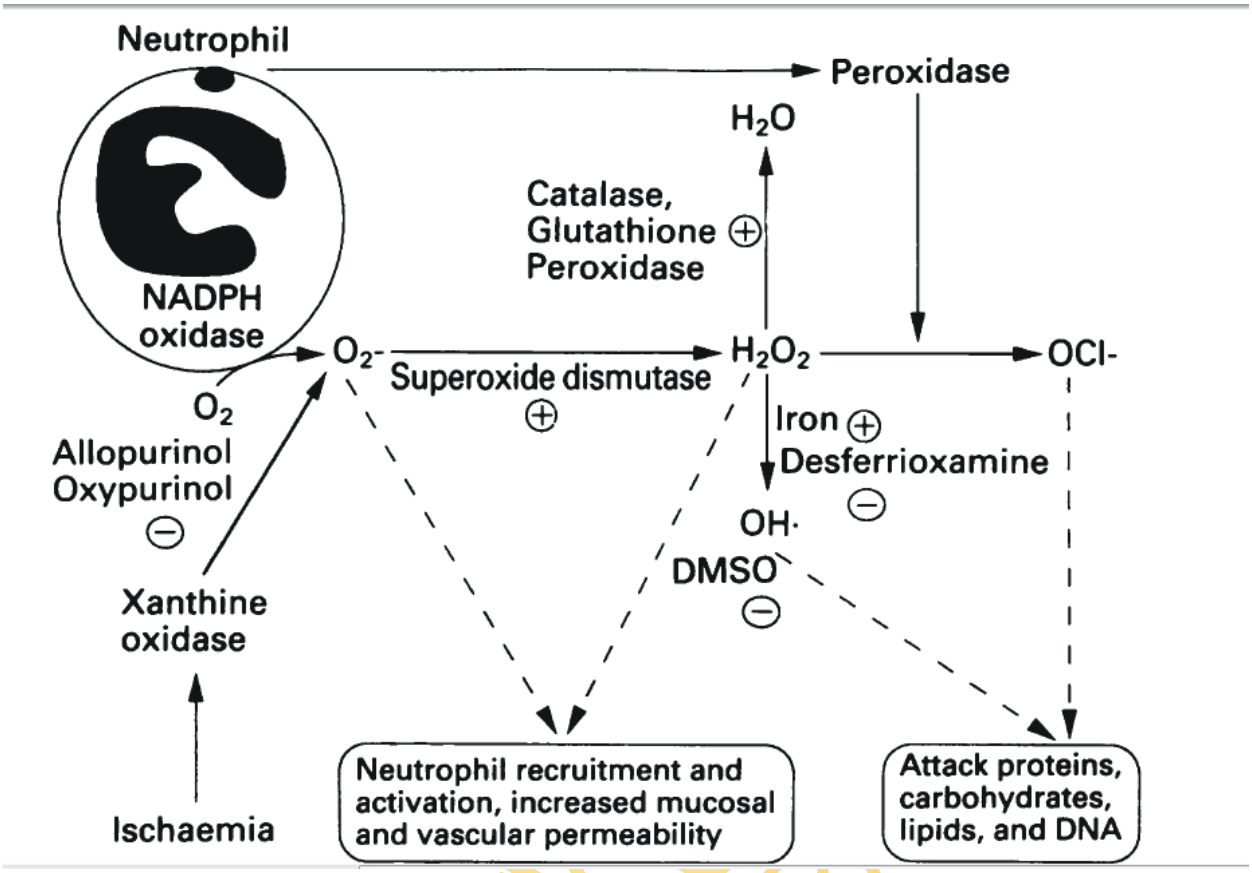


Figure 3: Reactive oxygen metabolites (ROM) in biological tissues. Simplified schematic representation showing the main sources and metabolic pathways involved in the production and removal of ROM. Superoxide dismutase catalyses the conversion of superoxide to hydrogen peroxide and also decreases the amount of iron available for Fenton chemistry. Catalase and glutathione peroxidase catalyse the breakdown of hydrogen peroxide to water. Allopurinol and oxypurinol inhibit xanthine oxidase, DMSO scavenges the hydroxylradical and desferrioxamine chelates iron. OE3 indicates catalysis, (e) indicates scavenging or inhibition. DMSO = dimethyl sulphoxide (Source: Muller *et al*., 2007).

Superoxide and hydrogen peroxide increase mucosal and vascular permeability, are involved in the recruitment and activation of neutrophils (Grisham and Granger, 1988), and are precursors of the more damaging hydroxyl radical (via Fenton chemistry) and hypochlorite (via the action of myeloperoxidase released by activated neutrophils). The hydroxyl radical can attack and damage almost every molecule found in living cells, including proteins, carbohydrates, lipids, and DNA. It is for this reason that the production of free radicals is normally tightly controlled and that concentrations of free transition metals are virtually non-existent. Hypochlorite is a powerful oxidant that may react directly against membrane associated targets or indirectly by forming less reactive chloramines that may diffuse across the membrane and attack cytosolic components (Weiss, 1989). Increased mucosal production of reactive oxygen metabolites related to disease activity has been shown in colorectal biopsy specimens" and stimulated mucosal phagocytes' from patients with inflammatory bowel disease compared with controls (Keshavarzian *et al.*,1992). Quantification is difficult, but evidence that sufficient ROM are produced to cause mucosal damage is supported by the findings of increased lipid peroxides in rectal biopsy specimens of patients with active ulcerative colitis. Low values of mucosal trypsin inhibitor, superoxide dismutase, metallothionein, and glutathione, and of circulating superoxide dismutase and glutathione peroxidase, and increased activities of circulating leukocyte elastase and colorectal mucosal collagenase may also reflect the activity of ROM in patients with active inflammatory bowel disease (Playford *et al.*, 1990; Mulden *et al.*, 1991). However, D'Odorico *et al.*, 2001, examined signs of oxidative stress and plasma antioxidant levels in controls compared to patients with ulcerative colitis and Crohn's disease. Oxidative DNA damage was noted in both inflammatory bowel disease groups compared to controls, measured by production of 8-hydroxy-deoxyguanosine (8-OHdG). Ulcerative colitis patients were found to have significantly lower

plasma levels of vitamins A and E and several carotenoids compared to controls; there were no differences between ulcerative colitis and Crohn's disease groups. Other researchers have also found increased oxidative stress in ulcerative colitis patients. Mucosal biopsies of ulcerative colitis patients were analysed and shown to have increased reactive oxygen intermediates, DNA oxidation products (8-OHdG), and iron in inflamed tissue compared to controls. Decreased levels of copper and zinc, cofactors for the endogenous antioxidant superoxide dismutase, were also observed (Lih-Brody *et al.*, 1996). In addition, increased protein carbonyls in inflamed mucosa were noted. The authors speculate this supports the theory that free radicals can produce damage to mucosal proteins in inflammatory bowel disease.

There are two main sources of ROM production in the gut. Firstly, phagocytes are prominent in the mucosa of patients with active inflammatory bowel disease and can produce ROM via both the respiratory burst and prostaglandin and leukotriene metabolism. Secondly, xanthine oxidase, which produces superoxide, is formed from xanthine dehydrogenase during ischaemia and is implicated in the pathogenesis of ischaemia reperfusion injury; this seems a likely route of ROM production in Crohn's disease if multifocal gastrointestinal infarction does indeed contribute to its pathogenesis (Wakefield *et al.*, 1989). Conversion of xanthine dehydrogenase to xanthine oxidase, however, can also take place in the presence of various proinflammatory substances such as formyl-leucyl-methionyl phenylalanine (FMLP) and tumour necrosis factor alpha, and can be induced by activated neutrophils (Friedl *et al.*, 1989). Superoxide and hydrogen peroxide are not only precursors of more harmful substances, but, by increasing mucosal permeability and recruitment and activation of further neutrophils, may help establish a vicious cycle of inflammation and tissue damage (Grisham and Granger 1988).

2.2.6.1. NOXIOUS INVOLVEMENT OF REACTIVE OXYGEN SPECIES IN COLITIS

Direct measurement of ROS in cells and tissues is quite difficult because of their short biological half-lives (Rumley and Paterson, 1998). However, direct quantification of ROS levels in colon biopsy specimens from UC and CD patients using chemiluminescence assays showed that ROS levels in these tissues are considerably increased compared to those in normal mucosa and positively correlate with IBD (Keshavarzian *et al.,* 1992; Simmonds *et al.,* 1992; Naito *et al.,* 2007). Mounting evidences indicate that there are increased levels of reactive nitrogen metabolites (RNM) such as NO in the inflamed IBD mucosa based on analysis of nitric oxide synthase activity (Singer *et al.*, 1996). Thus, increased levels of both ROS and RNM are closely correlated with the clinical development of IBD.

2.2.6.2. LIPID PEROXIDATION

Lipid peroxidation (LPO) is the most extensively studied manifestation of oxygen activation in biology. LPO is broadly defined as "oxidative deterioration of polyunsaturated fatty acids (PUFA)", which are fatty acids that contain more than two carbon-carbon double bonds (Halliwel, 1990). In general, the most significant effect of LPO in all cells is the perturbation of membrane (cellular and organellar) structure and function (transport processes, maintenance of ion and metabolite gradients, receptor mediated signal transduction, etc.).

Besides membrane effects, LPO can damage DNA and proteins, either through oxidation of DNA bases (primarily guanine via lipid peroxyl or alkoxyl radicals) or through covalent binding to malondialdehyde (MDA) resulting in strand breaks and cross-linking. ROS can also induce oxidation of critical -SH groups in proteins and DNA, which will alter structure and function of spermatozoa with an increased susceptibility to attack by macrophages (Aitken and Fisher,

1994). The oxidative damage to mitochondrial DNA is well known to occur in all aerobic cells which are rich in mitochondria and this may include spermatozoa.

The reactions of lipid peroxidation proceed through three main steps- initiation, propagation, and termination. During initiation, the free radicals react with fatty acid chains and release lipid free radicals. This lipid free radical may further react with molecular oxygen to form the lipid peroxyl radical. Peroxyl radicals can react with fatty acids to produce lipid free radicals, thus propagating the reaction (Aitken and Fisher,1994). One of the by-products of lipid peroxidation is malondialdehyde (MDA). This byproduct has been used in various biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa (Aitken and Fisher, 1994).

There are a lot of other products of lipid peroxidation such as: conjugated dienes, and secondary peroxidation products, which include saturated and unsaturated aldehydes, ketones, oxo- and hydroxy acids, and saturated and unsaturated hydrocarbons (e.g. ethane, pentane). Lipid peroxidation in biological membranes causes impairment of membrane functioning, decreased fluidity, inactivation of membrane-bound receptors and enzymes, and increased non-specific permeability to ions. Moreover, lipid hydroperoxides decompose upon exposure to copper while iron chelates the other factors including metals as haem, haemoglobin or myoglobin. Cytotoxic aldehydes are formed as a consequence of lipid hydroperoxide degradation (Valko *et al.*, 2007). The varieties of intermolecular reactions that occur in lipid peroxidation are illustrated in the figure below.

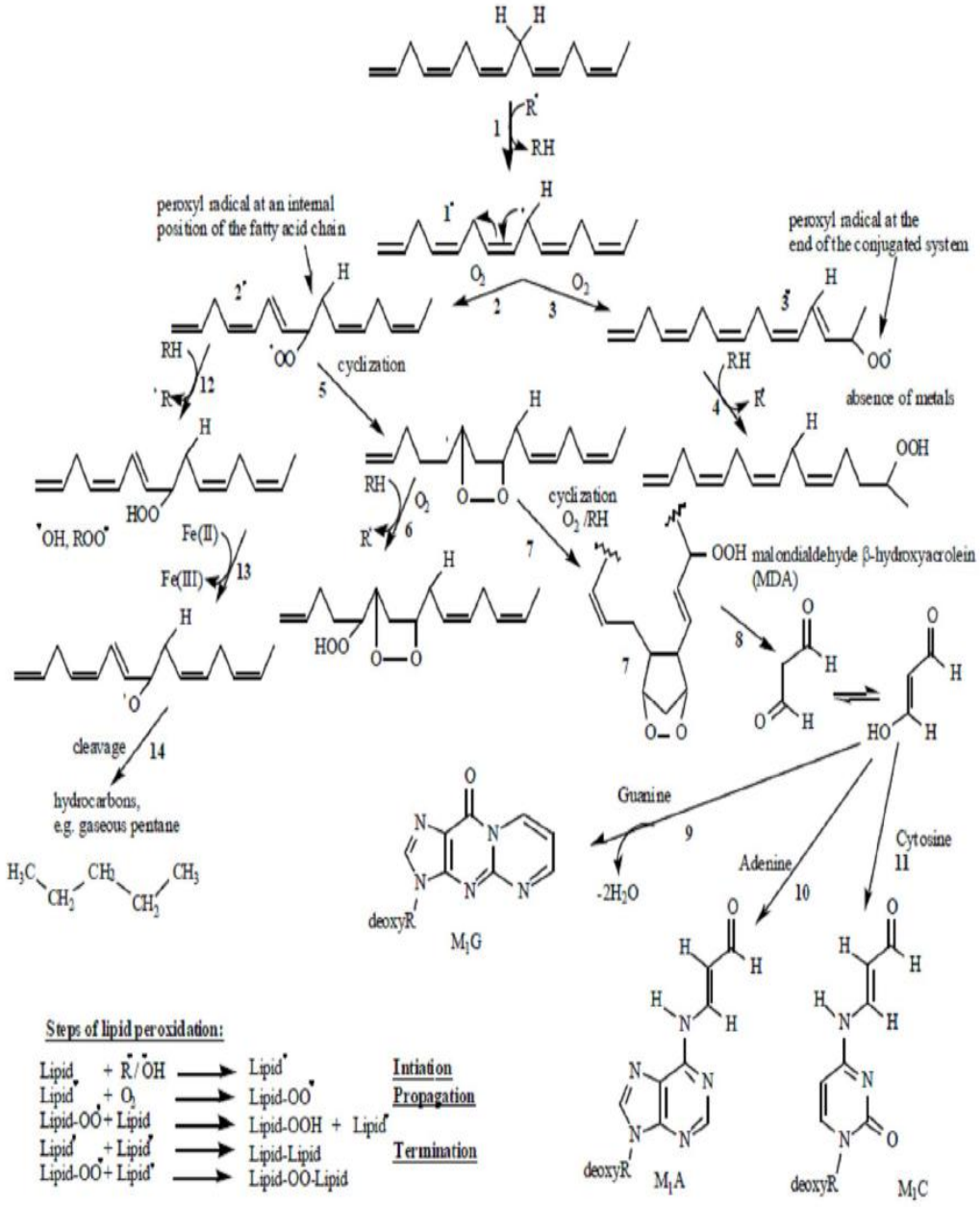


Figure 4: Intermolecular reactions in Lipid peroxidation (Source: Valko *et al.*, 2007)

The methylene groups of polyunsaturated fatty acids are highly susceptible to oxidation and their hydrogen atoms, after the interaction with radical R, are removed to form carbon-centered radicals 1 (reaction 1). Carbon centered radicals react with molecular di-oxygen to form peroxyl radicals (reactions 2 and 3). If the peroxyl radical is located at the end of a conjugated system (3), it is reduced to a hydroperoxide which is relatively stable in the absence of metals (reaction 4). A peroxyl radical located at an internal position of the fatty acid chain (2) can either react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centered radical (reaction 5). This can then either be reduced to form a hydroperoxide (reaction 6) or, by reaction 7, can undergo a second cyclisation to form bi-cyclic peroxide which after coupling to di-oxygen and reduction yields a molecule structurally analogous to the endoperoxide. Compound 7 is an intermediate product in the production of malondialdehyde (reaction 8). Malondialdehyde can react with DNA bases dG, dA, and dC to form adducts M1G, M1A and M1C, (reactions 9, 10, 11). Peroxyl radicals located in the internal position of the fatty acid (2) can, in addition to the cyclisation reactions (reaction 5), also abstract hydrogen from the neighboring fatty acid molecule, thus creating lipid hydroperoxides (reaction 12). They can further react with redox metals (e.g. iron) to produce reactive alkoxyl radicals (RO) (reaction 13) which after cleavage (reaction 14) and depending on the chain length may form e.g. gaseous pentane a good marker of lipid peroxidation.

2.2.7. GUT PERMEABILITY

An impaired colonic mucosal barrier leading to increased intestinal permeability has been demonstrated in patients with ulcerative colitis. Local leaks due to apoptosis of colonic epithelium comprise the primary lesion in mild ulcerative colitis. Moderate-to-severe ulcerative colitis is characterised not only by extensive local leaks but also by highly permeable ulcerous

lesions. Patients with ulcerative colitis have also demonstrated decreased colonic mucin species IV compared to biopsy specimens from normal controls. An *in vitro* study demonstrated a possible interaction between bacterial peptides and the mucosa in UC, resulting in depletion of mucus secretion by goblet cells. Medical therapy (unspecified) leading to remission not only results in decreased inflammation but also improved gut barrier integrity (Fasano, 2012).

2.2.7.1. COLONIC MILLEU: THE SULFUR-BUTYRIC ACID CONNECTION

The healthy colon produces butyric acid, a four-carbon short chain fatty acid, and several other SCFAs, including propionic and acetic acids by fermentation of fiber and other carbohydrates.

Butyric acid provides the primary fuel for colonocytes. Proper ion transfer, mucus synthesis, phase II detoxification, and lipid synthesis for cell membrane integrity in the colonocytes depend on butyrate oxidation (Roediger *et al.*, 1993). Impaired metabolism of SCFAs has been implicated as a factor in UC. Den *et al.* 1998 compared butyrate metabolism in healthy controls with that of 25 hospitalised patients with severe ulcerative colitis and 11 UC patients in remission. They measured butyrate metabolism after rectal instillation of ^{14}C-labeled butyrate by measuring $^{14}CO_2$ in the breath. Patients with active ulcerative colitis had significantly lower butyrate oxidation than patients in remission (who had normal butyrate oxidation) or controls. Three patients with inactive disease had decreased butyrate oxidation and interestingly, all three relapsed within a few weeks. Perhaps decreased oxidation of SCFAs is a good predictor of possible relapse and occurs before other signs of inflammation. Because normal oxidation was observed in patients in remission, faulty SCFA oxidation is likely to be a result rather than a primary cause of ulcerative colitis (Roediger *et al.*, 1993).

Other researchers compared the rate of butyrate, glucose, and glutamine oxidation to carbon dioxide in colonoscopy biopsy specimens from 15 patients with quiescent or mild colitis to

specimens from 28 controls with normal colonic mucosa. Butyrate, but not glucose or glutamine, oxidation was significantly impaired in the UC patients compared to controls, even though the disease was mild (Chapman *et al.,* 1994).

High concentrations of sulfate-reducing bacteria with concomitant elevation of hydrogen sulfide have been noted in patients with ulcerative colitis. Hydrogen sulfide can potentially damage the gut mucosa by inhibiting butyrate oxidation in the mitochondria, essentially starving the colonocyte. In experiments on human colonocytes isolated from colectomy patients, hydrogen sulfide and other sulfur compounds inhibited butyrate oxidation by 75 percent in the distal colon and 43 percent in the ascending colon (Den *et al.* 1998). The authors of the study conclude that the "metabolic effects of sodium hydrogen sulfide on butyrate oxidation along the length of the colon closely mirror metabolic abnormalities observed in active ulcerative colitis (Roediger *et al.,* 1993)." Animal studies on rabbits and guinea pigs have demonstrated that feeding sulfated polysaccharides (such as carrageenan), but not unsulfated polysaccharides, can induce lesions similar to ulcerative colitis (Pitcher and Cummings, 1996).

2.3. DIAGNOSIS

Ulcerative colitis characteristically commences in the rectum and extends proximally in a continuous, confluent and concentric manner to affect a variable extent of the colon, or its entire mucosal surface. Symptoms depend on the extent and severity of disease, extra-intestinal manifestations and concurrent therapy. The primary presenting symptom of ulcerative colitis is visible blood in the stools and is reported by more than 90% of patients. Associated symptoms generally reflect the endoscopic severity of the disease as a measure of mucosal damage and may differ according to disease extent. Loose stools (or a decrease in stool consistency) for more than six weeks differentiates UC from most infectious diarrhoea. Patients with extensive active

Ulcerative colitis present with chronic diarrhoea almost invariably associated with rectal bleeding, or at least visible blood in the stools. Such patients also describe rectal urgency, tenesmus, passage of mucopurulent exudates, nocturnal defaecation and crampy abdominal pain, or ache over the left iliac fossa prior to and relieved by defaecation (Abraham and Cho, 2009).

The onset of UC is usually insidious and symptoms are often present for weeks or even months before medical advice is sought. The disease may present with intermittent episodes of symptoms or as a severe attack (in about 15%) with systemic symptoms including weight loss, fever and tachycardia, or even nausea and vomiting (Baumgart and Sandborn, 2007). Extra-intestinal manifestations, especially an axial or peripheral arthropathy, episcleritis and erythema nodosum may accompany the presentation in about 10% and rarely precede intestinal symptoms. Thromboembolism is more frequent in UC than the general population, but is generally associated with active disease and pancolitis.

2.4. ANTIMALARIALS.

2.4.1. CHLOROQUINE PHOSPHATE.

2.4.1.1. History and Structure:

Chloroquine is a 4- aminoquinoline drug used in the treatment or prevention of malaria.

Chloroquine (CQ) was discovered in 1934 by Hans Andersag and co-workers at the Bayer laboratories who named it “Resochin”. It was ignored for a decade because it was considered too toxic for human use. During World War II United States government sponsored clinical trials for antimalarial drug development showed unequivocally that CQ has a significant therapeutic value as an antimalarial drug. It was introduced into clinical practice in 1947 for the prophylactic treatment for malaria.

2.4.1.2. Usage:

It has long been used in the treatment or prevention of malaria. After the malaria parasite plasmodium falciparum started to develop widespread resistance to chloroquine (Plowe 2005; Uhlemann and Krishna, 2005), new potential utilisations of this cheap and widely available drug ppphave been investigated. Chloroquine has been extensively used in mass drug administration which may have contributed to the emergence and spread of resistance.

As it mildly suppresses the immune system, it is used in some autoimmune disorders such as rheumatoid arthritis and lupus erythematosus (Gordon and Klinkhoff, 2005; Leecharoen *et al.*, 2007). Chloroquine is in clinical trials as an investigational antiretroviral in humans with HIV-1/AIDS and as a potential antiviral agent against Chikungunya fever (Savarino *et al.*,2003). The radiosensitising and chemosensitising properties of chloroquine are beginning to be exploited in anticancer strategies in humans (Soleto *et al.*, 2006; Sasaki *et al.*, 2010).

2.4.1.3. Actions:

Antimalarial

Inside red blood cells, the malaria parasite must degrade hemoglobin to acquire essential amino acids, which the parasite requires to construct its own protein and for energy metabolism. Digestion is carried out in a vacuole of the parasite cell. During this process, the parasite produces the toxic and soluble molecule heme. The heme moiety consists of porphyrin ring called Fe(II)-protoporphyrin IX (FP). To avoid destruction by this molecule, the parasite biocrystallises heme to form hemozoin, a non- toxic molecule. Hemozoin collects in the digestive vacuole as insoluble crystals.

Chloroquine enters the red blood cell, inhabiting parasite cell and digestive vacuole by simple diffusion. Chloroquine then becomes protonated (to CQ^{2+}) as the digestive vacuole is known to

be acidic (pH 4.7); chloroquine then cannot leave by diffusion. Chloroquine caps hemozoin molecules to prevent further biocrystallization of heme, thus leading to heme buildup. Chloroquine binds to heme (or FP) to form what is known as the FP- chloroquine complex; this complex is highly toxic to the cell and disrupts membrane function. Action of the toxic FP-Chloroquine and FP results in cell lysis and ultimately parasite cell autodigestion. In essence, the parasite cell drowns in its own metabolic products (Hempelmann, 2007).

The effectiveness of chloroquine against the parasite has declined as resistant strains of the parasite evolved. They effectively neutralise the drug via a mechanism that drains chloroquine away from the digestive vacuole. CQ- resistant cells efflux chloroquine at 40- times the rate of CQ- sensitive cells; the related mutations trace back to transmembrane proteins of the digestive vacuole, including sets of critical mutations in the Plasmodium falciparum Chloroquine Resistant Transporter (PfCRT) gene. The mutated protein, but not the wild type transporter, transports chloroquine when expressed in Xenopus oocytes and is thought to mediate chloroquine leak from its site of action in the digestive vacuole (Martin *et al.*, 2009). Resistant parasites also frequently have mutated products of the ABC transporter PfMDRI (Plasmodium falciparum Multi –Drug Resistance gene) although these mutations are thought to be of secondary importance compared to PfCRT. Research on the mechanism of chloroquine and how the parasite has acquired chloroquine resistance is still ongoing and there are likely to be other mechanisms of resistance (Martin *et al.*, 2009)

Antirheumatic

Chloroquine and Hydroxychloroquine have been used widely for the treatment of rheumatoid arthritis and systemic lupus erythematosus. These compounds had led to improvement of clinical and laboratory parameters but their slow onset of action distinguishes

them from glucocorticoids and nonsteroidal anti – inflammatory agents. Chloroquine and its analog hydroxychloroquine increases pH within intracellular vacuoles and alter processes such as protein degradation by acidic hydrolases in the lysosome, assembly of macromolecules in the endosomes and post translational modification of proteins in the Golgi apparatus (Gorjatschko and Betts, 1988). The anti-rheumatic properties of these compounds result from their interference with "antigen processing" in macrophages and other antigen presenting cells. Acidic cytoplasmic compartments are required for the antigenic protein to be digested and for the peptides to assemble with the alpha and beta chains of major histocompatibility complex (MHC) class II proteins. As a result, antimalarials diminish the formation of peptide- MHC protein complexes required to stimulate CD4+T cells and result in down- regulation of the immune response against autoantigenic peptides. Because this mechanism differs from other anti-rheumatic drugs, antimalarials are well suited to complement these other compounds in combination drug therapy (Fox, 1993).

Antiviral.

Chloroquine exerts direct antiviral effect, inhibiting pH- dependent steps of the replication of several viruses including members of the flaviviruses, retroviruses and coronaviruses. Its best studied effects are those against HIV replication, which are being tested in clinical trials. Moreover, chloroquine has immunomodulatory effects suppressing the production/release of tumor necrosis factor α and interleukin 6, which mediate the inflammatory complications of several viral diseases (Savarino *et al.*,2003).

Antitumor

The mechanisms behind the effects of chloroquine on cancer are currently being investigated. The best- known effects (investigated in clinical and pre-clinical studies) include radiosensitising

effects through inhibition of drug efflux pumps, ATP-binding cassette transporters (Soleto *et al.*, 2006; Sasaki *et al.*, 2010).

2.4.1.4. Pharmacokinetics:

Chloroquine has a very high volume of distribution, as it diffuses into the body's adipose tissue. Chloroquine and related quinine have been associated with cases of retinal toxicity, particularly when provided at higher doses for longer time frames (Bjelle *et al.*,1983). Accumulation of the drug may result in deposits that can lead to blurred vision and blindness (Leecharoen *et al.*, 2007).

Chloroquine is also a lysosomotropic agent. It accumulates preferentially in the lysosomes of cells in the body. The pKa for the quinoline nitrogen of chloroquine is 8.5, meaning that it is 10% deprotonated at physiological pH as calculated by the Henderson – Hasselbach equation. This decreases to 0.2% at a lysosomal pH of 4.6. Because the deprotonated form is more membrane permeable than the protonated form, a quantitative "trapping" of the compound in lysosomes results. The lysosomotropic character of chloroquine is believed to account for much of its anti malarial activity; the drug concentrates in the acidic food vacuole of the parasite and interferes with essential processes.

2.4.1.5. Adverse Effects:

At the doses used for prevention of malaria, side effects include gastrointestinal problems such as stomach ache, itch, headache and blurred vision. Chloroquine- induced itching is very common among black Africans (70%) and may be because of the high affinity of chloroquine for melanocytes, but much less common in other races. It increases with age and is so severe as to stop compliance with drug therapy. It is increased during malaria fever, its severity correlated to

the malaria parasite load in blood. There is evidence that it has genetic basis and is related to chloroquine action with opiate receptors centrally or peripherally (Ajayi, 2000).

When doses are extended over a number of months, it is important to watch out for a slow onset of "changes in moods" (i.e. depression, anxiety). These may be more pronounced with higher doses used for treatment. Chloroquine tablets have an unpleasant metallic taste.

A serious side effect is also a rare toxicity in the eye (generally with chronic use) and requires regular monitoring even hen symptoms free (Yam and Kwok, 2006). Chloroquine is very dangerous in overdose. Cann and Verhulst (1961) published a work showing three children who took overdoses died $2^1/_2$ hours of taking the drug. While the amount of the overdose was not cited, it is known that the therapeutic index for chloroquine is small. According to Davidson *et al.*, 2008, an overuse of chloroquine treatment has led to the development of a specific strain of E.coli that is now resisitant to the powerful antibiotic ciproflaxin. Also, at therapeutic dose, it potentiates both indomethacin and acidified ethanol induced ulceration in rats by enhancing lipid peroxidation and decreasing the endogenous antioxidant in the gastric mucosa (Ajeigbe *et al*., 2008b).

Pruritogenicity

The pathogensis of chloroquine - induced pruritus remains unclear. Genetic background seems to be a strong predisposing factor, as this symptom is observed mainly in Black Africans. Chloroquine has been shown to release histamine, and antihistaminic drugs have been demonstrated to be effective in a subgroup of patients (Osifo, 1995; Adebayo *et al.*, 1997; Ajayi *et al.*, 2004). Severity of pruritus also correlated with the antecedent malaria parasite density in the blood (Adebayo *et al.*,1997). In addition, it was suggested that subjects with pruritus may present slower metabolism of chloroquine, leading to higher plasma concentration of

chloroquine, although the overall pharmacokinetic patterns were comparable in both pruritic and non pruritic patients (Ademowo *et al.,* 2000; Onyeji and Ogunbona, 2001). Another possibility is mediation of pruritus in malaria individuals by endogenous opioid peptides via μ - opioid receptors (Onigbogi *et al.,* 2000; Ajayi *et al.,* 2004). Based on these, chloroquine – induced pruritus is considered as a multifactorial phenomenon (Osifo, 1989).

Ocular toxicity

Chronic use of chloroquine has been shown to induce numerous pathophysiological defects in the retina. This drug has the ability to alter P^H of intracellular compartments and lysosomal function of the retina pigment epithelium (RPE) and retinal neurons may constitute the basis of chloroquine retinopathy (Mahon *et al.,* 2004). The disturbances in serum protein pattern and free amino acid levels accompanying ultrastructural changes in retina have been implicated in the retinopathy after chloroquine toxicity (El Sayed *et al.,* 1998). Aside the lamellar lysosome – like structures formed within the photoreceptive layer, as well as the pigment epithelium and neuro –retinal layers by chloroquine, hypolipidemia in the serum was observed mainly due to the decrease in phospholipid portion. It is then hypothesised that due to the inhibition of the degradation process in the defective lysosomes, the retinal cells were denied the re- use of their own phospholipids and thereby result to their uptake from the serum (Gaafar *et al.,* 1995). Hence, lysosomal dysfunction, aberrations in serum proteins and free amino acids and various lipidemic effects are all chloroquine's toxicological consequences on the retina.

Antifertility

Chloroquine may cause antifertility in female rats (Okanlawon and Ashiru, 1992) and its antifertility in male rats may result from reduction in circulating plasma testosterone (Adeeko *et al.,* 1993). Testosterone is required for spermatogenesis and maturation of sperm. The Leydig

cell through luteinizing hormone receptor regulates testosterone synthesis. The Sertoli cells, seminiferous tubules, interstitial cells provide support for germinal cells to synthesise follicle stimulating hormone, androgen receptors and act as blood – barrier. It possesses other reversible antifertility activities including reductions in sperm motility and viability (Adeeko and Dada, 1998; Salman and Ajayi 2007), increase in the number of abnormal spermatozoa and a reduction in the weight of the testes and accessory sex organs (Nicola *et al.,* 1997). All these effects including testicular lesions observed by Oforah *et al.,* 2004 in chloroquine – treated rats could precipitate sterility as they affect spermatogenesis.

2.4.2. ARTEMISININ DERIVATIVES.

HISTORY

Artesunate is part of the artemisinin group of drugs that treat malaria, a semi – synthetic derivative that is water soluble. The Chinese medicine has used the plant, *Artemisia annua* extensively for over a thousand year as an antimalarial but developed in the beginning in 1960s (Woodrow *et al.*,2005). Artemisinin, a poorly soluble chemical extract from Artemisia, is a fast acting blood schizonticide effective in treating the acute attack of malaria including chloroquine-resistant and cerebral malaria (Boulangier *et al.*,2007). It is a rapid acting and potent antimalarial (White 1997).

Artesunate is prepared from dihydroartemisinin (DHA) by reacting it with succinic acid anhydride in basic medium. Pyride as base /solvent, sodium bicarbonate in chloroform and catalyst DMAP (N, N- dimethylaminopyridine) and triethylamine in 1,2-dichloroethane have been used with yields of up to 100%.

Artemisinin can be given orally, intramuscularly or by suppository. They are rapidly absorbed and widely distributed and are converted in the liver to the active metabolite dihydroartemisinin. The half life of artemisinin is about 4 hours, of artesunate 45 minutes and of artemether 4-11 hours. Bioavailability with rectal suppository formulations is 30% less than with oral administration, although there is large inter-individual variation. Studies comparing parasite clearance times following oral and rectal administration have led to the conclusion that therapeutic concentrations should be achieved with suppositoried (Sidhu, 1998). Suppositories have been shown to be effective as parenteral antimalarial drugs in clinical trials for the treatment of severe malaria (Cao, 1997).

Adverse Effects

Toxic effects have been reported less frequently with the artemisinins than with other antimalarial agents (Taylor and White, 2004). The most common toxic effects that have been identified are nausea, vomiting, anorexia and dizziness; these are probably due, in many patients to acute malaria rather than to the drugs (Price *et al.*,1999). More serious toxic effects, including neutropenia, anemia, hemolysis and elevated levels of liver enzymes, have been noted rarely (Ribeiro and Olliaro, 1998).

Neurotoxicity is the greatest concern regarding artemisinins, since the administration of high doses in laboratory animals has led to severe and irreversible changes in the brain (Brewer *et al.,* 1994). Extensive studies in many species showed the intramuscular dosing was more toxic than oral dosing and that, by any route; fat- soluble artemisinins were more toxic than artesunate (Nontprasert *et al.*,2000). In humans, an episode of ataxia was reported after treatment with oral artesunate (Miller *et al.*,1986) and one case- control study showed hearing loss after the use of

artemether/lumefantrine (Toovey and Jamieson, 2004). Most reported toxic effects may have been due to underlying malaria or other factors that were independent of artemisinin use. Multiple studies have shown that neurologic findings are fairly common with acute malaria but there is no convincing evidence of neurotoxic effects resulting from standard oral or intravenous therapy with artemisinins (Kissinger *et al.*, 2000, van Vugt *et al.*, 2000).

Another concern about artemisinins is embryotoxic effects, which have been demonstrated in animals (Clark *et al.*, 2004).

2.5 METHODS OF INDUCING ULCERATIVE COLITIS

Several models of experimental ulcerative colitis have been reported. This is as a result of attempts to reproduce experimental inflammatory disease in the colon. These models can be broadly divided into spontaneous colitis model, inducible colitis models, genetically modified models and adaptive transfer model. Although these models do not represent the complexity of human disease, they are valuable and indispensible tools that provide a wide range of options for investigating involvement of various factors into the pathogenesis of IBD and evaluate different therapeutic options (Wirtz and Neurath, 2007). Currently, models of exogenous induction of experimental colitis have been used more extensively due to their technical simplicity, reproducibility and lower cost. Chemically induced murine models of intestinal inflammation are one of the most commonly used models because they are simple to induce, the onset, duration and severity of inflammation are immediate and contyrollable (Wirtz *et al.*, 2007).

The most frequently used agents are acetic acid, ethanol, dextran sulfate sodium and trinitrobenzene sulfonic acid (TBNS) (Strober, 1985; Wirtz and Neurath, 2007). The most widely used models are induced by administering toxic chemicals such as dextran sulphate sodium (DSS) or trinitrobenzene sulfonic acid (TNBS) (Morris *et al*., 1989, Gaudio *et al*., 1999).

The dextran sulfate sodium induced colitis model has some advantages when compared to other animal models of colitis. It is well appreciated and widely used model of inflammatory bowel disease because of its simplicity. For example, an acute, chronic or relapsing model can be produced easily by changing the concentration of administration of DSS (and cycle in rats and other strains of mice). It has many similarities to human inflammatory bowel disease. Although DSS induced colitis results in inflammation, resembling ulcerative colitis, an obvious obstacle is that DSS is very expensive (Chen *et al.*, 2007).

Trinitrobenzene sulfonic acid (TNBS) induced colitis is a common method for studying IBD in animal models. Several factors may however affect its reproducibility, rate of animal mortality and macroscopic and histopathological outcomes. The major advantages of this model include proposing a simple process and reproducible colnic damage, short duration of the experiment, long-lasting damage accompanied by inflammatory cell infiltration and ulcers. TNBS model of colitis suffer some disadvantages such as absence of spontaneous relapse which is the hallmark of ulcerative colitis as found in many other methods. The reproducibility of the model is dependent upon the dose of TNBS (Neurath *et al.,* 2000, Zhang, 2000). Also the inflammation of TNBS induced colitis is transmural and includes the formation of granulomas and Langhan's type giant cells. The mucosal frequently has a "cobble-stone" like appearance. The evidence suggests that this model is histopathologically relevant to the features of Crohn's disease, not ulcerative colitis (Morris *et al.*, 1989).

Experimental colitis induced by acetic acid has been used extensively as a model for intestinal inflammatory disease and it serve as a useful model (Bertevello *et al.,* 2005). The use of acetic acid provokes nonspecific inflammation similar to ulcerative colitis with easily reproducible lesions and low mortality. The model of induced colitis through acetic acid enema

present advantages over other experimental models of ulcerative colitis. Such advantages include easy availability of the aggressor reagent, low cost, reproducibility and similarities to IBD in humans principally in terms of histological and metabolic aspects (Sharon and Stenson, 1985).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS AND CHEMICALS USED

i. Wistar rats
ii. Chloroquine
iii. Artesunate
iv. Lonart^{R} (Arthemeter + Lumefantrine)
v. Oral cannula
vi. Microscopes
vii. Syringes and needles
viii. Dissecting sets
ix. Cotton wool
x. Dissecting board
xi. Weighing balance
xii. Hand gloves
xiii. Blade
xiv. Slides
xv. Animal cages
xvi. Feeding bottles
xvii. Rubber catheter
xviii. Acetic acid
xix. Formalin
xx. Homogenizer
xxi. Centrifuge
xxii. Spectrophotometer

3.2. ANIMALS AND GROUPINGS

Growing male albino rats of the Wistar strain (weight range 160-180g) were used. The animals were housed in the preclinical animal house, College of Medicine, University of Ibadan. They were maintained under standard laboratory conditions and were fed with standard commercial rat feed and allowed water *ad libitum* throughout the period of the experiment.

A total of 80 rats were used. The animals were randomly divided into four groups of 20 animals each as follows:

Control Group: Animals in this group received distilled water for 3 days before induction of colitis

Group B: Animals in this group received chloroquine (dissolved in distilled water) orally for 3 days before induction of colitis.

Group C: Animals in this group received artesunate (dissolved in distilled water) orally for 5 days before induction of colitis.

Group D: Animals in this group received Lonart^{R} 'artemether + lumefantrine' (dissolved in distilled water) orally for 3 days before induction of colitis.

3.3. DRUGS

Chloroquine, artesunate and lonart^{R} tablets were purchased from Danax Pharmaceutical store Dugbe, Ibadan. . Acetic acid, formalin,thiobarbituric acid and other analytical grade chemicals used were obtained Sigma line, St Louis USA, through a local agent. Distilled water was obtained from the Department of Physiology, University of Ibadan.

3.4. INDUCTION OF COLITIS.

Under light ether anaesthesia, a flexible plastic catheter (outer diameter of 2 mm) was inserted rectally into the colon 8 cm proximal to the anus. Colitis was then induced by administering 1ml/ 200g 6% acetic acid. The animals were inspected and scored for the presence of diarrhea.

Rats were sacrificed on daysthree (3), six (6), nine (9) and twelve (12) after induction of colitis.

3.5 DIARRHEA SCORING

The animals were inspected and scored for the presence of diarrhea using the method of Masonobu *et al.*, (2002). Scoring of diarrhea started 24 hours after the induction of colitis. Table 3.1 shows the scoring method used.

Table 1: Scoring system for comparative analysis of intestinal bleeding.

Score	**Stool consistency**
0	Normal stool
1	Normal stool with blood
2	Loose stool without blood
3	Loose stool with visible blood
4	Bloody diarrhea

3.6. ORGAN COLLECTIONAND PREPARATION OF POST MITOCHONDRIAL SUPERNATANT (PMS) OF COLON SAMPLES

The animals were sacrificed by cervical dislocation and dissected (cut open from the abdominal cavity to the thoracic cavity) to collect the colon. The colon was isolated, 8 cm segment of the distal colon proximal to the anus was resected, its lumen rinsed with ice cold saline.

The colon was homogenised in ice – cold phosphate buffer 7.4 using a Teflon homogenizer. The resulting homogenate was centrifuged at 10,000g for 10 minutes in a cold centrifuge (4^{o}C), to obtain the post mitochondrial fraction. The supernatant was collected and used for biochemical analyses. A cross section of the colon was fixed in formalin and processed for histopathological assessment.

3.7. PROTEIN DETERMINATION

The protein concentrations of the various samples were determined by means of the Biuret method as described by Gornal *et al.* (1949) with some modifications. Potassium iodide was added to the reagent to prevent precipitation of Cu_2^+ ions as cuprous oxide.

Principle

Proteins form a colored complex with cupric ions in an alkaline solution as exemplified by the Biuret reagent containing copper sulphate ($CuSO_{4)}$, potassium iodide (KI) and sodium potassium tartarate. The protein and Biuret reagent form complex with maximum absorbance at 540nm. The procedure is usually calibrated with a standard BSA curve.

Reagents

1. 0.2M sodium hydroxide

8 g of NaOH (BDH, England) was dissolved in distilled water and made up to 1litre.

2. Stock Bovine Serum Albumin (standard)

20 mg of BSA (Sigma Chemical Co., USA) was dissolved in 2 ml distilled water to give a stock solution of 10mg protein/ml.

3. Biuret Reagent

3 g $CuSO_4$. $5H_2O$ (BDH Chemicals, England) were dissolved in 500 ml of 0.2M NaOH, 9 g of Sodium tartarate and 5 g of potassium iodide, KI (BDH Chemicals,England) was added and the solution made up to a litre with 0.2M NaOH.

4. Estimation of protein in the samples

0.1 ml of the post mitochondrial fractions of the colon supernatants was added to 0.9 ml of distilled water to make a 1 in ten dilution in order to reduce the sensitivity of the Biuret reagent. 1 ml of the diluted sample was taken and added to 3 ml of Biuret reagent in triplicate. The mixture was incubated at room temperature for 30 minutes after which the absorbance was read at 540nm using distilled water as blank. The protein content of the samples were usually extrapolated from the standard curve and multiplied by 100 to get the actual amount in the fraction.

3.8. LIPID PEROXIDATION ASSESSMENT.

Lipid peroxidation (LPO) was assayed for, by measuring the thiobarbituric acid reactive product (TBARS) present in the test sample using the procedure of Varshney and Kale (1990) and expressed as micromolar of malondialdehyde (MDA/gtissue).

Principle: The assay is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) under acidic condition to yield a stable pink complex with maximum absorption at 532nm wavelength. Small amount of malondialdehyde are produced during lipid peroxidation and these reacts with thiobarbituric acid.

Reagents

30% Trichloroacetic acid (TCA) solution: 4.5 g of TCA was dissolved in 15 ml of distilled water and stored at $4^{o}C$.

0.75% Thiobarbituric acid (TBA) solution: 0.1M HCl was prepared by adding 0.04ml of concentrated HCl to 14.96 ml of distilled water. 0.1125 g of thiobarbituric acid was dissolved in 15 ml of 0.1M HCl. Dissolution was aided by shaking in a boiling water bath.

0.575 g of KCl and 1.18 g of Tris base were dissolved in distilled water and made up to 50 ml and the pH adjusted to 7.4

Procedure

0.40 ml of reaction mixture i.e sample already quenched with 0.5 ml of 30% TCA was added to 1.6 ml of Tris KCl. The reaction mixture was placed in a water bath for 45 minutes at $80^{o}C$, cooled in ice and then centrifuged at 3500rpm for 5 minutes. The clear pink supernatant was collected and the absorbance measured against a reference blank (distilled water) at 532nm. The MDA level was calculated according to the method of Adam- Vizi and Seregi (1982).

Calculation

MDA (unit/ mg protein) = absorbance x volume of mixture

E_{532nm} x volume of sample x mg protein

Where E_{532nm} is $1.56 \times 10^{-5} M^{-1} cm^{-1}$

3.9. HISTOLOGICAL ANALYSIS

Procedure:

Step 1 (Fixation): The colon was placed in a sample bottle containing 10% formalin. Small sections were taken from the distal part of each colon. This was immediately immersed in neutral buffered 10% formal- saline for 48 hours.

Step 2 (Dehydration): Water was removed from the tissue by putting it in ascending grades of alcohol (70%, 80%, 90%, 2 changes of 100%) one hour each. Ascending concentrations of alcohol was used to prevent sudden rush out of water from the tissues, so that the cell will not be distorted or damaged.

Step 3 (**Clearing)**: Alcohol was then removed from the tissues because it is not miscible with paraffin. The tissues were infiltrated with xylene, which replaced the alcohol and was also miscible with paraffin. Xylene also made the opaque tissue transparent, therefore the name clearing stage. The tissue was passed twice through xylene, and it spent about 2 hours each time.

Step 4 **(Embedding)**: The tissue was infiltrated and impregnated in 2 changes molten paraffin wax one hour each. It was allowed to cool on a frozen surface then removed from mold.

Step 5 **(Microtomy):** The tissue was trimmed to expose tissue surface with mocrotome, and was cooled on ice. 5µ of tissue was sectioned.

Step 6 **(Mounting Of Paraffin Sections):** Float using 2% alcohol into a warm water of about 2^0C below melting point of wax. Use clean, grease free slide to pick the floating section. The

other side of the slide was cleaned and placed on hot plate after proper labeling for about 3 hours for the section to be completely fixed and the slide to dry.

Step 7 **Staining**: The section was deparaffinized in 2 changes of xylene for 4 minute each so that the stains can permeate. The slide was then immersed in a descending concentration of alcohol (ie. 100%, 90%, 80%, and 70%) for about 1 minute in each alcohol solution so as to dehydrate it. The slides were rinsed in water and placed in Erhlich haematoxylin for about 15 minutes. The slides were dipped in 1% acid-alcohol (2 dips) and rinsed in running water for about 3 minutes till the colour of the section to become blue. The slides were counterstained in eosin for about 2 minutes and briefly rinsed in water. The slides were immersed in ascending grades of alcohol (70%, 80%, 90%, and 100%) for about 30 seconds so as to dehydrate the preparation. The preparation was cleared of alcohol by dipping it in xylene for 1minute. After these, the slide was blotted and mounted under a cover slip using dibutylphthalate xylene (DPX), and air bubbles were prevented from getting in. The slide was then read under the microscope using x100 and x400 magnifications, and lesions were noted. A photomicrograph of the slide preparation was then taken. The extent of tissue injury was scored on a scale of Tissues contraction, regeneration of the ulcerated mucosa, and inflammatory exudates was observed under stereomicroscope.

3.10. STATISTICAL ANALYSIS

The mean, standard deviation (S.D) and standard error of mean (S.E.M) of all values were calculated. Student's t- test for variables was employed to compare differences between control and test groups. P-value was set at 0.05 to determine the level of significance.

CHAPTER FOUR

4.0 RESULTS

4.1 EFFECT OF ARTESUNATE, CHLOROQUINE AND ARTEMETHER/ LUMEFANTRINE ON BODY WEIGHT

There was no significant difference in the mean body weight of animals in all the groups before the induction of colitis. However by day three after colitis induction, weight loss was evident in all the groups and was significantly higher in the chloroquine group (150 ± 6.81) when compared with the control (170 ± 3.78) and the artesunate treated group (175 ± 6.81). This was consistent throughout the period of the study. By day nine, significant differences were observed between the body weight of the artesunate treated group (179 ± 6.39) and the artemether/ lumefantrine treated group (160 ± 4.99) at $P < 0.05$. The artesunate treated group started regaining weight by day six. By day twelve the mean weight has exceeded the initial mean weight while the other groups did not attain the initial mean weight till the end of the study. This is shown in Figure 5

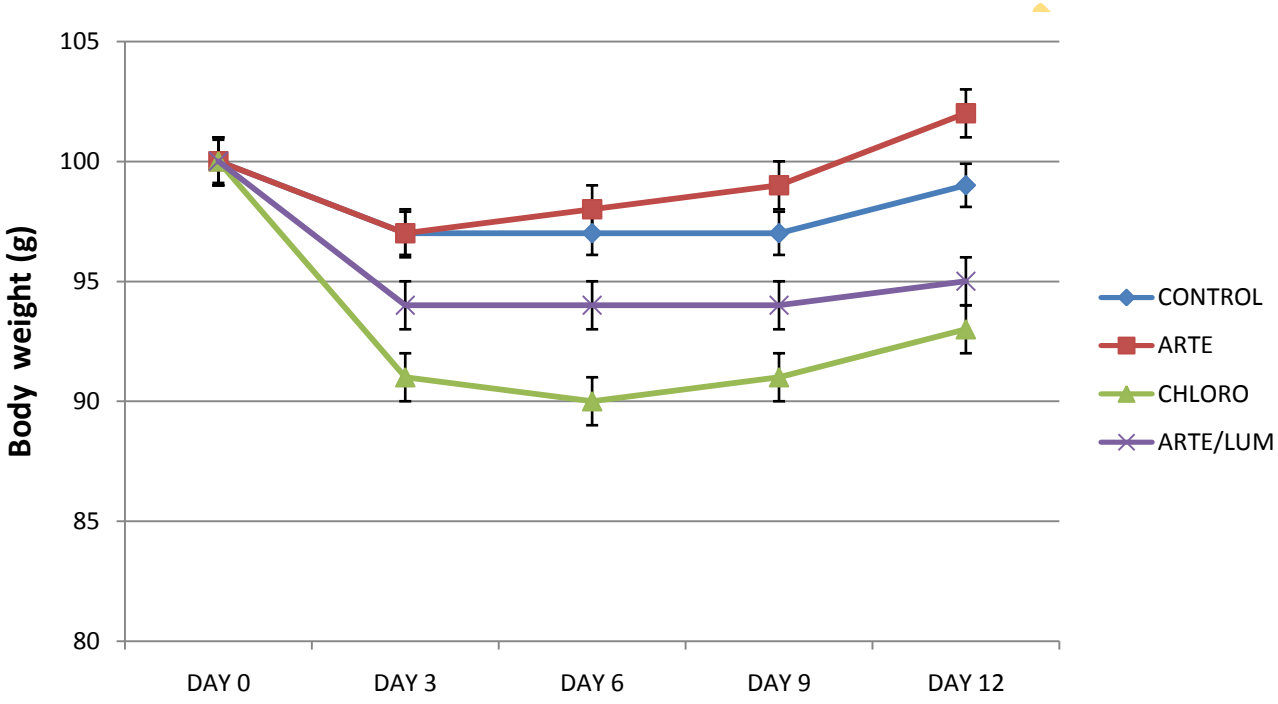


Fig 5: Body weight changes in the control and experimental groups following acetic acid colitis induction in rats.

4.2 EFFECT OF ARTESUNATE, CHLOROQUINE AND ARTEMETHER/ LUMEFANTRINE ON DIARRHEA

Animals in group Cpretreated with chloroquine had the highest mean diarrhea score (3.10 ± 0.31) when compared to the other groups on day one. The difference was not significant when compared with control group (2.4 ± 0.16). There was significant difference between the chloroquine group and the other treated groups with Artesunate group having the lowest mean score (1.6 ± 0.27). By day three the diarrhea score for control, chloroquine and lonart^{R}(2.3± 0.15, 2.1± 0.28, and 2.1± 0.41 respectively) were similar and not significantly different but there was significant difference when control was compared with the artesunate group. By day six the control group had the highest diarrhea score and there was significant difference between the control, artesunate and chloroquine groups. The diarrhea score for the artesunate group (0.5± 0.3) was significantly low when compared with chloroquine group (1.5± 0.33). By day nine the diarrhea score for all antimalarial treated groups increased from 0.50± 0.33, 0.88 ± 0.35 and 1.5± 0.33 to 1.00± 0.45, 1.67± 0.33 and 1.67± 0.33 for artesunate, chloroquine and lonart respectively though there was no signifant difference in any of the groups.By day twelve there was significant difference between artesunate and chloroquine group though other groups were not significantly different. The result is presented further in fig 6

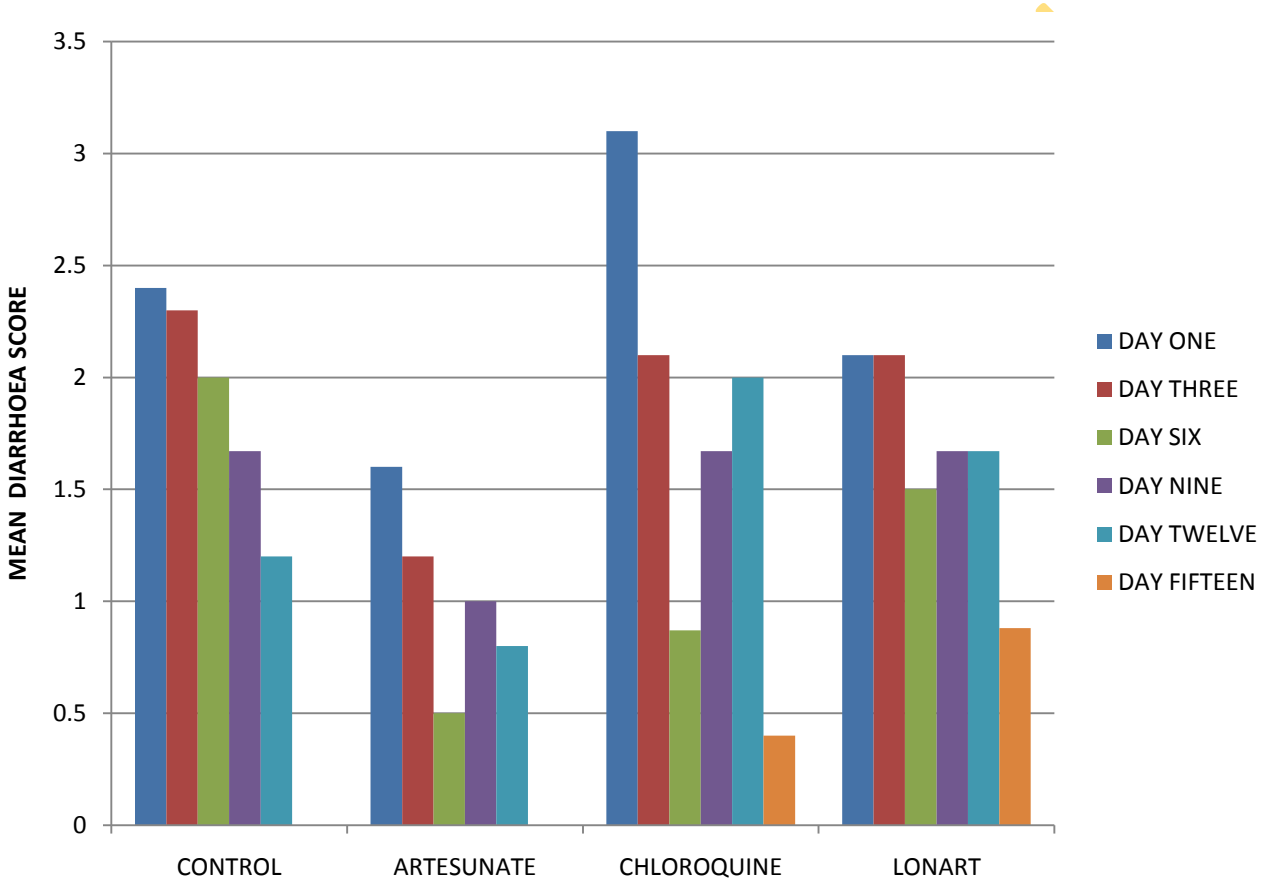


Fig. 6: Mean diarrhoea scores for different days after induction of colitis

4.3. EFFECT OF ARTESUNATE, CHLOROQUINE AND LONART^{R} ON COLONIC PROTEIN CONCENTRATION.

The protein concentration increased in all the groups as the day increases. The lowest concentration on day three was seen in the lonart^{R}group with 0.5± 0.02. There was significant difference when the control group was compared with the artesunate and lonart groups but was not significant when compared with chloroquine group. There was significant difference when all the treated groups were compared with each other with the highest value noticed in the artesunate group (0.99± 0.12) on day three.There was significant difference when all the treated groups were compared with the control on day six with the artesunate group still having the highest protein conentration.By day nine significant differences was noticed between the control group and the chloroquine and lonart^{R} groups with the chloroquine group having the highest value(1.23± 0.05).No significant difference was observed when the treated groups were compared. The concentration of protein on day twelve was highest in the lonart^{R} group (1.31± 0.02) while the control group had the lowest concentration (0.78± 0). The difference was significant ($p<0.05$) when control group was compared with all the treated groups. This is as shown in fig 7.

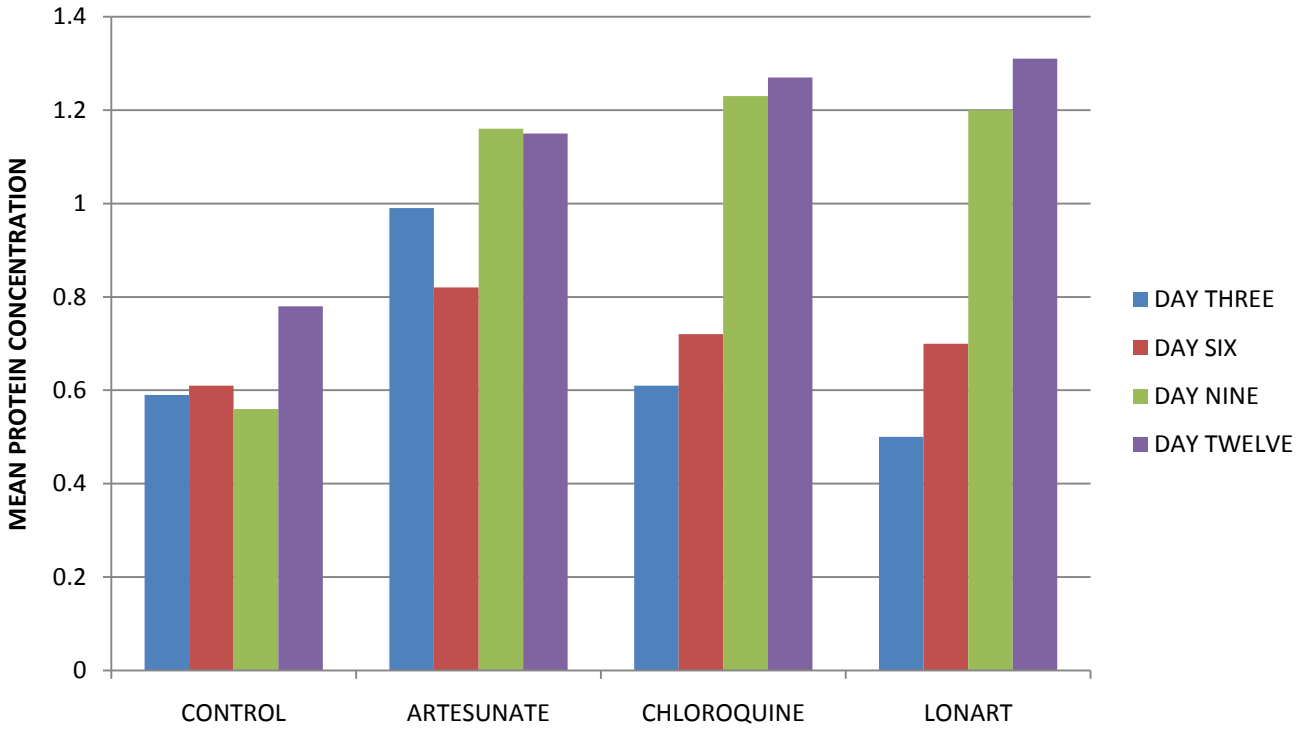


Fig 7: Mean protein concentration for different days after induction of colitis

4.4. EFFECT OF ARTESUNATE, CHLOROQUINE AND LONART^{R} ON COLONIC LIPID PEROXIDATION.

The artesunate treated group had the lowest concentration of malondialdehyde (MDA an index of lipid peroxidation) when compared to the other treatment groups and the control. This was consistent throughout the period of study. The chloroquine treated group had the highest concentration on day three and day twelve which mark the end of the experiment. There was significant difference in all the groups throughout the study except on day nine when the MDA concentration was not significantly different in the control group compared to the chloroquine and lonart groups ($p<0.05$). The artesunate treated group was significantly lower than the control and other treated groups on day twelve while there was no significant difference between the control and lonart^{R} group. The result is presented in fig 8.

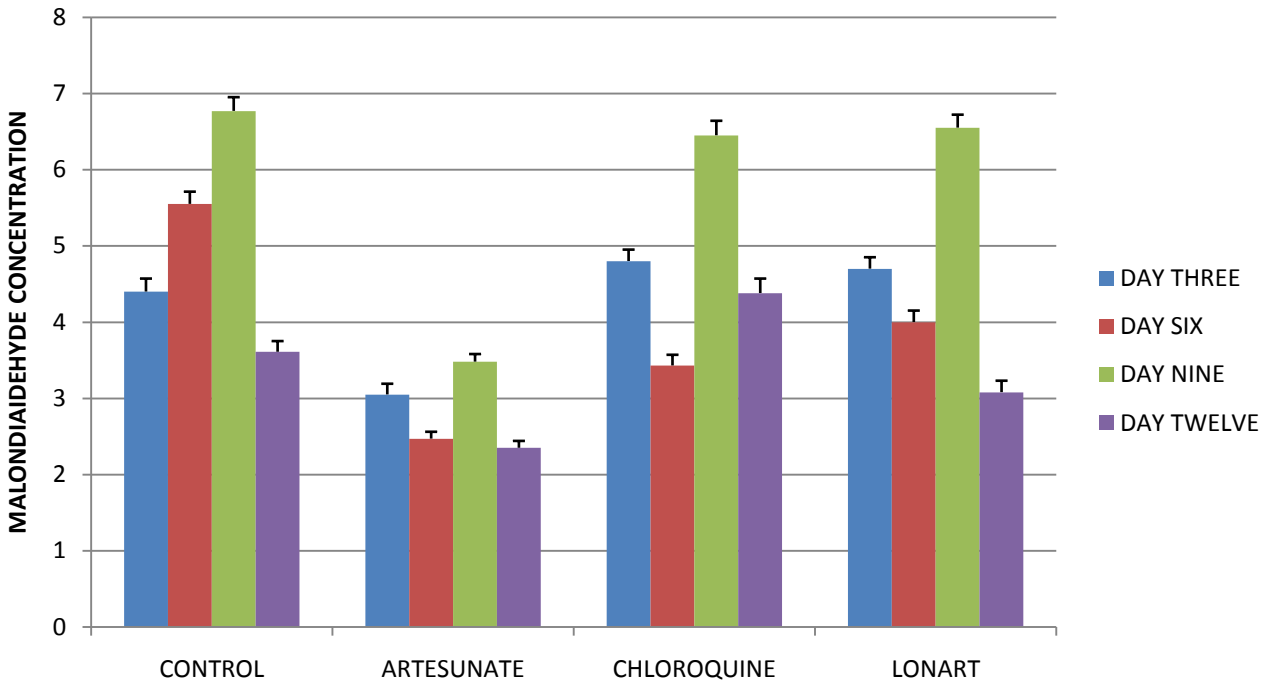
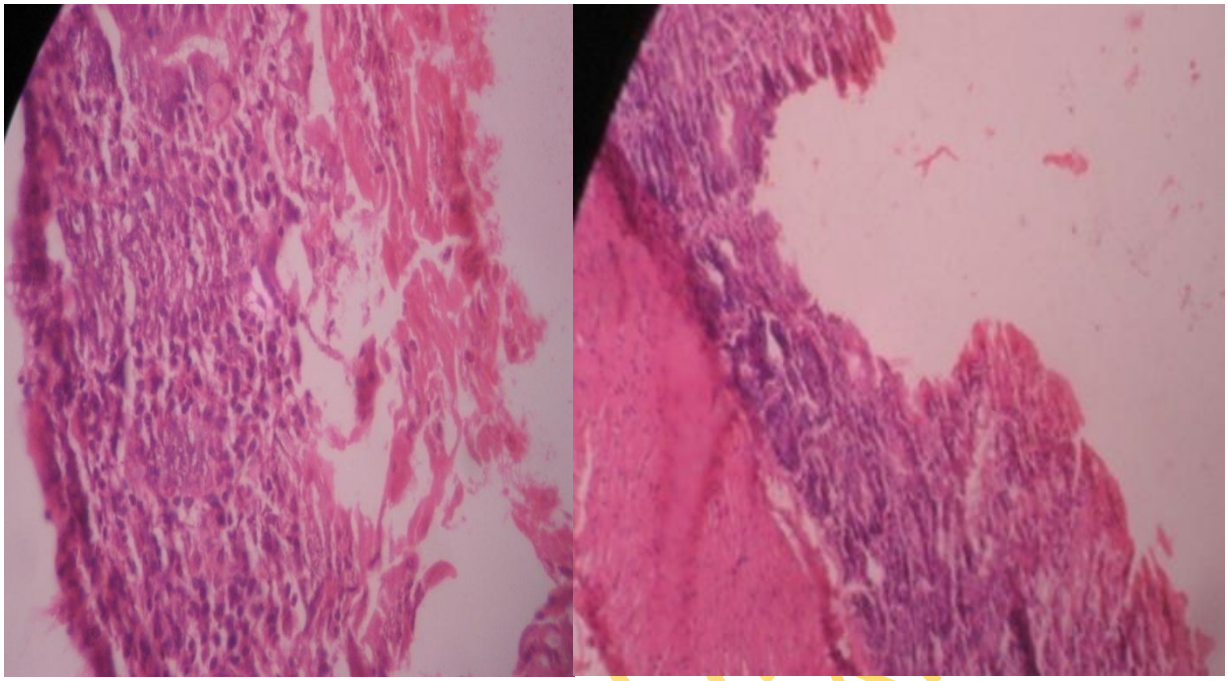


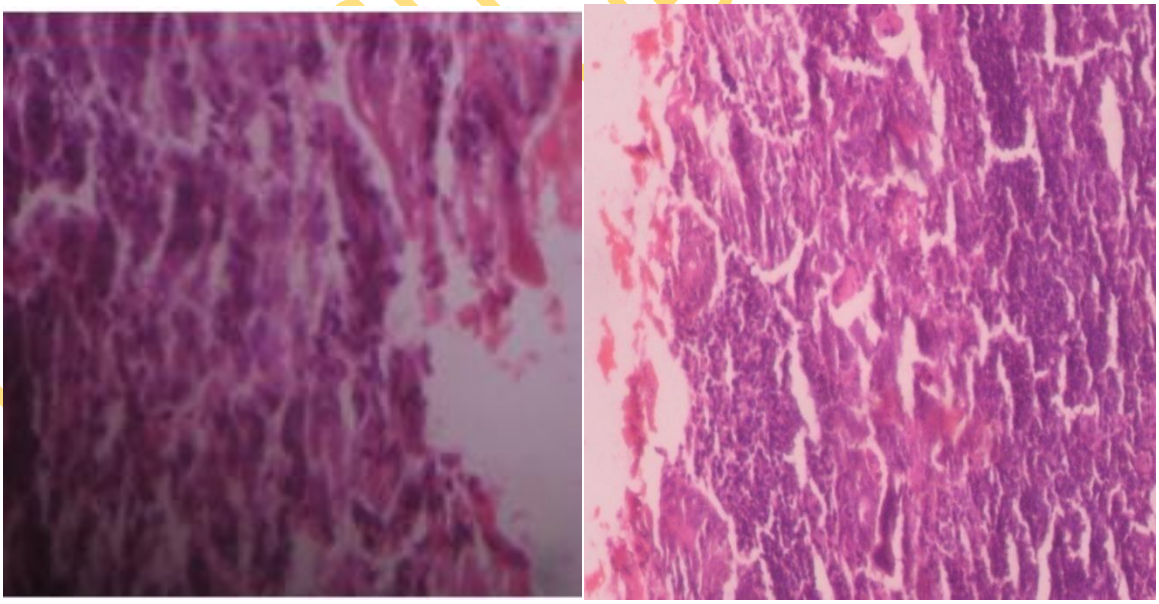
Fig 8: Mean malondialdehyde concentration for different days after induction of colitis (x 10^{-6})

4.5. EFFECT OF ARTESUNATE, CHLOROQUINE AND LONART^{R} ON HISTOLOGY OF COLON

Induction of colitis by acetic acid resulted in severe infiltration of inflammatory aggregates (majorly neutrophils, macrophages, plasma cells and lymphocytes), erosion of epithelial lining and glandular degeneration. By day three, colitis was marked in all the groups as evident by the abundance of inflammatory cells found. The histological photomicrograph on day six still showed evidence of colitis in all the groups but the colitis was marked and hemorrhage evident in the control, arthemeter/lumefantrine and chloroquine groups, necrotic debris was found in the lumen of the chloroquine and arthemeter/lumefantrine groups while signs of healing was seen in the artesunate group with few inflammatory cells seen. By day nine, colitis was healed in the artesunate group while colitis was still healing in the other groups with inflammation and hemorrhage still evident in some groups. There are degenerated neutrophils and swollen epithelial cells.Colitis was completely healed in the artesunate group by day twelve with lots of goblet cells found in the lamina propria while the other groups are still at various stages of healing with some inflammatory cells, macrophages and plasma cells seen.

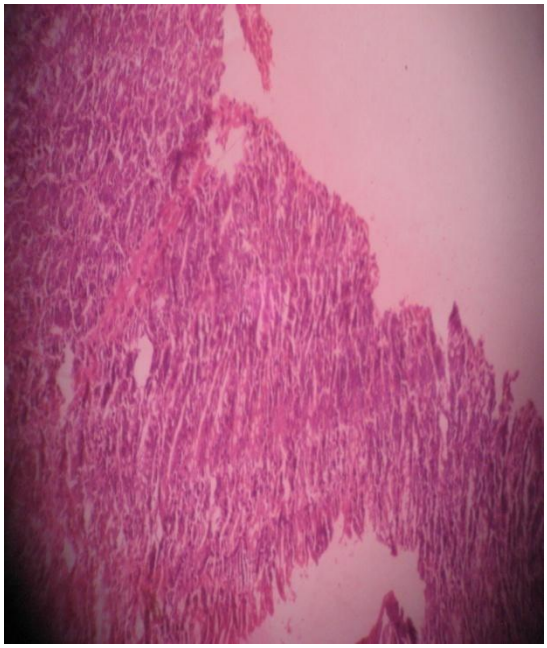


ChloroD6 Art D6

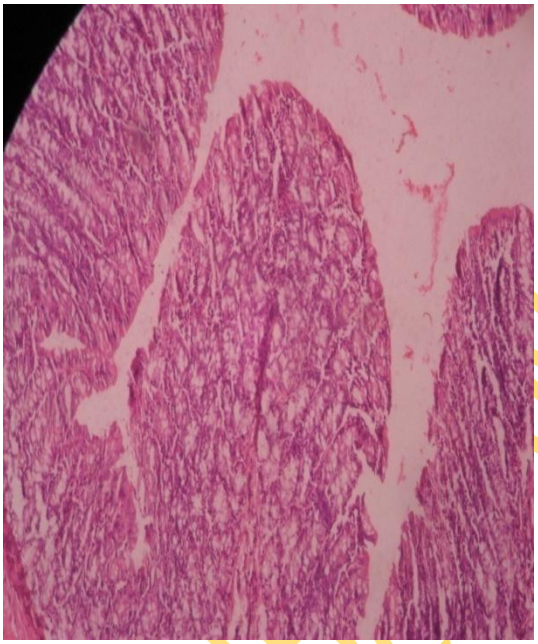


Control D6 Lonart D6

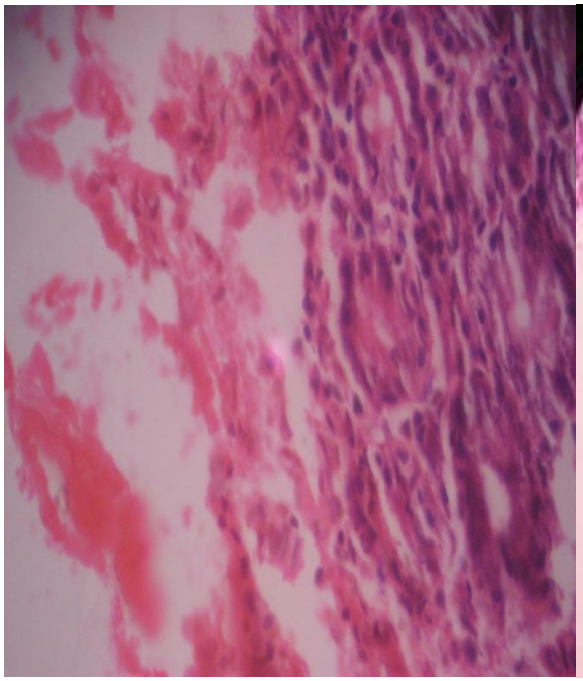
Plate 1: Photomicrograph of control and treated rats on day six, showing marked colitis, hemorrhage and lots of inflammatory cells. X 100 (H&E)



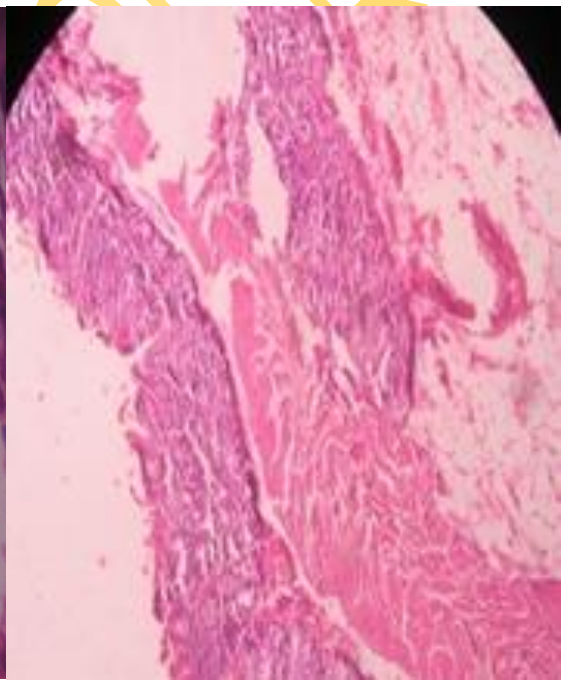
Control D12



Art D12



Chloro D12



Lonart D12

Plate 2: Photomicrograph of control and treated rats on day twelve, colitis completely healed in the artesunate group while the other groups are at different stages of healing. X 100 (H&E)

CHAPTER FIVE

DISCUSSION AND CONCLUSION

In this study, the effect of artesunate, chloroquine and artemether/ lumefantrine combination (Lonart^{R}) on experimental colitis was analyzed. This was accomplished by pre – treatment with the antimalarial drugs after which colitis was induced and studied for a period of twelve days during which various studies were carried out.

Ulcerative colitis is an autoimmune disease associated with altered immunological response, genetic susceptibility and intestinal microflora disorders. The main clinical manifestations are abdominal pain, diarrhea, mucous blood and purulent stool, recurrent attacks and relapse. Pathologic activation of the mucosal immune system in response to antigens is a key factor in the pathogenesis of ulcerative colitis with changes in leukocyte migration and cytokine production appearing to contribute to the perpetuation (Neurath and Schurmann, 2000).

The 6% acetic acid model of induction of colitis was used according to the range used by Fabia *et al.*,1992. This resulted in severe, deep colitis and high infiltration of inflammatory aggregates. This supports the report of Lowe and Noronha – Blob (1993) in which intracolonic administration of dilute acetic acid caused intense inflammation of the colon, neutrophil infiltration, hemorrhage, necrosis and denuding of the epithelium. However, there was no mortality which is in contrast to the study by Fabia *et al.,* 1992probably because 1ml of 6% of acetic acid was used. The stool consistency was studied over the following twelve days to monitor the healing process of the induced colitis since abdominal pain and bloody diarrhea are the commonest warning signs and the symptoms range from infrequent and mild to persistent and severe.

In the present study, diarrhea as evidenced indirectly by perianal fur soiling was prominent among the colitic animals. The incidence of diarrhea was used as an indicator of the effect of the tested drugs on colitis induced by acetic acid in rats. The chloroquine and the arthemeter/ lumefantrine treated groups showed significantly worse mucus and bloody diarrhea than the artesunate treated groupthroughout the period of the experiment. The study revealed that peak diarrhea score was lowest in the artesunate treated group while the highest score was seen in the chloroquine treated group.

The protein concentration in the colon was determined regularly throughout the period of the study. Control animals had the lowest colonic protein concentration which was significantly different from the treated groups. The artesunate treated group had the highest colonic protein concentration until day nine when significantly higher colonic protein was recorded in the chloroquine and arthemeter/ lumefantrine groups. This was maintained till the end of the study. According to Haapamaki *et al.*,1999, colonic protein increase is closely associated with the inflammatory activity of ulcerative colitis. This means the increased colonic protein concentration seen in chloroquine and arthemeter/ lumefantrine (Lonart^{R}) treated groups in this study were due to the treatment with the antimalarial drugs because low protein is present in the colon.This suggests that chloroquine and arthemeter/ lumefantrine combination triggered more inflammation of the induced colitis.This finding supported higher inflammatory activity in these groups which correlated well with their degree of histological inflammation since higher colonic protein is related to severe inflammation (Haapamaki *et al.*,1999). Elevated C- reactive protein concentrations as a marker of systemic low grade inflammation are related to a higher risk of colon cancer (Aleksandrova*et al.*,2010). The most feared longterm complication of ulcerative

colitis is cancer of the colon or long standing ulcerative colitis exposes patients to the risk of colorectal cancer (Eaden *et al.*,2001, Garrity – Park*et al.*, 2012).

Reactive oxygen metabolites are potent inflammatory mediators that may be involved in tissue injury in inflammatory bowel disease (Keshavarzian *et al.*,1990). Reactive oxygen metabolites are generated intracellularly by either NADPH oxidase in the neutrophils or by xanthine oxidase present in the gut mucosa (Weiss, 1989). Reactive oxygen species (ROS) generation is a normal component of oxidative phosphorylation and plays a role in normal redox control of physiological signaling pathways (Murdoch *et al.*,2006, Giordano, 2005). However, excessive ROS generation triggers cell dysfunction, lipid peroxidation and DNA mutagenesis and can lead to irreversible cell damage or death (Murdoch *et al.*,2006, Sawyer *et al.*,2002). ROS are small oxygen based molecules that are highly reactive because of unpaired electrons (Papa and Skulachev, 1997). The most prominent ROS are the superoxide anion ($O2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl ion (OH^-) (Turner and Lysiak, 2008). Severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis while more intense stresses may cause necrosis (Lennon *et al.*,1991). Reactive oxygen specieshas been implicated as a major cause of tissue injury in ulcerative colitis and a possible mechanism of the ROS mediated tissue injury is lipid peroxidation (Sedghi *et al.*,1994). Chloroquine had the highest malondialdehyde (MDA) concentration and since high level of MDA in colitis is an indication of oxidative stress (Mohammed *et al.*,2007). This study agrees with the finding of Toler and Steven (2004) that chloroquine induces oxidative stress both in man and animals. The value of MDA in the artemether/ lumefantrine (Lonart^R) group also was very high and similar to chloroquine throughout the study, this agrees with the work of Otuechere *et al.*,2012 in which artemether/ lumefantrine was found to increase lipid peroxidation. However artesunate on the other hand had

the lowest concentration of MDA throughout the study. This disagrees with the finding of Otuechere *et al.,* (2012) in which artesunate – amodiaquine combination increased lipid peroxidation. The discrepancy in this study and the finding of Otuechere *et al.,*(2012)might be as a result of combination with amodiaquine because amodiaquine alone has been reported to worsen lipid peroxidation (Farombi *et al.,*2001). In this study, chloroquine and arthemeter/ lumefantrine may have acted indirectly through generation of high levels of ROS or directly as toxins to the cells of the colon affecting the cellular integrity and causing defect in membrane permeability and cell homeostasis.

Increased neutrophil infiltration in inflammatory mucosa has been severally reported (Otamiri *et al.,*1988). Neutrophils are among the first cell type to arrive at a site of inflammation. In ulcerative colitis, neutrophil activation, migration and degranulation are important effector mechanisms of intestinal damage (Verspaget *et al.,*1988). Neutrophil have been implicated to be the major effector cells of tissue injury in both human inflammatory bowel diseases (Dallegri, 1990) and animal models of colitis (Wallace *et al.,*1992). Disease activity in ulcerative colitis is linked to an influx of neutrophils in the mucosa and subsequently in the intestinal lumen, resulting in the formation of crypt abscesses. Circulating and activating neutrophils, a major source of inflammatory cytokines are elevated in ulcerative colitis patients (Cui *et al.,*2010). The tissue injury produced by these cells has been attributed to their ability to liberate a variety of reactive oxygen metabolites (Wallace *et al.,*1992) and to the distribution of epithelial barrier function when these cells migrate into the lumen. Ulcerative colitis is characterised by presence of neutrophil in association with epithelial damage (Goldman, 1991). Such infiltration might be regarded as a trigger of free radical release. Increased production of free radicals and impaired antioxidant defense mechanisms are postulated to be causative factors in inflammatory diseases

(Han and Meydani, 2000). In active ulcerative colitis, the lamina propria of the mucosa is heavily infiltrated with mixture of acute and chronic inflammatory cells. The cellular infiltrate in the lamina propria is homogenously increased and mixed in composition with predominance of lymphocytes and plasma cells (Neer and Appleman, 1998).

This present study displayed all the most consistent variables in the histology of ulcerative colitis as stated by Geboes *et al.*,(2000) which include location of neutrophils in the lamina propria or between the epithelial cells, occurrence of crypt destruction and presence of erosion or ulcers (Geboes *et al.*,2000).

Accumulation of inflammatory cells seen in the chloroquine and lonart groups caused the increased reactive oxygen species indicating the high lipid peroxidation. This agree with the finding of Robinson *et al.*,(1997) in which mucosal injury in ulcerative colitis is said to involve enhanced migration and activation of neutrophils which are known to be pro- inflammatory in ulcerative colitis.

Conclusion

Pretreatment with artesunate attenuated diarrhea and microscopic colonic damage. It also reduced the lipid peroxidation and concentration of formed protein even in colitis. On the other hand, pretreatment with chloroquine and arthemeter/lumefantrine triggered more colonic damage and increased the release of reactive oxygen species by increasing the rate of oxidative stress in colitis.

This study suggests that pretreatment with artesunate protect against formation of colitis and also hastened the healing of acetic acid induced colitis while chloroquine and arthemeter/ lumefantrine (lonart^{R}) did not prevent colitis formation and also delayed the healing process.

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APPENDIX

Table 4.1a: Mean Weight of rats.

DAY O INITIAL WEIGHT

ANIMAL		CONTROL	ARTE	CHLORO	ART/LUM
	1	170	180	150	140
	2	190	180	160	170
	3	200	200	150	180
	4	160	160	130	160
	5	180	150	180	200
	6	170	160	190	170
	7	160	220	170	160
	8	170	190	190	180
MEAN		175	180	165	170
S.D		14.14214	23.29929	21.3809	17.72811
SEM		4.997221	8.232966	7.555088	6.264348

DAY 3

ANIMAL		CONTROL	ARTE	CHLORO	ART/LUM
	1	170	180	140	130
	2	180	180	140	170
	3	190	170	140	170
	4	160	160	120	160
	5	170	150	150	150
	6	160	160	180	170
	7	160	210	160	160
	8	170	190	170	160
MEAN		170	175	150	158.75
SD		10.69045	19.27248	19.27248	13.56203
SEM		3.777544	6.810064	6.810064	4.792236

DAY 6

ANIMAL	CONTROL	ARTE	CHLORO	ART/LUM
1	170	180	130	130
2	180	180	140	170
3	180	180	140	170
4	160	160	120	160
5	170	150	150	150
6	160	160	180	170
7	160	210	160	160
8	170	190	160	160
MEAN	168.75	176.25	147.5	158.75
SD	8.34523	19.2261	19.08627	13.56203
SEM	2.948844	6.793674	6.744265	4.792236

DAY 9

ANIMAL	CONTROL	ARTE	CHLORO	ART/LUM
1	170	180	130	130
2	180	190	150	170
3	180	180	140	170
4	170	160	120	160
5	170	160	150	150
6	160	160	180	170
7	160	210	170	170
8	170	190	160	160
MEAN	170	178.75	150	160
SD	7.559289	18.07722	20	14.14214
SEM	2.671127	6.387709	7.067138	4.997221

DAY 12	CONTROL			
ANIMAL		ARTE	CHLORO	ART/LUM
1	170	180	130	130
2	190	200	150	170
3	190	180	140	170
4	170	170	130	160
5	170	170	150	150
6	170	160	180	170
7	160	210	170	170
8	170	190	170	170
MEAN	173.75	182.5	152.5	161.25
SD	10.6066	16.69046	19.08627	14.57738
SEM	3.747916	5.897689	6.744265	5.151018

Diarrhea Scoring		DAY ONE		
Animal	Control	Artesunat	Chloro	Lonart
1	2	2	2	2
2	2	2	2	2
3	2	2	2	2
4	3	0	4	2
5	2	2	3	2
6	3	0	4	0
7	2	2	4	2
8	3	2	2	3
9	2	2	4	4
10	3	2	4	2
Mean	2.4	1.6	3.1	2.1
SD	0.516398	0.843274	0.994429	0.994429
SEM	0.163299	0.266667	0.314466	0.314466

Diarrhea Scoring		DAY THREE		
Animal	Control	Artesunat	Chloro	Lonart
1	3	0	0	3
2	2	0	3	3
3	2	2	2	2
4	2	0	2	0
5	3	3	2	2
6	2	0	2	3
7	2	2	2	0
8	2	2	2	2
9	2	2	3	4
10	3	1	3	2
Mean	2.3	1.2	2.1	2.1
SD	0.483046	1.135292	0.875595	1.286684
SEM	0.152753	0.359011	0.276887	0.406885

Diarrhea Scoring		DAY NINE		
Animal	Control	Artesunat	Chloro	Lonart
1	2	2	2	2
2	0	2	2	2
3	2	0	2	2
4	2	0	2	0
5	2	0	2	2
6	2	2	0	2
Mean	1.666667	1	1.666667	1.666667
SD	0.816497	1.095445	0.816497	0.816497
SEM	0.333333	0.447214	0.333333	0.333333

Diarrhea Scoring		DAY TWELVE		
Animal	Control	Artesunat	Chloro	Lonart
1	2	2	2	2
2	0	0	2	2
3	2	0	2	2
4	2	2	2	0
5	0	0	2	2
Mean	1.2	0.8	2	1.6
SD	1.095445	1.095445	0	0.894427
SEM	0.489898	0.489898	0	0.4

PROTEIN
DETERMINATION

CONT	CONC	ART	CONC	CHLORO	CONC		CONC
c13	0.561514	A13	1.287066	CH13	0.620121	LON13	0.520121
c23	0.618297	A23	0.700315	CH23	0.588506	LON23	0.458506
c33	0.587269	A33	0.987215	CH33	0.631461	LON33	0.530461
c34	0.587151	A34	0.989713	CH34	0.590991	LON34	0.49099
MEAN	0.588558	MEAN	0.991077	MEAN	0.60777	MEAN	0.50002
SD	0.023234	SD	0.239561	SD	0.021342	SD	0.03233

PROTEIN DETERMINATION DAY 6

c16	0.598914	A16	0.850016	CH16	0.728361
c26	0.629237	A26	0.796054	CH26	0.750132
c36	0.589914	A36	0.789945	CH36	0.669981
c46	0.609131	A46	0.850478	CH46	0.731785
MEAN	0.606799	MEAN	0.821623	MEAN	0.720065
SD	0.016894	SD	0.033146	SD	0.03473
SEM	0.008447	SEM	0.016573	SEM	0.017365

PROTEIN DETERMINATION DAY 9

C19		0.545589	A19		1.350158	CH19		1.343849
C29		0.561524	A29		0.971609	CH29		11. 1041
C39		0.574657	A39		1.351714	CH39		1.219756
C49		0.539897	A49		0.958814	CH49		1.237129
	MEAN	0.555417		MEAN	1.158074		MEAN	1.227786
	SD	0.015758		SD	0.22276		SD	0.095568