CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

The structure and function of the kidneys

The kidney were two small retoperitoneal organs and located roughly between T12 and L3 [25]. The section of a kidney reveals three distinct regions called the pelvis, medulla, and cortex. The renal pelvis is the large collecting space within the kidney, formed from the expanded upper portion of the ureter. It connects the structures of the medulla with the ureter. The pelvis branches into two smaller cavities, the major calyces and minor calyces. The renal medulla is the middle portion of the kidney. It consists of renal pyramids, which are cone shaped areas. The base of each pyramid is adjacent to the outer cortex. The apex of each renal pyramid ends in the papilla, which opens into a minor calyx. Renal pyramid consist of tubules and collecting ducts of the nephrons. The tubules of the pyramids are involved with the reabsorption of filtered materials. Urine passes from the collecting ducts in the pyramid to the minor calyces, major calyces, and renal pelvis. From there, the urine drains into the ureter and is transported to the urinary bladder. The renal cortex is the outer portion of the kidney. It is divided into two regions, the outer cortical and the inner juxtamedullary. Within the cortex, the granular appearance is caused by spherical bundles of capillaries and associated structures of the nephron that help to filter blood [25] as shown in Figure 1.

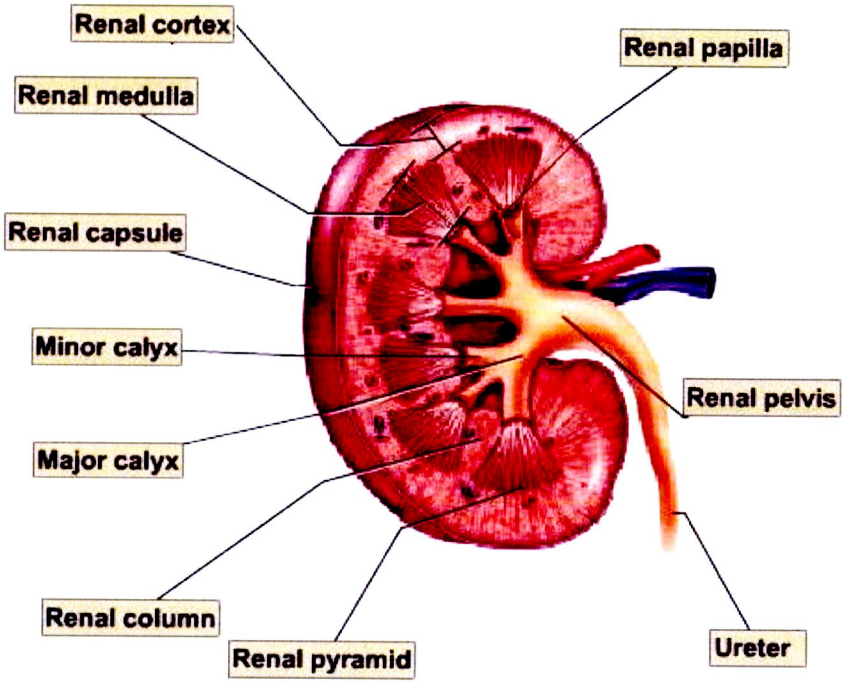

Figure 1 The Structure of the kidney

Source: www.comprehensive-kidney-facts.com/kidney-anatomy.html

The kidneys receive more blood in proportion to their weight than any other organ in the body. About 20 to 25 percent of cardiac output goes to the kidneys [26]. Approximately 1200 ml of blood per minute, and the body's entire blood volume (4L to 6L) is filtered through the kidneys about 340 times a day [25]. Blood comes to the kidneys directly from the abdominal aorta through the renal artery. As the renal artery enters the renal sinus, it provides blood to the segmental arteries. Segmental arteries further divide into a series of interlobar arteries that radiate outward, penetrating the renal capsule and extending through the renal columns between the renal pyramids. The interlobular arteries reach the juncture of the cortex and medulla, they turn and run parallel to the bases of the renal pyramids. At the turning point, the arteries are the arcuate arteries, which make small arcs around the boundary between the cortex and medulla. The arcuate arteries branch further into the interlobular arteries, which ascent into the cortex to supply the renal corpuscle (the renal capsule and glomerulus). The

branching produces numerous small afferent arterioles, which carry blood to the site of the filtration (the glomerulus). Each nephron receives one afferent arteriole, which divides into a tangled and branches extensively to from a ball- shaped capillary called the glomerulus. This capillary bed is where the blood is filtered. Glomerular capillary loops join together to form an efferent arteriole that drains blood out of the glomerulus. The afferent - efferent arteriole situation is unique because blood usually flows out of capillaries into venules and not into other arterioles. The glomeruli are part of the cardiovascular system as well as the urinary system. The efferent arterioles eventually branches to from a network of capillaries, call the peritubular capillaries, which surround tubular portions of the nephron in the renal cortex. These capillaries reabsorb some of the water and solutes that were filtered from the blood in the glomerulus. Extending from some efferent arterioles are long loop-shaped capillaries calls vasa recta, which supply tubular portion of the nephron in the renal medulla. The peritubular capillaries unite to form the interlobular veins, which carry blood out of the cortex to the arcuate veins. The small arcuate veins join to form the larger interlobar veins in the renal columns, and the interlobar veins eventually come together to form the single renal vein as shown in figure 2 that to cleansed blood from each kidney to the interior vena cava and into the circulatory system [27].

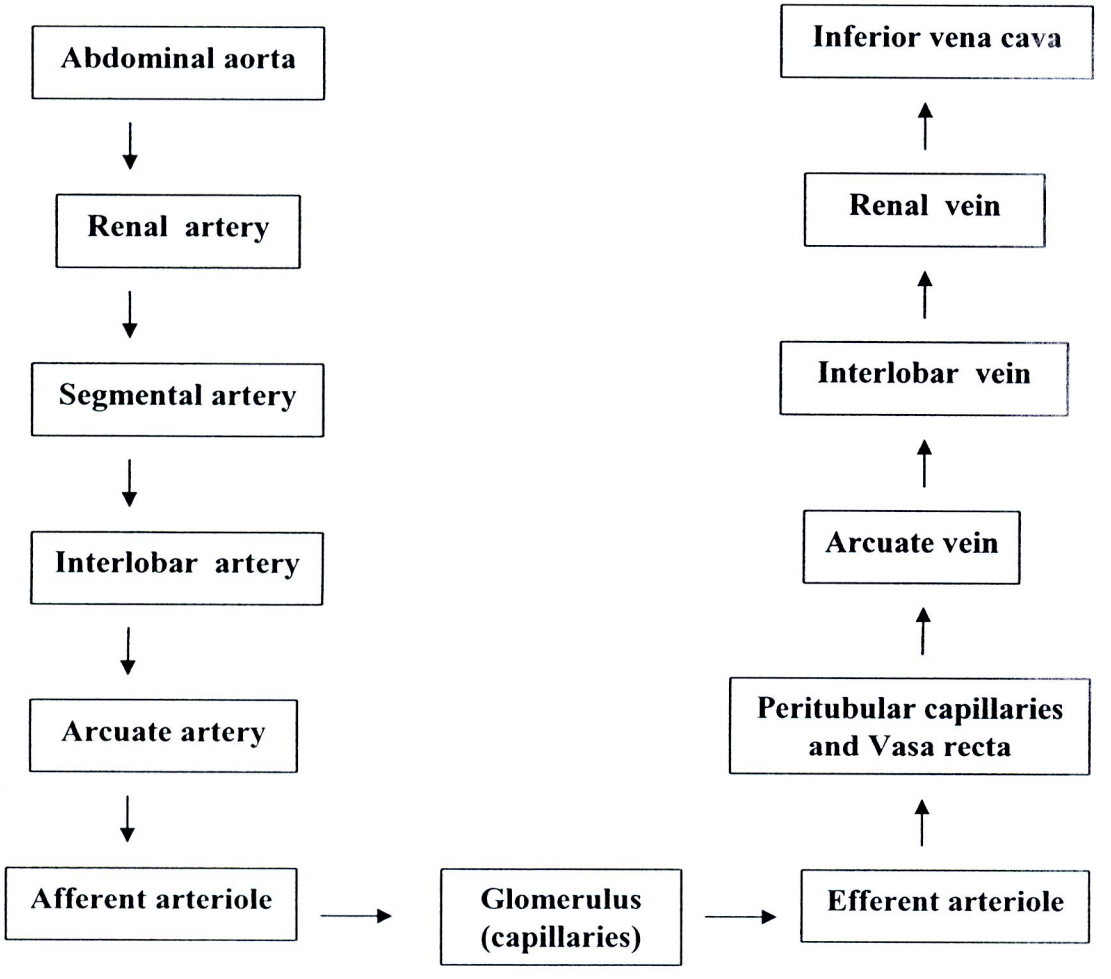

Figure 2 The flow chart summarizes the pattern of renal circulation

Source: www.usi.edu/science/biology/mkho.

Kidney composed two major portions 1) cortex: The renal cortex is the outer layer of the kidney in connect with the capsule and under the fibrous capsule a 90% of renal blood flow goes to the cortex, most highly perfused tissue per gram of any organ 2) Medulla: The medulla is consists of 6 to 18 distinct conical or triangular structure called renal pyramids. The base of each pyramid faces the cortex and the tip or renal papilla, projects into the renal sinus. Each pyramid has a series of fine grooves that converge at the papilla adjacent renal pyramids are separated by bands of cortical tissue, called renal columns. The columns have a distinctly granular texture, similar to

that of the cortex. A renal lobe contains a renal pyramid, the overlying area of renal cortex and adjacent tissues of the renal columns. The function units of the kidneys are the nephrons, which have three function-filtration, secretion, and reabsorption. The filtration, some substances are permitted to pass from the blood into the nephrons, while other are kept out. Then, as the filtered liquid moves through the nephrons, it gains some additional materials (wastes and excess substances) is call secretion. Other substances are returned to the blood this is reabsorption [27].

The nephrons have two types, cortical and juxtamedullary [26] [Figure 3]. The tubular structures of the cortical nephron the loop of Henle extend only into the base of the renal pyramid of the medulla. About 80-85% of nephrons have short loops of Henle that penetrate only into the superficial region of the renal medulla. These neprons usually have glomeruli in the superficial region of the renal cortex. They receive their blood supply from peritubular capillaries that arise from efferent arterioles. While the longer loop of the the nephron of the juxtamedullary nephron projects deep into the renal pyramid. The remaining 15-20% were juxtamedullary nephron. They receive their blood supply from peritubular capillaries and vasa recta that arise from efferent arterioles. Cortical nephrons are about seven times more numerous than juxtamedullary nephrons [25].

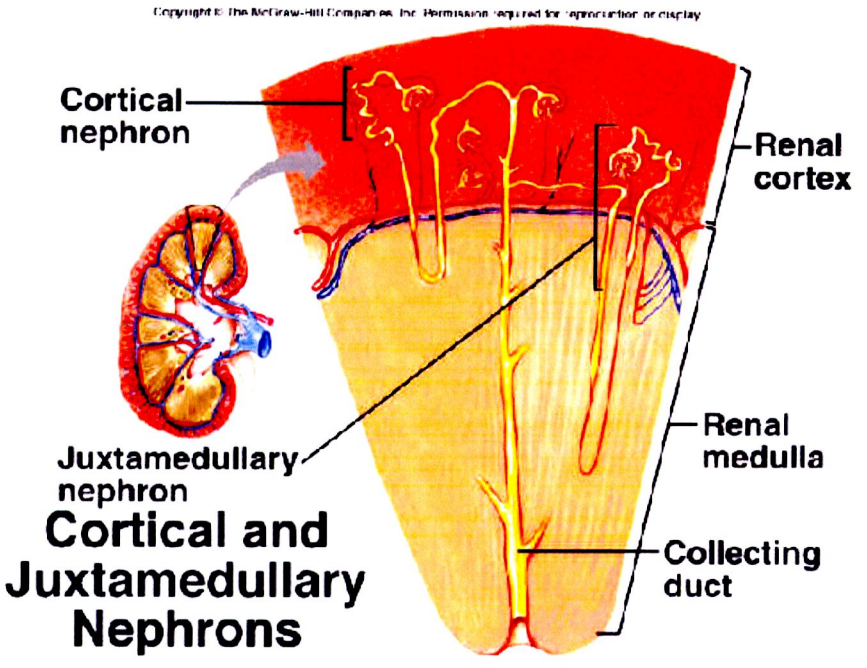

Figure 3 The Structure of cortical and juxtamedullary nephrons

Source: www.flashcardmachine.com/bio-120-2.html

A nephron consists of (1) a renal corpuscle, which the filter of plasma and (2) a renal tubule, which the filtered fluid passes [Figure 4].

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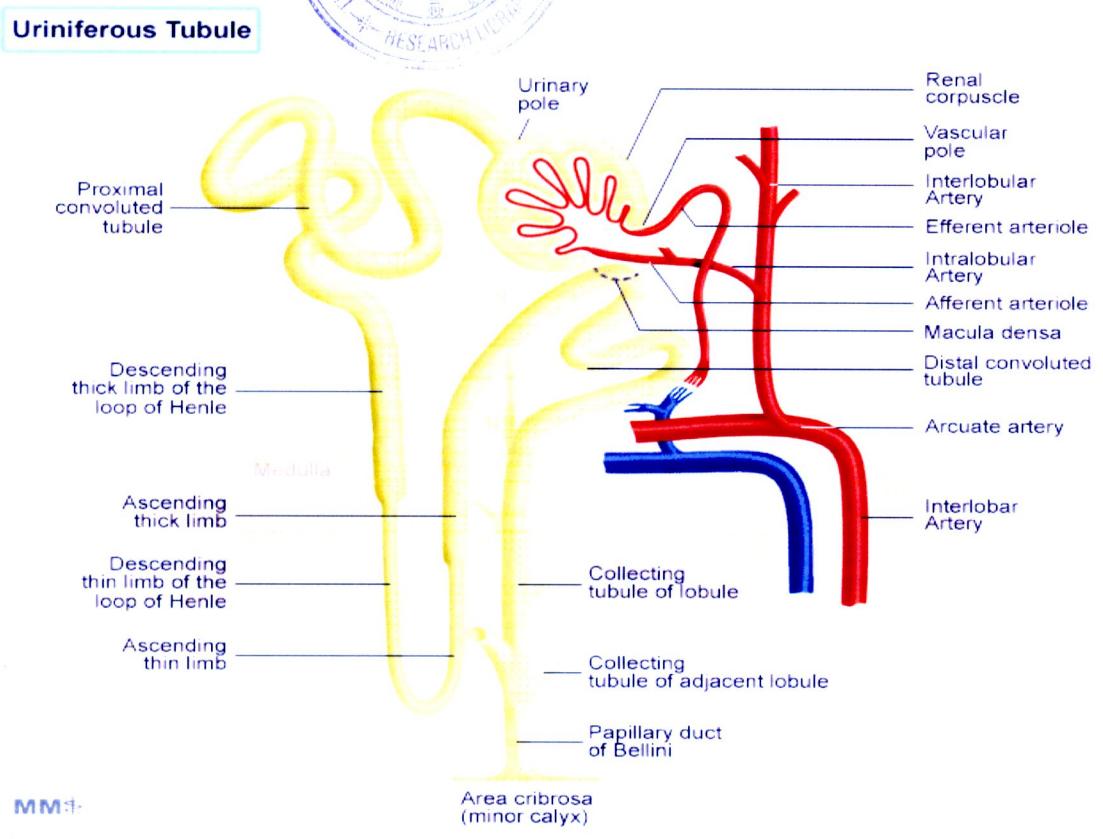

Figure 4 The uriniferous tubule and its vascular supply and drainage

Source: www.alexandria.healthlibrary.ca/documents/notes/bom/unit_2/L45%20Renal%20System.xml

A renal corpuscle has two components – a tuft of capillary loops, called the glomerulus, and the double walled epithelial cup that surrounds the glomerlus, called the glomerular (Bowman's) capsule [Figure 5]. Blood enters a glomerulus through an afferent arteriole and exits through an efferent arteriole.

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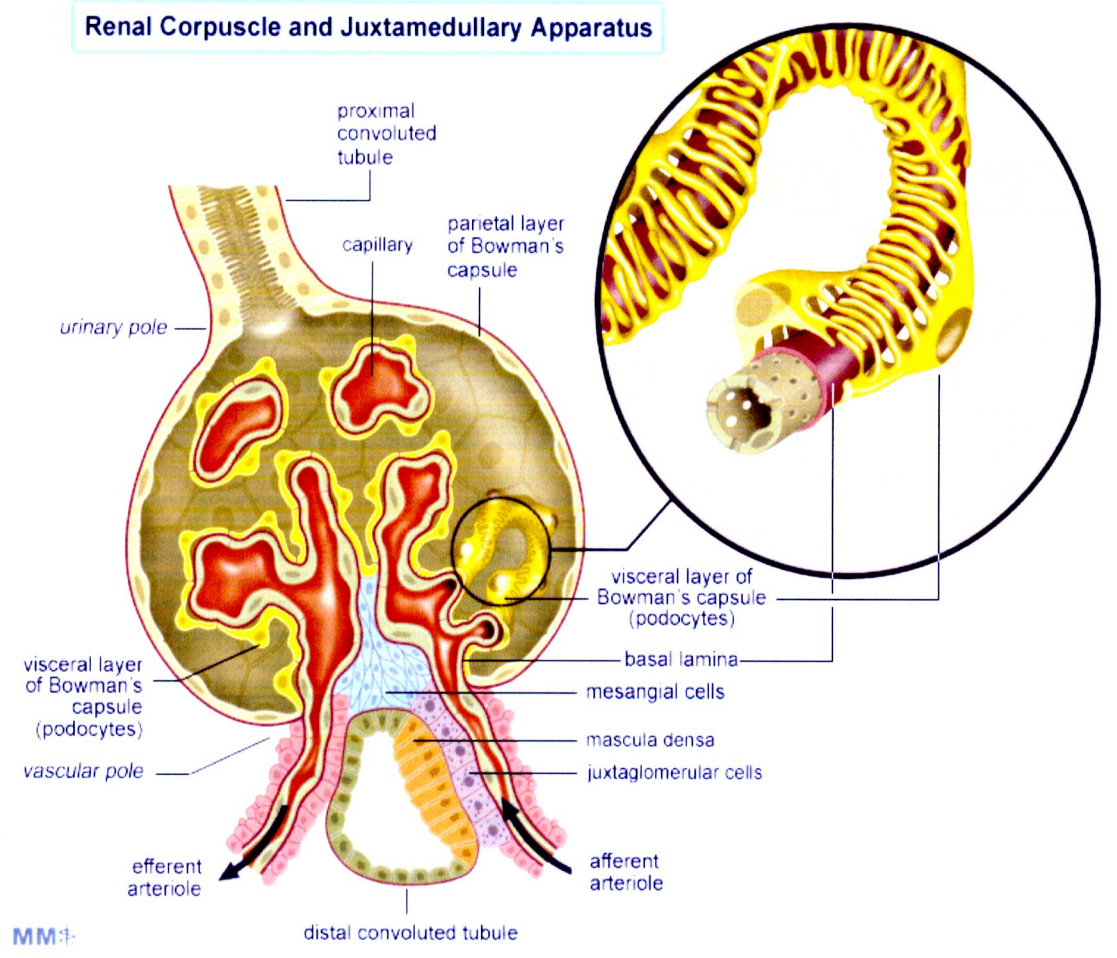

Figure 5 Diagram of renal corpuscle and juxtamedullary apparatus

Source: www.alexandria.healthlibrary.ca/documents/notes/bom/unit_2/L45%20Renal%20System.xml

The outer wall of the Bowman capsule is separated from the inner wall by the capsular (Bowman's) space. As blood flows through the glomerular capillaries, water and most kinds of solutes filter from blood plasma into the capsular space. Large plasma proteins and the formed elements in blood do not normally pass through. From the capsular space, filtered fluid passes into the renal tubule.

The renal tubule consists of a (1) proximal convoluted tubule (PCT), (2) Loop of Henle (nephron loop), and (3) distal convoluted tubule (DCT). Convoluted means the tubule is coiled rather than straight. Proximal tubule sinifies the tubule portion

attached to the glomerular capsule, and distal tubule the portion that is farther away. The renal corpuscle and both convoluted tubules lie in the renal cortex of the kidney, whereas the loop of Henle extends in to the renal medulla, makes a hairpin turn, then returns to the renal cortex. The distal convoluted tubules of several nephrons empty into a single collecting duct. Within a renal lobe, a collecting duct and all the nephrons that drain into it constitute a renal lobule. Collecting ducts, in turn, unite and converge until eventually there are only several hundred large papillary ducts, which drain into minor calyces. The collecting ducts and papillary ducts stretch from the renal cortex through the renal medulla to the renal pelvis. Each kidney has about 1 million nephrons but a much smaller number of collecting ducts and even fewer papillary ducts [27].

The kidney nephrons formed urine by using three process: 1) filtration by the glomerulus, 2) reabsorption with the renal tubule, and 3) secretion by the tubular cells. The filtration is the first step in urine formation. In this process, blood from form the renal artery enters the smaller afferent arteriole, enters the even smaller capillaries of the glomerulus, blood pressure is about 25 mm/Hg in the body and in the glomerulus has been blood pressure between 60-90 mm/Hg. The high blood pressure forces are plasma fluid to filter from the blood in the glomerulus into bowman's capsule. This fluid is called the filtrate which consists of water, glucose, amino acids, some salts, and urea. The filtrate does not contain plasma protein or red blood cell because they are large to pass the blood in a single minute. The Bowman's capsule filters out of 125 ml of fluid from blood in single minute and 1 hour 7500 ml of filtrate leave the blood, so amount 1800 ml in 24 hour period. The seconed step in urine formation: reabsorption in this process includes of useful substances form the filtrate within the renal tubules in to the capillaries around the tubules. These include water, glucose, amino acid, vitamins and bicarbonate ions (HCO_3^-), and the chloride salts of calcium, magnesium, sodium, and potassium. Reabsorption starts in the proximal convoluted tubules continues through the loop of Henle, distal convoluted tubule, and the collecting tubules. The term used to be describe the limit of reabsorption is the threshold. And the last step in urine formation process: secretion in this process is the opposite of reabsorption. Some substances are actively secreted into the tubule. Secretion transports substances from the blood in the peritubular capillaries into the

urine in the distal and collecting tubules [10]. So the function of the kidney is of filter the plasma, selectively reabsorption solutes and water in order to maintain internal homeostasis, and secrete metabolic waste products and toxin [28].

Principle of Renal pathophysiology

Renal injury can be temporally characterized as either acute or chronic. Each has a distinctive clinical expression.

Acute renal failure [25] is the total or near- total stoppage of kidney function. Little or no urine is produced, and substances that are normally eliminated from the body are retained. It is often caused by a diminished blood supply to the kidney, which may be brought on by a serious blood loss due to an injury or hemorrhage, a heart attack, or a thrombosis. Another common cause of acute renal failure is a high level of toxin materials, such as mercury, arsenic, carbon tetrachloride, and insecticides, which build up in the kidneys. And damage to the kidneys themselves. Recovery usually takes from 7 to 10 day and can be assisted by dialysis. Athough complete recovery is not uncommon, residual kidney damage may lead to chronic renal failure [28].

Chronic renal failure develops slowly and progresses over many years. Its most common causes are bacterial inflammation of the interstitial area and renal pelvis, renal imflammation involving the structures around the renal pelvis or glomeruli, and renal damage due to high blood pressure or obstructions in the lower urinary tract [25]. The condition is characterized by progressive destruction of nephrons, which may lead to reduced amounts of urine, dilute urine, thirstiness, severe high blood pressure, poor appetite and vomiting, frequent urination, depletion of bone calcium, coma, and convulsion. A low protein diet is usually prescribed, and dialysis may be necessary in some case [28].

The Anatomy of renal failure

The causes of renal failure, whether acute or chronic can be conveniently compartmentalized as pre-renal, renal and, post-renal. Pre-renal causes of uremia center upon the renal vasculature and its perfusion. Impaired cadiac contractility or severe vasoconstriction of the arteries can result in a decrease or even a cessation of glomerular filtration and urine formation. Persistent poor renal perfusion because of

hypovolemia, vasoconstriction, or low cardiac output may result in oliguria and azotemia. Acute pathophysiology of the renal vasculature includes embolism or thrombosis with subsequent renal infarction. Renal causes of uremia center upon the renal vasculature and its perfusion. When a sufficiently large proportion of nephrons is involved by this process, the glomerular filtration rate falls to critical levels, and renal function fails. Post-renal causes of kidney failure involve occlusion of the urinary tract beyond the distal nephron. Obstruction can occur in the renal pelvis, throughout the cause of the ureter, in the bladder, or in the urethra [29].

The main function of the kidneys is to remove waste from the blood and return the cleaned blood back to the body [10]. Kidney failure means the kidneys are no longer able to remove waste and maintain the level of fluid and salts that the body needs [29]. One cause of kidney failure is diabetes mellitus, a condition characterized by high blood glucose (sugar) levels. Over time, the high levels of sugar in the blood damage the millions of tiny filtering units within each kidney. This eventually leads to kidney failure [1]. Around 20 to 30 % of people with diabetes are at a higher risk of developing kidney disease (diabetic nephropathy).

Diabetes mellitus is a common metabolic diseases characterized by high blood sugar (glucose) levels, that result from defects in insulin secretion or action or both, is characterized by hyperglycemia often accompanied by glycosuria, polyuria, and proteinuria . Insulin is a hormone produced in the pancreas, an organ near the stomach. Insulin is needed to turn sugar and other food into energy. Diabetes mellitus is caused by absolute or relative insulin deficiency, sometimes associated with insulin resistance, β-cell dysfunction, impaired glucose tolerance. Insulin resistance is an inability of some of the cells of the body to respond to insulin, especially by muscle and fat (adipose) tissues. Diabetic nephropathy (DN) is currently the leading cause of end stage renal disease (ESRD) in the United States and most other developed nation. Approximately 40 % of new patients who entered ESRD programs in the United States in 2003 had DN and the overall rate ESRD resulting from diabetes in the United States has risen 68% since 1992 [30]. Moreover even the earliest clinical detected stage of renal injury in diabetes (microalbuminuria) is associatedwith an increased prevalence of macrovascular disease [31].

In this regard, there is accumulating evidence to support a role for oxidative and carbonyl group in the pathogenesis of DN, as well as in other diabetic complications [32]. Diabetes is usually accompanied by increased production of free radicals or impaired antioxidant defenses. Mechanisms which increased oxidative stress is involved in the diabetic complications are partly known, including activation of transcription factors, advanced glycation end products (AGEs). Protein glycation and advanced glycation end products (AGEs) results from the formation of a covalent binding between the aldehyde glucose function and the free amino groups of protein. Glycation protein can give an electron to the molecular oxygen, leading to oxygenated free radical [16].

Marker of tubular damage

The marker of glomerular damage, creatinine and urea, were measure using commercial kits. As marker localized in the brush border of tubular damage, measured urinary excretion of NAG activity [12]. NAG activity was measured using p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate. N-acetyl-β-D-glucosaminide is a high molecular weight lysosomal enzyme. Then it cannot pass into glomerular and urinary NAG is of renal origin [33]. This enzyme are show high activity in renal proximal tubular cell, and leak into the tubular fluid. Urinary NAG activity to be useful early marker of renal injury such as nephrotoxicity, glomerular proteinuria, nephrolithiasis, hyperglycemia [12].

In our research, we use the modified method of the spectrophotometric assay for urine NAG activity [34]. Urine NAG activity was separated from urine added to an enzyme reaction mixture that consist of the substrate (p-nitrophenyl-N-acetyl-β-D-glucosaminide) in sodium citrate buffer (pH 4.4). Thus, incubation at 37 °C for 15 min. The reaction is stopped by add 2-amino-2-methyl-1-propanol (AMP) buffer (pH 10.25), and the reaction product is measured by spectrophotometry at 405 nm.

Role of free radical reactions and mechanism of injury [4]

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals. The reactivity of free radicals caused from the present of unpaired electrons. The unpaired electron being

one that is alone in an orbital, but electrons usually associate in pairs in orbitals of atoms and molecules. Then free radicals are generally more reactive than non-radicals due to their unpaired electron [15, 18, 25, 26, 27, 28]. Free radical will grasp electron from other molecules because they require another electron to fill the orbital and become stable [29]. The simplest free radical is an atom of the element hydrogen. The hydrogen atom contains one proton and a single unpaired electron, but different types of free radicals vary widely in their reactivity [15, 18, 25, 26, 27, 28]. The example of free radical are superoxide superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$). When the O_2 oxidized, it can be cause the formation of reactive oxygen species (ROS). Innordinate generation of reactive oxygen species (ROS) is widely incriminated in the pathogenesis of tissue injury [8]. Some of the $O_2^{\bullet -}$ production that occurs in vivo appears to be a chemical accident, due to autoxidation reactions and the "leak" of electrons from electron-transport chains to oxygen [8]. Excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids. Reactive oxygen species are formed continuously as consequence of biochemical reactions as well as external factors which cause lipid peroxidation of cell and organelle membranes and, disruption of the structural integrity and imbalance in cell capacity for cell transport and energy production, especially in the proximal tubule segment of kidney [3]. The superoxide dismutase (SOD) enzyme is an important physiological antioxidant defense mechanism in aerobic organism although too much SOD (in relation to the activities of H_2O_2 - removing enzymes such as catalase and glutathione peroxidases) may sometimes be deleterious.

The generation of free radical and antioxidant mechanism are shown in Figure 6. The superoxide anion radical is formed by the process of reduction of molecular oxygen mediayed by NADPH oxidases and xanthine oxidase or non enzymatically by redox- reactive compounds of the mitochondrial electron transport chain. Superoxide radical ($O_2^{\bullet -}$) is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2) and hydrogen peroxide is scavenged by the enzyme glutathione peroxidase (GPx) which requires GSH as the electron donor. The oxidized glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (Gred) which uses NADPH as the electron donor. Some transition metal e.g. ferrous iron (Fe^{2+}), Cu^{+} can breakdown hydrogen peroxide to the reactive hydroxyl radical

(Fenton reaction). The hydroxyl radical can abstract an electron from polyunsaturated fatty acid (LH) to give rise to a carbon- center lipid radical ($L^{\bullet}$) and lipid radical can interact with molecular oxygen to give the lipid peroxyl radical ($LOO^{\bullet}$). And lipid peroxyl radical ($LOO^{\bullet}$) is reduced within the membrane by the reduced form of Vitamin E resulting in the formation of lipid hydroperoxide and a radical of vitamin E. The generation of vitamin E by vitamin C (the vitamin E radical is reduced back to vitamin E by vitaminC) and GSH (the oxidized vitamin E radical is reduced by GSH). The lipid hydroperoxides are reduced to alcohols and dioxygen by GPx using GSH as the electron donor lipid peroxidation process and lipid hydroperoxides can react fast by Fe^{2+} to form lipid alkoxyl radical ($LO^{\bullet}$) or much slower with Fe^{3+} to form lipid peroxyl radicals ($LOO^{\bullet}$). If the result lipid peroxyl radical ($LOO^{\bullet}$) is not reduced by antioxidants, the peroxyl radical located in the internal position of the fatty acid can react by cyclisation to produce a cyclic peroxide adjacent to carbon center radical. This radical can then either be reduced to form a hydroperoxide. Formed compound is an intermediate product for the production of malondialdehyde (MDA) and it can react with DNA base Cytosine, ademine, and Guanine, respectively [4].

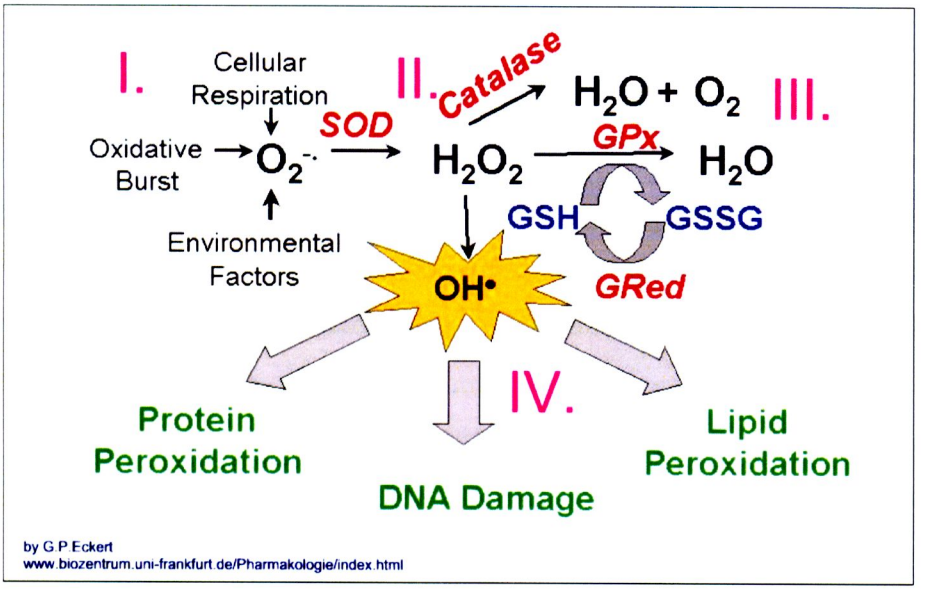

Figure 6 The pathway of free radical and antioxidant mechanism

Source: www.biozentrum.uni-frankfurt.de/...ethe.htm

ROS is incriminated in the pathogenesis of renal and forms of tissue injury include the superoxide anion, hydrogen peroxide, and the hydroxyl radical are recognized for their capacity to induce oxidant injury. The superoxide anion is generated as oxygen accepts a single electron and the dismutation of the superoxide ion yields hydrogen peroxide, the hydroxyl radical is generated when hydrogen peroxide react with the superoxide ion under the catalytic effect of the transition metal, iron. The hydroxyl ion is highly reactive dissipating itself and the immediate vicinity of its site of generation. Hydrogen peroxide lack such reactive, freely diffusing across lipid bilayers and thus instigation oxidant effects from the site of generation. In recent years numerous clinical and experimental studies focused on detection of signs of oxidative stress in renal patients [3]. There is good evidence indicating that ureamia in general is associated with enhanced oxidative stress.

Free radical and Oxidative Stress in renal failure patient

The kidney has an organ that can generate ROS and is vulnerable to the damage effect of ROS. The intact disease free kidney generates small amounts of ROS in the course of renal oxidative metabolism. Healthy kidneys comprise less than 1% of body weight, consume relatively large amounts of oxygen, accounting for some 10% of total consumption of oxygen by the body and oxygen is used in heightened by healthy kidney to sustain oxidative phosphorylation and synthesis of ATP, the latter needed in copious quantities for renal tubular transport process [18]. However, ROS produced excessively by endogenous or infiltrating cells in the injured kidney, can contribute to depending on the circumstance, acute renal injury and progressive renal damage [4].

Renal sources for ROS are activated macrophages, vascular cells and various glomerular cells. This effect plays a role in a variety of renal diseases contribute to the pathogenesis of ischemia reperfusion injury in the kidney such as glomerulonephritis and tubulointerstitial nephritis, which can contribute to proteinuria and other conditions. This suggests that an increase in oxidative stress is considered an important pathogenic mechanism in the development of ischemic and toxic renal tubular injury [18]. ROS also impair enzymatic and structural protein molecules affect cellular function and vitality. For example, peroxidation of lipids in plasma and

intracellular membranes perturbs membrane fluidity, permeability, and ion and solute transport, hydrogen peroxide compromises mitochondrial ATP synthesis by inhibiting the ATP- synthetase complex. These changes are by the elevation in intracellular calcium, disruption of the cytoskeleton, foam cell formation of the plasma membrane, and finally cell death. Augmentation in renal production of ROS may be derived from metabolic and other processes engendered in endogenous cells in the injured kidney, or from activated leukocytes in the kidney with ATN that reactive oxygen species contribute to post ischemic renal injury [3].

In health the relatively low amounts of reactive oxygen species (ROS) generated by the kidney are tolerated without any apparent adverse effects. However ROS produced excessively by endogenous or infiltrating cell in the injured kidney, can contribute to depending on the circumstance and acute renal injury or progressive renal damage. ROS commonly incriminated in the pathogenesis of renal and other forms of tissue injury include the superoxide anion, hydrogen peroxide and the hydroxyl radical. The more recently, nitric oxide and the peroxynitrite anion are recognized for their capacity to induce oxidant injury. The harmful effect of free radicals causing potential biological damage is termed oxidative stress and nitrosative stress [4]. Oxidative stress was results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of oxidant/antioxidant reactions in living organism. The excess ROS can damage cellular lipids, proteins, or DNA inhibiting their normal function. By products of lipid peroxidations such as conjugated dienes and malondialdehyde (thio barbituric acid reaction substance; TBARS) have been found to have increased in serum.

Oxidative stress induced lipid hydroperoxides and lipid peroxidation (LPO)

Hydroperoxides are recognized reactive oxygen species which are associated with oxidative stress, and are major products of these reaction with generated by mixture of superoxide superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) [35].

Lipid peroxidation (LPO) reactions are free radical driven chain reactions in which one radical can induce the oxidation of a comparatively large number of substrate, and it involves the oxidation of polyunsaturated fatty acids (PUFAs), which are basic biological membrane components. Many unsaturated components, mainly

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aldehydes, are then formed [36]. Fundamental reactions occurring during peroxidation are showing in figure 7.

The lipid peroxidation chain reaction has three stages; initiation of lipid peroxidation is caused by attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group ($-CH_2-$) upon a PUFA [7, 37, 38]. Species that can abstract the first hydrogen atom include the hydroxyl radical ($OH^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), and possibly $HO_2^{\bullet}$ but not H_2O_2 or O_2^{-} [42]. Since hydrogen atom in principle is a free radical with a single unpaired electron, its removal leaves behind an unpaired electron on the carbon atom to which it was originally attached. The carbon-center radical was stabilized by a molecular rearrangement to form a conjugated diene, followed by reaction with oxygen to give a peroxyl radical. Peroxyl radicals are capable of abstracting a hydrogen atom from another adjacent fatty acid side-chain to form a lipid hydroperoxide, but can also combine with each other or attack membrane proteins. When the peroxyl radical abstracts a hydrogen atom from a fatty acid, the new carbon-center radical can react with oxygen to form another peroxyl radical, and so the propagation of the chain reaction of lipid peroxidation can continue. A single substrate radical may result in conversion of multiple fatty acid side chains into lipid hydroperoxides [39]. The length of the propagation chain before termination depends on several factors e.g. the oxygen concentration and the amount of chain-breaking antioxidants present. Hydroperoxides are fairly stable molecules, but their decomposition can be stimulated by high temperatures or by exposure to transition metal ions (iron and copper ions). Decomposition of hydroperoxides generates a complex mixture of secondary lipid peroxidation products such as hydrocarbon gases (e.g. ethane and penthane) and aldehydes (e.g. malondialdehyde; MDA, and 4-hydroxynonenal).

The reduction of a peroxyl radical by another fatty acid molecule results in the formation of a new carbon-centered radical which propagates the fatty acid oxidation. In this way, an oxidized molecule can induce the oxidation of other fatty acid molecules. Approximately 60 linoleic acid molecules and 200 molecules of arachidonic acid are oxidized as the result of transformations initiated by one free-radical reaction [40]. The length of the free-radical reaction chain depends on many factors.

It is known that transition metal ions initiate free-radical reactions including LPO because they participate in generating reactive oxygen species ($O_2^{\cdot-}$, $HO^{\cdot}$). At the same time, they contribute to the propagation of the process by reducing lipid hydroperoxides [38]. These compounds are formed from fatty acids in various amounts depending on their structures and oxidation conditions.

Lipid peroxidation can proceed not only non enzymatic free radicals induced pathways, but also through processes that are enzymatically catalyzed [7, 37, 38]. Enzymatic lipid peroxidation may be referred only to the generation of lipid hydroperoxides achieved by insertion of an oxygen molecule at the active centre of an enzyme [7, 37, 38]. Free radicals are probably important intermediates in the enzymatically - catalyzed reaction, but are localized to the active sites of the enzyme cyclooxygenase (COX) and lipoxygenase and can produce enzymatic lipid peroxidation process. The hydroperoxides and endoperoxides produced from enzymatic lipid peroxidation become stereospecific and have important biological functions upon conversion to stable active compounds. Both enzymes are involved in the formation of eicosanoids, which comprise a large and complex family of biologically active lipids derived from PUFAs with 20 carbon atoms. Prostaglandins are formed by COX catalyzed the peroxidation of arachidonic acid. COX exists in at least two isoforms. COX-1 is present in cells under physiological conditions, whereas COX-2 is induced in macrophages, epithelial cells and fibroblasts by several inflammatory stimuli leading to release of prostaglandins.

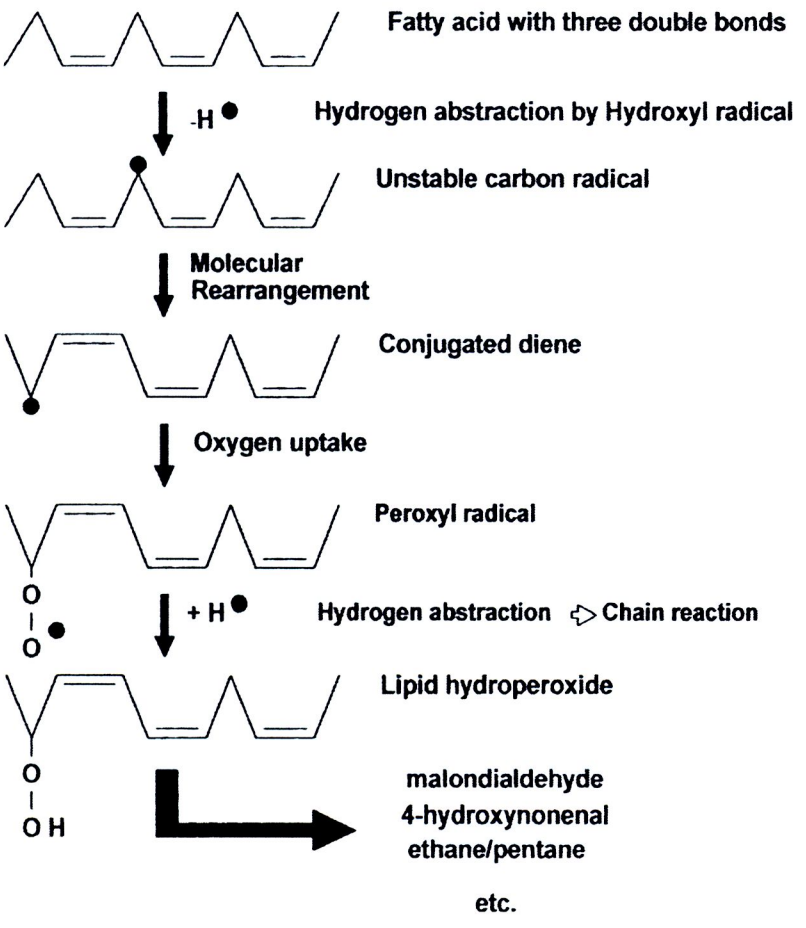

Figure 7 Basic reaction sequence of lipid peroxidation

Source: www.biochemsoctrans.org/bst/029/0358/bst0290358.htm

Oxidative stress can evaluate by measure oxidative stress biomarker from lipid, protein and DNA damaged. Lipid is an important compound of cell membrane. The damage of lipid cause cell lose of function [7, 37, 38] and lead to chronic illness. Lipid peroxidation was the mechanism of lipid damaged generated many biomarkers. Some examples of method for measurement of lipid peroxidation

Malondialdehyde (MDA)

Malondialdehyde is product from polyunsaturated fatty acids with three or more double bonds mainly arachidonic acid (20:4) and docosahexaenoic acid (22:6). Bicyclic peroxides with an oxygen bridge located inside the molecule are involved as

intermediates in the pathway of MDA formation; at higher temperatures or in an acid medium, they undergo decomposition to free MDA [41].

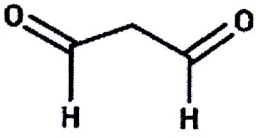

Figure 8 Malondialdehyde

Malonyldialdehyde (MDA) is a highly reactive three carbon dialdehyde produced as a by product of polyunsaturated fatty acid peroxidation [42]. MDA can combine with several functional groups on molecules including proteins, lipoproteins, RNA and, DNA. The measurement of MDA is a convenient and sensitive method for quantitative estimation of lipid peroxide concentration in many types of samples including drugs, food products and biological tissues from human and animal. The most common method of measuring MDA is based on the reaction with thiobarbituric acid (TBA) to form the thiobarbituric acid reactive substances (TBARS).

Thiobarbituric Acid Reactive Substances (TBARS) Assay

The TBARS assay was first introduced in 1944, and is now of the oldest and most frequency used methods for monitoring peroxidation of biological samples. The TBARS assay is still widely used. The success of this assay is due, in part, to the ease with which it can be performed. The reagents are inexpensive and easy to purchases, and most laboratories have the instrumentation of experiment today, it is oftentimes used in conjunction with another type of analysis such as chromatography, electrophoresis, fluorescence detection, other assays, or electron spin resonance [43]. The concentrations of MDA, one of the short aldehydes produce by lipid peroxidation, in a sample in believed to be proportional to the amount of oxidation. In the TBARS assay, MDA reacts with thiobarbituric acid (TBA) in an acidic environment to produce a red pigment. Under high temperature (100°C) and acidic condition, the reaction yields a pink MDA-TBA add the product of 2 ml of TBA plus 1 ml of MDA figure 9,

the color complex can be measured by spectrophotometry using wavelength 532 nm [44].

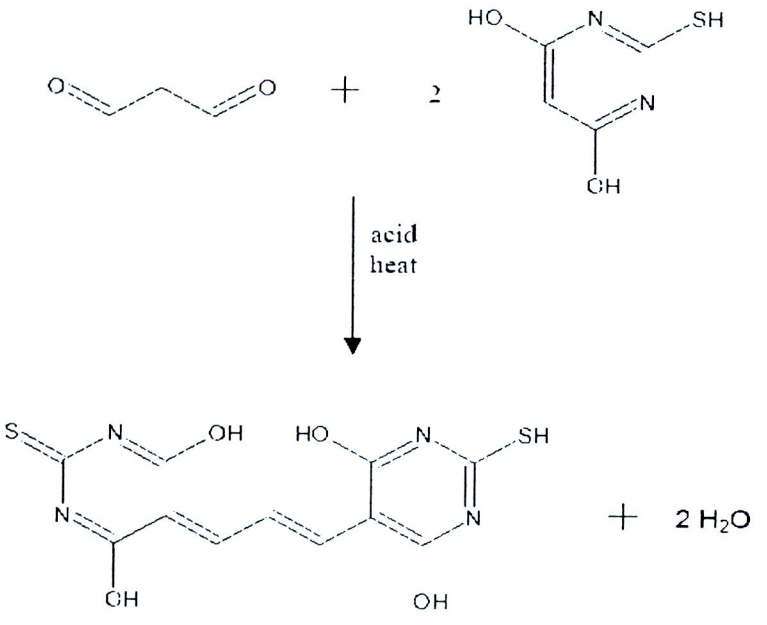

Figure 9 Formation of the TBARS pigment

Source: www.genprice.com/tbars.htm

Ferrous ion oxidation xylenol orange (FOX) assays for lipid hydroperoxide

Total hydroperoxides may also be determined using the ferrous oxidation in xylenol orange (FOX) assay, which can be used for hydroperoxides present in the aqueous (FOX1) and in the lipid (FOX2) phases [39]. The ferrous oxidation of xylenol (FOX) assay was developed by Wolff and co-workers to analyze the hydroperoxides formed [39]. The FOX method is based on the oxidation of ferrous (II) to ferric (III) ions by hydroperoxides under acidic conditions [39]. Ferric ions are detected by UV absorbance at 560 nm after reaction with the ferric ion indicator, xylenol orange, generating a blue-purple complex with an absorbance maximum at 550–600 nm.

1. Fe^{2+} + hydroperoxides → Fe^{3+} alkoxyl radical + OH–
2. Fe^{3+} + XO → blue-purple complex (550-600 nm)

In our research, we use the modified method of Tangvarasittichai S., et al. for measurement of MDA [45]. Briefly; a 50 µL of TEP standards, plasma specimens was

pipette in to the 13 ml polypropylene test tube. And 0.75 ml of phosphoric acid (0.44 mol/l) solution was added into the respective tubes and vortex-mix. Then 0.25 ml of TBA (42 mmol/l) was added to each tube. The test tube were capped tightly and heated at 100°C for 60 minutes after which the sample was cooled in an ice water bath (0°C). Then 100 % TCA was added and standby in ice water bath for 10 minutes. Last Distilled water (0.40 ml) was added to adjust the final volume to 1.5 ml and centrifuged at 3,000 rpm for 10 minutes. The optical density of the pink chromogen was read at 532 nm by a spectrophotometer. Total plasma hydroperoxide concentration were measured using method as described by Eva So dergren et al. [6] with minor modification. Fox reagent prepare by dissovlve 90 mM of xylenol orange and 120 mM of ammonium ferrous in 25 mM sulfuric acid solution. Triphenyl phosphine was prepared by dissolved triphenyl phosphine in methanol to 100 mM. BHT was prepared by dissolved BHT in methnol to 100 mM. FOX reagent was daily prepared. Briefly; 10 µL of plasma sample was incubated with 90 µL of working triphenyl phosphine at room temperature for 15 minutes. Add 990 µL of working FOX reagent and incubrated at room temperature for 30 minuted. Read absorbance at 560 nm.

Antioxidant defense mechanism

An antioxidant has been defined by Halliwel and Gutteridge as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substate" [46]. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Living organisms have evolved antioxidant defend systems and repair systems for protection against free radicals and free radical damages at different sites [4, 8, 47, 48]. There are a number of antioxidants present in the body and derived from the diet. Based on their location in the body, they can be divided into enzymatic and non-enzymatic antioxidants [49, 50]. The first groups are enzymatic antioxidants intracellular antioxidants include low molecular weight scavengers of oxidizing species, and enzymes which degrade superoxide and hydroperoxides [51]. Some of the antioxidant enzymes exist in several forms for example, membrane, cytosolic, and plasma forms. The level and locations of these

antioxidants must be tightly regulated for cell survival. Several antioxidant enzymes exist that convert ROS into less noxious compounds, for example, superoxide dismutase (SOD), catalase, thioredoxin reductase, peroxiredoxin and glutathione peroxidase (GPx) [4, 52]. Collectively, these enzymes provide a first line of defense against superoxide and hydrogen peroxides. And the second groups are major non-enzymatic antioxidant defense system includes ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH) and β-carotene [53]. Non enzymatic antioxidants are exert their protective effect by quenching potentially damaging oxidants to a slow reacting radical, or is converted to a new non-reactive product before damage to cellular components [9]. In addition, major of the non enzymatic antioxidant, uric acid, bilirubin, are scavenging antioxidant. Ubiquinone is chain breaking antioxidant. Chelating agent, transferrin, lactoferrin, ceruloplasmin, and albumin are inhibit fenton reaction. In normal physiological condition, there is a balance between both the activities and the intracellular levels of antioxidants. This balance are essential for the survival of organisms and their health if this balance is perturb, it's lead to oxidative stress and damage to DNA, lipids and protein. The cooperation among different antioxidant provides greater protection against cellular damage by reactive oxygen Thus, the overall of antioxidant capacity may provide more information compared to that obtained by the measurement of individual components. The measure of cooperation of antioxidant is call total antioxidant capacity.

The two most commonly used methods to evaluate the levels of many of these antioxidants are those utilizing ether high performance liquid chromatography (HPLC) or gas chromatography (GC), very frequency used in combinations with mass spectrometry (MS) [43]. Both theses methods are used for determination of the concentrations of various antioxidants in body fluids as well as in tissue samples. The commonly monitored antioxidants are the determination of total antioxidant activity has been used for scientific purposes, to examine the medical importance of free oxygen radicals and antioxidative defense. Potency tests include various methods that were developed to assess the reduction capacity of body fluids (e.g. plasma), as well as the capacity of these fluids to inhibits oxidation. The major assays in this group are [9].

1. TRAP (total radical trapping parameter) assays, based on the concept described by Wayner, et al. (TRPO), have recently been improved with respect to both

the generator of ROS and the methods used to evaluate the results. For this purpose, the most frequently used targets of oxidation include luminol (TRPL), phycoerthin (TRPP) and 2'-7'-Dichlorofluorescin (TRPD).

2. ORAC (oxygen radical absorbance capacity) is based on the oxidation-induced decolorization of ABTS (2, 2' - azinobis-3-ethylbenzothiazoline-6-sulfonic acid) cation. This assay is also referred to as TAS (total antioxidant status), can be conducted with commercial kits available from both Calbiochem and Randox Labs.

3. FRAP (ferric reducing ability of plasma/ferric reducing antioxidant power) is based on the reduction of ferric tripyridyltriazine to ferrous tripyridyltriazine.

4. TOSC (total oxyradical scavenging capacity) is based on the formation of gases in biological solution.

Antioxidant Assay kit

The survival of organisms and their health have a large number of antioxidants, including macro and micro molecule of proteins, and enzymes, which represent the total antioxidant activity of the system and play a central role in preventing oxidative stress. Therefore, quantitative measurement of the cumulative antioxidant capacity of body fluids, tissues, and cells, following different stimuli, may provide important biological information.

A number of assays have been introduced for the measurement of the total antioxidant activity of body fluids [7, 54], food extracts [48]. Two types of approach have been taken, namely, the inhibition assays in that the extent of the scavenging by hydrogen or electron-donation of a pre-formed free radical is the marker of antioxidant activity, as well as assays involving the presence of antioxidant system during the generation of the radical. From the ABTS [2, 29-azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid)] radical generation as the cation form that the basis of the spectrophotometric methods [55] that have been applied to the measurement of the total antioxidant activity of solutions of pure substances. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS [2, 29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] to $ABTS^{\cdot+}$ by metmyoglobin [56]. The amount of $ABTS^{\cdot+}$ produced can be monitored by reading the absorbance at 705 nm or 405 nm. Color is reverse correlated to total antioxidant.