The effect of ageing on antioxidant status in different regions of the rat kidney

By

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In the name of Allah, the Entirely Merciful, the Especially Merciful.

Recite in the name of your Lord who created. Created man from a clinging substance. Recite and your Lord is the most Generous, Who taught by the pen, Taught man that which he knew not.

Declaration

I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree or qualification.

I certify that any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

Signature: 

Noor Riyadh Thiab

Data: 23/04/2015

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Publications arising from this thesis.

- Noor Riyadh Thiab, Graham L. Jones, and Nicola King (2013). Effect of ageing and oxidative stress on the glutathione concentration in different kidney regions. Amino Acids, 45(3) p.587.

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- Noor Riyadh Thiab, Nicola King, Mary McMillan, Amer Almashhadany and Graham L Jones (2015). Age-related protein and transcriptional expression of glutathione peroxidases (GPX) and Hsp-70 in different rat kidney regions with and without stressor. Biochimie. (Under review). **Attached as Appendix III.**

Abstract

Under control physiological conditions, the use of oxygen by cells of aerobic organisms generates potentially toxic reactive oxygen metabolites. A chronic state of oxidative stress may exist in cells because of an imbalance between pro-oxidants and antioxidants. The amount of oxidative damage increases as an organism ages and this is postulated to be a major causal factor of senescence (Sohal & Weindruch, 1996). Oxidative stress probably plays a major role in the progressive compromise in the ability to maintain homeostasis characteristic of the ageing process. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defence mechanisms can lead to damage of cellular organelles, enzymes, as well as increased lipid peroxidation and altered protein and gene expression (Maritim *et al.*, 2003).

The aim of this study was to investigate how the antioxidant status is affected by age and oxidative stress (±0.2 mM H_2O_2) in different functional regions of the rat kidney.

Antioxidant enzyme activities were shown to be attenuated with age under both control and stress conditions after peaking at 12 weeks old (young adult). Antioxidant enzyme activities were higher in the cortex by comparison with the outer and inner medulla respectively. GSH concentrations followed a similar pattern to the levels of antioxidant enzymes.

In all regions and under both stress and non-stress conditions the TBARS and lactate concentration showed a similar pattern with 12 weeks old < 36 weeks old < 5 weeks old

and < 60 weeks old. The greatest concentration was measured in the 60 weeks rat under stress condition with the superficial cortex greater than the outer and inner medulla.

Protein expression of GPX1 detected by Western blot was shown to decrease with age under both control and stress conditions after peaking at 12 weeks (young adult). GPX1 expression was greater in the cortex by comparison with the outer and inner medulla respectively. On the other hand, GPX4 expression did not show much variation across the different regions of the kidney under control or stress conditions, although it did show a significant increase in expression in the inner medulla at 12 weeks under control. GPX2 expression was not detected across the different regions of the kidney under control or stress conditions.

mRNA was extracted from the superficial cortex, outer medulla and inner medulla. The expression of *Gpx1*, *Gpx4* and *Hsp-70* was analysed by quantitative reverse transcribed polymerase chain reaction (QRT-PCR).

Gene expression of *Gpx1* was highest in the cortex, with a trend to decrease with age, after reaching a maximum at 12 weeks. By contrast, *Gpx4* gene expression did not vary in a consistently significant fashion across the different regions of the kidney in all age groups, with the one exception of a significantly higher level of expression in the inner medulla at 12 weeks. In general there was a good correlation between enzyme activity, protein expression and gene expression for the antioxidant enzymes studied. The

negative correlation between gene expression of *Gpx* and *Hsp70* was striking although there is no data on protein expression of HSP70.

Thus, in stark contrast with the expression of both *Gpx1* and *4*, gene expression of stress protein *Hsp-70* was elevated in the inner medulla, and was increased overall in the ageing rats at 60 weeks.

Overall then, the thesis provides general support for the free radical theory of ageing particularly as this pertains to kidney function.

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Abbreviations

GBM	Glomerular Basement Membrane
ROS	Free radicals
TBARS	Thiobarbituric acid reactive substances
GSH	Glutathione
BSA	Bovine serum albumin
GPX	Glutathione Peroxidase
GSH-red	Glutathione Reductase
GSH-ST	Glutathione S-Transferase
CAT	Catalase
SOD	Superoxide dismutase
β-ME	β-mercaptoethanol
DTT	dithiothreitol
TAE	Tris acetate EDTA
GSSG	Oxidized glutathione
RT-PCR	Reverse transcription polymerase chain reaction
H_2O_2	Hydrogen peroxide
PBS	Phosphate Buffered Saline
CDNB	1-Chloro-2, 4-dinitrobenzene

NBT	Nitroblue tetrazolium
$O2^{\cdot -}$	Superoxide radical
MBF	Medulla blood flow
$OH^{\cdot}$	Hydroxyl radical
O^1_2	Snglet oxygen
MDA	Malondialdehyde
TBA	Thiobarbituric acid
GFR	Glomerular filtration rate
AAPH	2,2-Azobis-di-hydrochloride
BSA	Bovine serum albumin

Chapter 1 : Literature review

1.1. Introduction

The kidneys constitute a paired organ located in the abdominal cavity and are considered to be retroperitoneal organs on both sides of the vertebral column. The upper part of each kidney lies at the level of the twelfth thoracic vertebra, and the lower pole lies opposite the third lumbar vertebra. The left human kidney is more proximal in position in relation to the right kidney (Moore *et al.*, 2009). The weight of each kidney is about 125 g to 170 g in the adult human male and about 115 g to 155 g in the adult human female. The human kidney is around 11 cm to 12 cm in length, 5.0 cm to 7.5 cm in width, and 2.5 cm to 3.0 cm in thickness. The hilus is a slit like structure located at the medial surface of each kidney, through which the renal pelvis, the renal artery and vein, the lymphatics, and a nerve plexus pass into the kidney. The kidney is surrounded by a tough fibrous lining which is normally smooth and can be easily removed (Barry & Brenner, 2008; Søren Nielsen *et al.*, 2011). A single renal artery supplies each kidney, although the presence of one or more accessory renal arteries is common. The renal artery enters the hilar region and then divides to an anterior and a posterior branch. Three lobar or segmental arteries arise from the anterior branch giving blood supply to the upper, middle, and lower thirds of the anterior surface of the kidney (Fig 1-1) (Barry & Brenner, 2008). The posterior branch supplies more than half of

the posterior surface and sometimes gives rise to a small apical branch. However, the apical segmental or lobar branch arises mostly from the anterior division. No collateral circulation has been shown between individual segmental or lobar arteries or their subdivisions (Barry & Brenner, 2008). Occasionally the kidneys receive aberrant arteries from the superior mesenteric, suprarenal, testicular, or ovarian arteries .The lower pole of the kidney receives blood from true accessory arteries arising from the abdominal aorta.

Figure 1-1: Diagram of the vascular supply of the human kidney (Graves, 1954).

Figure 1-1 shows that the anterior half of the kidney can be divided into upper (U), middle (M), and lower (L) segments, each supplied by a segmental branch of the anterior division of the renal artery. A small apical segment (A) is usually supplied by a division of the anterior segmental branch. The posterior half of the kidney is divided into apical (A), posterior (P), and lower (L) segments, each supplied by branches of the posterior division of the renal artery. Two distinct regions can be identified on the cut surface of the kidney: a pale outer region, the cortex, and a darker inner region, the medulla (Graves, 1954).

The medulla in the human kidney is divided into 8 to 18 striated conical masses, the renal pyramids. The base of each pyramid is located at the corticomedullary boundary, and the apex extends toward the renal pelvis to form a papilla. On the tip of each papilla are 10 to 25 small openings that represent the distal ends of the collecting ducts of Bellini. The kidney of the rat and of many other laboratory animals, in contrast to the human kidney, has a single renal pyramid and is therefore termed “unipapillate.” On the other hand, these kidneys resemble the human kidney in their gross appearance. The renal cortex in humans, about 1 cm in thickness, forms a cap over the base of each renal pyramid, and extends downward between the individual pyramids to form the renal columns of Bellini. From the base of the renal pyramid, at the corticomedullary junction, longitudinal elements termed the “medullary rays of Ferrein” extend into the cortex. The medullary rays, despite their name are actually considered a part of the cortex and are formed by the collecting ducts and the straight segments of the proximal and distal tubules.

Figure 1-2: Anatomy of the kidney (Graves, 1954).

The renal pelvis represents the expanded portion of the upper urinary tract and is lined by transitional epithelium. In humans, two and sometimes three outpouchings, the major calyces, extend outward from the upper dilated end of the renal pelvis. From each of the major calyces, several minor calyces extend toward the papillae of the pyramids and drain the urine formed by each pyramidal unit. In mammals such as the rat possessing a unipapillate kidney, the papilla is directly surrounded by the renal pelvis. The ureters originate from the lower portion of the renal pelvis at the ureteropelvic junction, and in humans they descend a distance of approximately 28 cm to 34 cm to open into the fundus of the bladder. The walls of the calyces, pelvis, and ureters contain smooth muscle that contracts at regular intervals to propel the urine to the bladder.

1.2. The Nephron

The functional unit of the kidney is the nephron. Each human kidney contains between 0.6×10^6 to 1.4×10^6 nephrons (Hughson *et al.*, 2003; Keller *et al.*, 2003; Nyengaard & Bendtsen, 1992),which contrasts with the approximately 30,000 nephrons in each adult rat kidney (Bertram *et al.*, 1992; Nyengaard 1993). The important parts of the nephron include the renal or Malpighian corpuscle (glomerulus and Bowman's capsule), the proximal tubule, the thin limbs, the distal tubule, and the connecting tubule. The embryonic origin of the nephron is from the metanephric blastula (Neiss, 1982). Although is no consensus on the origin of the connecting tubule, it is now commonly believed to derive from the metanephric blastema. The collecting duct system, which includes the initial collecting tubule, the cortical collecting duct (CCD) in the medullary ray, the outer medullary collecting duct (OMCD), and the inner medullary collecting duct (IMCD), is not specifically considered as a part of the nephron as embryologically it arises from the ureteric bud. On the other hand, all of the components of the nephron and the collecting duct system are correlated functionally.

Two main populations of nephrons are identifiable in the kidney: those having a short loop of Henle and those with a long loop of Henle. The loop of Henle is composed of the straight part of the proximal tubule (pars recta), the thin limb segments, and the straight portion of the distal tubule (thick ascending limb, or

pars recta). The length of the loop of Henle is commonly related to the position of its parent glomerulus in the cortex. Nephrons originating from superficial and midcortical locations mostly have short loops of Henle that bend within the inner stripe of the outer medulla close to the inner medulla. A few species, including humans, also possess cortical nephrons with extremely short loops that never enter the medulla but turn back within the cortex. Nephrons originating from the juxtamedullary region near the corticomedullary boundary have long loops of Henle with long descending and ascending thin limb segments that enter the inner medulla (Eaton & Pooler, 2009). There are many variations found between the two basic types of nephrons, depending on their relative position in the cortex. The ratio between long and short loops varies among species. Humans and most rodents have a larger number of short-looped by comparison with long-looped nephrons (Pannabecker, 2008; Pannabecker & Dantzler, 2006, 2007; Zhai *et al.*, 2006)

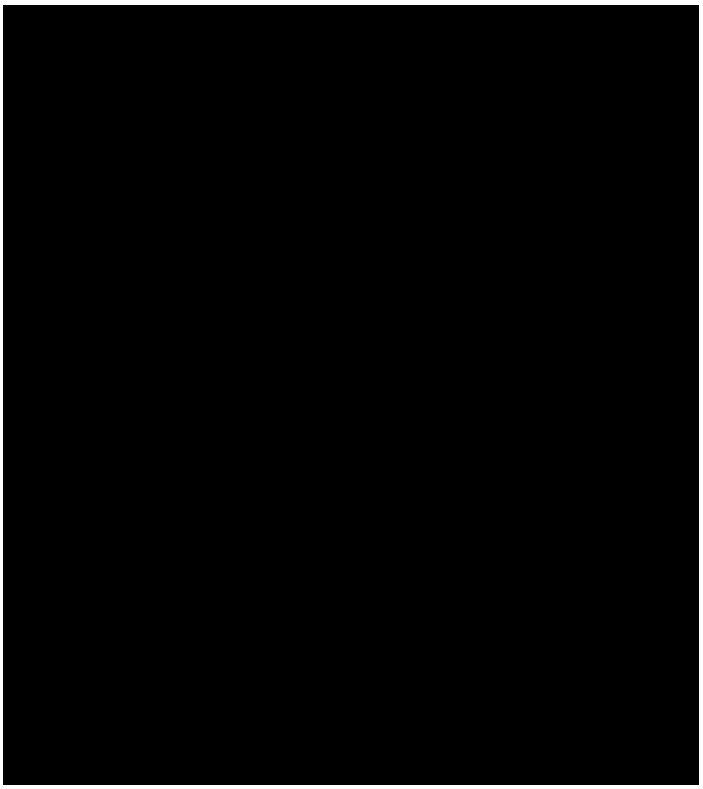

Figure 1-3: Anatomy of the nephron (Eaton & Pooler, 2009).

Figure 1-3 shows the relationships of component parts of a long-looped nephron, which has been “uncoiled” for clarity (relative lengths of the different segments are not drawn to scale). The combination of glomerulus and Bowman’s capsule is the renal corpuscle (Eaton & Pooler, 2009).

1.3. Renal Corpuscle (Glomerulus)

The renal corpuscle constitutes; 1) A capillary network that is lined by a thin layer of endothelial cells; 2) A central region of mesangial cells with surrounding mesangial matrix material; 3) The parietal layer of Bowman's capsule with its basement membrane; 4) The visceral epithelial cells and the associated basement membrane (Guasch & Myers, 1994; Neiss, 1982; Samuel *et al.*, 2005). Between the two epithelial layers is a narrow cavity that is called Bowman's space. Despite the fact that the term renal corpuscle is more precise anatomically than the term glomerulus when referring to that portion of the nephron composed of the glomerular tuft and Bowman's capsule, the term glomerulus is used throughout this chapter because of its common use. The visceral epithelium is continuous with the parietal epithelium at the vascular pole, where the afferent arteriole enters and the efferent arteriole exits the glomerulus. The parietal layer of Bowman's capsule continues into the epithelium of the proximal tubule at the so-called urinary pole. The average diameter of the glomerulus is approximately 200 μm in the human kidney and 120 μm in the rat kidney. The average glomerular volume has been reported to be 3 to 7 million μm^3 in humans and 0.6 to 1 million mm^3 in the rat. The glomerular number and size vary significantly with age and gender as well as birth weight. (Hughson *et al.*, 2003; Keller *et al.*, 2003; Nyengaard & Bendtsen, 1992). In the rat, juxtamedullary glomeruli are larger than glomeruli in the

superficial cortex, although, this is not the case in the human kidney (Samuel *et al.*, 2005).

Figure 1-4: A, Anatomy of the glomerulus. B, Cross-section of glomerular membranes. US, “urinary” (Bowman’s) space; E, epithelial foot processes; GBM, glomerular basement membranes; End, capillary endothelium; Cap, lumen of capillary. C, scanning electron micrograph of podocytes covering glomerular capillary loops; the view is from inside Bowman’s space (Eaton & Pooler, 2009).

In Figure 1-4, the large mass is a cell body. Note the remarkable interdigitation of the foot processes from adjacent podocytes and the slits between them (Eaton & Pooler, 2009).

1.4. Glomerular Basement Membrane (GBM).

The GBM constitutes a central dense layer, the lamina densa, and two thinner, more electron-lucent layers, the lamina rara externa and the lamina rara interna. The last two layers measure approximately 20 nm to 40 nm in thickness (Friederici, 1967). The layered configuration of the GBM results in part from the fusion of endothelial and epithelial basement membranes during development (Abrahamson, 1987). Several researchers have provided estimates of the width of the GBM of peripheral glomerular capillary loops. Jørgensen and Bentzon (1968) measured a mean width of 310 nm. Steffes and co-workers (1983) cited in calculated a mean width of 310 nm. Steffes and co-workers (1983) determined the GBM width in a large group of donor kidneys for transplantation and found a considerably thicker basement membrane in men (373 nm) than in women (326 nm). The thickness of the GBM in the rat was found to be 132 nm (Rasch, 1979). The GBM possesses fixed, negatively charged sites that may control the filtration of macromolecules. Caulfield and Farquhar (1976) showed the existence of anionic sites in all three layers of the GBM with the use of the cationic protein lysozyme. Additional studies employing cationic ferritin and ruthenium red, a cationic dye, showed a network of anionic sites with a spacing of approximately 60 nm throughout the lamina rara interna and lamina rara externa, which might contribute to the formation of the charge barrier (Kanwar *et.al.*, 1979). Kanwar and Farquhar (1979) confirmed that the anionic sites in the GBM consist of

glycosaminoglycans rich in heparan sulphate. Their removal by enzymatic digestion resulted in an increase in permeability of the GBM to ferritin (Kanwar *et al.*, 1980), and to bovine serum albumin, (Rosenzweig & Kanwar, 1982) resulting in the proposition that glycosaminoglycans play a role in creating the permeability properties of the GBM to plasma proteins. It has also been assumed that the glycosaminoglycans might serve as anticlogging agents in the GBM (Kanwar & Rosenzweig, 1982).

1.5. Juxtaglomerular Apparatus

The juxtaglomerular apparatus is situated at the vascular pole of the glomerulus, where a part of the distal nephron comes into contact with its parent glomerulus. It has a vascular and a tubule component. The vascular part is composed of the terminal portion of the afferent arteriole, the first portion of the efferent arteriole, and the extraglomerular mesangial region. The tubule part is the macula densa, which is that portion of the thick ascending limb that is in contact with the vascular component (Barajas, 1970; Barajas, 1979). The extra glomerular mesangial region, which has also been referred to as the polar cushion (polkissen) or the lacis, is surrounded by the cells of the macula densa, the specialized regions of the afferent and efferent glomerular arterioles, and the mesangial cells of the glomerular tuft (the intraglomerular mesangial cells). Two distinct cell types

can be recognized inside the vascular component of the juxtaglomerular apparatus: the juxtaglomerular granular cells, also called epithelioid or the myoepithelial cells, and the agranular extraglomerular mesangial cells, which are also, referred to as the lacis cells or pseudo-meissnerian cells of Goormaghtigh.

1.6. Juxtaglomerular Granular Cells

The granular cells are positioned principally in the walls of the afferent and efferent arterioles, but they are also present in the extra glomerular mesangial region (Barajas, 1970; Barajas, 1979; Hackenthal *et al.*, 1990; Tisher *et al.*, 1968). They show features of both smooth muscle cells and secretory epithelial cells and therefore have been called myoepithelial cells (Barajas, 1979). The juxtaglomerular granular cells are believed to represent modified smooth muscle cells as they have myofilaments in the cytoplasm and, except for the presence of granules, are difficult to distinguish from the nearby arteriolar smooth muscle cells. They also exhibit features suggestive of secretory activity, including a well-developed endoplasmic reticulum and a Golgi complex containing small granules with a crystalline substructure (Barajas, 1979; Biava & West, 1966). The juxtaglomerular granular cells are known by the presence of many membrane-bound granules of variable size and shape (Barajas, 1979; Biava & West, 1966).

1.7. The Renal Circulations and Glomerular Ultrafiltration

About 20% of the overall cardiac output in humans perfuses the kidneys under resting conditions, organs that constitute only about 0.5% of the overall human body mass. Thus the rate of blood flow, approximately 400 mL/100 g of tissue per minute, is much greater than that observed in other vascular beds ordinarily considered to be well perfused, such as liver, brain, and heart (Stein *et al.*, 1978). Only a small quantity of urine is formed (about 1 mL/minute) from this enormous blood flow (about 1 L/minute). Despite this, the metabolic energy requirement of urine production is great constituting about 10% of basal O_2 consumption. Examination of the renal arteriovenous O_2 difference shows the blood flow to be far beyond metabolic demands. In fact, the high rate of blood flow is crucial to the process of urine formation.

1.8. Proximal Tubule

The proximal tubule begins immediately at the urinary pole of the glomerulus. It is composed of an initial convoluted portion, the pars convoluta, which is a direct continuation of the parietal epithelium of Bowman's capsule, and a straight portion, the pars recta, which is situated in the medullary ray. The length of the proximal tubule is about 10 mm in the rabbit (Madsen & Park, 1987), 8 mm in the rat, and 4

nm to 5 mm in the mouse, (Zhai *et al.*, 2003) compared with approximately 14 mm in the human. Three morphologically distinct segments—S1, S2, and S3—have been identified in the rat (Maunsbach, 1966). The S1 segment is the first portion of the proximal tubule; it begins at the glomerulus and forms about two thirds of the pars convoluta. The S2 segment consists of the remainder of the pars convoluta and the first portion of the pars recta. The S3, located in the deep inner cortex and the outer stripe of the outer medulla segment, represents the remainder of the proximal tubule (Zhai *et al.*, 2006). The structural features that differentiate the cells in the three segments in the rat have been elucidated in detail by Maunsbach (Maunsbach, 1966). The S1 segment has a well-developed vacuolar-lysosomal system and a tall brush border. Elongated mitochondria are located in the lateral cell processes in proximity to the plasma membrane. The basolateral plasma membrane forms extensive lateral invaginations. Lateral cell processes extending from the apical to the basal surface interdigitate with similar processes from adjacent cells. The ultrastructure of the S2 segment is identical to that of the S1 segment. On the other hand, the brush border is shorter, the basolateral invaginations are less prominent, and the mitochondria are smaller. Numerous small lateral processes are located close to the base of the cell. The number and size of the lysosomes vary among species and between males and females, and numerous large lysosomes are often observed in the S2 segment of the male rat. The endocytic compartment is less prominent than in the S1 segment (Madsen &

Park, 1987; Maunsbach, 1966). In the S3 segment, mitochondria are small and randomly distributed within the cell and lateral cell processes and invaginations are essentially absent. The length of the brush border in the S3 segment varies among species. It is fairly short in the rabbit, intermediate in length in the human kidney and long in the rat. Considerable species variation is also seen in the vacuolar-lysosomal compartment in the S3 segment. In the rat (Maunsbach, 1966) and the human,(Tisher *et al.*, 1966) endocytic vacuoles and lysosomes are small and sparse, whereas in the rabbit, large endocytic vacuoles and numerous small lysosomes are present in the S3 segment (Madsen & Park, 1987). Peroxisomes increase in number along the length of the proximal tubule and are most prominent in the S3 segment. They are present throughout the proximal tubule.

1.9. Function of the kidneys

Although there are significant differences between various structural elements of the human and rat kidney some of which have already been mentioned the overall function is nearly identical.

1.9.1. Regulation of Water and Electrolyte Balance.

The balance concept states that the organisms/cells are in balance for any substance when the inputs and outputs of that substance are matched. Any difference between output and input leads to a decrease or increase in the amount of that substance within the body (Eaton & Pooler, 2013; Fusch & Jochum, 2014; Pang, 2014). The input of water and electrolytes is extremely variable and is only sometimes driven in response to body needs. For example, animals drink water when thirsty; they also consume food to provide energy, but food often contains large amounts of water. They drink much more because it is a component of beverages that we consume for reasons other than hydration. The kidneys respond by varying the output of water in the urine, thereby maintaining water balance (i.e. constant total body water content) (Pang, 2014). Minerals like magnesium, sodium, potassium, and so on are components of foods and generally present far in excess of body needs. As with water, the kidneys excrete minerals at a highly variable rate that, in aggregate, matches input. One of the significant characteristics of the kidneys is their special ability to regulate each of these minerals independently (e.g., one can be on a low-potassium, high-sodium diet or low-sodium, high-potassium diet, and the kidneys will appropriately adjust excretion of each of these substances) (Eaton & Pooler, 2009; Fusch & Jochum, 2014; Pollock *et al.*, 2014).

1.9.2. Excretion of Metabolic Waste

End products of metabolic processes in many cases, have no function and are harmful at high concentrations (Ahmed & Latif, 2014; Weiner *et al.*, 2014). Some of these waste products include uric acid (from nucleic acids), creatinine (from muscle creatine), uroporphyrin end products of haemoglobin breakdown (which give urine much of its colour), urea (from protein), and the metabolites of various hormones, among many others (Eaton & Pooler, 2009). In addition, foreign substances, including many common drugs, are excreted by the kidneys. In many cases the kidneys work in concert with the liver. The liver metabolises many organic molecules into water-soluble forms that are more easily handled by the kidneys (Eaton & Pooler, 2013; Jensen-Jarolim *et al.*, 2014). Drugs and hormones in the blood that affect body function are removed in many ways, mostly in the liver, but a number of them are removed in parallel by renal processes. Medical doctors have to be mindful of how fast the drugs are excreted in order to prescribe a dose that achieves the appropriate body levels (Chillistone & Hardman, 2014).

1.9.3. Regulation of Arterial Blood Pressure

Although it is widely known that the kidneys excrete waste substances like urea and salts, the kidneys' crucial role in controlling blood pressure is less well

recognised. Blood pressure eventually depends on blood volume, and the kidneys' maintenance of sodium and water balance helps regulate blood volume (Vitzthum *et al.*, 2014). Thus, the kidneys participate in blood pressure control through volume control (Lakshmikanthan *et al.*, 2014). They also contribute to the regulation of blood pressure via the generation of vasoactive substances such as renin that regulate smooth muscle in the peripheral vasculature (Eaton & Pooler, 2009; Waeckel *et al.*, 2014).

1.9.4. Regulation of Red Blood Cell Production

Erythropoietin is a peptide hormone that has a role in the control of erythrocyte (red blood cell) production by the bone marrow. It is produced mainly in the kidneys, although the liver also secretes small amounts. The renal cells that secrete erythropoietin are a particular group of cells in the interstitium. The stimulus for secretion is a reduction in the partial pressure of oxygen in the kidneys, as occurs, e.g., in arterial hypoxia, anaemia, and inadequate renal blood flow (Bouvy *et al.*, 2014). An increase in the production of erythrocytes by the bone marrow is stimulated by erythropoietin. Renal disease may result in diminished erythropoietin secretion, and the resulting decrease in bone marrow activity is one important causal factor of anaemia associated with chronic renal disease (Eaton & Pooler, 2009; Nasri, 2014).

1.9.5. Regulation of Vitamin D Production

In vivo vitamin D synthesis involves a series of biochemical transformations, the last of which occurs in the kidneys. The active form of vitamin D (1,25-dihydroxyvitamin D3) is actually made in the kidneys, and its rate of synthesis is regulated by hormones that control calcium and phosphate balance (Eaton & Poole, 2013; Vogiatzi *et al.*, 2014).

1.9.6. Gluconeogenesis

The human central nervous system (CNS) has an absolute requirement for blood glucose regardless of whether the body is in a fed or fasted state. The body begins to synthesise new glucose (the process of gluconeogenesis) from non-carbohydrate sources (amino acids from protein and glycerol from triglycerides) whenever the intake of carbohydrate is stopped for much more than half a day (Clar *et al.*, 2014; McGill, 2014; Rhee, 2014). Most gluconeogenesis occurs in the liver, but a substantial fraction occurs in the kidneys, particularly during a prolonged fast (Eaton & Pooler, 2009; Tucci *et al.*, 2014).

1.10. Free radicals and ROS

Ageing is characterised by a compromised ability to maintain homeostasis, and this is evident when considering the normal processes of oxidation. Oxidation leads to the creation of highly reactive substances called free radicals which rapidly react with and lead to the deterioration of other critical bioactive molecules (Andriantsitohaina *et al.*, 2012). When free radicals start attacking the body's own cells, this may initiate ageing processes (Friederici, 1967; Kirkwood & Kowald, 2012). The concept that free radicals may play a role in the ageing process has been anticipated and widely discussed (Davies, 1995; Pacifici & Davies, 1991; Speakman & Selman, 2011). It has been postulated that antioxidants may positively influence the ageing process, protecting organisms against free radical-induced damage (Forman *et al.*, 2014; You *et al.*, 2014). Antioxidants are free radical scavengers that protect living organisms against oxidative damage to tissues (Jagetia & Rajanikant, 2015). On the other hands pro-oxidants are chemical compounds and reactions capable of generating potentially toxic reactive oxygen species (ROS). The term antioxidant initially referred to a chemical that prevented the consumption of molecular oxygen (Andriantsitohaina *et al.*, 2012). In a normal cell, there is an appropriate pro- and antioxidant balance. However, this balance can be shifted towards the pro-oxidant state when production of oxygen species is increased or when levels of antioxidants are diminished, and this imbalance can result in severe cell damage if it is large or prolonged (Irshad &

Chaudhuri, 2002). An organism's ability to maintain cellular homeostasis declines during the ageing process (Kowald, 2002). Aerobes are provided with intrinsic antioxidant defence systems, consisting of both enzymatic and non-enzymatic components, in order to protect cellular macromolecules against the highly reactive and potentially damaging oxygen metabolites (Kalyanaraman, 2013). The enzymatic antioxidant defence system includes the enzymes catalase, superoxide dismutase and glutathione peroxidase, which offer primary defence against reactive oxygen species (Andriantsitohaina *et al.*, 2012). In addition, ascorbate, reduced glutathione, and alpha-tocopherol form a set of cellular antioxidants, which react with reactive oxygen species to form lesser-reactive radical species (Andriantsitohaina *et al.*, 2012). Each component of the antioxidant system is found in specific cellular and sub-cellular locations, and the individual components function together in a complementary manner (Bandyopadhyay *et al.*, 1999). Free radical damage accumulates and speeds up the ageing process and disease if antioxidants do not control free radicals. Ultimately, free radical production exceeds the body's natural supply of antioxidants and in order to maintain cellular homeostasis more antioxidants need to be obtained through diet. According to the oxidative stress hypothesis of ageing, the senescence-associated loss of functional capacity is a result of the accumulation of molecular oxidative damage (Sohal & Forster, 2007; Subramanian & James, 2010) caused by toxic free radicals produced during normal respiration. Oxidative damage may be generally

responsible for the ageing process and have a role in the neuropath genesis of several diseases including stroke, Parkinson's, and Alzheimer's. Reactive oxygen species are believed to be usually generated in aerobic cells and aerobic organisms which are supplied with antioxidant defence systems that can prevent damage due to oxidative stress. As mentioned before, the major antioxidant defence systems constitute macromolecular antioxidant enzymes and small molecule biological antioxidants. The former include superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX), and the latter include reduced glutathione (GSH), vitamin C and vitamin E. The insufficiency of antioxidants might lead to the increased oxidative damage observed during ageing (Reiter, 1995). Age-related decline in overall proteolytic activity occurs in almost all organisms and the progressive intracellular accumulation of damaged proteins with age has been widely reported (Ward, 2002) and this underwrites the importance of cell chaperone production such as HSP-70.

A large number of chemicals are released into blood and these act as molecular markers during cell damage and ageing. A trial was made to check the effect of deprenyl on the levels of these diagnostic marker enzymes along with antioxidant enzymes (Ward, 2002). The lack of reliable, well defined and precise cellular and biochemical markers of ageing has delayed efforts to identify the primary causal mechanisms and separate those from secondary associated effects. Many theories about the causes of ageing have been proposed (Weinert & Timiras,

2003). Such theories may be divided into two broad categories (Troen, 2003); the stochastic theories reflecting the accumulation of cellular and molecular damage throughout the life span and the genetic theories which concentrate on genetically transmitted cellular and organismal ageing programmes which, while they differ from individual to individual in the precise tempo nonetheless reflect a general pattern in much the same way as other aspects of foetal, infant and adult development.

The build-up of “damage” that occurs throughout the entire lifespan of cells is the essential concept behind the stochastic theories (Rattan, 2008). Such damage may accumulate from toxic by-products of routine metabolism or inefficient repair/defensive systems. The excessive production of ROS in the cells is involved in the pathogenesis of various diseases including stroke, diabetes mellitus, atherosclerosis and inflammatory diseases such as end-stage renal disease (ESRD) and chronic renal failure (Laviano *et al.*, 2010; Obay *et al.*, 2008). ROS, such as hydrogen peroxide, superoxide anion and hydroxyl radical, which are generated as by-products of oxidative metabolism in mitochondria, can interact with biomolecules such as RNA, DNA, lipids, and protein subsequently damaging various cellular components (Ergüder *et al.*, 2005; Kheradmand *et al.*, 2010; Pajović & Saicić, 2008; Turner & Lysiak, 2008) . Oxidative stress can also affect insulin sensitivity of peripheral tissues (Laviano *et al.*, 2010). In this regard, ESRD is associated with a state of insulin resistance (Ikizler, 2007). Interestingly,

inflammation has been demonstrated to derange mitochondrial function thereby favouring leakage of ROS (Laviano *et al.*, 2010; Victor *et al.*, 2009). This may occur either indirectly, that is favouring the development of oxidative stress and insulin resistance and is the main mediator of wasting in diseases with chronic renal failure such as ESRD and also considering its role in anorexia or directly, that is activating the proteolytic systems (Laviano *et al.*, 2008).

Figure 1-5: Reactive oxygen species (ROS) and their contribution to development of oxidative stress-related diseases (Georgiev *et al.*, 2014).

The presence of antioxidants may prevent diseases development by modulating damaging effects of ROS by suppressing multiple process steps (Georgiev *et al.*, 2014).

1.11. Mitochondria

The main function of mitochondria is energy production. Highly reactive oxygen radicals are generated during oxidative phosphorylation. It has been estimated that the release of reactive intermediates accounts for about 2% of the oxygen consumed during respiration. One major site of oxidant production is in the mitochondrial electron transport chain in which O_2 is reduced to H_2O. In this process, electrons from succinate are donated to complex II (succinate dehydrogenase complex) and electrons from NADH are donated to complex I (NADH dehydrogenase complex). Ubiquinone, also known as co-enzyme Q or CoQ, accepts electrons from both complexes and is sequentially reduced, one electron at a time, to ubisemiquinone and ubiquinol. Ultimately, electrons are transferred through complex III (CoQ-cytochrome c reductase), cytochrome c, and complex IV (cytochrome-c oxidase) which leads to reduction of O_2 to H_2O. Mitochondrial electron transport, however, is imperfect and results in the production of O_2^- from the one-electron reduction of O_2. Some contemplate that this electron results from the free radical ubisemiquinone: instead of accepting

another electron and proton to form ubiquinol, one of the electrons may leak from ubisemiquinone and reduce O_2 to O_2^-. Enzymatic dismutation of O_2^- results in H_2O_2, another important biological oxidant (Pollack & Leeuwenburgh, 1999; Sen *et al.*, 2000). In state 4 respirations, which can be described by a high degree of reduction of the electron carriers and a limiting supply of ADP, H_2O_2 production is at its maximum. It has been estimated that under these conditions the release of reactive intermediates accounts for 2% of the oxygen consumed during respiration (Boveris & Chance, 1973; Pollack & Leeuwenburgh, 1999).

ROS production by mitochondria can lead to oxidative damage to mitochondrial proteins, membranes, and DNA, impairing the ability of mitochondria to synthesize ATP and other essential functions. Mitochondrial oxidative damage can also increase the tendency of mitochondria to release cytochrome c (cyt c) into the cytosol by mitochondrial outer membrane permeabilization (MOMP), leading to apoptosis. Mitochondrial ROS production leads to induction of the mitochondrial permeability transition pore (PTP), which makes the inner membrane permeable to small molecules. Mitochondrial oxidative damage contributes to a wide range of pathologies, and mitochondrial ROS act as a reversible redox signal modulating the activity of a range of cellular functions (Figure1-6) (Galley, 2010).

Figure1-6: Overview of mitochondrial reactive oxygen species (ROS) production (Galley, 2010).

1.12. Mitochondria and Ageing

Numerous studies have investigated age-associated increases in the generation of oxidants by mitochondria (Andriantsitohaina *et al.*, 2012; Barja, 2014). For examples an experiment using Mongolian gerbils (*Meriones unguiculatus*) demonstrated that O_2^- and H_2O_2 production increased with age, especially in isolated mitochondria and sub mitochondrial particles from the aged heart (Pollack & Leeuwenburgh, 1999; Sanchez-Roman *et al.*, 2012). In another study, carried

out using whole hepatocytes from old rats, it was found that older hepatocytes had a 30% decrease in the mitochondrial membrane potential and a 23% increase in mitochondrial hydrogen peroxide generation. Hydrogen peroxide levels within the liver cells were also increased (Hagen *et al.*, 1998; Pollack & Leeuwenburgh, 1999; Sanchez-Roman *et al.*, 2012). Additionally, another study carried out using intact, isolated rat hepatocytes reached the same conclusion: cellular oxidant generation by mitochondria increases with age (Barja, 2014; Pollack & Leeuwenburgh, 1999; Sastre *et al.*, 1996; Small *et al.*, 2012). Several other studies using intact muscle mitochondria from house flies have shown that the rate of H_2O_2 generation progressively increases 2-fold as the house fly ages (Sohal & Sohal,1991). Santos *et al.* (2006), compared whether mt DNA accumulates more oxidative DNA damage relative to nuclear DNA. This study also looked for significant differences in repair between nuclear and mt DNA. More specifically, the researchers studied the formation and repair of H_2O_2-induced DNA damage in a 16.2 kb mitochondrial fragment and a 17.7 kb nuclear fragment flanking the b-globin gene. Fibroblasts treated with a relatively high dose of hydrogen peroxide exhibited 3-fold greater damage to the mitochondrial fragment compared with the nuclear fragment. Moreover, damage to the nuclear fragment was completely repaired within 1.5 h; whereas, no DNA repair in the mitochondrial fragment was observed (Santos *et al.*, 2006). These data support the conclusion that mt DNA is

more susceptible to oxidants, and mitochondrial repair mechanisms may be non-existent or less efficient.

1.13. Ageing and Antioxidant defences

Various therapeutic agents that scavenge ROS (reactive oxygen species) may be useful in the controlling of several diseases related to free radical damage. The majority of free radicals are generated by mitochondria as a consequence of the mitochondrial cycle, whereas free radical accumulation is limited by the action of a variety of antioxidant processes that reside in every cell (Andriantsitohaina *et al.*, 2012). Antioxidants are exogenous (natural or synthetic) or endogenous compounds acting in several ways, including removal of O_2, scavenging ROS or their precursors, inhibiting ROS formation and binding metal ions needed for catalysis of ROS generation. The natural antioxidant systems can be classified into two major groups: enzymatic and non-enzymatic antioxidants (Andriantsitohaina *et al.*, 2012).

The possibility that oxidative stress and ageing is mostly due to a decline in antioxidant defences is still not well understood. In fact, most researchers would suggest that antioxidant defences are, in general, not different between old and young animals. This topic has been widely reviewed (Pollack & Leeuwenburgh,

1999; Yu, 1996), and therefore not covered in detail here. Some of the major antioxidants that may undergo age-related changes include SOD, Cat, GPX, GSH-ST, vitamin C, GSH, and vitamin E. However, there are a multitude of other known and possibly unknown antioxidant defences which may change with age.

1.13.1. Glutathione peroxidase (GPX)

Glutathione peroxidase is the main hydrogen peroxide scavenger enzyme, and it is present in both the cytosol and mitochondria (Folbergrová *et al.*, 2013). In the presence of its substrate GSH, it forms a strong defence against hydrogen peroxides and lipid peroxides. No change in glutathione peroxidase levels has been detected with increasing age in the rat brain (Marosi *et al.*, 2012; Pollack & Leeuwenburgh, 1999; Yu, 1996). In the heart and the liver, there are a similar number of studies reporting no change in GPX levels as there are reporting a decrease in enzyme activity; thus, there is no consensus as to whether GPX levels decrease with age or not (Anandan *et al.*, 2013; Öztürk *et al.*, 2012; Yu, 1996). In skeletal muscle GPX activity increases with age in rats, indicating an adaptive change to mitigate against oxidative stress in this tissue (Table 1-1) (Corbi *et al.*, 2012; Ji, 1995).

1.13.2. GSH reductase (GSH-red)

GSH reductase (EC 1.6.4.2) catalyses the reduction of GSSG using NADPH as cofactor. The role of GSH reductase is to raise the availability of reduced glutathione for reaction with peroxides, and free radicals, an essential feature in the metabolism of xenobiotics. GSH reductase is involved in cellular detoxification (Arrick & Nathan, 1984). Due to the sensitivity of this enzyme toward drugs and environmental pollutants (Milano *et al.*, 1988; Vernie *et al.*, 1988), GSH reduct has been used as a marker of the toxic effects of ingestion or inhalation of various xenobiotics. Glutathione reductase is present in many animals and plants (Jocelyn, 1972).

As an example GSH-red, plays an important role in the detoxification of ROS that are generated by Cisplatin (CP) a heavy metal complex that is one of the most active drugs used in the treatment of a variety of cancers. (Ognjanović *et al.*, 2012) so as to protect cells from the potential toxicity. The activities of GSH-red were found to decrease after CP administration, resulting in the decreased ability of the kidney to scavenge toxic hydrogen peroxide (H_2O_2) and lipid peroxides in the rat kidney (Ognjanović *et al.*, 2012).

1.13.3. Glutathione-S transferases (GSH-STs)

Glutathione-S transferases (GSH-STs) are a group of enzymes that are important in the detoxification of many different xenobiotics in mammals (Cummins *et al.*, 2011; Jakoby & Ziegler, 1990; Lushchak, 2012; Morrow & Cowan, 1990). These enzymes protect cells against toxicants by conjugating the thiol group of the glutathione to electrophilic xenobiotics, and thereby defend cells against the mutagenic, toxic, and carcinogenic effects of these compounds (Backos *et al.*, 2012; Coles *et al.*, 1990; Jerina & Bend, 1977; Lushchak, 2012; Sies & Ketterer, 1988). GSH-ST activity is found to be present in plants (Cummins *et al.*, 2011; Mohsenzadeh *et al.*, 2013), insect (Robert *et al.*, 2013), yeast, bacteria, and in most mammalian tissues (Klaus *et al.*, 2013), especially in the liver, which plays a key role in detoxification (Klaus *et al.*, 2013; Sies, 1999). There are several classes of GSH-ST isozymes that differ in their specificity toward xenobiotic or endogenous substrates (Mannervik *et al.*, 1992; Takahashi *et al.*, 1993; Zhong *et al.*, 1993).

1.13.4. Catalase (CAT.)

Another H_2O_2 metabolising enzyme catalase is an antioxidant enzyme ubiquitously present in mammalian and non-mammalian aerobic cells containing a cytochrome system (Sofic *et al.*, 2014). It is mainly found in the peroxisomes, but it is also located, less plentifully, in mitochondria. It was initially isolated from ox liver and later from blood, bacterial, and plant sources (Deisseroth & Dounce, 1970). Catalase contains 4 ferrihaemoprotein groups per molecule and has a molecular mass of 240 kDa. Catalase activity varies greatly between tissues. The activity is highest in the liver and kidney (Jones & Masters, 1976), and lowest in connective tissues. Catalase promotes the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. Hydrogen peroxide is formed in the eukaryotic cell as a by-product of various oxidases and superoxide dismutase reactions (Sofic *et al.*, 2014). Hydrogen peroxide is highly deleterious to the cell and its accumulation causes oxidation of cellular targets such as DNA, proteins, and lipids leading to mutagenesis and cell death. (Bai & Cederbaum, 2001; Kowaltowski *et al.*, 2001; Kowaltowski *et al.*, 2000; Yamada *et al.*, 1999). Removal of H_2O_2 from the cell by catalase provides protection against oxidative damage to the cell. Its role in oxidative stress related diseases has been widely studied (Sofic *et al.*, 2014). The literature shows no consistent trend in changes of CAT activity in brain tissues with ageing; there are an equal amount of studies showing a decrease and an increase in activity with age (Manikonda & Jagota, 2012; Movahed *et al.*, 2012; Yu, 1996).

This may indicate difficulties in the measurement of CAT activity. CAT activity in the liver appears to decline consistently in ageing rats (Carney *et al.*, 2012; Farahmand *et al.*, 2013; Ramesh *et al.*, 2012). In striking contrast, Cat activity increases in the heart and skeletal muscle of ageing rats (De & Darad, 1991; Hollander *et al.*, 2001; Ji, 1995; Vanella *et al.*, 1982; Yu, 1996). For example, Leeuwenbugh *et al.* (1994) found that there was a 40% increase in Cat activity in very old animals compared to middle-aged animals (14.5 months); and that skeletal muscle CAT activity was increased by 150% in very old rats (26.5 month old) compared to young (4.5 month old) rats (Hollander *et al.*, 2001) (Table1-1).

Table1-1: Activity of antioxidant enzyme in deep vastus lateralis (DVL) skeletal muscle of young and old rats (Hollander *et al.*, 2001).

	GPX DVL	CAT DVL	SOD DVL
Young	4.25 ± 0.29	15.8 ± 2.2	1960 ± 143
Adult	5.70 ± 0.34[a]	29.4 ± 3.8[b]	2190 ± 118
Old	5.88 ± 0.36[a]	39.6 ± 4.2[b]	2510 ± 120[a]

Values are means ± SE; GPX: glutathione peroxidase; SOD: superoxide dismutase (units/g wet weight); CAT: catalase ($K \times 10^{-2}$/g wet weight); GSH: glutathione µmol/g wet weight; Young = 4.5 months; Adult = 14.5 months: Old = 26.5 months. [a]$p < 0.05$, Adult or Old vs. Young; [b]$p < 0.001$, Adult or Old vs. Young.

1.13.5. Superoxide Dismutase (SOD)

SOD exists as a cytosolic (copper/zinc) or mitochondrial (manganese) isoenzyme, which dismutates superoxide radicals to oxygen and hydrogen peroxide (Chelack & Petkau, 1981; Schriner *et al.*, 2009; Sun *et al.*, 1988; Winterbourn *et al.*, 1975; Yin *et al.*, 2015). Most of the studies on total SOD activity in the brains and heart of rats and mice, have found small changes in the activity of this enzyme with age (Haider *et al.*, 2014; Öztürk *et al.*, 2012; Warner, 1994; Yu, 1996). One study, actually found an increase in mitochondrial SOD activity in the brain of rats with age, which is not what one would expect if decreased antioxidant enzymes were a major factor in ageing (Öztürk *et al.*, 2012; Vanella *et al.*, 1982; Warner, 1994). On other hand, many studies in rats have shown an age-related decrease SOD activity in liver (Manikonda & Jagota, 2012; Ramesh, Kim, Sung*, et al.*, 2012; Yu, 1996). This apparent discrepancy might give an indication of variable adaptations between species. Skeletal muscle SOD, however, does increase with age in old rats compared to either middle-aged or young rats (Table 1-1) (Ji, 1995; Pollack & Leeuwenburgh, 1999). Some of the inductions of SOD with age could reflect a chronic adaptation to the increase in superoxide production.

1.13.6. Small molecular weight antioxidants

Vitamin C is a critical water soluble antioxidant which interacts with GSH and vitamin E in maintaining a reduced intracellular environment. Levels of vitamin C show a steady decline with age in various species. For example, researchers from Sweden determined that the nucleus accumbens in 3-, 6- and 18-month-old Sprague-Dawley rats showed a significant age-related decrease in basal extracellular vitamin C concentration (Svensson *et al.*, 1993). Several studies show that there is a significant age-related decline in vitamin C levels in the liver of rats (Liu & Mori, 1993; Nicolson & Ellithorpe, 2006; Rikans, 1988) Vitamin C concentration also decreases with age in toad skeletal muscle (Kara, 1994). One difficulty is that these data are complex to translate to humans as humans lack the ability to synthesise vitamin C and are dependent on dietary vitamin C. Many epidemiological studies clearly show that inadequate vitamin C levels correlate highly with diseases such as cataracts and cancer (Shigenaga *et al.*, 1994) providing evidence that this antioxidant may have anti-ageing properties. GSH (l-g-glutamyl-l-cysteinyl-glycine; GSH) is a tripeptide (Figure 1-7) found in practically all animal cells. It is an important reducing agent that maintains enzyme activity and functions to maintain compounds like dehydroascorbate (vitamin C) and a-tocopherol, vitamin E, (Figure 1-8) in the functional reduced state. GSH has several characteristics that determine its metabolism and antioxidant function. One is that glutamate and cysteine peptide linkage is linked through a gamma-carboxyl

group of glutamate instead of the more common a-carboxyl peptide linkage (DeLeve & Kaplowitz, 1991). This distinctive property makes the gamma -carboxyl bond resistant to all peptidases except gamma-glutamyltranspeptidase (GGT), which is bound to the external surface of the cell membrane. As a result, GGT does not affect the intracellular breakdown of GSH (DeLeve & Kaplowitz, 1991). The most important moiety of GSH is the cysteinyl reactive thiol group, which accounts for many of the antioxidant functions of GSH metabolism. These characteristics, along with the fact that GSH is the most abundant non-protein thiol source and antioxidant in the cell, give GSH multiple functions for antioxidant defence, such as, GSH conjugates with harmful exogenous and endogenous toxic compounds; GSH provides a substrate for GSH peroxidase wherein GSH is used to reduce hydrogen and organic peroxides to water and alcohol; GSH reduces disulphide linkage of proteins and other molecules maintaining glycolytic and antioxidant enzymes in the reduced state. GSH is a main nontoxic storage form of cysteine providing a vehicle for transport between organs; GSH is a major thiol source maintaining essential redox status of the cell; and GSH has an important role in the reduction of ribonucleotides to deoxyribonucleotides (DeLeve & Kaplowitz, 1991).

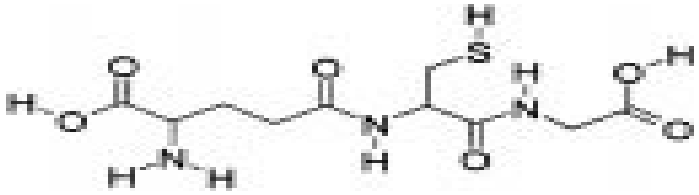

Figure 1-7: Structure of glutathione.

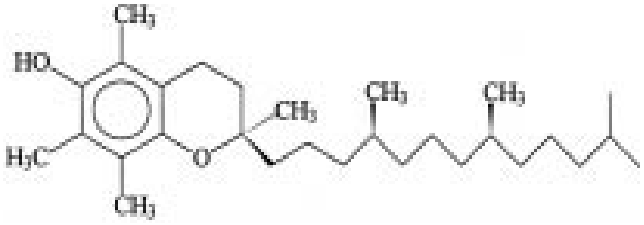

Figure 1-8: Structure of vitamin E.

There are several studies that have investigated the effect of age on GSH levels in different tissues. One study investigated GSH and several major antioxidant enzymes. Contrary to what one might expect, GSH concentrations were found to increase with age in the mice cerebellum and brain stem (Hussain *et al.*, 1995). Most studies do agree that there is no significant age-related decrease in GSH concentration in the brain. Other reports found no change in GSH concentration in the brains of senescent rats, while another found an increase in GSH in the brain (Barja *et al.*, 1990). These studies showed increases in heart, liver, and plasma GSH concentration in old rats (Barja *et al.*, 1990). There are, however, some reports that show a decrease in brain GSH levels with age (Ravindranath *et al.*, 1989). These studies found no significant change or an increase in liver GSH

concentration (Rikans & Moore, 1988). Liver GSH is crucial as it provides GSH to all tissues. The liver contains the highest GSH concentration and continuously exports GSH to extra-hepatic tissues. Changes in GSH levels in skeletal muscle have also been investigated with age. Leeuwenburgh *et al* (1994) found a significant increase in GSH content in the soleus muscle and no difference in GSH concentration in deep vastus lateralis muscle (Table 1-2) (Leeuwenburgh *et al.*, 1994), indicating that ageing does not result in a significant decline in this antioxidant and that there are fiber-specific adaptations of the GSH system in skeletal muscle with age (Table 1-2).

Table 1-2: Concentration of the antioxidant glutathione (GSH) in deep vastus lateralis (DVL) and in soleus skeletal muscle of young and old rats (Leeuwenburgh *et al.*, 1994).

	GSH DVL	GSH Soleus
Young	1.94 ± 0.21	2.10 ± 0.13
Adult	1.91 ± 0.16	2.64 ± 0.06
Old	1.76 ± 0.15	2.88 ± 0.11[a]

Values are means ± SE; GPX: glutathione peroxidase; SOD: superoxide dismutase (units/g wet weight); CAT: catalase (K × 10^{-2}/g wet weight). GSH: glutathione µmol/g wet weight. Young = 4.5 months; Adult = 14.5 months; Old = 26.5 months. [a]$p < 0.001$, Adult or old vs. young

Studies investigating subcellular components and changes of GSH content with age have found that GSH may decrease with aging. One such study investigating GSH in rat cerebral cortex synaptosomes as a function of age, found a decrease

in GSH concentration (Favilli *et al.*, 1994). Another study investigating mitochondrial GSH showed a general decrease in GSH levels with ageing in the brain, kidney, and liver of rats and mice (Asunción *et al.*, 1996). This would suggest that there may be differences in GSH concentration among different sub-cellular compartments during the ageing process. Such changes may not be detected when looking at the overall levels of GSH in tissues in some of the previously selected studies. As a result, there is a need to investigate specific organelles within the cell to determine changes in antioxidant levels under different conditions and with age.

Overall then, a critical examination of the relevant literature on antioxidant status and ageing in the kidney revealed a need to comprehensively examine key metabolite concentrations in different regions of the kidney at different ages, to correlate this with the activity of key antioxidant enzymes and to relate this to age and kidney region variation in expression at the protein and gene level. Here, the rat is used as a model organism but, where appropriate, implications for human health will be mentioned.

Chapter 2 : Effect of ageing and oxidative stress on antioxidant enzymes in different regions of the kidney.

2.1. Abstract

Oxidative stress has been implicated in ageing and the pathogenesis of chronic kidney disease (Dalle-Donne *et al.*, 2003), glutathione peroxidase (GPX) is a major H_2O_2 scavenger enzyme, and is located in both the cytosol and mitochondria (Drevet, 2006). Together with its glutathione (GSH), they form a formidable defence against H_2O_2 and lipid peroxidation (Pollack & Leeuwenburgh, 1999), Organic peroxides are reduced by the glutathione peroxidase (GPX) and also, specifically, by selenium-independent enzymes the glutathione S-transferases (GSH-ST) (Bauché *et al.*, 1994; Lawrence & Burk, 1976; Prohaska, 1980). It has been suggested that selenium dependent GPX (Se-GPx) (Mirault *et al.*, 1991; Raes *et al.*, 1987) and GSH-ST (Carrillo *et al.*, 2012; Kolm *et al.*, 1995; Lavoie *et al.*, 1992; Morrow & Cowan, 1990) play key roles in cellular defence against oxidative damage. When peroxides are detoxified, glutathione (GSH) is converted to oxidized glutathione (GSSG) and the glutathione reductase (GSH-red), thus maintaining the homeostatic GSH/GSSG ratio. Several studies have demonstrated an important role of this enzyme in cellular resistance to reactive oxygen species (Baker *et al.*, 1988; Bauché *et al.*, 1994; Miller & Blakely, 1992);

The aim of this study was to investigate how the antioxidant enzymes GPX, GSH-red, GSH-ST, CAT, and SOD are affected by age and oxidative stress in different regions of the rat kidney.

Kidneys from male Wistar rats of different ages (5, 12, 36 and 60 weeks) were dissected into cortex, outer medulla and inner medulla. Tissues were incubated for 30 minutes with/without 0.2 mM H_2O_2 to induce oxidative stress. Levels of each antioxidant enzyme were assayed before and after stress exposure. All of the antioxidant enzymes assayed were shown to decrease with age under both control and stress conditions, after peaking at 12 weeks (young adult). The activity of the antioxidant enzymes was greater in the cortex by comparison with the outer and inner medulla respectively.

2.2. Introduction

Oxidative stress has been implicated in ageing and the pathogenesis of chronic kidney disease (Chandran *et al.*, 2014). Semsei *et al.* (1991) have proposed that enzymes scavenging active oxygen species are key factors in determining the longevity of animals. Indeed, there are correlations between levels of these enzymes and the maximum life span potential of different species (Sohal *et al.*, 1990) There is evidence for example that Superoxide dismutase (SOD) (superoxide: superoxide oxidoreductase, E.C 1.15.1.1) and catalase (Chabory *et al.*, 2009) (hydrogen-peroxide: hydrogen-peroxide oxidoreductase, E.C 1.11.1.6), which defend the components of cells against reactive oxygen species, may play a role in the ageing process (Holmström & Finkel, 2014). Cellular GPX is the major hydrogen peroxide scavenger, and it is found in both the cytosol and mitochondria. Together with its substrate GSH-, it forms a formidable defence against hydrogen peroxides and lipid peroxides (Pollack & Leeuwenburgh, 1999), Organic peroxides are reduced by the latter enzyme and also, specifically, by selenium-independent enzymes named glutathione S-transferases (GST) (Bauché *et al.*, 1994; Lawrence & Burk, 1976; Prohaska, 1980; Zhu *et al.*, 2012). It has been suggested that Se-GPx (Mirault *et al.,* 1991; Raes *et al.*, 1987) and GST (Carrillo *et al.*, 2012; Kolm *et al.*, 1995; Lavoie *et al.*, 1992; Morrow & Cowan, 1990) play a key role in cellular defence against oxidative damage. When peroxides are detoxified, glutathione (GSH) is converted to oxidized glutathione (GSSG), and the activity of glutathione

reductase (GSH-red) (E C 1.8.1), then maintains homeostasis (GSH/GSSG) ratio (Zhang *et al.*, 2014). Several studies have also demonstrated an important role for this enzyme in cellular resistance to reactive oxygen species (Baker *et al.*, 1988; Bauché *et al.*, 1994; Miller & Blakely, 1992; Sena & Chandel, 2012; Sharma *et al.*, 2012). In the light of this previous work, it is hypothesised here that the activity of antioxidant enzymes in the kidney would decrease with age under control and stressful conditions. Furthermore it also hypothesised that enzyme activity will decrease preferentially in the medulla with respect to the cortex (Zou & Cowley, 2003). This is due to the known effects of chronic exposure to hypoxia with attendant reactive oxygen species production that occurs in the inner renal medulla, which has also been linked to altered Na+ regulation and hypertension (Zou & Cowley, 2003).

The aim of this study was to investigate how antioxidant enzyme activities are affected by age and oxidative stress in different regions of the rat kidney.

2.3. Methods

2.3.1. Sample Collection

Male Wistar rats were supplied by the UNE animal house. All experiments were approved by the ethics committee of the UNE (AEC09/152, EC10/036 and AEC11/100). Five rats were used in each age group; very young (5 weeks), young mature (12 weeks), mature (36 weeks) and ageing (60 weeks). Animals were stunned and sacrificed by cervical dislocation. Kidneys were removed, snap frozen in liquid nitrogen and stored at -80°C until use. Here it is worth noting that different groups of rats of each age cohort were used in the experiments described in this chapter and the following 3 chapters (Chapters 3, 4, and 5).

2.3.2. Tissue preparation

Kidneys were dissected to yield; a) cortex b) outer medulla and c) inner medulla. Fifty mg of each tissue incubated at room temperature 25 ºC for 30 min with phosphate buffered saline (PBS) at a ratio of 1:1000 prior to homogenise for 2 min (control condition). To model the effects of oxidative stress additional 50 mg sections of tissue were exposed to 0.2 mM H_2O_2 for 30 minutes at room temperature prior to homogenisation (stress condition). The resulting homogenates were centrifuged at 10000 g for 10 minute at 4 ºC. The supernatant was divided into small aliquots, and frozen until use.

2.3.3. Protein assay

Total protein concentration was determined using a Bradford reagent assay (Sigma, USA: catalog number B6916). A standard curve was prepared using varying concentrations of bovine serum albumin (BSA) (2000, 1500, 1000, 750, 500, 250 and 125 µg/ml BSA/water). 20 µl of each sample was added to 1 ml of Bradford reagent and vortexed. Reactions were incubated for 15 min at room temperature, and absorbance measured at 595 nm. Protein concentration (mg/ml) of kidney extracts was determined by extrapolation from the BSA standard curve.

2.3.4. Measurement of antioxidant enzymes

In general it should be noted that all enzyme and metabolite concentrations assayed here and in Chapter 3 were normalised relative to protein concentration in order to provide consistent meaningful comparisons. In the same vein, most of the commercial assay kits used provided positive controls to ensure consistency in absorbance measurements.

2.3.4.1. Glutathione peroxidase (GPx)

GPx activity was determined using a commercially available kit (Sigma, USA: catalog number CGP1), according to the manufacturer's instructions. Briefly, 10 µl samples of kidney homogenate were added to assay buffer, and 50 µl of NADPH reagent added. The reaction was started by adding 10 µl of 30 mM tert-butyl hydroperoxide solution, and the change in absorbance at 340 nm measured by spectrophotometry. Enzyme activity (U/mg protein) of each sample was calculated using the formula:

($\triangle A_{340}$ x DF)/ (6.22 x V), where DF = dilution factor of sample prior to reaction, and V = sample volume in ml and 6.22 is the extinction coefficient of NADP (εmM).

It is possible that the measured GPX activity may include a minor contribution by some glutathione transferases.

2.3.4.2. Glutathione reductase (GSH-red)

Glutathione reductase (GSH-red) activity was determined using a commercially available kit (Signma, USA, catalog number GRSA) according to the manufacturer's instructions. Briefly, 50 µl samples of kidney extract were added to 450 µl assay buffer, and 500 µl of 2 mM oxidised glutathione (GSSG) added. The reduction of glutathione was initiated by adding 50 µl of 2 mM NADPH in 250 µl of 3 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and the change in absorbance at

412 nm monitored by spectrophotometry. Enzyme activity (U/mg protein) of each sample was calculated using the formula below:

$$\text{units/ml} = \frac{(\Delta A_{sample} - \Delta A_{blank}) \times (\text{dilution factor})}{\varepsilon^{mM} \times (\text{volume of sample in ml})}$$

An extinction coefficient (εmM) for NADPH = 6.22 $mM^{-1}cm^{-1}$ and an extinction coefficient (εmM) for TNB = 14.15 $mM^{-1}cm^{-1}$.

2.3.4.3. Glutathione S-transferase (GSH-ST)

Glutathione S-transferase (GSH-ST) activity was determined using a commercially available kit (Signma, USA, catalog number CS0410) according to the manufacturer's instructions. Briefly, 50 µl samples were added to the master mix solution containing; Dulbecco's phosphate buffered saline, 10 µl of 200 mM L-glutathione reduced (GSH) and 10 µl 100 mM of 1-chloro-2, 4-dinitrobenzene (CDNB). The change in absorbance at 340 nm was measured by spectrophotometry employing a kinetic program read every 30 s over a period of 5 min. Enzyme activity (U/mg protein) of each sample was calculated using the formula below:

$$\frac{(\Delta A_{340})/\min \times V(ml) \times dil}{\varepsilon_{mM} \times V_{enz}(ml)} = \mu mol/ml/\min$$

Where, dil = the dilution factor of the original sample εmM ($mM^{-1}cm^{-1}$) the extinction coefficient for CDNB conjugate at 340 nm = 9.6 mM^{-1} (path length $^{-1}$ cm), (V) the reaction volume =1 ml and $V_{enz.}$ = the volume of the enzyme sample tested.

2.3.4.4. Catalase

Catalase was determined using a colourimetric assay to determine residual H_2O_2 concentration after a calibrated enzymatic reaction according to the method described in the kit (Sigma, Catalog Number CAT100). Briefly, different concentrations of H_2O_2 standards (0, 125, 250, 500, and 750 µl) of 10 mM H_2O_2 solution were used in the preparation of a standard curve representing the increase in A_{520} with increasing concentrations of H_2O_2 as the peroxidatic reaction using substrate quinoneimine dye catalysed by the horse radish peroxidase (HRP) included with the kit proceeds to completion. The standard curve was used to calibrate concentrations of H_2O_2 remaining after the action of catalase in the kidney extracts. The catalatic activity (resulting in the disproportionation of H_2O_2 to water and oxygen and therefore progressive decrease in H_2O_2 concentration) of catalase is then determined as indicated by the decrease in H_2O_2 concentration over a fixed time period (the reaction being terminated by adding azide solution).

Enzyme activity (U/mg protein) of each sample was calculated using the formula below:

$$\text{Activity} = \frac{\Delta\mu\text{moles } (H_2O_2) \times d \times 100}{V \times t}$$
(µmoles/min/ml)

Where, the amount of H_2O_2 (mmoles) remaining in the colorimetric reaction mixture using the H_2O_2 standard curve OD_{520} of 1.4 is equivalent to 0.082 mmole of H_2O_2, A_{520}(Blank) = µmoles of H_2O_2 in Blank A_{520}(Sample) = mmoles of H_2O_2 in Sample D µmoles (H_2O_2) = µmoles of H_2O_2 (Blank) – mmoles of H_2O_2 (Sample), d = dilution of original sample for catalase reaction, t = catalase reaction duration (minutes), V = sample volume in catalase reaction (x ml = 0.00x ml), 100= dilution of aliquot from catalase reaction in colorimetric reaction (10 ml from 1 ml).

2.3.4.5. Superoxide dismutase SOD

SOD activity was determined using a commercially available kit (Sigma 19160), according to the manufacturer's instructions. Each sample reaction was set up according to volumes shown in Table 2-1. Kidney extract samples were compared to three blank samples, as shown in Table 2-1.

Table 2-1: Reaction components for the SOD activity assay

	Sample	Blank 1	Blank2*	Blank 3
Sample solution	20 µl		20 µl	
ddH2O		20 µl		20 µl
WST working solution	200 µl	200 µl	200 µl	200 µl
Enzyme working solution	20 µl	20 µl		
Dilution buffer			20 µl	20 µl

Reactions were incubated at 37 °C for 20 min, and the change in absorbance at 450 nm monitored using a microplate reader. Enzyme activity (U/mg protein) of each sample was calculated using the formula:

SOD activity (inhibition rate %) = [(S1-S3)-(SS-S2)] / (S1-S3)] * 100

2.3.5. Statistical analysis

Data were given as normalised to mean ±, standard error and n=5. Statistically significant differences between group means were determined using a one-way ANOVA with an appropriate post hoc test (Tukey's range test) using Instat Instant Biostatistics software v 3.0 sourced from Graphical software La Jolla, CA, USA.

2.4. Results

2.4.1. Glutathione peroxidase activity (GPX)

GPX activity in the outer and inner medulla was significantly lower ($P < 0.01$) by contrast with the cortex under control; while at 60 weeks rat, the GPX activity was significantly lower ($P < 0.05$) by 3.8-fold in the inner medulla only by comparison with the cortex under the same condition (Figure 2-1). Under oxidative stress condition, GPX activity was much lower in the inner medulla by comparison with the cortex at 5 and 60 weeks. In addition, there was a significant decrease in the GPX activity at 36 ($P < 0.001$) and 60 ($P < 0.05$) weeks in the outer medulla by comparison with the cortex. No significant change was recorded in the GPX activity between the cortex, outer and inner medulla at 12 weeks old under the oxidative stress condition (Figure 2-1).

The activity of GPX in the cortex, and the outer and inner medulla showed a consistent pattern of activity under control and oxidative stress condition. Overall, the activity of GPX was significantly elevated at 12 and 36 weeks by comparison with 5 and 60 weeks (Figure 2-1).

At 12 weeks, the GPX activity was significantly lower ($P < 0.05$) under stress condition by comparison with control in all regions of the kidney (Figure 2-1). In addition, the GPX activity was significantly lower ($P < 0.01$) in the inner medulla by 4.8-fold under the oxidative stress conditions by comparison with control at 36

weeks while in the cortex and outer medulla differences under control and oxidative stress conditions in the GPX activity at 36 weeks did not quite reach significance. At 5 and 60 weeks, despite a very obvious lowering trend in the stress conditions this decrease did not quite reach significance in any region of the kidney (Figure 2-1).

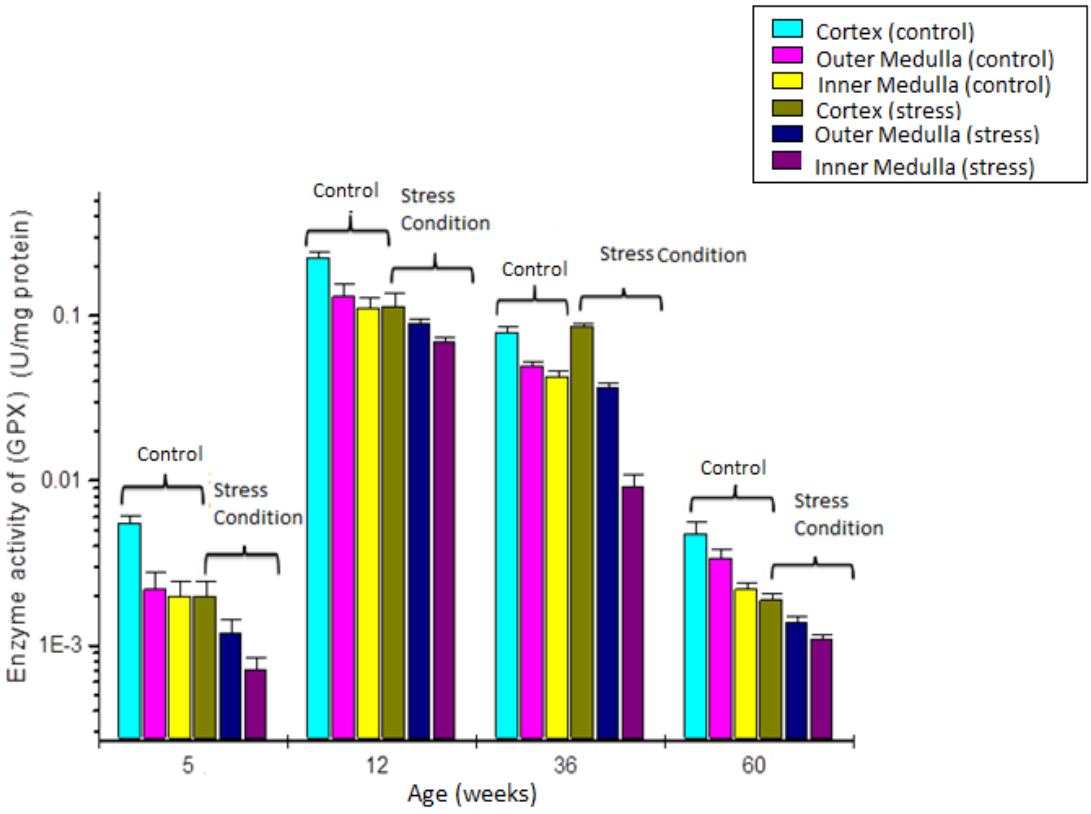

Figure 2-1: GPX activity in the cortex, outer medula and inner medulla under control and oxidative stress conditions at 5, 12, 36 and 60 weeks old. Data are presented as mean ± SE for n=5 individual animals in each age group. Between group levels of statistical significance (ANOVA) are explored in the text.

2.4.2. GSH-reductase (GSH-red)

No significant differences were observed in the GSH-red activity at 5 and 12 weeks in the cortex, outer and inner medulla under control, as well as at 60 weeks under oxidative stress conditions (Figure 2-2). At 5 weeks, the GSH-red activity was significantly lower in the outer medulla and inner medulla ($P < 0.01$ and $P < 0.001$ respectively) by 1.6- and 1.8-fold respectively by comparison with the cortex under the oxidative stress condition. In the oxidative stress condition, the GSH-red activities were significantly higher ($P < 0.05$) in the cortex by 2-fold by comparison with the inner medulla at 12 weeks (Figure 2-2). The GSH-red activities were significantly higher in the cortex ($P < 0.01$) by comparison with the inner medulla in both control and stress conditions at 36 weeks (Figure 2-2).

Overall the GSH-red activity showed a similar pattern to the other enzymes. In this case at 12 weeks cohort all showed a significant increase ($P < 0.05$) when compared with the enzyme activity at 5 and 60 weeks. The activity at 36 weeks cohort was slightly elevated by comparison with that at 5 and 60 weeks cohorts except in the case of the control inner medulla. Stressful conditions significantly ($P < 0.05$) decreased the GSH-red activity in the inner medulla at all ages (Figure 2-2).

The GSH-red activity was significantly ($P < 0.05$) lower in the cortex, outer and inner medulla at 12 weeks; and in the outer medulla at 36 weeks ($P < 0.05$) under

the stress condition by comparison with the control. Despite the overall obvious general trend toward lower values in the stress condition other changes did not reach statistical significant (Figure 2-2).

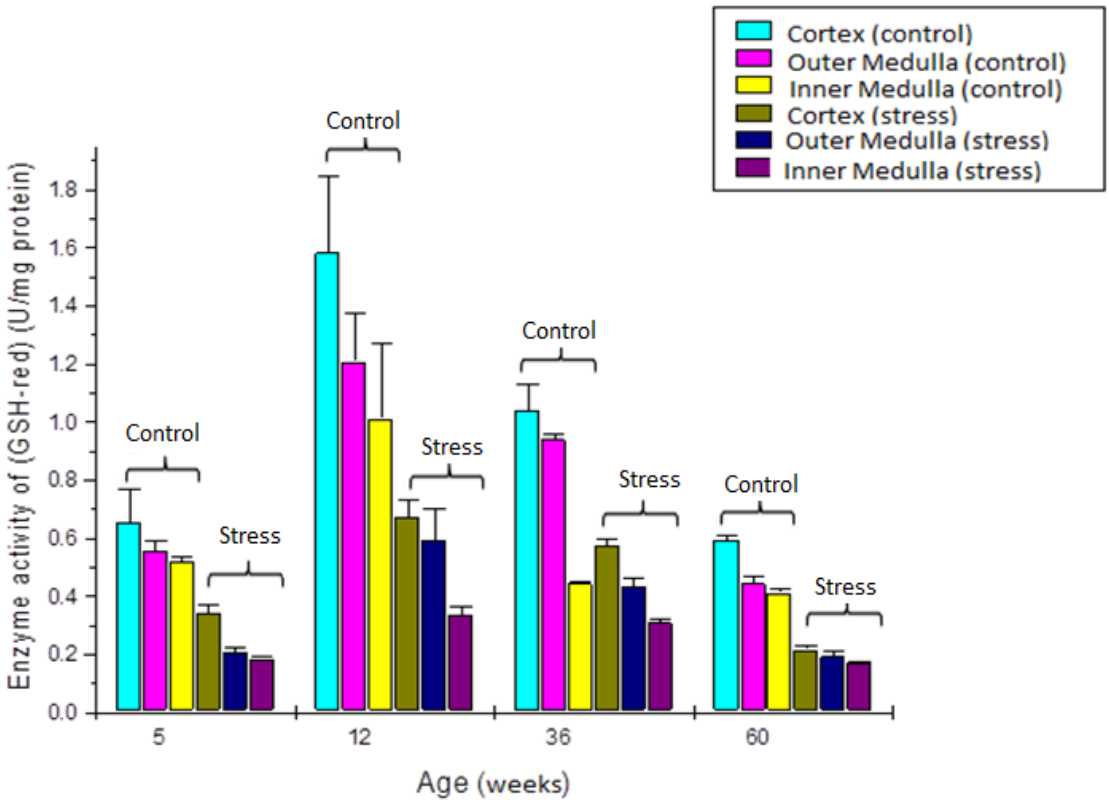

Figure 2-2: GSH-red activity in the cortex, outer medula and inner medulla under control and oxidative stress conditions at 5, 12, 36 and 60 weeks old. Data are presented as mean ± SE for n=5 individual animals in each age group. Between group levels of statistical significance (ANOVA) are explored in the text.

2.4.3. Glutathione-S-transferase (GSH-ST)

Figure 2-3 shows that the distribution of the GSH-ST activity had a similar trend under control and oxidative stress conditions as the activity was significantly lower ($P < 0.05$) in the inner medulla by comparison with the cortex and outer medulla at 5 weeks old. At 12 weeks there was no significant difference between the different region of the kidney in the GSH-ST activity under control and oxidative stress conditions (Figure 2-3). At 36 weeks, the GSH-ST activity was lower in the outer ($P < 0.001$) and inner ($P < 0.001$) medulla by comparison with the cortex under control and oxidative stress conditions. At 60 weeks there were no significant differences between the regions of the kidney under control. However, the GSH-ST activity was significantly lower ($P < 0.05$) in the outer and inner medulla by 1.4-fold by comparison with the cortex at 60 weeks under stress conditions (Figure 2-3). Overall GSH-ST was higher at 12 and 36 weeks by compared with 5 and 60 weeks.

Figure 2-3 shows that the GSH-ST activity under control was significantly higher at 12 and 36 weeks in the cortex ($P < 0.001$) by 2.6-and 2-fold respectively by comparison with the value at 5 and 60 weeks. In the outer and inner medulla at 12 weeks cohort showed a significantly ($P < 0.05$) elevated GSH-ST activity by comparison with all other ages under the control. At 36 weeks cohort showed a significant ($P < 0.05$) elevation in the outer medulla only (Figure 2-3). In the

oxidative stress condition as in the control the GSH-ST activity was significantly ($P < 0.05$) higher at 12 weeks cohort in the cortex by comparison with other age groups. Again in the outer medulla at 12 weeks group shows a significantly ($P < 0.05$) higher activity compared with other age groups. This was also the case with the inner medulla under oxidative stress conditions (Figure 2-3).

At 12 weeks, the GSH-ST activity was significantly lower ($P < 0.05$) in the cortex by 1.4-fold and by 2.5-fold ($P < 0.001$) in the outer medulla under stress conditions by comparison with the control. At 36 weeks, the GSH-ST activity was considerably lower ($P < 0.001$) in the cortex by 1.7-fold and in the outer medulla ($P < 0.01$) under oxidative stress by comparison with the control (Figure 3). There was no significant change in the GSH-ST activity under the stress condition by comparison with the control at 5 and 60 weeks in the cortex, outer and inner medulla (Figure 2-3).

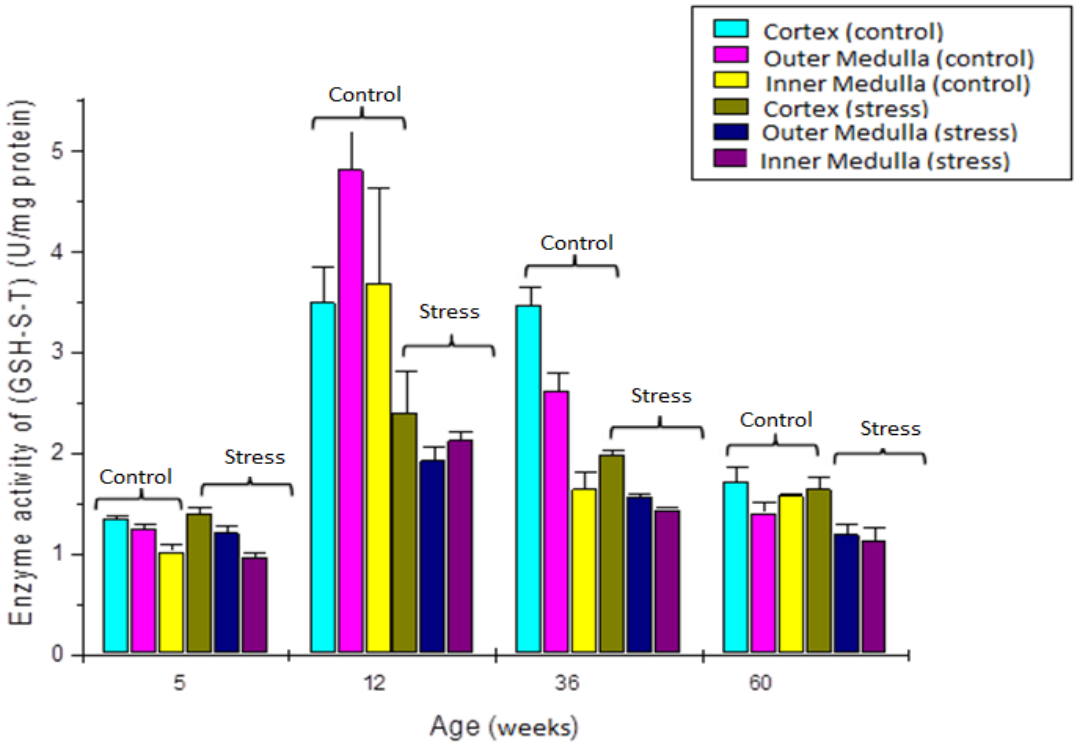

Figure 2-3: GSH-ST activity in the cortex, outer medula and inner medulla under control and oxidative stress conditions at 5, 12, 36 and 60 weeks old. Data are presented as mean ± SE for n=5 individual animals in each age group. Between group levels of statistical significance (ANOVA) are explored in the text.

2.4.4. Catalase activity (CAT)

The data shows that CAT activity in the outer and inner medulla under control and oxidative stress conditions were significantly reduced ($P < 0.001$) by comparison with the cortex at 5 weeks. As shown in Figure 2-4, the highest CAT activity was seen at 12 weeks under control in the cortex, and the outer and inner medulla. However, there was no significant difference in the CAT activity between the different regions of the kidney under both control and stress condition at 12 weeks. At 36 weeks, the CAT activity was significantly lower in the outer and inner medulla ($P < 0.05$ and $P < 0.001$ respectively) by comparison with the cortex under control. No significant differences were seen in the CAT activity under the stress condition (Figure 2-4). Under control and stress conditions, the CAT activity appears to be reduced ($P < 0.001$) in the outer and inner medulla by comparison with the activity in the cortex at 60 weeks (Figure 2-4).

It was noteworthy that the highest CAT activity was recorded at 12 weeks in all regions of the kidney under both control and stress conditions and that the second highest CAT level was at 36 weeks (Figure 2-4). At 5 and 60 weeks, there were no significant change in the CAT activity in the cortex, and the outer and inner medulla under control and stress conditions. While, at 12 and 36 weeks, the CAT activity was significantly lower ($P < 0.05$) under stress conditions by comparison with control in all regions of the kidney (Figure 2-4).

Although from a cursory examination of (Figure 2-4) it appears as if very little intra and inter group variation is evident between the 5 and 60 weeks cohorts' detailed statistical analysis does show the differences alluded to earlier. It is worthwhile pointing out that the very small SE values here are not immediately evident in (Figure 2-4) due to the scale of the graph.

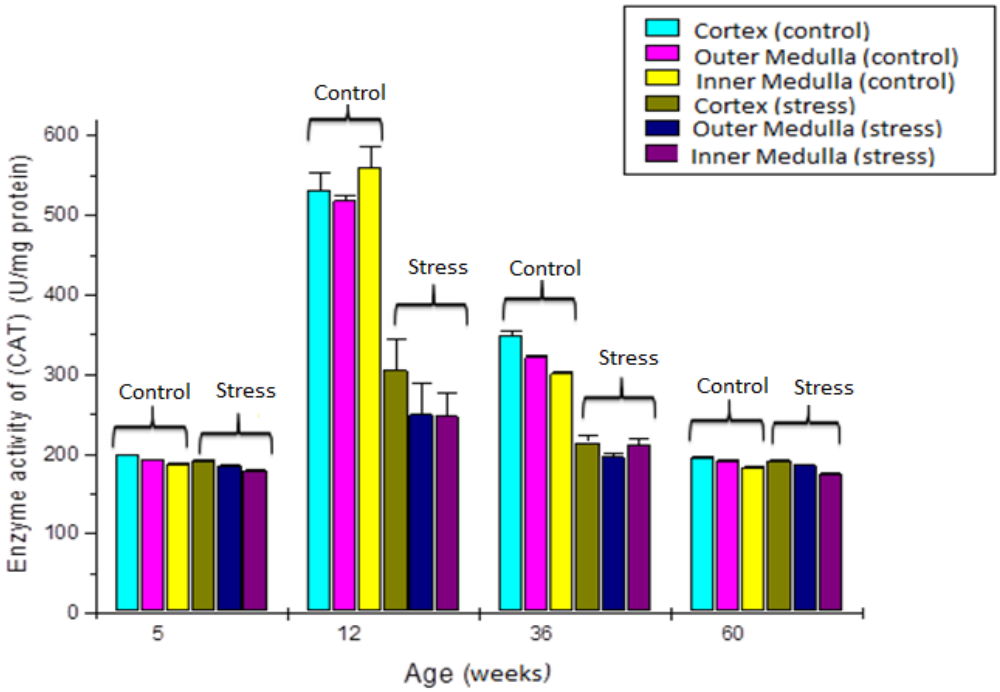

Figure 2-4: CAT activity in the cortex, outer medula and inner medulla under control and oxidative stress conditions at 5, 12, 36 and 60 weeks. Data are presented as mean ± SE for n=5 individual animals in each age group. Between group levels of statistical significance (ANOVA) are explored in the text.

2.4.5. Superoxide dismutase (SOD)

Figure 2-5 shows that at 5 weeks the SOD activity was significantly lower ($P < 0.001$) in the outer and inner medulla by comparison with the cortex under both control and stress conditions. At 12 weeks the SOD activity was significantly higher ($P < 0.05$) in the cortex by comparison with the inner medulla under the control, and by comparison with the outer medulla and inner medulla ($P < 0.001$) under oxidative stress condition (Figure 2-5). At 36 weeks the SOD activity was significantly lower ($P < 0.001$) in the outer and inner medulla by comparison with the cortex under both control and stress conditions (Figure 5). By 60 weeks the SOD activity in the inner medulla was significantly less ($P < 0.05$) than the outer medulla and cortex under both control and stress conditions.

The activity of SOD in the cortex, outer and inner medulla displayed a similar pattern of activity under control and stress conditions. The overall pattern was similar to that already seen for the other enzymes. The activity of SOD showed a significant increase at 12 and 36 weeks by comparison with 5 and 60 weeks (Figure 2-5) although, under the control, in the outer and inner medulla, the increase did not quite reach the level of statistical significance (Figure 2-5).

Even though the overall pattern was similar to the other enzymes studied, it was only in the cortex and outer medulla at 12 weeks that the SOD activity was significantly lower ($P < 0.05$) under the oxidative stress condition by comparison

with the control (Figure 2-5). There was no significant change in the SOD activity under stress condition at 5, 36 and 60 weeks (Figure 2-5).

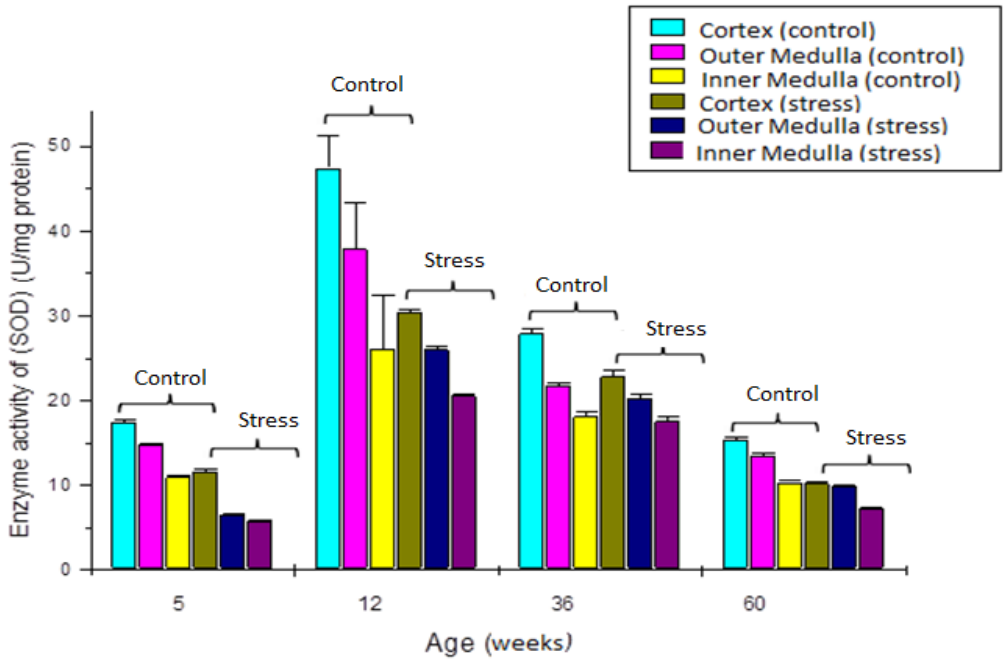

Figure 2-5: SOD activity in the cortex, outer medula and inner medulla under control and oxidative stress conditions at 5, 12, 36 and 60 weeks. Data are presented as mean ± SE for n=5 individual animals in each age group. Between group levels of statistical significance (ANOVA) are explored in the text.

2.5. Discussion

Resistance to oxidative agents is controlled by several key antioxidant enzymes, including GPX, GSH-red, GSH-ST, CAT and SOD. Measuring the activity of these enzymes is therefore crucial to understanding antioxidant defence mechanisms. The aim of the present study was, therefore, to evaluate the antioxidant defence capacity of the different regions of the kidney at different ages. While confirming key overall age related findings of previous authors (Rao *et al.*, 1990) the novel aspect to our work involves the discrimination between different kidney regions.

Overall the results of our study indicate that after reaching a maximum at maturity (12 weeks), the activity of GPX, GSH-red, GSH-ST catalase, and SOD decreased significantly with age, in all regions of the kidney (cortex, outer and inner medulla). As previously mentioned the overall conclusions of the present study are in broad agreement with many earlier studies (Baird & Samis, 1971; Carney *et al.*, 2012; Farahmand *et al.*, 2013; Hazelton & Lang, 1985; Ramesh *et al.*, 2012) which suggest that there is an age-related decrease in the activity of catalase, in liver and kidney tissues from mice and rat. In addition, the findings of the present study are in general agreement with many earlier studies (Hazelton & Lang 1985; Ramesh *et al.*, 2012) demonstrating that GPX and GSH-red activity also decrease with increasing age in the kidney. Furthermore the observed age-related reduction in the activity of SOD, GPX and GSH-ST is in agreement with earlier reports (Ito *et*

al., 1998; Subramanian & James, 2010). There is much evidence associating a decrease in antioxidant status with age (Čejková *et al.*, 2004; Shih & Yen, 2007).

It has been proposed that the ageing process involves an increased susceptibility to free radicals (Harman, 1955; Rao *et al.*, 1999; Rao *et al.*, 1990; Rao *et al.*, 2003). SOD, CAT, and GPX comprise the major enzymatic antioxidants involved in neutralizing the ill effects of free radicals produced during ageing. SOD, a family of intracellular enzyme that catalyses dismutation, protects against oxygen free radicals by catalysing the removal of a superoxide radical ($O_2^{\cdot -}$), which damages the membrane and biological structures. CAT has been shown to be responsible for the detoxification of significant amounts of H_2O_2 (Jones & Masters, 1976; Subramanian & James, 2010). A decrease in the activity of SOD contributes toward attenuation in the efficacy of mitochondrial protection mechanisms against the disorganizing effects of free radicals. The results presented here are in accordance with the earlier study done by Subramanian and James (2010) indicating that SOD and GPX activities decreased slightly with age although the current study extends this work by providing a more comprehensive view of age related changes placing emphasis on the concerted response of the whole gamut of the antioxidant enzymes as well as critically analysing the importance of the regional distribution of the enzyme activities.

In the present study it was also observed that under stress condition following H_2O_2 exposure, the activity of all antioxidant enzymes (GPX, GSH-red GHS-ST,

CAT and SOD) was decreased in comparison with the control condition. Exposure to 0.2 mM H_2O_2 is a standard model for exposing tissues to oxidative stress (King *et al.*, 2010; King *et al.*, 2003) and is accompanied by abundant ROS production. The model of oxidative stress employed here does have some limitations. Most importantly here we examine the effect of H_2O_2 on reconstituted frozen tissue extracts which presumably are not capable of the full gamut of metabolic processes. Having expressed this caveat, here we explore the effects that this has on the different kidney regions knowing they have different enzyme activities and taking into account their different functions. The cortex for example is heavily involved in transport activities and has abundant mitochondria so one might therefore expect a high level of antioxidant enzyme expression and activity in this region. The outer medulla is more involved with water and Na^+ balance while the inner medulla is mainly concerned with collection of the end products of filtration and passing it onto the bladder and, as such, a low rate of metabolic activity might be expected to be attended by low antioxidant enzyme activity.

Our working hypothesis here is that as the kidney matures and then ages the response to oxidative stress will vary from region to region. This will be reflected in different antioxidant enzyme activities.

Our results indicate that an increase in oxidative stress results in a systematic decrease in antioxidant enzymes levels in the kidney. This observed reduction in the activity of antioxidant enzymes under stress conditions is in agreement with

earlier reports using a range of oxidative challenges (Chandran *et al.*, 2014; Gecit *et al.*, 2011; Sheng *et al.*, 2011), The increased production of ROS that results from oxidative stress leads to modifications in lipids, carbohydrates, proteins and nucleic acids, resulting in cytotoxicity and dysfunction in several tissues (Sindhu *et al.*, 2004). Given the time scale of the treatment with H_2O_2 (30 min.) it is most likely that the effects on enzyme activity reflect the overall cytotoxicity of the treatment.

The results of the present study demonstrate that antioxidant enzyme activities were higher in the cortex by comparison with the outer and inner medulla. These results should be interpreted with regard to earlier studies carried out by Chen *et al.* (2003) which showed that the basal level of H_2O_2 in the renal medulla was twofold higher than in the renal cortex suggesting that, under physiological (control) conditions, the renal medulla is exposed to higher levels of oxidative stress than the cortex possibly due to the lower antioxidant enzyme levels in the renal medulla and this may eventually result in a reduction of medullary blood flow (MBF), water and Na+ maintenance, leading to hypertension and renal dysfunction. In this regard, Meng *et al.* (2002) propose that the increase of oxidative stress can be caused by an increase in $O_{2^{-\cdot}}$ production due to increased oxidase activity or by a decrease in antioxidant levels. The oxidases that could have led to the increased oxidative stress in this previous study were not determined, but the data indicate that a decrease in renal medullary and cortical

SOD levels could have contributed to the higher oxidative stress in high Na-fed Dahl salt-sensitive rats.

Previous studies (Chen *et al.*, 2003; Cowley, 2008; Makino *et al.*, 2003; Pallone, 2006) examined whether H_2O_2 has direct effects on renal dysfunction. It was found that direct delivery of H_2O_2 into the renal medulla significantly increased the concentration of H_2O_2 in this kidney region and largely decreased medulla blood flow (MBF) and water and Na^+ excretion. These results suggest that excessive H_2O_2 in the renal medulla produces vasoconstriction and antinatriuresis. Given the importance of renal MBF and Na^+ excretion in the development of hypertension, elevations of H_2O_2 in the region could contribute to medullary vasoconstriction and Na+ retention, thereby contributing to the development of hypertension. In this regard Zou *et al* (2001) investigated both the mitochondrial enzyme system and NADH oxidase primarily responsible for $O_2^{2\cdot}$ production in the renal medulla showing that the renal outer medulla is a major region for $O_2^{2\cdot}$ production in the renal medulla. Inhibition of SOD activity reduced renal MBF and water/sodium excretion, whereas scavenging of $O_2^{2\cdot}$ by TEMPOL increased renal MBF and water/sodium excretion. These results further support the proposition that endogenously produced $O_2^{2\cdot}$ participates in the control of renal MBF and water/sodium excretion.

In conclusion, the current study has demonstrated that, in the rat kidney, activity of the antioxidant enzymes GPX, GSH-red, GSH-ST, CAT and SOD reaches peak

levels in young, sexually mature animals (12 weeks). The lowest activities of antioxidant enzymes were observed in immature and ageing animals (5 and 60 weeks). These antioxidant enzymes were found to have the highest activity in the renal cortex compared to the medulla, and levels of the antioxidant enzyme activity decreased with age in all regions of the kidney.

The enzymatic antioxidant system works in concert with the small molecule antioxidant system and this we consider in the next chapter (Chapter 3)

In Chapter 4 we investigate expression of several of the antioxidant proteins whose enzyme activity we have explored here using Western blot detection to confirm aspects of the results in this chapter while in Chapter 5 we investigate gene expression of these same proteins to expand and confirm aspects of the results in Chapters 2 and 4 and at the same time providing preliminary evidence of a reciprocal relationship between antioxidant gene expression and the stress protein response.

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(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

	Author's Name (please print clearly)	**% of contribution**
Candidate	Noor Riyadh Thiab	60
Other Authors	Graham L. Jones	20
	Nicola King	20

Name of Candidate: **Noor Riyadh Thiab**

Name/title of Principal Supervisor: **Associate Professor/ Graham L. Jones**

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23 /04 / 2015

Date

Candidate

[redacted]

23 /04 / 2015

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Type of work	Page number/s
Figure 1	53
Figure 2	55
Figure 3	58
Figure 4	60
Figure 5	62

Name of Candidate: Noor Riyadh Thiab

Name/title of Principal Supervisor: Associate professor/ Graham L. Jones

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23 /04 / 2015

Candidate Date

[redacted]

23 /04 / 2015

Principal Supervisor Date

Chapter 3 : Effects of ageing on metabolite and oxidant concentrations in different regions of rat kidney under control and stress conditions.

3.1. Abstract

Oxidative stress has been implicated in ageing and the pathogenesis of chronic kidney disease (Dalle-Donne *et al.*, 2003). Glutathione (GSH) is the major intracellular thiol in the kidney and an indicator of this organ's redox status. The purpose of this study was to investigate how the GSH concentration, thiobarbituric acid reactive substances (TBARS), and lactate concentration are affected by age and oxidative stress in different regions of the kidney.

Kidneys were dissected from Male Wistar rats at 5, 12, 36 and 60 weeks. Slices of superficial cortex, outer or inner medulla were incubated at RT for 30 min ± 0.2mM H_2O_2 (control and stress condition) prior to homogenisation and centrifugation. Data presented in nmol/mg protein, were means ± SE of n=5 and statistical comparisons were carried out using ANOVA with an appropriate post-test (Tukey's honestly significant difference).

In all regions and all conditions the GSH concentration showed a similar pattern with 12 weeks > 36 weeks > 60 weeks and 5 weeks. The highest concentration

was measured at 12 weeks in the cortex which was significantly greater than the outer ($P < 0.01$) and the inner medulla ($P < 0.01$). H_2O_2 exposure significantly reduced ($P < 0.05$) the GSH concentration at all ages and all regions except the inner medulla which had the lowest concentrations overall and where oxidative stress had little effect at 5 and 60 weeks. These results suggest that the antioxidant capacity in different kidney regions peaks at maturity (12 weeks) and then decreases with increasing age. In all regions and all conditions the TBARS and the lactate concentration showed a similar pattern with 12 weeks < 36 weeks < 5 weeks and < 60 weeks. The highest TBARS concentration was measured at the 60 weeks under oxidative stress conditions with the superficial cortex significantly higher than the outer ($P < 0.0001$) and inner medulla ($P < 0.0001$), and the highest lactate concentration was at 60 weeks old under oxidative stress conditions with the inner medulla than outer medulla and cortex ($P < 0.0001$).

3.2. Introduction

Reactive oxygen species (ROS), including hydrogen peroxide and organic peroxides (H_2O_2, ROOH), superoxide ($O_2^{-\cdot}$), hydroxyl radical ($OH^{\cdot}$), singlet oxygen (O^1_2), are overproduced by cells exposed to radiation (Gill & Tuteja, 2010; Sonntag, 1987), redox-cycling drugs (Halliwell, 1994). These intermediates are known to be cytotoxic and are often a cause of tissue injury in diseases, such as cancer, atherosclerosis, rheumatoid arthritis, and in a variety of situations, such as ischaemia reperfusion, chemo and radio therapeutic treatment and hyperoxia (Jomova *et al.*, 2012; Sahiner *et al.*, 2012; Sies, 1991). Superoxide $O_2^{-\cdot}$ and peroxides are thought to be moderately active, and much of their toxicity is considered to result from their subsequent metal ion catalyzed transformation to the highly reactive $OH^{\cdot}$ (Halliwell & Gutteridge, 1986). The hydroxyl radical is a very powerful oxidant that can attack all types of biological molecules, including lipids (Gajewski *et al.*, 1990; Jomova *et al.*, 2012), proteins (Jomova *et al.*, 2012; Taylor & Davies, 1987) and DNA (Bauché *et al.*, 1994; Jomova *et al.*, 2012). Cells are normally protected against oxidative damage by multiple enzymatic mechanisms and by antioxidant molecules (Sahiner *et al.*, 2012). Among the antioxidant molecules, glutathione appears to be a key component. Reduced glutathione (GSH) is changed to oxidized glutathione (GSSG). This disulphide is subsequently reduced by a NADPH-dependent enzyme (Anathy *et al.*, 2012). This tripeptide has different functions which contribute to cell protection and defence;

free radical scavenger, coenzyme for several antioxidant enzymes, maintenance of the thiol-disulfide status and detoxication of electrophilic xenobiotics via conjugation (Mates *et al.*, 2012; Meister & Anderson, 1983). The importance of glutathione in protecting various cells against free radical injury or chemically induced damage is now well established (Mates *et al.*, 2012; Meister & Anderson, 1983; Reed, 1990; Simic & Nygaard, 1983).

Lipid peroxidation products generate malondialdehyde (MDA) invariably at low yields. MDA is one of several low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products but is neither the sole end product of fatty peroxide formation and breakdown nor a substance generated exclusively through lipid peroxidation. The TBA test is intrinsically nonspecific for MDA. Nonlipid-related materials as well as fatty peroxide-derived decomposition products other than MDA are also TBA positive. The thiobarbituric acid (TBARS) test has been utilised to identify and quantify lipid peroxidation in biological matrices as well as in a variety of chemical transformations (Delfino *et al.*, 2011; Halliwell & Whiteman, 2004; Lunec, 1989; Pryor, 1989)

The kidney has a most important role in lactate metabolism, and under normal physiological conditions, the kidney is second only to the liver in removing lactate from circulation (Bellomo, 2002; Mizock & Falk, 1992). Lactic acidosis is an important metabolic disorder with a poor prognostic outcome (Bellomo, 2002;

Mizock & Falk, 1992). It is not surprising, then, that its pathogenesis has been, and continues to be, a source of interest to critical care physicians (Gutierrez & Wulf, 1996). Lactate is fully filtered at the glomerulus (Bellomo, 2002; Höhmann *et al.*, 1974). However, it is also almost completely reabsorbed in the proximal tubule (Bellomo, 2002; Höhmann *et al.*, 1974). Only a marked rise in blood lactate levels results in an increase in urinary excretion. Even then, lactate urinary losses are small by comparison with overall renal lactate metabolism, with only 2% of total lactate removal during exercise (plasma lactate > 20 mmol/l) being realized by urinary excretion (Bellomo, 2002). Lactate uptake is the major mechanism of renal lactate removal and appears to be essentially a function of the cortex (Bartlett *et al.*, 1984), as shown by radio isotopic methods in isolated, perfused rat kidney (Bartlett *et al.*, 1984). In the absence of glucose and in the presence of starvation, the cortex produces tiny amounts of lactate. Once glucose returned to normal levels, the cortex continues to produce little, if any, lactate. The medulla, on the other hand, uses glucose and generates lactate from glycolysis. The cortex at the same time takes up the lactate released by the medulla and uses it in aerobic metabolism and gluconeogenesis. The cortex does not oxidize glucose directly and hence the presence of a cortical-medullary glucose–lactate recycling system. The medulla consumes glucose via glycolysis and generates lactate. The cortex takes up lactate to oxidize it for energy production and to generate glucose by

gluconeogenesis for release back to the medulla for medullary glycolysis and energy production (Bellomo, 2002).

The overall rationale for the regional dissection of the rat kidney has already been elaborated in the discussion to Chapter 2 as has the rationale for the use of 0.2 mM H_2O_2 as a model oxidant challenge

On the basis of previous work it is hypothesised that the concentration of GSH in the kidney would decrease with age, under oxidative stress conditions, and would be decreased in the medulla compared to other regions of the kidney. Furthermore, it is hypothesised that TBARS and lactate would increase with age, under oxidative stress conditions, and would be higher in the medulla than in other regions of the kidney.

The aim of this study was to investigate how the concentration of GSH, TBARS, and lactate are affected by age and oxidative stress in different regions of the rat kidney.

3.3. Method

3.3.1. Sample Collection

Animals were treated in the same manner as described in section 2.3.1.

3.3.2. Tissue preparation

Tissues were prepared in the same manner as described in section 2.3.2.

3.3.3. Protein assay

The protein concentration was determined in the same manner as described in section 2.3.3.

3.3.4. Measurement of glutathione concentration

Glutathione concentration was determined using a commercially available kit (Sigma, USA, catalog number CS0260), following the manufacturer's instructions. 10 μl of sample was mixed with 150 μl working mixture and incubated for 5 min at room temperature. To start the reaction 50 μl NADPH solutions was added to each sample and absorbance measured at 412 nm against a SSA/working mixture/NADPH solution blank. Absorbance was read at 1 min intervals for 5 min. Results were compared to a standard curve containing 3.125, 6.25, 12.5, 25 and

50 μM glutathione. The concentration of glutathione was calculated using the following equation below:

$$\text{nmoles GSH per ml of sample} = \frac{\Delta A_{412}/\text{min(sample)} \times \text{dil}}{\Delta A_{412}/\text{min(1 nmole)} \times \text{vol}}$$

Where ΔA412/min (sample) = slope generated by sample (after subtracting the values generated by the blank reaction). ΔA412/min (1 nmole) = slope calculated from standard curve for 1 nmole of GSH, dil = dilution factor of original sample and vol = volume of sample in the reaction in ml.

3.3.5. Estimation of lipid peroxidation by thiobarbituric acid reactive substances (TBARS)

The amount of lipid peroxidation in the various samples before and after oxidative stress was determined as malondialdehyde (MDA),which reacts with thiobarbituric acid (TBA) to form a coloured TBA-MDA complex (Buege , 1978). Tissue samples in the presence of 50 mM 2,2'-azobis (2-methylpropionamidine) dichloride (AAPH) (300μl) were combined with 1 ml of a solution containing trichloroacetic acid (15% w/v) and Thiobarbituric acid (0.375% w/v) in 0.25 M HCl. The mixture was boiled for ten minutes and allowed to cool on ice followed by centrifugation at 12,000 xg for 5 min. The absorbance of the supernatant was determined at 535nm against a blank solution containing all the reagents except

tissue extract. The MDA concentration was calculated using a molar extinction coefficient of $1.56*10^5$ M^{-1} cm^{-1} (Ohkawa *et al.*, 1979). TBA-reactive material is expressed as nmoles of malondialdehyde/mg protein.

3.3.6. Lactate assay

The method for obtaining neutralised extracts prior to the measurement of cellular contents such as lactate has been described elsewhere (Williams *et al.*, 2001). Briefly, tissue samples (50 mg) were ground under liquid nitrogen using a mortar and pestle. The resulting fragments were scooped into ice cold tubes containing 500 ml of (4.8% v/v) per-chloric acid. The tubes were then centrifuged at 4,000 rpm, 4 C, for 10 minutes. Three hundred ml of supernatant was added to 300 ml of pre-cooled 0.44M K_2CO_3. Samples were vortexed then placed on ice for 4 min, with an additional mix by vortex every 2 min. Samples were centrifuged at 4000 rpm, 4 C, for 10 min and the supernatant retained. For each sample a β-NAD vial (Sigma-Aldrich catalogue number N8285) as reconstituted by adding 2 ml glycine buffer (Sigma-Aldrich catalogue number G5418) 4 ml H_2O and 0.1 ml lactate dehydrogenase (Sigma-Aldrich catalogue number L3916-2.5ml). 0.1 ml of sample was added to 2.9 ml of the reconstituted β-NAD and incubated at 37 ^oC for 15 min. Absorbance was read at 340 nm against a set of standards containing 0, 0.05, 1, 2, 4 and 8 mM lactate.

3.3.7. Statistical analysis

Data were given as normalised to mean±, Standard error and n=5 of different region and different ageing of groups. Statistically significant differences between group means were determined using a one-way ANOVA with appropriate post hoc test (Tukey's honestly significant difference) using Instat Instant Biostatistics software v 3.0 sourced from Graphical software La Jolla, CA, USA. Data is presented as mean ± standard error.

3.4. Results

3.4.1. Glutathione concentration as an indicator of redox status.

Figure 3-1 shows that the GSH concentration exhibits a generally lowering trend in the oxidative stress condition. In all age groups GSH concentration in the cortex was significantly lower ($P < 0.001$) under the stressful condition. In the outer medulla GSH concentration under the stress condition was significantly lower ($P < 0.05$) at 5, 12 and 36 weeks whereas this difference did not quite reach statistcal significance at 60 weeks rats. In the inner medulla GSH concentration under the stress condition was significantly lower ($P < 0.001$) at 12 and 36 weeks rats whereas this did not reach significance at 5 and 60 weeks (Figure 3-1).

Clearly GSH concentration was highest at 12 weeks rats under control and oxidative stress condition in all regions of the kidney while 36 weeks was second highest. The lowest control level of GSH concentration was seen at 60 weeks rats ($P < 0.001$). However the levels of GSH concentration in the stress condition was lowest in both the very young animals (5 weeks) and the ageing animals (60 weeks) (Figure 3-1).

Generally GSH concentration appears to rise to a maximum at early maturity (12 weeks) and decline progressively to 60 weeks which shows a similar level to the 5 weeks.

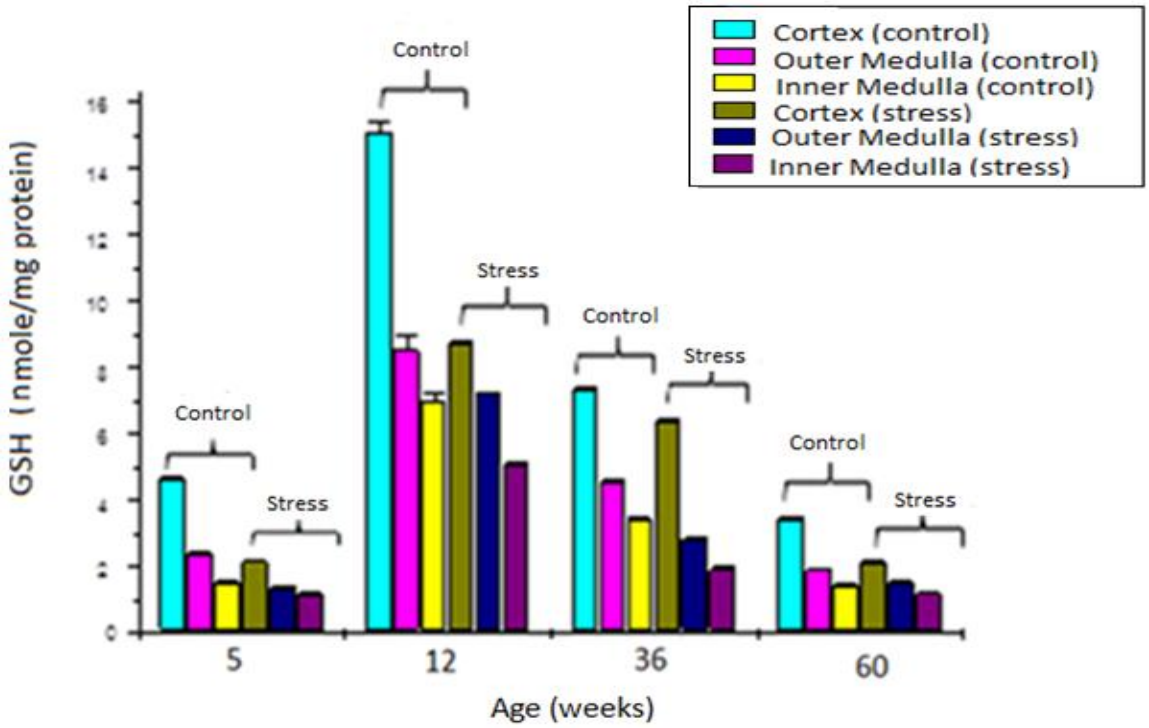

Figure 3-1: GSH concentration in the cortex, outer medula and inner medulla under control and stress conditions at 5, 12, 36 and 60 weeks. The data are shown as mean ± SE for n=5 individual animals in each age group. Between group levels of statistical significance (ANOVA) are explored in the text.

3.4.2. Effect of ageing on TBARS concentration in different regions of the kidney under control and stress conditions.

Figure 3-2 show that, generally speaking, TBARS levels were significantly elevated ($P < 0.001$) in the ageing animals (60 weeks). Furthermore the TBARS concentration did not change in the cortex, outer and inner medulla under the stress condition by comparison with the control at 5, 12 and 36 weeks, whilst, a significant increase was seen in TBARS concentration ($P < 0.001$) under the stress condition at 60 weeks by 1.7-, 1.9- and 1.6-fold in the cortex, outer and inner medulla respectively by comparison with the control (Figure 3-2).

Figure 3-2 shows that the TBARS concentration was significantly lower in both the outer and inner medulla at 60 weeks by comparison with the cortex under both control and stress conditions, while there was no significant difference between the different kidney regions at any of the other ages.

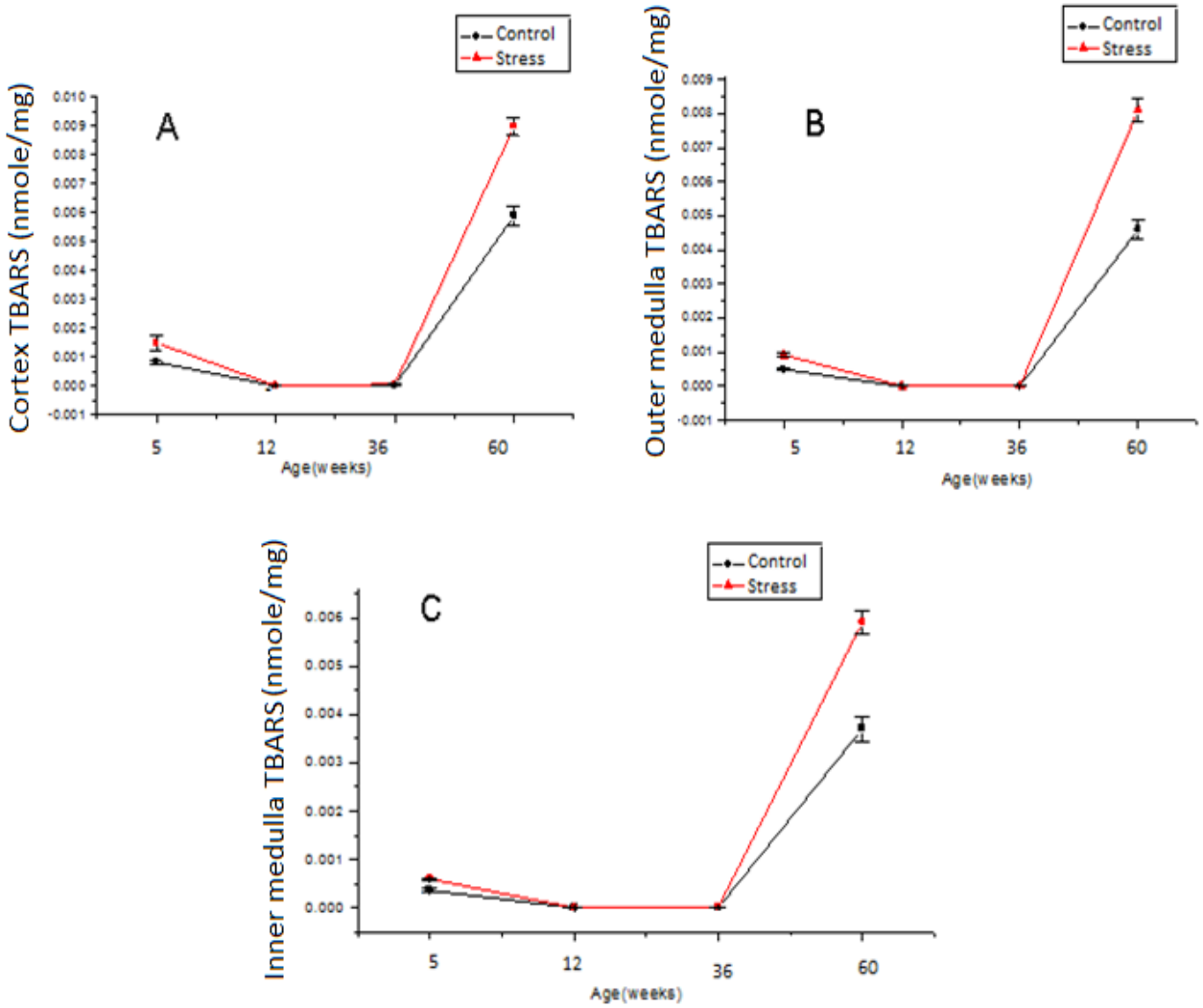

Figure 3-2: TBARS concentration under control and oxidative stress conditions at 5, 12, 36 and 60 weeks. Panel (A) cortex. Panel (B) outer medulla. Panel (C) inner medulla. The results are expressed as nmole TBARS/mg protein and are shown as mean ± SE for n=5 individual animals in each age group. Between group levels of statistical significance (ANOVA) are explored in the text.

3.4.3. Effect of ageing on lactate concentration in different kidney regions in control and stress condition.

Figure 3-3, Panel A shows that the lactate concentration was significantly higher ($P < 0.001$) under the stress condition in the cortex by 1.9-, 1.2-,1.3- and 1.8-fold at 5, 12, 36 and 60 weeks respectively. A similar pattern of increase was seen in the lactate concentration ($P < 0.001$) at 5, 12, 36 and 60 weeks by 1.8- ,1.2- ,1.4- and 1.8-fold respectively in the outer medulla under the stress condition by comparison with the control (Figure 3-3, Panel B). Figure 3, Panel C, shows the lactate concentration was significantly augmented under the stress condition by 1.7-, 1.2-, 1.3- and 1.7-fold at 5, 12, 36 and 60 weeks respectively in the inner medulla by comparison with control ($P < 0.001$).

Figure 3-3, shows that the lactate concentration in the control varied in the order : cortex < outer medulla < inner medulla ($P < 0.001$). Under the stress condition, except at 60 weeks, the lactate concentration also varied in the same order ($P < 0.001$) (Figure 3-3). At 60 weeks, the lactate concentration was not significantly different between the outer and inner medulla, however, both of these were significantly higher ($P < 0.001$) by comparison with cortex (Figure 3-3).

Figure 3-3 shows that generally the lactate concentration in the young mature (12 weeks) and older mature (36 weeks) was significantly lower ($P < 0.001$) by comparison with either the young immature rats (5 weeks) or the ageing rats (60

weeks) under both control and stress conditions. In addition, the lactate concentration at 60 weeks was significantly increased ($P < 0.001$) by comparison with 5, 12 and 36 weeks respectively in the cortex, outer and inner medulla under the control (Figure 3-3).

Under the stress condition, the lactate concentration seems to have a parallel pattern of reduction ($P < 0.001$) at 12 weeks and a comparable increase pattern ($P < 0.001$) at 60 weeks by comparison with 5 and 36 weeks in the cortex, outer and inner medulla (Figure 3-3).

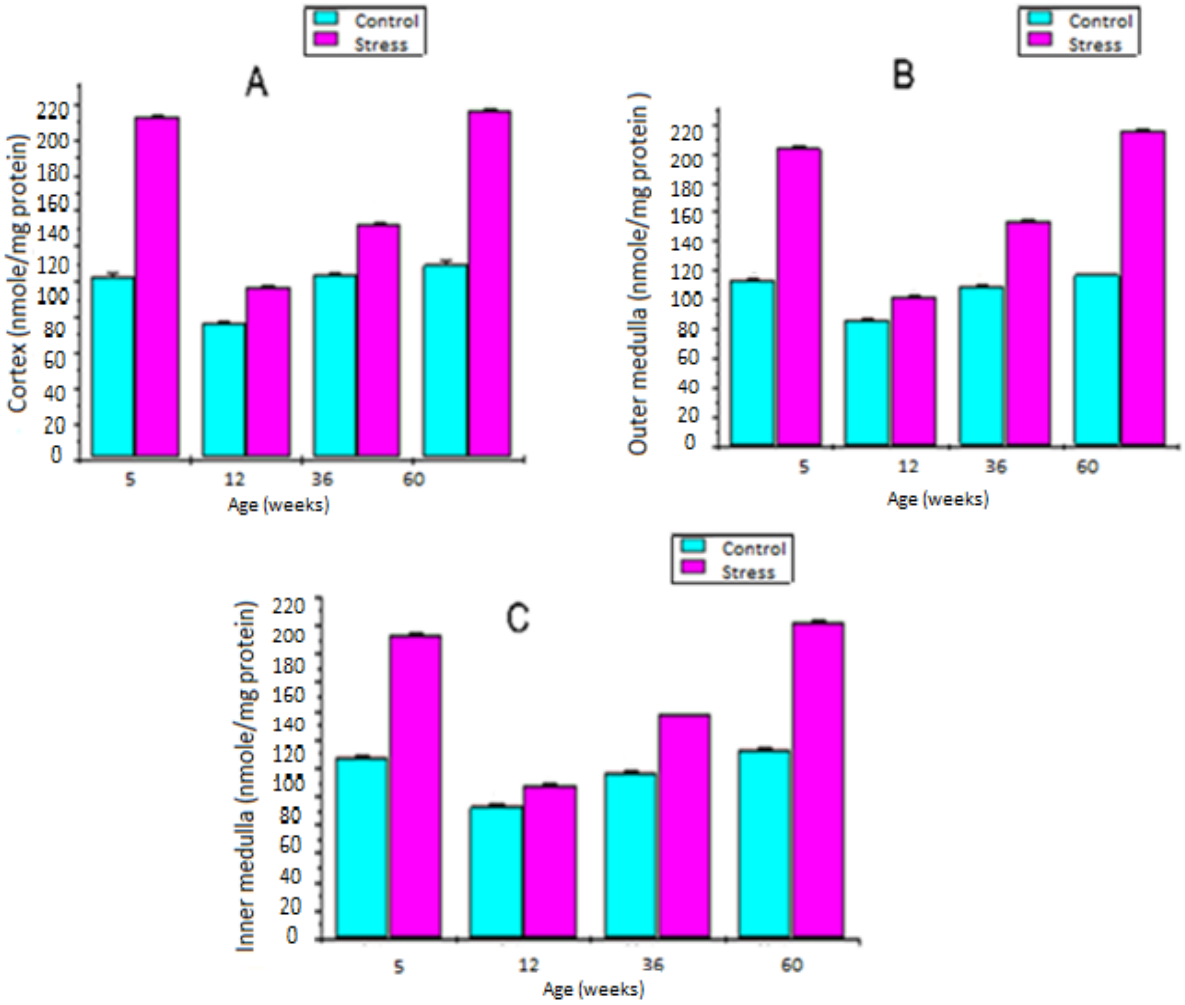

Figure 3-3: Lactate concentration under control and oxidative stress conditions at 5, 12, 36 and 60 weeks. Panel (A) cortex. Panel (B) outer medulla. Panel (C) inner medulla. Data are shown as mean ± SE for n=5 individual animals in each age group. Between group levels of statistical significance (ANOVA) are explored in the text.

3.5. Discussion

GSH is an essential antioxidant tri-peptide found in all animal cells. Intracellular GSH status appears to be a sensitive indicator of the oxidant homeostasis of the cell and therefore its ability to resist toxic challenge. GSH has direct antioxidant activity (Schulz *et al.*, 2000).

The results of this investigation indicate that reduced GSH content may be a general phenomenon of ageing in the kidney. Such age-related reduction in GSH could have a marked effect on the detoxification capacity of the senescent organism, since the primary physiological role of GSH is the detoxification of peroxides generated during normal metabolism. Such detoxification reactions may be nonenzymatic or enzymatic mediated by GSH-S-transferases and glutathione peroxidases. Therefore this decreased capacity may provide a toxicological basis for senescence (Farooqui *et al.*, 1987), Indeed, it has been shown that brain glutathione (GSH) decreases with age in humans, and that a loss of GSH may affect cognitive function (Currais & Maher, 2013).

The prevalence of lower concentrations of GSH observed in the kidney of very young (5 weeks) and ageing rats (60 weeks) animals observed here may be explained by the following possibilities. Firstly, the loss of GSH may be a result of the increased rate of oxidation in ageing rats. As such the reduced GSH concentration may be due to either augmented degradation or diminished

synthesis of the total GSH. In fact, the activity of enzymes of GSH metabolism has been found to decrease with age in human erythrocytes (Imanishi *et al.*, 1985; Jozwiak & Jasnowska, 1985; Louboutin & Strayer, 2014) and as shown in the previous chapter. In the very young rat there appears to be a rapid turnover of GSH compared to adults (Jain *et al.*, 1991; Rook & Goudoever, 2014) and this may account for lower levels in the very young rats (5 weeks old) observed here.

Here we observe apparently enhanced lipid peroxidation (TBARS) in the ageing rat. Excessive lipid peroxidation has been shown to result in augmented GSH consumption (Anandan *et al.*, 2013; Subramanian & James, 2010). GSH plays an important role in reactions that destroy free radicals, and in the metabolism of H_2O_2, organic peroxides, and certain foreign compounds (Rana *et al.*, 2002). Reduction of GSH below a certain threshold can trigger suicide of the cell by the process of apoptosis (Rana *et al.*, 2002).

Activities of GSH-dependent antioxidant enzymes were significantly reduced with age (see previous Chapter). Previous reports demonstrated that activities of GSH-dependent antioxidant enzymes were significantly reduced with age (Baird & Samis, 1971; Hazelton & Lang, 1985). The observed reduction of GSH reported here is in agreement with earlier reports (Anandan *et al.*, 2013; Ito *et al.*, 1998; Subramanian & James, 2010). Furthermore a decrease in GSH may be related to destruction of mitochondria (Jain *et al.*, 1991; Marí *et al.*, 2013) and a decrease in the number of mitochondria in the brain, liver, and lungs of newborn rats

(Martensson *et al.*, 1991). The decrease with age may be due to an augmented production of H_2O_2 in aged rats where GPX causes the oxidation of GSH, which in turn is reduced by GR at the expense of NADPH. The measured apparent decrease in GSH may reflect a net loss as GSSG is exported from the cell. In addition it is possible that with age more GSH is bound as mixed protein disulphide.

The current study also observes that under stress conditions after H_2O_2 exposure, the GSH levels decreased in comparison with control, and this may be a consequence of an increase in free radical production and oxidative stress in the kidney. This observed reduction in the level of GSH under stress condition is in agreement with earlier reports (Gecit *et al.*, 2011).

Our study also indicates that levels of the antioxidant molecule GSH was higher in the cortex in comparison with the outer and inner medulla, which is in agreement with earlier reports (Chen *et al.*, 2003; Zou *et al.,* 2001). This is consistent with the proposition that, under physiological situations, the renal medulla is exposed to higher levels of oxidative stress than the cortex resulting in differential depletion of GSH and this may have important consequences for age related changes in the function of the kidney (Chen *et al.*, 2003; Zou *et al.*, 2001). It is worth considering the possibility that the different levels of GSH observed in the different kidney regions could be due to differences biosynthesis but equally

differences in the breakdown of GSH by the enzyme γ GT may also contribute and this leads to a suggestion mentioned in the section of future work.

It is well known that H_2O_2 freely crosses biological membranes, and the rate of its production is closely related to levels of $O_2^{\cdot-}$ and $^{\cdot}OOH$ (Halliwell & Gutteridge, 1989). Therefore, the measurement of H_2O_2 has been often used to reflect the oxidative status of tissues or cells. Although there have been recent reservations expressed regarding the overall utility of the MDA assay in determining oxidant homeostasis (Delfino *et al.*, 2011) and although it is known that measurement of TBARS can be problematic (Choi & Yu, 1990) the assay itself is still widely used and serves as a robust and reliable indicator of free radical mediated lipid peroxidation (Boaz *et al.*, 1999; Sheng *et al.*, 2011).

To determine whether the age-related changes in the expression of antioxidant enzymes (Chapter 4) have any physiological relevance, a set of experiments were performed to evaluate free radical damage by measuring TBA-reactive substances (TBARS) in the tissue homogenates. These experiments indicate that TBARS increase significantly with age (Shih & Yen, 2007). Interestingly, the tissues that showed an increase in TBARS also showed a significant reduction in antioxidant enzyme activity with increasing age. The present findings are in agreement with previous studies (Shih & Yen, 2007) in which an age related increase in lipid peroxidation was reported in the kidney of mice and rats (Farooqui *et al.*, 1987; Rao *et al.*, 1990; Subramanian & James, 2010).

The result presented here show that under oxidative stress conditions induced by H_2O_2 exposure TBARS levels were significantly elevated at 5 and 60 weeks for all regions in comparison to control. This result demonstrates that increased free radical production and enhanced oxidative stress in the kidney could be mitigated at age 12 and 36 weeks possibly due to the observed higher levels of antioxidants by comparison with 5 and 60 weeks which is in agreement with earlier reports (Gecit *et al.*, 2011; Sheng *et al.*, 2011).

This study also investigated lactate concentration in each kidney region (cortex, outer and inner medulla), and found that there was a significant elevation of lactate under oxidative stress conditions in comparison to control. The concentration of lactate was higher in the inner medulla compared with the cortex and outer medulla, which is in agreement with earlier reports (Hervy & Thomas, 2003; Thomas, 2000). At 60 weeks old the lactate concentration was higher by comparison with other age groups (5, 12 and 36 weeks). We conclude, therefore, that a switch to anaerobic metabolism may be a general response to increased oxidative stress in the ageing rat kidney. In this respect it would be useful in subsequent studies to examine the effects of pre-incubation with GSH on lactate levels as well as examining the effect of H_2O_2 treatment on the expression of the enzyme lactate dehydrogenase (LDH).

In summary, in this investigation we have demonstrated that there is a significant reduction in GSH concentration with increasing age in different regions

of the kidney. The GSH concentration is also low in immature rats but this may reflect a higher rate of GSH turnover rather than increased oxidative stress *per se* (Jain *et al.*, 1991). The highest GSH concentration was measured at 12 weeks. Interestingly, the tissues that had a lower level of GSH also showed a significant increase in TBARS and lactate. This observation suggests that during the period of low GSH concentrations various tissues of the rat could become vulnerable to lipid peroxidation and lactate accumulation reflecting a switch to anaerobic metabolism.

STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*

	Author's Name (please print clearly)	**% of contribution**
Candidate	Noor Riyadh Thiab	60
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Name/title of Principal Supervisor: **Associate Professor/ Graham L. Jones**

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23 /04 / 2015

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Principal Supervisor

23 /04 / 2015

Date

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STATEMENT OF ORIGINALITY

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

Type of work	Page number/s
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Figure 2	84
Figure 3	87

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Name/title of Principal Supervisor: Associate Professor/ Graham L. Jones

[redacted]

23/04 / 2015

Candidate Date

[redacted]

23 / 04 / 2015

Principal Supervisor Date

Chapter 4 : Age related expression of glutathione peroxidases (GPX) in different kidney regions with and without stressor using normalised Western blotting analysis.

4.1. Abstract

Small molecular free radical species involved in oxidative stress include superoxide, singlet oxygen and hydroxyl radicals. These species can cause damage to cellular macromolecules, including proteins, DNA and lipids. There is some suggestion that continual damage inflicted by endogenously produced oxidants contributes to cellular ageing (Daehn *et al.*, 2014; Fraga *et al.*, 1990; Shigenaga *et al.*, 1994; Stadtman, 1992). The cellular changes and molecular mechanisms underlying the functional and structural changes associated with renal ageing are currently under investigation (Gomes *et al.*, 2009). One of the most important enzymes involved in ROS detoxification is glutathione peroxidase (GPX). Here we study the age-related expression of GPX isoenzymes in various regions of the rat kidney before and after oxidative stress (H_2O_2) treatment.

Expression of GPX1 was shown to decrease with age under both control and stress conditions after peaking at 12 weeks (young adult). GPX1 expression was greater in the cortex by comparison with the outer and inner medulla respectively. On the other hand, GPX4 expression did not show much variation across the

different regions of the kidney under control or stress conditions, although it did show a significant increase ($P < 0.001$) in expression in the inner medulla at 12 weeks under control. GPX2 expression was not detected across the different regions of the kidney under control or stress conditions.

4.2. Introduction

Ageing is a complex process characterised by a general decline in physiological function, leading to increasing morbidity and eventual mortality (Gomes *et al.*, 2009; Miyoshi *et al.*, 2006). There is increasing evidence for an age-related decline in renal function, both in animal models and in humans (Clark, 2000; Davies *et al.*, 1989; Epstein, 1996; Hirokawa, 1975; Lim *et al.*, 2014; Maurer *et al.*, 2013; Zhou *et al.*, 2008). Several studies have demonstrated that this deterioration is manifested both in structural (tubular atrophy, glomerulosclerosis and interstitial fibrosis) and functional (reduced drug excretion, proteinuria, reduced ability to concentrate or dilute urine, impairment of electrolyte and ion transport, decreases in glomerular filtration rate (GFR), alteration in hormonal functions) changes in the kidney. The molecular mechanisms and the cellular changes underlying these changes are currently under active investigation (Gomes *et al.*, 2009).

It is hypothesised here that the production of antioxidant enzymes in the kidney may decrease with age and under stress conditions as oxidant homeostasis is compromised. The aim of this study was to investigate the effects of ageing on expression of major antioxidant enzymes under control and oxidative stress conditions and to corroborate the results of the antioxidant enzyme activity presented in Chapter 3, relating these enzyme activities with the level of protein expression using Western blot analysis.

4.3. Methods

4.3.1.Sample Collection

Animals were treated in the same manner as described in section 2.3.1.

4.3.2.Tissue preparation

Tissues were prepared in the same manner as described in section 2.3.2.

4.3.3. Protein assay

The protein concentration was determined in the same manner as described in section 2.3.3.

4.3.4. Western blotting technique

Denatured dissolved protein in tissue homogenates (total protein load 50 µg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE) and the separated proteins electro-blotted onto nitrocellulose membrane (Life Science, catalogue number 10402594). Membranes were blocked in 5% non-fat milk powder dissolved in TBST for 1 hr, Membranes were incubated with primary antibody solutions of: 1) GPX1 (Abnova catalogue no. 110-00845) (1:6000, mouse monoclonal antibody), 2) GPX4 (Abnova catalogue no. PAB7065) (1:5000, goat polyclonal antibody) 3) GPX2 (Abnova catalogue no. H00002877-K, rabbit monocloncal) at various concentrations and 4) 'housekeeping' standard protein Anti-β-Actin mouse monoclonal (Sigma-Aldrich catalogue no. A2228) (1:5000 mouse monoclonal) 5) Molecular weight marker proteins (Precision Plus Protein ™ Kaleidoscope ™ Standards no.161-0375, Bio-Rad), in blocking solution at room temperature for 2 hr with constant shaking. Appropriate HRP-conjugated secondary antibodies (1:10000) were applied for 1 hr followed by chemiluminescent detection after application of chemiluminescent HRP substrate. Bands indicative of the concentration of the target protein developed on exposed X-ray film after suitable periods of development (1-15 min). Relative band densities were visualised and quantified on a Bio-Rad scanning densitometer (Bio-Rad Laboratories, Hemel Hempstead, UK) with appropriate software molecular

analyst, V.2.1, Bio-Rad). Target band migration positions were compared with the position of migration of standard molecular weight marker proteins (kalidescope).

4.3.5. Statistical analysis

Data were given as normalized to mean+, Standard error of different region and different aging of groups. The calculations of statistical significance between groups that was determined by one-way ANOVA with appropriate post hoc test (Tukey's Honestly Significant Difference) using Instat Instant Biostatistics software v 3.0 sourced from Graphical software La Jolla, CA, USA.

4.4. Results

4.4.1. Effect of region on the GPX1 protein expression at different ages under control and stress condition

Figure 4-1 shows the changes in the expression of GPX1 protein with respect to ageing in the control and stressed condition. The Western blots for 5 individual animals were scanned and the relative density expressed as a ratio of the relative density for the housekeeping protein beta actin. In this manner, the protein expression of GPX1 was normalised to account for loading differences in concentration. No consistent significant age related changes were observed in beta actin expression *per se.*

Figure 4-1 shows that, generally speaking, expression of GPX1 protein rises from a low level at 5 weeks to reach a maximum in young adult rats at 12 weeks and declines thereafter with age under both control and stressed tissues. The lowest expression level therefore, was in immature very young (5 weeks) and also in ageing rats (60 weeks). Under control conditions, at 5 weeks, GPX1 was expressed at similar levels in all regions of the kidney. At 12 and 36 weeks there was an increase in GPX1 expression in the cortex compared to other regions of the kindey. At 60 weeks, GPX1 expression was higher in the outer medulla compared with the cortex and inner medulla under control conditions. Under oxidative stress conditions (H_2O_2), there was a signficant decrease in the

expression of GPX1 in the cortex and inner medulla at 12 weeks. There was also a signficant decrease in the outer medulla at 60 weeks. The outstanding feature is the high level of expression at 12 weeks in all regions of the kidney.

Under control conditions, GPX1 protein expression was significantly increased at 12 weeks in all three regions of the kidney. In the cortex, GPX1 expression was also elevated at 36 weeks compared to 5 and 60 weeks (Figure 4-1).

Under oxidative stress conditions, GPX1 expression followed a similar pattern to that observed under control conditions. GPX1 expression was increased at 12 weeks in both the cortex and outer medulla. Expression was also increaed in the outer medulla at 36 weeks compared to 5 and 60 weeks. The only significant difference in expression of GPX1 in the inner medulla was a decrease in expression at 60 weeks compared to the other age groups (Figure 4-1).

Interestingly, whereas the treatment with stressor led to a lower apparent expression of GPX1 at 12 weeks, on the whole, other age groups were either unchanged or raised after treament with stressor.

GPX1 expression in the cortex was significantly decreased at 12 weeks when kidney sections were exposed to oxidative stress conditions. No change in GPX1 expression was observed between control and stress conditions in the cortex at other ages. (Figure 4-1, Panel A). In the outer medulla, GPX1 expression was decreased under oxidative stress conditions at 60 weeks only. No difference in the

GPX1 expression was observed in the outer medulla at other ages (Figure 4-1, Panel B). In the inner medulla, GPX1 expression was decreased at 12 weeks under oxidative stress conditions but increased at 36 weeks under such conditions. No change in expression between control and stress conditions was observed at other ages (Figure 4-1, Panel C).

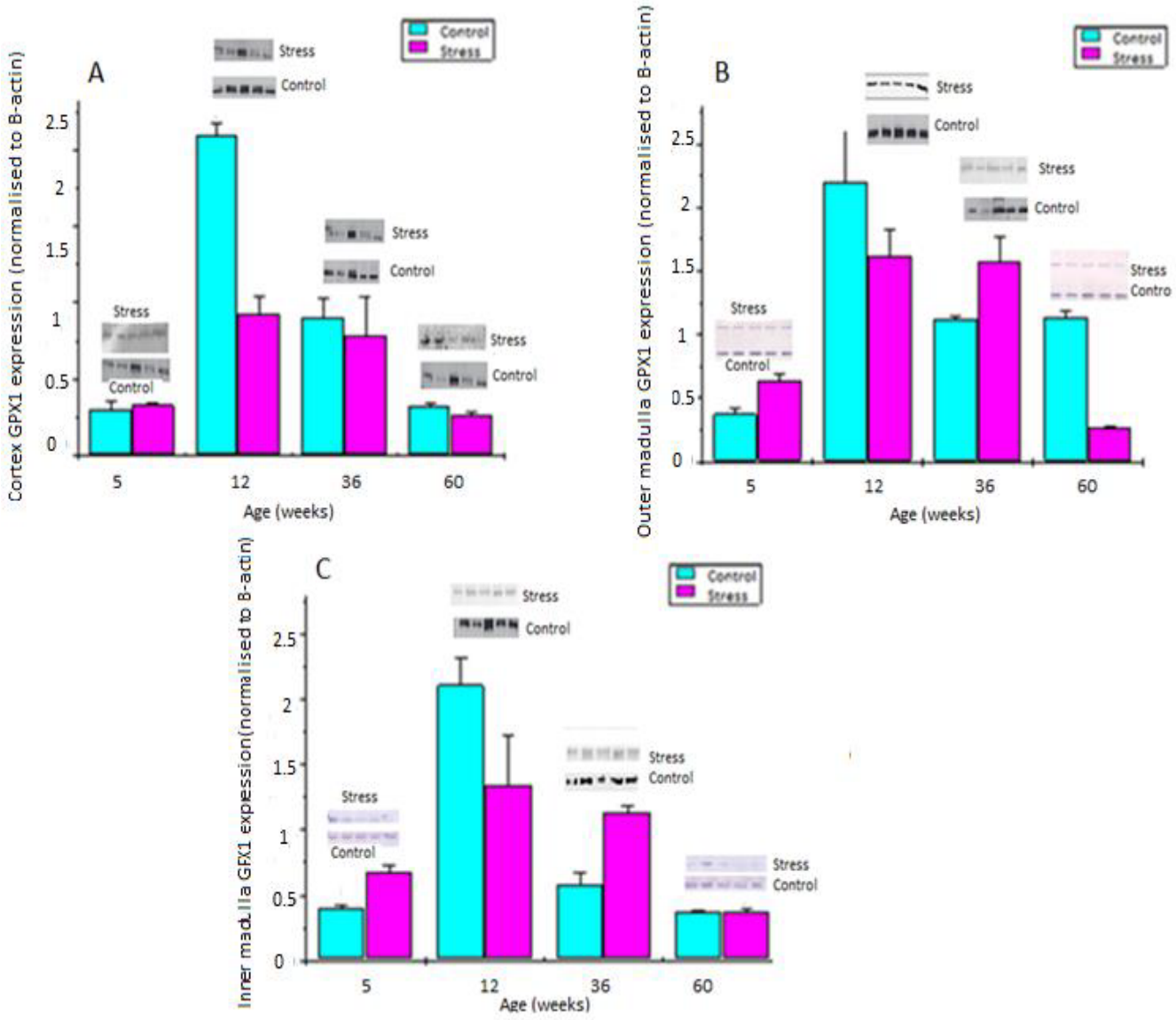

Figure 4-1: Kidney GPX1 protein expression under control and stress conditions. Panel (A) kidney cortex. Panel (B) kidney outer medulla. Panel (C) kidney inner medulla. Inserts above each column show Western blot detection of GPX1 protein band in each case. Data is presented as mean +/- SE for n=5 biological replicates in each age group. Between group levels of statistical significance (ANOVA) are explored in the text.

4.4.2. Effect of region on the GPX4 protein expression at different ages in control and stress condition.

Unlike GPX1, Figure 4-2 shows that there was little extensive age related difference in the apparent expression of GPX4.

Figure 4-2 shows the changes in the expression of the GPX4 protein with respect to ageing in the control and stressed condition. The Western blots for 5 individual animals were scanned and the relative density expressed as a ratio of the relative density for the housekeeping protein beta actin. In this manner, the protein expression of GPX4 was normalised to account for loading differences in concentration. No consistent significant age related changes were observed in beta actin expression *per se.*

Under control condition, GPX4 protein expression did not vary substantially between kidney cortex, outer and inner medulla at all ages (5, 12, 36 and 60 weeks) (Figure 4-2). With the exception of the 60 weeks cohort, similar expression patterns were seen under oxidative stress condition (Figure 4-2) although here it is noteworthy that overall the GPX4 protein expression was significantly lower ($P < 0.01$) in the outer medulla and inner medulla ($P < 0.01$) by 1.3-fold and 2.1-fold by contrast with the cortex respectively as shown in (Figure 4-2).

The outstanding feature was the relatively small change in the expression of GPX4 observed by comparison to that observed with GPX1.

GPX4 protein expression did not change with age in the cortex and outer medulla under control condition. By contrast, in the inner medulla, the GPX4 protein expression was significantly raised ($P < 0.05$) by 1.9-fold between 5 and 12 weeks. Furthermore, the GPX4 protein expression was significantly higher ($P < 0.01$) at 12 weeks by 2.6-fold by comparison with 60 weeks (Figure 4-2).

As shown in (Figure 4-2), the GPX4 protein expression after treatment with stressor (H_2O_2) was similar in the cortex, outer and inner medulla at 12 and 36 weeks by contrast with 5 weeks. In addition, the GPX4 protein expression was considerably lower ($P < 0.01$) by 3.3-fold at 60 weeks by comparison with 12 weeks (Figure 4-2).

Figure 4-2 also shows changes due to stressor treatment in different regions of the kidney (panels A (cortex), B (outer medulla) and C (inner medulla)) for each age group.

In the cortex there was no difference in the GPX4 protein expression under control and oxidative stress condition in all age groups (Figure 4-2, Panel A).

In the outer medulla, the GPX4 protein expression under stress condition was significantly reduced ($P < 0.01$) at 5 weeks by 10.9-fold and at 36 weeks by 2.7-fold compared with the control condition. By contrast, there was no significant stressor effect on GPX4 protein expression in the outer medulla at 12 and 60 weeks (Figure 4-2, Panel B).

In the inner medulla, as shown in Figure 4-2, (Panel C), except at 60 weeks, the GPX4 protein expression was considerably reduced ($P < 0.05$) by 9.6-, 2- and 2.5-fold at 5, 12 and 36 weeks respectively under the oxidative stress condition by comparison with the control condition (Figure 4-2, Panel C).

GPX2 expression was not detected in any of the different regions of the kidney under control or stress conditions in any age groups.

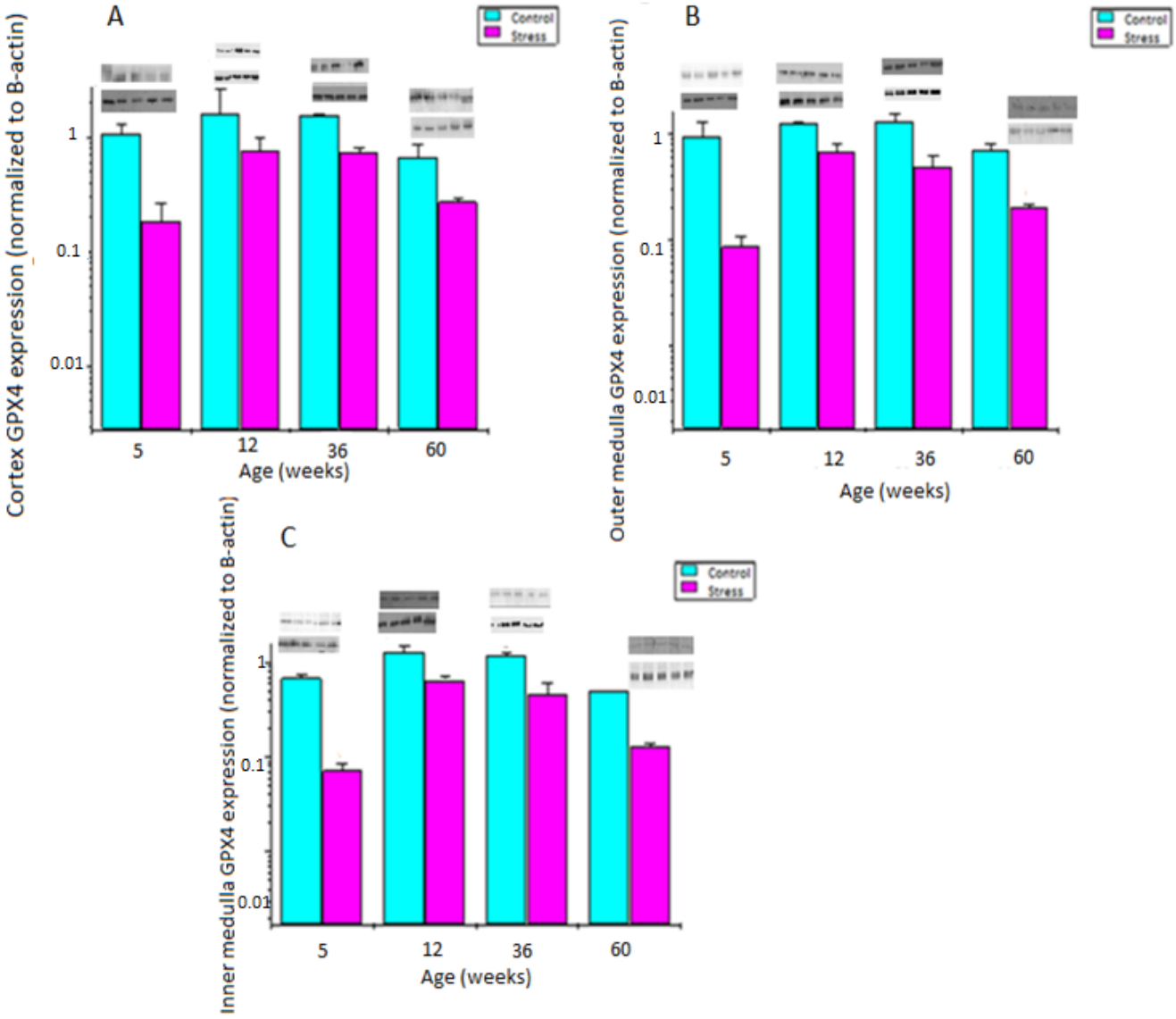

Figure 4-2: GPX4 protein expression under control and stress conditions. Panel (A) kidney cortex. Panel (B) kidney outer medulla. Panel (C) kidney inner medulla. Inserts above each column show Western blot detection of GPX4 protein band in each case. Data is presented as mean +/- SE for n=5 biological replicates in each age group. Between group levels of statistical significance (ANOVA) are explored in the text.

4.5. Discussion

Ageing is accompanied by changes in the structure and function of different organs and tissues (Gomes *et al.*, 2009). In the rat kidney, ageing has been related to oxidative stress and enhanced ROS generation (Cand & Verdetti, 1989; Jung & Henke, 1996; Rikans & Hornbrook, 1997). Several researchers have shown that ageing is associated with decreased expression of antioxidant enzymes in the rat hepatocytes of varying ages (Sanz *et al.*, 2002; Shih & Yen, 2007).

The results of this study indicate that GPX1 expression was higher in the cortex than in the outer or inner medulla in the control and at 60 weeks under oxidative stress conditions. This is in agreement with earlier reports (Chen *et al.*, 2003; Zou *et al.*, 2001) that demonstrate that the outer medulla is subject to higher levels of oxidative stress by comparison with the renal cortex under control. This suggests that, under physiological situations, the renal medulla is exposed to higher levels of oxidative stress than the cortex.

The current study demonstrates that exposure to oxidative stress results in a decrease in GPX1 protein expression compared with control. Exposure to 0.2 mM H_2O_2 is a standard model for exposing tissues to oxidative stress (King *et al.*, 2010; King *et al.*, 2003) and is accompanied by abundant ROS production.

It is likely that endogenous oxidative stress may induce the expression of antioxidant enzymes via *ia* the NRF2 pathway.

Furthermore, in this investigation we have demonstrated that ageing rats display a reduction in the expression of GPX1 in their tissues with increasing age. The highest concentration for GPX1 expression occurred in young mature rats (12 weeks) and the lowest concentration in the immature rats (5 weeks) but this probably reflects lower (non-induced) GPX enzyme levels mentioned in Chapter 2 involved in GPX1 protein expression. Our results clearly demonstrate that, after reaching a maximum in the young mature rats, levels of GPX1 progressively decrease with age so that very old rats (60 weeks) demonstrate a similar level to the presumptively non induced levels recorded in the very young rats.

Interestingly, by contrast, expression of GPX4 shows no significant age related changes under control condition.

Having said this, the results of this study do indicate an age-related effect on expression of GPX4 under oxidative stress conditions, with expression declining with age after reaching a peak value in the young mature rat. This result should be considered in the context of the universal attenuation of GPX4 expression following treatment with stressor.

An earlier study (Gomes *et al.*, 2009) demonstrated that the expression of GPX remained unchanged during the aging process of male Wistar Kyoto (WKY) rats.

This previous study concerned the expression of whole GPX not the individual expression of GPX isoenzymes so it would be interesting to further investigate differential expression of GPX in the light of the current results particularly with respect to stressor effects. In this regard, here we show that GPX4 expression was decreased under oxidative stress conditions in both the outer and inner medulla. This result should be considered in the context of the suggestion that, under physiological situations, the renal medulla is exposed to higher levels of oxidative stress than the cortex.

From this study we observed no protein expression of GPX2 under control or stress conditions, in different regions of the kidney or at any age. Despite many attempts with varying concentrations of primary and secondary antibodies it is still possible that we may have encountered some unresolved methodological issues that compromised our attempts to detect this isozyme. However it is clear that GPX2 is predominantly a gastrointestinal isozyme (GI–GPX, GPX2) (Behne & Kyriakopoulos, 2001; Brigelius-Flohé, 1999; Chu *et al.*, 1993) and therefore, may simply be below the level of detection in the kidney.

In general the results of this study indicate that GPX1 protein expression was higher in the cortex than in the outer or inner medulla under control and at 60 weeks old under oxidative stress conditions. Under exposure to oxidative stress GPX1 protein expression was decrease in compared with control. Furthermore, in this investigation we have demonstrated that ageing rats display a reduction in the

GPX1 protein expression in their tissues with increasing age. The highest concentration for GPX1 protein expression occurs in young mature rats (12 weeks) and the lowest concentration in the immature rats (5 weeks). Interestingly, by contrast, expression of GPX4 shows no significant age related changes under control condition. Having said this, the results of this study do indicate an age-related effect on expression of GPX4 under oxidative stress conditions, with expression declining with age after reaching a peak value in the young mature rat at (12 weeks). This result should be considered in the context of the universal attenuation of GPX4 expression following treatment with stressor. In this regard, here we show that GPX4 expression was decreased under oxidative stress conditions in both the outer and inner medulla. From this study we observed that there was no observable protein expression for GPX2 under control and stress, in different kidney regions (cortex, outer medulla and inner medulla) at all ages (5, 12, 36 and 60) weeks. Despite many attempts with varying concentrations of primary and secondary antibodies it is still possible that we may have encountered some unresolved methodological issues that compromised our attempts to detect this isozyme but it is more likely that the levels of GPX2 in the kidney are simply too low to be detected.

STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

	Author's Name (please print clearly)	**% of contribution**
Candidate	**Noor Riyadh Thiab**	**60**
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	Graham L. Jones	**20**

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Name/title of Principal Supervisor: **Associate professor/ Graham L. Jones**

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23 / 04 / 2015

Date

[redacted]

Principal Supervisor

23 / 04 /2015

Date

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STATEMENT OF ORIGINALITY

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We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

Type of work	Page number/s
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Figure 4	129
Figure 5	131

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23 / 04 / 2015

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23/04 / 2015

Principal Supervisor Date

Chapter 5 : Age-related transcriptional expression of glutathione peroxidases *Gpx1* and *Gpx4*, and the chaperone protein *Hsp-70*

5.1. Abstract

Free radicals cause cumulative oxidative stress-induced ageing resulting in DNA, protein and lipid dysfunction. Thus, it is critical for organisms to maintain optimal antioxidant defence against oxidative stress (Zou *et al.*, 2001).

The aim of this study was to investigate the effects of ageing on mRNA expression of *Gpx1, Gpx4* and *Hsp-70* in different regions of the rat kidney.

Gene expression of *Gpx1, Gpx4* and *Hsp-7*0 was studied in male Wistar rats. Kidneys were separated from Male Wistar rats at different ages (5, 12, 36 and 60 weeks). mRNA was extracted from the superficial cortex, outer medulla and inner medulla. The expression of *Gpx1, Gpx4* and *Hsp-70* was analysed by quantitative reverse transcribed polymerase chain reaction (QRT-PCR).

The prevailing regional trend in the expression of *Gpx1* was highest in the cortex, and shows a trend decrease with age, after reaching a maximum at 12 weeks although these differences did not achieve statistical significance. By contrast, *Gpx4* expression did not vary across the different regions of the kidney in all age groups, with the one exception of a significantly higher level of expression in the inner medulla at 12 weeks.

In stark contrast with the expression of both *Gpx1* and *Gpx* 4, expression of *Hsp-70* was elevated in the inner medulla, and was significantly increased in the very old rats at 60 weeks.

In this investigation we have confirmed that ageing rats show an attenuated gene expression of antioxidant enzyme *Gpx1* and *Gpx4* in the kidney while expression of the chaperone gene *Hsp-70* was increased with age revealing therefore an interesting reciprocal relationship.

5.2. Introduction

Ageing is a biological process accompanied by gradual deterioration of physiological functions and metabolic processes. This multifactorial process, which is the result of genetic and environmental factors, affects different organs and tissues in different ways. There is increasing evidence for an age-related decline in renal function, both in animal models and in humans (Clark, 2000; Davies, 1989; Epstein, 1996; Hirokawa, 1975; Martin & Sheaff, 2007; Zhou *et al.*, 2008). The molecular mechanisms and cellular changes underlying the functional and structural changes associated with renal aging are currently under active investigation (Martin & Sheaff, 2007; Zhou *et al.*, 2008). The oxidative stress theory of ageing contends that declines in organism function, which characterise the ageing process, result from a progressive accrual of oxidative damage to cellular constituents (Harman, 1955, 1998, 2006). Living organisms produce reactive oxygen species (ROS) such as O´-2, H_2O_2, OH´, and other free radicals during physiological processes and in response to external stimuli (Halliwell & Gutteridge, 1999). These are highly reactive molecules that can potentially damage DNA, proteins, carbohydrates, and lipids (Beckman & Ames, 1998; Castro *et al.*, 2012). In order to minimise such damage, cells have evolved defence systems comprising both enzymatic and nonenzymatic processes. Examples of the former are antioxidant enzymes such as superoxide dismutase

(SOD), catalase, and glutathione peroxidase (GPX), and the latter include antioxidant molecules such as glutathione (GSH).

Glutathione peroxidases (GPX) are antioxidant enzymes using cofactor glutathione as an electron donor to mitigate the toxic effects of oxidants generated within cells (Brigelius-Flohé, 1999; Castro *et al.*, 2012). So far, eight isoforms of Gpx have been descrbed. Four isoforms including cytosolic Gpx (cGpx or Gpx1), gastrointestinal Gpx (GI–Gpx or Gpx2), plasma Gpx (pGpx or Gpx3), and phospholipid hydroperoxide Gpx (PHGpx or Gpx4) contain a selenocysteine in their structure (Behne & Kyriakopoulos, 2001; Chu *et al.*, 1993). Gpx6 contains a cysteine in rodents, but a selenocysteine in human (Kryukov *et al.*, 2003). Non-selenocysteine containing isoforms, Gpx5, Gpx7, and Gpx8, are also present in mammalian tissues (Chabory *et al.*, 2009; Nguyen *et al.*, 2011).

Gpx1 constitutes the most abundant and widespread glutathione peroxidase in mammals. This isoenzyme plays an important role in the protection of cells from oxidative stress by reducing hydrogen peroxide to water employing glutathione as cofactor (Kasaikina *et al.*, 2011). Gpx-1 has been sequenced, either directly or through complementary DNA (cDNA) cloning in a range of species including human, rat, mouse, rabbit, cattle and sheep (Sunde, 1990). In all species examined GPX-1 is a tetrameric protein with four identical subunits, each of which contains one selenocysteine residue per monomer molecular weight of 22–23 kDa. The three-dimensional structure of bovine Gpx-1 shows that it contains four

spherical subunits, each with a selenocysteine residue in a depression in the surface (Epp *et al.*, 1983).

Gpx-4 or phospholipid hydroperoxide Gpx is the fourth selenium-containing GPX which has been characterized. This is a 20–22 kDa protein that contrasts with Gpx1 in that it is a monomer that can react with phospholipid hydroperoxide as substrate. There are many differences between Gpx-4 and the other Gpxs (Nam *et al.*, 1997; Sunde *et al.*, 1993). Cloning of Gpx-4 showed it to be a polypeptide of 170 amino acids with a theoretical molecular weight of approximately 19 kDa. The sequence identity of Gpx-4 is between 30% and 40% of Gpx-1, dependent on the species being compared. Like the other Gpxs in the 3' untranslated region of the mRNA, there is a selenocysteine insertion sequence (SECIS) element which directs the incorporation of selenium into the protein at the UGA triplet within the coding region (Sunde *et al.*, 1993). Within the amino acid sequence of Gpx-4 there is a tyrosine which is thought to be phosphorylated, but the role of this in control of expression of enzyme activity is not known. In the full length Gpx-4 gene there are 7 exons and several hormone- responsive elements in the 5' untranslated region which may be related to the function of PHGPX in the testis (Nam *et al.*, 1997; Sunde *et al.*, 1993).

The so-called heat shock (HS) response is one of the primordial intracellular defence mechanisms against stress. Exposure of cells and organisms to stresses such as high temperature, caloric restriction, exercise, oxidative and osmotic

stress, heavy metals, proteasome inhibitors, amino acid analogues, ethanol, glutathione depletion, calcium ionophores and metabolic poisons induces the cellular stress response, leading to the preferential transcription and translation of heat shock proteins (HSPs) (Feder & Hofmann, 1999; Fehrenbach & Niess, 1998; Jolly & Morimoto, 2000) . Optimal HS response in terms of HSP synthesis and activity is essential for cell survival. In contrast, inefficient and altered HS response has been implicated in abnormal growth and development (Neuer *et al.*, 2000), ageing and apoptosis (Gabai *et al.*, 1998; Sőti & Csermely, 2000) and in the initiation and promotion of cancers (Boswell-Casteel *et al.*, 2015; Shipp *et al.*, 2011).

It is hypothesised we propose that the expression of antioxidant enzymes in the rat kidney might decrease with age and expression of Hsp70 increase with age as a response to increased oxidant challenge.

The aim of this study was to investigate the effects of aging on mRNA expression of the genes for Gpx1, Gpx4 and to correlate such age related changes with the expression of Hsp-70 mRNA.

5.3. Methods

5.3.1. Sample collection

Animal experiments were conducted under the guidelines of the University of New England Animal Ethics Committee (AEC09/152, AEC10/036 and AEC11/100). Twenty male Wistar rats in 4 groups of n=5 were stunned and sacrificed by cervical dislocation. Kidneys were removed and 30 mg sections of cortex, inner medulla and outer medulla were dissected and stored in RNAlater stabilization solution (Qiagen, Hilden, Germany cat no. 76104).

5.3.2. Isolation of total RNA and cDNA synthesis

Total RNA was extracted from tissue samples using an RNeasy Mini Kit (Qiagen, Hilden, Germany. Catalog no. 74106), following the manufacturer's instructions. RNA was quantified using spectrophotometry (spectra Max M_2e, bio-strategy). RNA integrity was assessed by gel electrophoresis on a 1.5% agarose gel in TAE buffer. Each gel included 0.5 GelRed nucleic acid stain (Biotium,USA), and bands visualized under UV light. Complementary DNA (cDNA) was synthesised using a QuantiTect Reverse Transcription kit with integrated genomic DNA removal (Qiagen, Hilden, Germany. Cat no. 205311), following the manufacturer's instructions.

5.3.3. QRT-PCR

QRT-PCR reactions were carried out in a Rotor-Gene Q thermal cycler (Qiagen, USA). Each reaction contained Rotor Gene SYBR Green PCR mastermix (Qiagen, Hilden, Germany. Cat. No. 204076), 0.5 µM each forward and reverse primer, and cDNA transcribed from 10 ng RNA. The cycling parameters are given in Table 5-1. The primers used in this study are shown in Table 5-2. The PCR reaction efficiency of each primer set was determined using a 5-fold serial dilution of pooled cDNA. The specificity of PCR products was determined by melt curve analysis and gel electrophoresis (as described previously) of the amplified products. Samples from five independent experiments were analyzed in duplicate, with negative controls included in each assay.

Table 5-1: cycling conditions for QRT-PCR.

Temperature (°C)	Time	Cycle no.
95	5 min	1
95 60	5 s 10 s	40
72	5 min	1

Table 5-2: Primer sequences of antioxidant enzymes, chaperone Hsp-70 and internal standard β-actin, GAPDH.

Gene	Forward primer	Reverse primer	Product size (bp)	Reference
Gpx1	CAGTTCGGACATCAGGAGAAT	AGAGCGGGTGAGCCTTCT	139	(Xiong *et al.*, 2010)
Gpx4	AACGTGGCCTCGCAATGA	GGGAAGGCCAGGATTCGTAA	101	(Xiong *et al.*, 2010)
Hsp70	TCATCTCCTGGCTGGACTCT	CTAGCCAACACCCTGAGAGC	228	This study
β -actin	CCTGCTTGCTGATCCACA	CTGACCGAGCGTGGCTAC	500	(Sadi *et al.*, 2012)

5.3.4. Data analysis

Threshold cycle (Ct) data was converted to expression data using the delta-delta Ct method by normalisation to β-actin as described by Livak and Schmittgen (2001). Statistical significance between groups was determined by a one-way ANOVA with appropriate post hoc test (Tukey's Honestly Significant Difference) using Instat Instant Biostatistics software v 3.0 sourced from Graphical software La Jolla, CA, USA, and ($P < 0.05$). Data is presented as normalised mean, +/- standard error.

5.4. Results

5.4.1. Sample preparation and analysis of PCR products.

Gel electrophoresis of extracted mRNA showed distinct 28S and 18S rRNA bands (Figure 5-1, Panel A), all samples had a A260/280 nm ratio of ~2, indicating high purity of samples (data not shown).

Electrophoresis of PCR products indicated that a single product of the expected size was produced from each set of primers (Figure 5-1, panel B). Melt peak analysis from each primer set showed a single peak, indicating formation of a single product (Figure 5-2).

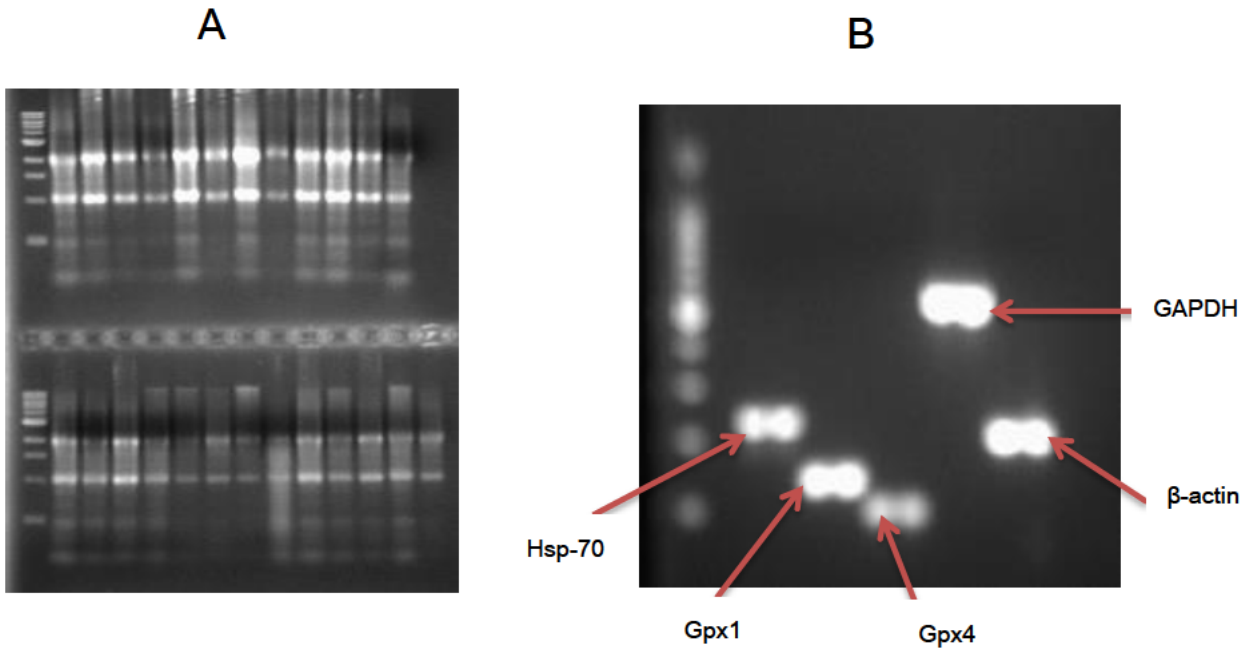

Figure 5-1: Panel A: mRNA integrity for all samples of the cortex, outer medulla and inner medulla. The first lane in each gel (extreme left) shows the RNA standard ladder. Panel B: gel of PCR products for *Hsp70*, *Gpx1*, *Gpx4*, *β-actin* and *GAPDH*.

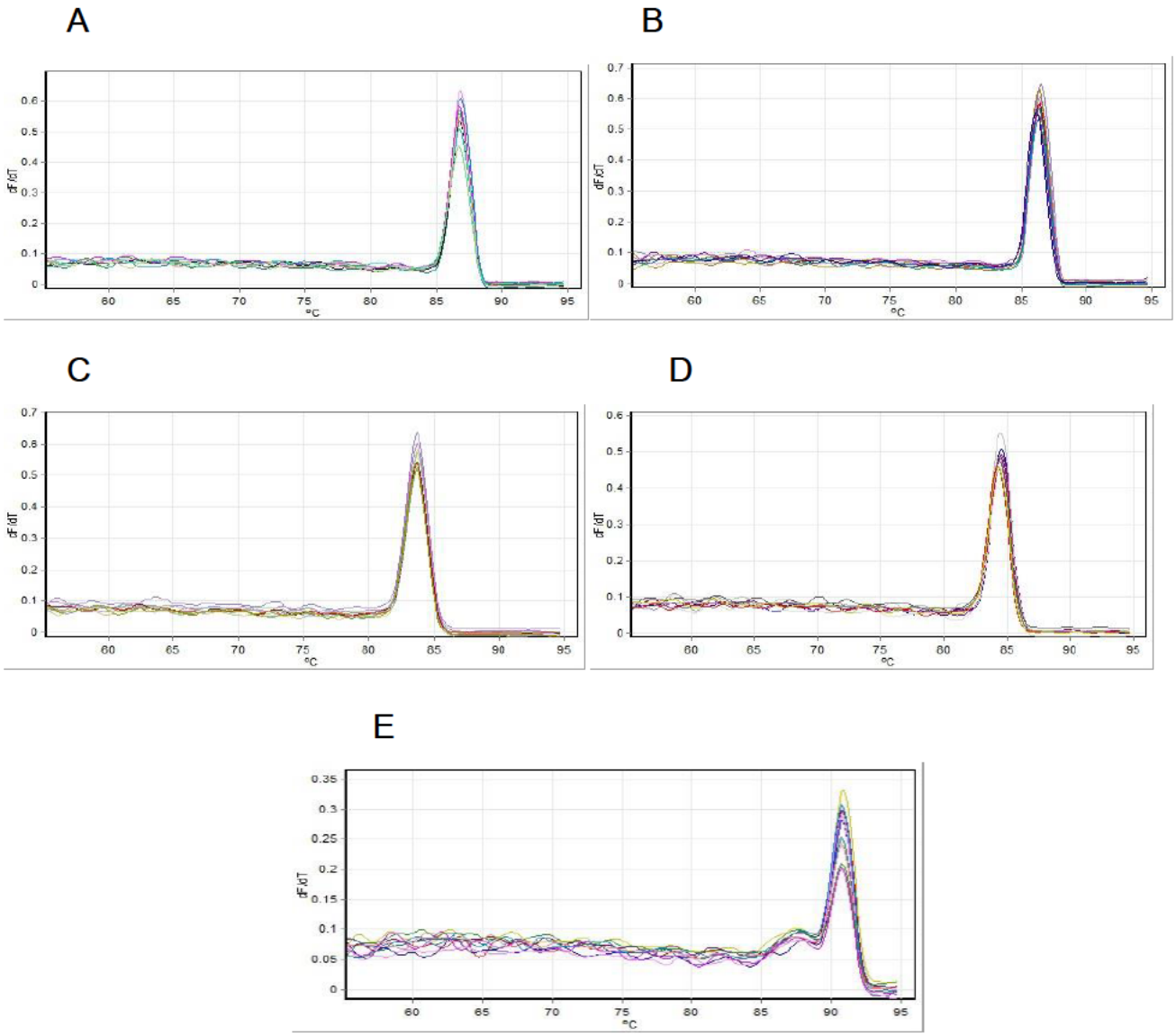

Figure 5-2: Melt curves ST. Panel (A) *β-actin*. Panel (B) *GAPDH*. Panel (C) *Gpx1*. Panel (D) *Gpx4*. Panel (E) *Hsp70*.

5.4.2. mRNA Expression of *Gpx1* gene in different regions of the kidney at different ages.

Overall the expression of the *Gpx1* gene was significantly higher ($P < 0.01$) in all age groups in the cortex than in the inner and outer medulla. At 12 and 60 weeks the expression of *Gpx1* gene was significantly higher in the outer medulla by comparison with the inner medulla ($P < 0.001$) although consideration of each age to age comparison in turn shows relatively modest effects of age on the mRNA expression of the *Gpx1* in the different regions of the kidney. Expression of the *Gpx1* showed a trend increase at 12 weeks in both the cortex and inner medulla by comparison with other age cohorts, however this did not reach statistical significance. On the other hand, expression of the *Gpx1* in the outer medulla was significantly increased at 12 weeks ($P < 0.001$): by 3.6-fold compared to 5 weeks, by: 2.2-fold compared to 36 weeks and by: 2-fold compared to 60 weeks. Expression of the *Gpx1* in the inner medulla was significantly lower ($P < 0.001$) by 1.8-fold at 60 weeks compared with 12 weeks (Figure 5-3).

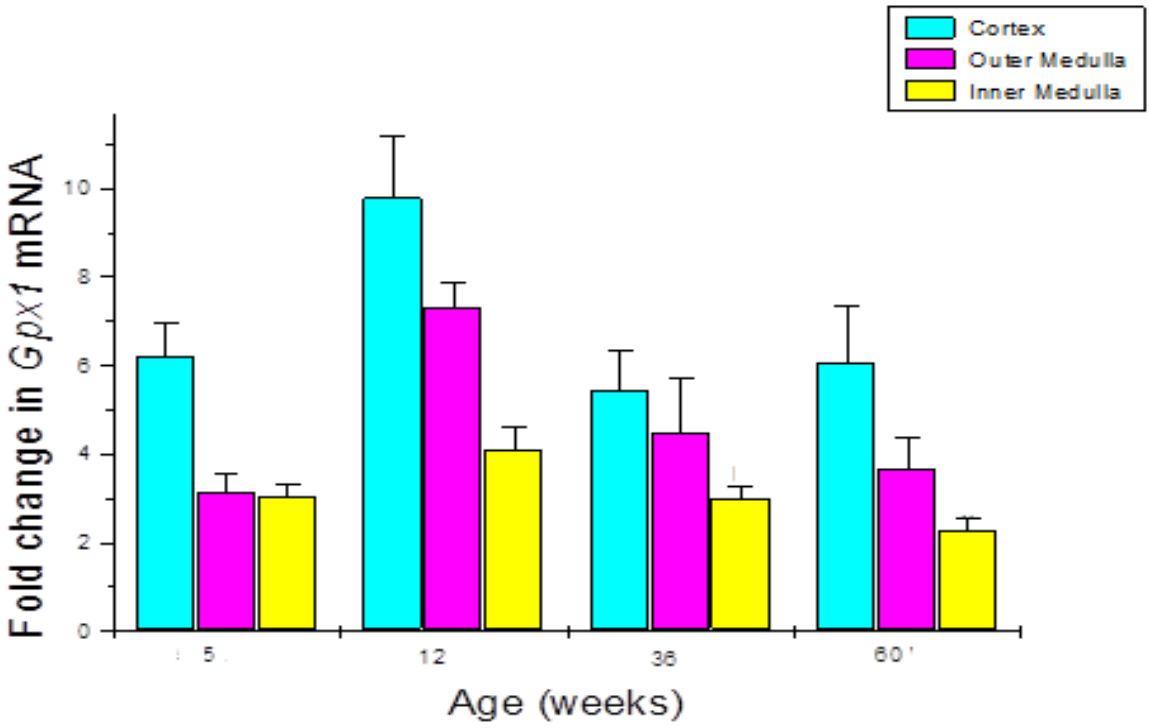

Figure 5-3: Relative mRNA expression of the *Gpx1* gene in the kidney cortex, the outer and inner medulla. Data are presented as mean +/- SE for n=5 biological replicates in each age group relative to β-actin as a house keeping gene. Between group levels of statistical significance (ANOVA) are explored in the text.

5.4.3. Effect of region on the mRNA Expression of *Gpx4* gene in different age cohorts.

No significant difference in the mRNA levels of *Gpx4* were detected between the cortex, outer and inner medulla at 5, 12, 36 or 60 weeks (Figure 5-4). The mRNA Expression of *Gpx4* in the inner medulla was significantly higher ($P < 0.05$) at 12 weeks by comparison with 36 weeks by 1.5-fold and 60 weeks by 1.4-fold (Figure 5-4), however, there was no significant difference in the mRNA Expression of *Gpx4* between different age groups in the cortex and outer medulla (Figure 5-4).

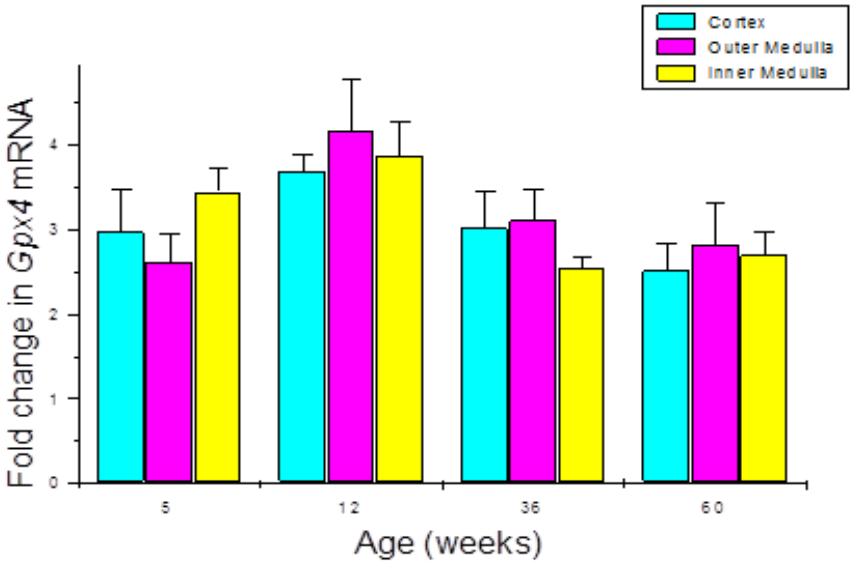

Figure 5-4 Relative mRNA expression of *Gpx4* in kidney cortex, outer and inner medulla. Data is presented as mean +/- SE for n=5 biological replicates in each age group relative to β-actin as a house keeping gene. Between group levels of statistical significance (ANOVA) are explored in the text.

5.4.4. Effect of region on the mRNA Expression of *Hsp-70* gene in different age cohorts.

Expression of *Hsp-70* was significantly higher in both the outer and inner medulla compared to the cortex at 5 weeks. A significant increase ($P < 0.01$) was also seen in the expression of *Hsp-70* in the inner medulla at both 12 and 60 weeks. No significant difference in the expression of *Hsp-70* was observed across the different kidney regions at 36 weeks. A dramatic increase ($P < 0.05$) was observed in the mRNA expression of *Hsp-70* in the inner medulla at 60 weeks by 1.9-fold compared to the cortex and by 1.7-fold compared to the outer medulla (Figure 5-5).

Hsp-70 expression increased with age in all three kidney regions, and was significantly higher ($P < 0.001$) at 60 weeks by comparison with 5, 12 and 36 weeks (Figure 5-5).

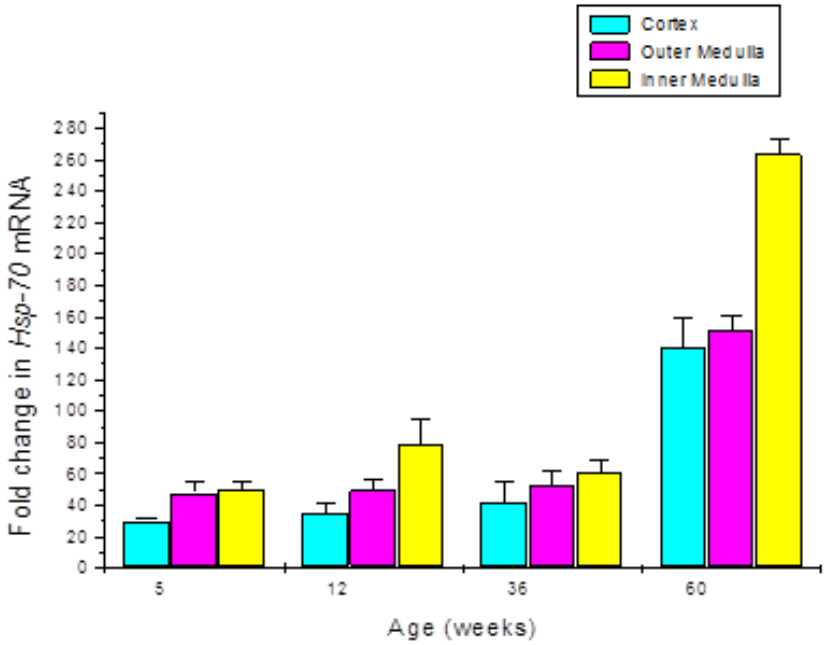

Figure 5-5: Relative mRNA expression of *Hsp-70* in kidney cortex, outer and inner medulla. Data is presented as mean +/- SE for n=5 biological replicates in each age group relative to β-actin as a house keeping gene. Between groups levels of statistical significance (ANOVA) are explored in the text.

5.5. Discussion

The aim of this study was to investigate the effects of ageing on mRNA (transcriptional) expression of *Gpx1*, *Gpx4* and *Hsp70* in different regions of the rat kidney.

Previously, it was observed that antioxidant enzyme activity GPX activity and level of protein expression for GPX1 and GPX4 in the rat kidney decreased with age (Chapter 2, 4). Here, we aim to determine if these age-related changes in the activities of the antioxidant enzymes correlate with changes in gene expression. We found that there was indeed an age-related decrease in the level of *Gpx1* gene expression in both the outer and inner medulla, but not in the cortex. No age-related change in *Gpx4* mRNA levels was observed. The age-related decrease of *Gpx1* expression is in agreement with earlier reports (Rao *et al.*, 1990) which demonstrated that the relative levels of *Gpx* mRNA in the brain, hepatocytes, and kidney of male Fischer F344 rats decreased with age. It is also in agreement with reports (Shih & Yen, 2007) that investigated antioxidant enzyme activity and gene expression of GPx, GSH-red and CAT in Sprague Dawley rats, and showed that aged rats exhibited lower enzymatic activities and gene expression than young rats.

It should be noted, however, that these studies did not discriminate between isoforms of *Gpx*. Therefore this previously observed age-related decrease in *Gpx*

mRNA and enzyme activity could be due to decreased production of specific isoforms of *Gpx*, such as *Gpx1*, rather than a general decrease in production of all isoforms. This would result in a decrease in overall GpX activity, in agreement with previous findings, and this is also consistent with unchanged Gpx4 levels and raised Gpx1 levels observed herein.

Expression of *Gpx1* was found to be higher in the cortex than in the outer and inner medulla. This may be because under physiological conditions, the renal medulla is exposed to higher levels of oxidative stress than the cortex. This is in agreement with earlier reports showing that the outer medulla had a higher concentration of oxidative stress than the renal cortex under physiological conditions (Chen *et al.*, 2003; Zou *et al.*, 2001).

The results in this Chapter confirm our previous results (Chapter 2 and 4) showing that GPX1 enzyme activity and protein expression were higher in the cortex when compared to the outer and inner medulla.

Given the well described nexus between oxidant homeostasis and stress protein expression, we were also interested in determining the effect of age on the heat shock response in the kidney. In this study we also show that the mRNA expression of *Hsp-70* level was substantially increased with increasing age. This may be in response to the progressive accumulation during the age of the kidney of various posttranslational modifications including glycation and protein oxidation

leading to damaged proteins and disturbed cellular homeostasis. Our result here is in general agreement with earlier reports (Sharma *et al.*, 2010; Sőti & Csermely, 2000) investigating an increase in the expression of *Hsp-70* in different brain regions of the ageing male Wistar rats. Seen in this context an elevated hsp response provides a homeostatic mechanism to counter accumulated metabolic and environmental insults (Chin *et al.*, 1996). Such specific age related increases in gene expression may contribute to the generally observed constitutively elevated chaperone levels in aged organisms (Schumpert *et al.*, 2014; Söti & Csermely, 2002). In another earlier study (Njemini *et al.*, 2007; Sharma *et al.*, 2010), age-related oxidative stress has been implicated in down-regulation of cytokine production with a concomitant up-regulation of HSP production in elderly humans as compared to younger counterparts. An increase of stress protein Hsp-70 plays a cytoprotective role and inhibits cell death in response to various stimuli (Gordon *et al.*, 1997; Guo & Mattson, 2000; Schumpert *et al.*, 2014; Sharp *et al.*, 1999). Furthermore, Hsp-70 helps damaged proteins regain their correct structure, function and/or location (Guo & Mattson, 2000). Given the contribution of HSP-70 to blocking apoptosis, an interesting avenue for further research would be to establish whether apoptosis is impaired as a result of age/oxidative stress, by examining expression of markers for apoptosis in the kidney. Overall we observed that expression of Hsp70 was higher in the inner medulla compared with the cortex and outer medulla, and the expression of Hsp-70 was significantly elevated in very

old rats at 60 weeks. This is in agreement with earlier reports (Borkan *et al.*, 1993; Gabai *et al.*, 1998; Lee *et al.*, 1996; Zou *et al.*, 1996; Verbeke *et al.*, 2001). The possibility exists that age-dependent changes in cellular redox status, as exemplified by the increased production of reactive oxygen intermediates and accumulation of oxidatively-modified proteins may be in part compensated by increased Hsp-70 expression.

In general, the age-related changes in the activities of the antioxidant enzymes observed in previous Chapter 2 were paralleled by changes in the relative levels of mRNAs expression. This suggests that changes in the expression of the antioxidant enzymes with age are largely regulated at the level of transcription, mRNA processing, or mRNA turnover. Because antioxidant enzymes play an important role in protection against free radical damage, changes in the expression of the antioxidant enzymes in kidney tissue could predispose that tissue to free radical damage. The preliminary findings of the present investigation support this concept.

STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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STATEMENT OF ORIGINALITY

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We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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Chapter 6 : Conclusion

In the investigations presented in this thesis, we have shown that, after reaching a peak of expression in the young sexually mature male rat (12 weeks), ageing male rats demonstrate a progressively reduced level of the antioxidant enzyme activities; GPX, GSH-red, GSH-ST, CAT and SOD, as well as the GSH molecule in different regions of the kidney under control and stress conditions. The levels of the antioxidant enzymes and GSH molecule were low in the immature rats (5 weeks). The highest concentration for the antioxidant enzymes and GSH molecule was at 12 weeks and the lowest concentration at 5 (immature) and 60 weeks (ageing). Interestingly, the tissues that showed an increase in TBARS and lactate concentration also showed a significant decrease in GSH level with increasing age. This observation suggests that during the period of low GSH concentration various tissues of the rat could become vulnerable to lipid peroxidation and lactate accumulation.

Furthermore, the results of this study indicate that GPX1 protein expression was higher in the cortex than in the outer or inner medulla under control and at 60 weeks under oxidative stress conditions. The current study demonstrates that exposure to oxidative stress may result in a decrease in GPX1 protein expression compared with control, however given the short time scale of hydrogen peroxide treatment (30 min), it is most likely that this decrease is a reflection of overall cytotoxicity rather than a specific effect on protein realisation *per se*.

Overall ageing rats display a reduction in GPX1 protein expression in their tissues with increasing age. The highest concentration for GPX1 protein expression occurs in young mature rats (12 weeks) and the lowest concentration in the immature rats (5 weeks). Interestingly, by contrast, expression of GPX4 shows no significant age related changes under control condition. Having said this, the results of this study do indicate an age-related effect on expression of GPX4 under oxidative stress conditions, with expression declining with age after reaching a peak value in the young mature rat at (12 weeks). This result should be considered in the context of the universal attenuation of GPX4 expression following treatment with stressor and may reflect a differential sensitivity between GPX1 and 4. In this regard, here we show that GPX4 expression was decreased under oxidative stress conditions in both the outer and inner medulla.

From this study, we observed that there was no apparent protein expression for GPX2 under control and stress, in different kidney regions (cortex, outer medulla and inner medulla) at all ages (5, 12, 36 and 60) weeks. Despite many attempts with varying concentrations of primary and secondary antibodies it is still possible that we may have encountered some unresolved methodological issues that compromised our attempts to detect this isozyme but it is more likely that the levels of GPX2 in the kidney are simply too low to be detected.

In general, the age-related changes in the activities of the antioxidant enzymes observed in Chapter 2 and the levels of protein expression detected by Western

blotting analysis in Chapter 4 were paralleled by changes in the relative levels of mRNA expression. This suggests that changes in the expression of the antioxidant enzymes with age are largely regulated at the level of transcription, mRNA processing, or mRNA turnover. Because antioxidant enzymes play an important role in protection against free radical damage, changes in the expression of the antioxidant enzymes in kidney tissue could predispose that tissue to free radical damage. The preliminary findings of the present investigation support this concept.

The novel aspect to our work is the critical separation of the different kidney regions which is important because of the different roles of the kidney regions. The cortex is heavily involved in transport activities and has abundant mitochondria so one might therefore expect a high level of antioxidant enzyme expression and activity in this region. The outer medulla is more involved with water and Na+ balance. The inner medulla is mainly concerned with collection of the end products of filtration and passing it onto the bladder. Such a low rate of metabolic activity might be expected to be attended by low antioxidant enzyme activity and indeed broadly speaking the results presented in Chapter 2 supports this expectation.

In summary then the results presented in this thesis basically confirm previous work on age-related changes in oxidant homeostasis in the rat kidney and extend the scope of this work by considering both the importance of regional distribution and the role of externally added stressor. Importantly, the qtPCR results provide

an intriguing insight into the reciprocal age relationship between Gpx and Hsp70 expression in the rat kidney.

Overall then, the results presented in this thesis provide general support for the free radical theory of ageing particularly as this pertains to compromised kidney function.

With respect to the implications for human health, it is worth noting here that chronic renal failure and end stage renal disease constitutes an increasing health burden in ageing Australians and that this is particularly true in indigenous populations.

Chapter 7 : Future work

Given the dynamic nature of GSH and GSSG and the ready interconversion in the kidney it would be good to measure the ratio in the different kidney regions in the stressed and control conditions. For the same reason it would be good to measure the levels of the enzyme gamma glutamyl transferase (γ GT).

In future work we would like to investigate possible molecular mechanisms underlying these age and kidney region dependant changes we have observed in key antioxidant enzymes. For example, it would be useful to examine the effects of a range of antioxidant treatments or antioxidant precursor treatments (eg N acetyl

cysteimine NAC) on renal function in young and ageing rats in a similar manner to functional techniques employed by King *et al.* (2010) with rat cardio-myocytes.

It would also be useful to explore the age and stress related expression of transcription factors like HSF and Nrf-2 involved in the overall coordination of the antioxidant and stress response in different regions of the kidney. Given the interesting finding of an apparent reciprocal age relationship between the gene expression of hsp70 and the antioxidant enzymes, it would be interesting to confirm this reciprocal expression relationship at the protein level using Western blotting and extend the comparison to other stress proteins and other antioxidant enzymes.

In future work we would like to investigate any changes in the morphology and functionality of the different regions of the rat kidney at different ages and try to relate this to our observed changes in oxidant status.

In this study the impairment of renal function as a result of exposure to the contrived oxidative stress was not measured directly. One avenue for further study would be to investigate whether renal function is indeed impaired as a result of oxidative stress exposure, and also to determine the mechanisms which could cause this impairment.

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Appendix III

Age-related protein and mRNA expression of glutathione peroxidases (GPx) and Hsp-70 in different rat kidney regions with and without stressor

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Abstract

Background: Small molecular weight oxygen free radical species (ROS) involved in oxidative stress can cause damage to cellular macromolecules, including proteins, DNA and lipids. One of the most important enzymes involved in ROS

detoxification is glutathione peroxidase (GPX). Here we study the age-related expression of GPX isoenzymes in various parts of the rat kidney with and without exposure to external oxidative stress. These results are correlated to the age dependent changes in the expression of the chaperone, HSP-70.

Protein and mRNA expression of GPx1 and GPx4 was studied in different kidney regions of ageing rats in the presence and absence of the external stressor 0.2 mM H2O2. Protein levels were examined by Western blot analysis following detection with appropriate antibodies and mRNA levels were analysed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using appropriate primer sequences. mRNA expression for the chaperone Hsp70 was investigated in parallel.

Results: After reaching a peak at maturity (12 weeks), GPx1 protein and mRNA levels decreased with age under both control and stress conditions, and was higher in the cortex than in the outer and inner medulla. GPx4 protein and mRNA levels showed few comparable age-related changes. By contrast with the observed age-related decrease in GPx1 expression, chaperone Hsp-70 mRNA expression greatly increased with age.

Conclusions: These results are consistent with the oxidative stress theory of ageing.

Keywords: Ageing, kidney region, oxidative stress, GPx1, GPx4, HSP-70.

1. Background

Ageing is a complex process characterised by a general decline in physiological function, leading to increasing morbidity and eventual mortality [1, 2]. There is increasing evidence for an age-related decline in renal function, both in animal models and in humans [3-9]. Several studies have demonstrated that this deterioration is manifested both in structural (tubular atrophy, glomerulosclerosis and interstitial fibrosis) and functional (reduced drug excretion, proteinuria, reduced ability to concentrate or dilute urine, impairment of electrolyte and ion transport, decreases in glomerular filtration rate (GFR), alteration in hormonal functions) changes in the kidney. The molecular and cellular mechanisms underlying these changes are currently under active investigation [2, 10-12]. There is some suggestion that continued damage by endogenously produced oxidants may contribute to the development of these age-related changes [13-16]. Such oxidants including superoxide, hydrogen peroxide, hydroxyl radicals, and possibly singlet oxygen can induce damage to cellular macromolecules, including proteins, lipids and DNA [14, 15, 17-19]. The antioxidant enzymes scavenging active oxygen species have been suggested as key factors in determining the longevity of animals [19-21]. Indeed, there are correlations between levels of these enzymes

and the maximum life span potential of different species [22, 23]. Glutathione peroxidase is a major hydrogen peroxide scavenger enzyme, found in both the cytosol and mitochondria. The enzyme and its substrate GSH, form a formidable defence against hydrogen peroxides and lipid peroxides [24, 25].

The so-called heat shock (HS) response is one of the primordial intracellular defence mechanisms against stress. Exposure of cells and organisms to stresses such as high temperature, caloric restriction, exercise, oxidative and osmotic stress, heavy metals, proteasome inhibitors, amino acid analogues, ethanol, glutathione depletion, calcium ionophores and metabolic poisons among many factors induces the cellular stress response, leading to the preferential transcription and translation of heat shock proteins (HSPs) [26-28]. Optimal HS response in terms of HSP synthesis and activity is essential for cell survival. By contrast, inefficient and altered HS response has been implicated in abnormal growth and development [29], accelerated ageing and apoptosis [30, 31] and in the initiation and promotion of cancers [32, 33]. Seen in this context, an elevated HSP response provides a homeostatic mechanism to counter accumulated metabolic and environmental insults [34]. The age related attenuation of the expression of key anti-oxidant enzymes could thus be seen as amplifying such insults and therefore leading to a concomitant age related sustained increase in the expression of HSP-70 and indeed this reciprocal relationship has been previously observed in the ageing rat brain [35]. The physiological cellular redox

state is a balance between the levels of oxidising and reducing equivalents, such as reactive oxygen species (ROS) and endogenous antioxidants. It appears that during the ageing process an imbalance between oxidants and antioxidants may occur, leading to oxidative stress [24, 36]. The free radical theory of ageing proposes that ageing itself and some age-related diseases are, at least in part, a consequence of oxidative stress [36, 37].

It is hypothesised here that expression of antioxidant enzymes in the kidney may decrease either with age or under conditions of oxidative stress as oxidant homeostasis is compromised. The aim of this study was to investigate the effect of ageing on the expression of the major antioxidant enzyme GPX (1 and 4) in different regions of the kidney under control and oxidative stress conditions and to correlate such age related changes with the expression of HSP-70 mRNA.

2. Methods.

2.1. Sample Collection.

All experiments were approved and carried out under conditions complying with the Animal Ethics Committee of the University of New England (AEC09/152, EC10/036 and AEC11/100).

Male Wistar rats in varying age cohorts (5 weeks; 12 weeks; 36 weeks; and 60 weeks of age) were used. For each experiment, five animals from each age cohort were stunned and sacrificed by cervical dislocation. For protein assays kidneys were removed, snap frozen in liquid nitrogen and stored at -80oC until use. For mRNA extraction kidneys were dissected into 30 mg sections from the cortex, inner and outer medulla, and placed into RNA later stabilisation solution (Qiagen, Hilden, Germany catalogue no. 76104) and stored at 4 oC.

2.2. Tissue preparation

For SDS/PAGE and subsequent Western blotting analysis, frozen kidneys after thawing were dissected to yield samples from cortex, outer medulla and inner medulla. Fifty mg of each tissue incubated at room temperature 25 ºC for 30 min with phosphate buffered saline (PBS) at a ratio of 1:1000 prior to homogenisation for 2 min (control condition). To model oxidative stress, parallel 50 mg tissue samples were placed in a 0.2 mM H2O2 solution for 30 min at room temperature (stress condition). In both cases samples were then homogenised. The resultant homogenate was centrifuged at 4000 rpm for 10 min at 4 oC, and the supernatant frozen until later use. A TBARS assay was carried out to confirm the oxidative stress state of the tissue and this data is included in a separate publication.

2.3. Protein assay and Western Blots

Total protein concentration in the tissue homogenates from the control or stressed condition was determined using a Bradford reagent assay (Sigma, USA: catalog number B6916), as per the manufacturer's instructions.

For Western blots denatured dissolved protein in tissue homogenates (total protein load 50 µg) was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE) alongside molecular weight marker proteins (Precision Plus Protein TM Kaleidoscope TM Standards No.161-0375, Bio-Rad). The separated proteins were electro-blotted onto nitrocellulose membrane (Life Science, Whatman, catalogue number 10402594). Membranes were blocked in 5% non-fat milk powder dissolved in TRIS buffered saline containing Triton (TBST) for one hour, Membranes were incubated variously with primary antibody solutions of 1) Anti-GPx1 (Abnova catalogue no. 110-00845) (1:6000, mouse monoclonal antibody), 2) Anti-GPx4 (Abnova catalogue no. PAB7065) (1:5000, goat polyclonal antibody and 3) 'housekeeping' standard protein Anti-β-Actin mouse monoclonal (Sigma-Aldrich catalogue no. A2228) (1:5000 mouse monoclonal), in blocking solution at room temperature for 2 hr with constant shaking. Appropriate Horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000) were applied for 1 hr followed by chemiluminescent detection after application of the chemiluminescent HRP substrate. Bands with a relative intensity indicative of the

concentration of the target protein were developed on exposed X-ray film after suitable periods of development (1-15 min). Relative band densities were visualised and quantified on a Bio-Rad scanning densitometer (Bio-Rad Laboratories, Hemel Hempstead, UK) with incorporation into Molecular Analyst software (version 2.1 for Macintosh, Bio-Rad). Target band migration positions were compared with the position of migration of standard molecular weight marker proteins (Precision Plus™ Kaleidoscope™ , Bio-Rad).

2.4. Isolation of total RNA and cDNA synthesis

Total RNA was extracted from tissue samples using an RNeasy Mini Kit (Qiagen, Hilden, Germany. catalogue no. 74106), following the manufacturer's instructions. RNA integrity was assessed by gel electrophoresis and relative concentration quantified by spectrophotometry. Complementary DNA (cDNA) was synthesised using a QuantiTect Reverse Transcription kit with integrated genomic DNA removal (Qiagen, Hilden, Germany. catalogue no. 205311), following the manufacturer's instructions.

2.5. Quantitative RT-PCR

qRT-PCR reactions were carried out in a Rotor-Gene Q thermal cycler (Qiagen, USA). Each reaction contained Rotor Gene SYBR Green PCR kit (Qiagen, Hilden, Germany. catalogue no. 204076), 0.5 μM each forward and reverse primer, and cDNA transcribed from 10 ng RNA. The cycling parameters are given in Table 1. The primers used in this study are shown in Table 2. The PCR reaction efficiency of each primer set was determined using a 5-fold serial dilution of pooled cDNA. The specificity of PCR products was determined by melt curve analysis and gel electrophoresis. Samples from five independent experiments were analysed in duplicate, with negative controls included in each assay.

2.6. Statistical analysis

Western blot protein expression data were normalised to the expression of housekeeping protein β-Actin and expressed as mean ± Standard error of different kidney regions and different ageing cohorts.

For mRNA expression, threshold cycle (Ct) data was first converted to expression data using the delta-delta Ct method by normalisation to housekeeping gene β-actin as described by Livak & Schmittgen [40].

Statistical significance between groups was determined using a one-way ANOVA with appropriate post hoc test (Tukey's Honestly Significant Difference). Results are presented as mean, ± S.E. Differences of $P < 0.05$ were considered significant.

3. Results

3.1. Effect of region on GPx1 protein expression at different ages in the kidney in control and stress conditions

Figure 1 shows the changes in protein expression for GPx1 with respect to ageing in the control and stressed condition. The Western blots for 5 individual animals were scanned and the relative density expressed as a ratio of the relative density for the housekeeping protein beta actin. There were no signficant differences in β-actin expression.

The greatest level of GPx1 protein expression occurred at 12 weeks under control conditions. Also under these conditions, there was a similar level of GPx1 expression in all regions of the kidney at 5 weeks. At 12 and 36 weeks there was greater GPx1 expression in the cortex compared to other kidney regions ($P < 0.05$), whereas at 60 weeks the expression was highest in the outer medulla.

Expression of GPx1 followed a similar pattern under stressful conditions. GPx1 expression was greatest at 12 and 36 weeks in the cortex and outer medulla, whereas the only signficant difference in the inner medulla was a decrease in GPx1 expression at 60 weeks compared to the other age groups.

GPx1 expression in the cortex was significantly decreased at 12 weeks old when kidneys were exposed to oxidative stress (Figure 1a). No change in GPx1 expression was observed between control and stress conditions in the cortex at other ages. In the outer medulla GPx1 expression was decreased under oxidative stress conditions at 60 weeks old only (Figure 1b). In the inner medulla GPx1 expression was only decreased at 12 weeks old under oxidative stress conditions (Figure 1c).

3.2. Expression of mRNA for GPx1 in different regions of the kidney at different ages

At all ages the highest level of GPx1 mRNA expression occurred in the cortex ($p < 0.01$). At 12 and 60 weeks the expression of GPx1 mRNA was signficantly greater in the outer medulla compared to the inner medulla ($p < 0.001$). Expression of GPx1 mRNA in the outer medulla was significantly increased at 12 weeks old ($p < 0.001$): by 3.6-fold compared to 5 weeks old, by: 2.2-fold compared to 36 weeks old and by: 2-fold compared to 60 weeks old. Expression of GPx1

mRNA in the inner medulla was significantly lower ($p < 0.001$) by 1.8-fold at 60 weeks old compared with 12 weeks old (Figure 2).

3.3. Effect of region on GPx4 protein expression at different ages in control and stress conditions

Figure 3 shows the changes in expression of GPx4 protein with repsect to ageing in the control and stress conditions. The Western blots were normalised to β-actin as described previously. There were no consistent age related changes in the expression of β-actin.

GPx4 protein expression did not change with age in the cortex and outer medulla under control conditions (Figures 3a and 3b). In contrast, GPx4 expression was signficantly raised ($p < 0.05$) in the inner medulla by 1.9-fold between 5 and 12 weeks old (Figure 3c). GPx4 expression in the inner medulla was also signficantly higher ($p < 0.01$) at 12 weeks by 2.6-fold in comparison with 60 weeks old (Figure 3c).

Figure 3 also shows changes due to stressor treatment in the different kidney regions. In the cortex there were no differences in GPx4 protein expression under oxidative stress conditions compared to the control conditions (Figure 3a). In the outer medulla GPx4 expression under the stress condition was signficantly

reduced ($p < 0.01$) at 5 weeks by 10.9-fold and at 36 weeks by 2.7-fold compared with the control condition (Figure 3b). There were no signficant effects in the outer medulla at 12 and 60 weeks. In the inner medulla GPx4 expression was reduced ($p < 0.05$) by 9.6-, 2- and 2.5-fold at 5, 12 and 36 weeks old repsectively under the stress condition compared to the control condition (Figure 3c).

3.4. Effect of region on mRNA expression of GPx4 at different ages

No significant differences in the mRNA levels of GPx4 were detected between the cortex, outer and inner medulla at any age (Figure 4). The only differences that did occur were that the expression of GPx4 in the inner medulla was signficantly higher ($p < 0.05$) at 12 weeks compared to 36 weeks and 60 weeks.

3.5. Effect of region on mRNA expression of HSP-70 in different age cohorts

Expression of HSP-70 was significantly higher in both the outer and inner medulla compared to the cortex at 5 weeks old (Figure 5). A significant increase ($P < 0.01$) was also seen in the expression of HSP-70 in the inner medulla at both 12 and 60 weeks old. No significant difference in the expression of HSP-70 was observed across the different kidney regions at 36 weeks old. An increase ($P <$

0.05) was observed in the mRNA expression of HSP-70 in the inner medulla at 60 weeks old by 1.9-fold compared to the cortex and by 1.7-fold compared to the outer medulla (Figure 5).

HSP-70 expression increased with age in all three kidney regions, and was significantly higher ($P < 0.001$) at 60 weeks by comparison with 5, 12 and 36 weeks old (Figure 5).

4. Discussion

Ageing is accompanied by changes in the structure and function of different organs and tissues [2]. In the rat kidney, ageing has been related to oxidative stress and enhanced ROS generation [25, 41-43]. Several researchers have shown ageing is associated with decreased expression of antioxidant enzymes [44, 45]. In this study, we investigated the effect of age and oxidative stress on the expression of key antioxidant enzymes GPx1, GPx4 at the protein and mRNA level and key heat shock protein Hsp-70 at the mRNA level in the rat kidney. Importantly, we investigated the effect of ageing and oxidative stress on GPx1 and 4 protein expression in different kidney regions therefore emphasising the possible functional significance of these changes.

The results of this study indicate that, under control conditions, GPx expression varies both with age and region of the kidney. GPx1 was expressed at

a higher level in the cortex compared with the outer or inner medulla under control conditions (Figure 1 and 2). This may be a reflection of the renal medulla being exposed to higher levels of oxidative stress compared with the cortex and that some aspect of this increased stress attenuates GPx1 expression. This suggestion is consistent with earlier reports [46, 47] demonstrating that the outer medulla is subject to higher levels of oxidative stress by comparison with the renal cortex under control conditions. By contrast, GPx4 was not shown to be differentially expressed across the different regions of the kidney under control physiological conditions (Figures 3 and 4) indicating perhaps an increased resistance to the effects of oxidative stress by comparison with GPx1.

The current study further demonstrates that exposure to a contrived oxidative stress (H_2O_2) results in a decrease in both GPx1 and GPx4 protein expression compared with control conditions (Figures 1 and 3). Exposure to 0.2 mM H_2O_2 is a standard model for inducing tissue oxidative stress [48, 49] and is accompanied by abundant ROS production. In this study the impairment of renal function as a result of exposure to the contrived oxidative stress was not measured directly. One avenue for further study would be to investigate whether renal function is indeed impaired as a result of such oxidative stress exposure, and also to determine the mechanisms underlying such putative impairment.

The results of this study also indicate that there is a relationship between age and GPx1 expression. Expression of GPx1 was found to be highest in young

mature rats (12 weeks old) compared to older and younger animals (Figure 1). The age-related decrease of GPx1 expression is consistent with earlier reports [50]. Our results are also in agreement with reports [45] that investigated antioxidant enzyme activity and gene expression of GPx, GSH-red and CAT in Sprague Dawley rats, showing that aged rats exhibited lower enzymatic activities and diminished gene expression by comparison with young rats.

However, in contrast to the situation with GPx1, the results of this study indicate that expression of GPx4 did not vary with age under control physiological conditions (Figure 3). Interestingly, an earlier study by Gomes, Simão [2] found that the expression of GPx remained unchanged during the ageing process of male Wistar Kyoto (WKY) rats. It should be noted, however, that this study did not discriminate between isoforms of GPx. Therefore the age-related decrease in GPx mRNA and enzyme activity previously observed [50] could be due to decreased production of specific isoforms of GPx, such as GPx1, rather than a general decrease in production of all isoforms. This would result in a decrease in overall GPx activity, and also explain why no change may be seen in GPx4 levels.

Given the well described nexus between oxidant homeostasis and stress protein expression, we were also interested in determining the effect of age on the heat shock response in the kidney. The results of this study suggest that expression of HSP-70 varies with both kidney region and age (Figure 5). Overall, we observed that expression of HSP-70 was higher in the inner medulla compared with cortex

and outer medulla, and that expression was significantly elevated in older animals (60 weeks old) compared to younger animals. This is in agreement with earlier reports [30, 51-54], which confirm that expression of HSP-70 is a useful biomarker of the heat shock transcriptional response to heat and physiological stresses in a number of ageing mammals. These authors and others have raised the possibility that age-dependent changes in cellular redox status, as exemplified by the increased production of reactive oxygen intermediates result in the accumulation of oxidatively modified proteins with reduced activity. Age related increased expression of the inducible chaperone HSP-70, then, would be seen as a way of mitigating against these deleterious changes. In any event, our observations are in general agreement with earlier reports [35] showing increased expression of HSP-70 in different brain regions and peripheral organs in different age male Wistar rats. These authors suggest that this response may reflect the age related accumulation of various posttranslational modifications including protein oxidation and glycation leading to damaged proteins and disturbed cellular homeostasis. Such age related epigenetic protein modifications may contribute to the generally observed constitutively elevated chaperone levels in ageing organisms [31]. Given the contribution of HSP-70 to blocking apoptosis, an interesting avenue for further research might be to establish whether apoptosis is impaired as a result of age/oxidative stress, by examining expression of appropriate markers for apoptosis in the kidney.

5. Conclusions

After reaching a peak at maturity (12 weeks), GPx1 protein and mRNA levels decreased with age under both control and stress conditions, and was higher in the cortex than in the outer and inner medulla. GPx4 protein and mRNA levels showed few comparable age-related changes. By contrast with the observed age-related decrease in GPx1 expression, chaperone Hsp-70 mRNA expression greatly increased with age. These results are consistent with the oxidative stress theory of ageing.

Competing Interests

None declared

Author Contributions

NT carried out the Western Blotting and qRT-PCR and helped to draft the manuscript. NK conceived the GPx work and edited the manuscript. MMcM participated in the qRT-PCR analysis and helped edit and revise the manuscript. GLJ was the overall project leader who participated in data analysis and interpretation, conceived the HSP-70 experiments and helped edit and revise the manuscript. All authors read and approved the final manuscript.

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Figure Legends

Figure 1a – GPx1 Expression in the Cortex. Effect of ageing in control and stress conditions on GPx1 protein expression in the kidney cortex. Shown here are representative Western Blots for each age and each condition for GPx1 and the house-keeper β-actin. The graph shows the relative expression level of GPx1 at each age and in each condition based on band density and normalised to the expression of β-actin. Western blots are representative of 3 such blots. Data shown in the graph is mean ± SE of 5 kidneys from 5 different animals. 1b – GPx1 Expression in the outer medulla. All details as figure 1a except that the blots and data shown is for GPx1 expression in the outer medulla. 1c – GPx1 Expression in the inner medulla. All details as figure 1a except that the blots and data shown are for the inner medulla.

Figure 2 – Effect of ageing on GPx1 mRNA expression in different kidney regions. Bar graph of the levels of GPx1 mRNA expression relative to β-actin expression at 5, 12, 36 and 60 weeks in the kidney cortex, outer and inner medulla. Data shown are means ± SE of N=5.

Figure 3a – GPx4 Expression in the Cortex. Effect of ageing in control and stress conditions on GPx4 protein expression in the kidney cortex. Shown here are representative Western Blots for each age and each condition for GPx4 and the house-keeper β-actin. The graph shows the relative expression level of GPx4 at

each age and in each condition based on band density and normalised to the expression of β-actin. Western blots are representative of 3 such blots. Data shown in the graph is mean ± SE of 5 kidneys from 5 different animals. 3b – GPx4 Expression in the outer medulla. All details as figure 3a except that the blots and data shown is for GPx4 expression in the outer medulla. 3c – GPx1 Expression in the inner medulla. All details as figure 3a except that the blots and data shown are for the inner medulla.

Figure 4 – Effect of ageing on GPx4 mRNA expression in different kidney regions. Bar graph of the levels of GPx4 mRNA expression relative to β-actin expression at 5, 12, 36 and 60 weeks in the kidney cortex, outer and inner medulla. Data shown are means ± SE of N=5.

Figure 5 – Effect of ageing on HSP-70 mRNA expression in different kidney regions. Bar graph of the levels of HSP-70 mRNA expression relative to β-actin expression at 5, 12, 36 and 60 weeks in the kidney cortex, outer and inner medulla. Data shown are means ± SE of N=5.

Table 1: Cycling conditions for qRT-PCR.

Temperature	Time	Cycle no.
95	5 minute	1
95 60	5 second 10 second	 40
72	5 minute	1

Table 2: Primer sequences used in this study.

Gene	Forward primer	Reverse primer	Product size	Reference
Gpx1	CAGTTCGGACAT CAGGAGAAT	AGAGCGGGTGA GCCTTCT	139	[38]
Gpx4	AACGTGGCCTC GCAATGA	GGGAAGGCCAG GATTCGTAA	101	[38]
Hsp70	TCATCTCCTGGC TGGACTCT	CTAGCCAACACC CTGAGAGC	228	This study
B-actin	CCTGCTTGCTGA TCCACA	CTGACCGAGCG TGGCTAC	500	[39]

Figure 1a – GPx1 Expression in the Cortex

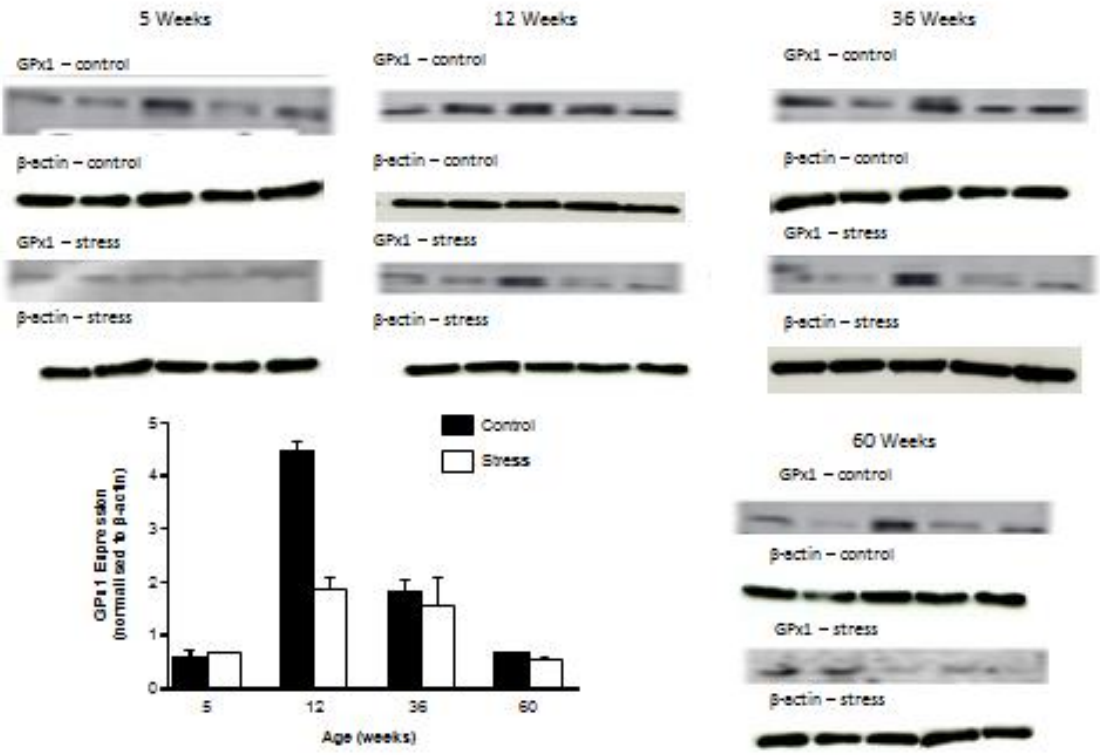

Figure 1b – GPx1 Expression in the Outer Medulla

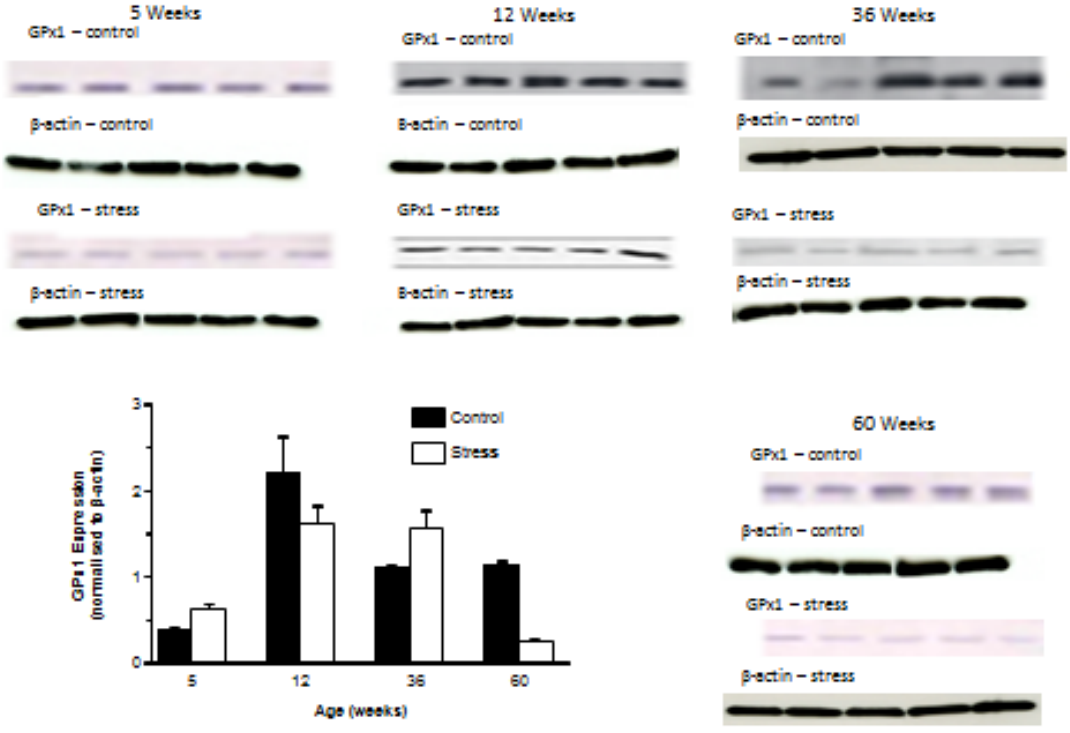

Figure 1c – GPx1 Expression in the Inner Medulla

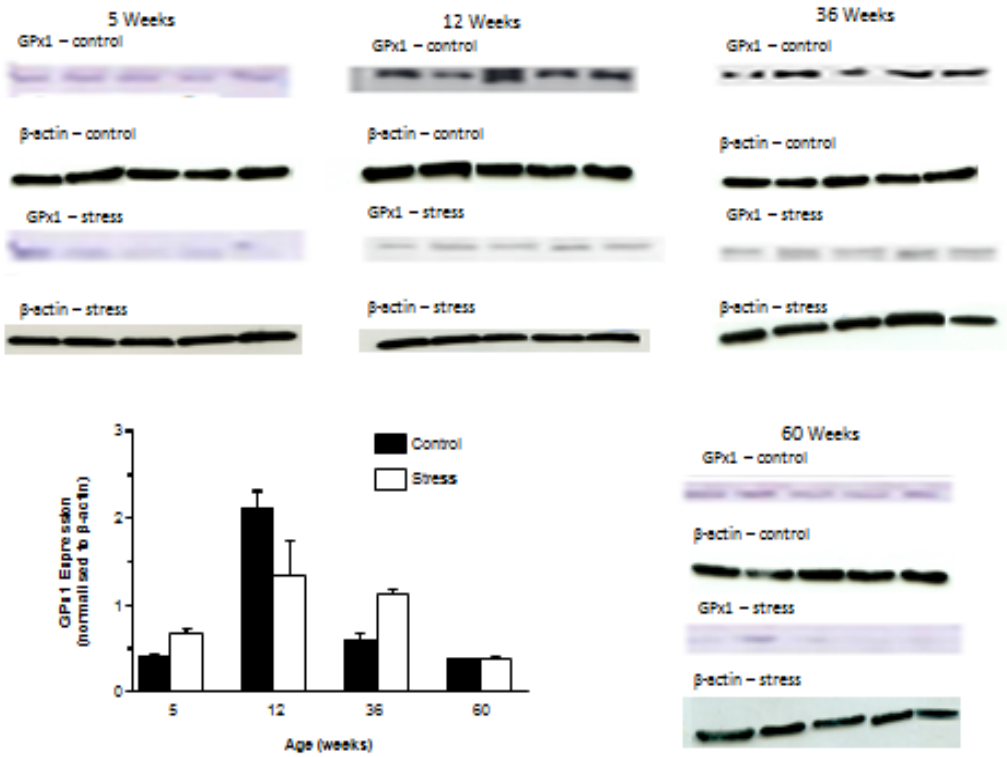

Figure 2 – GPx1 mRNA Expression

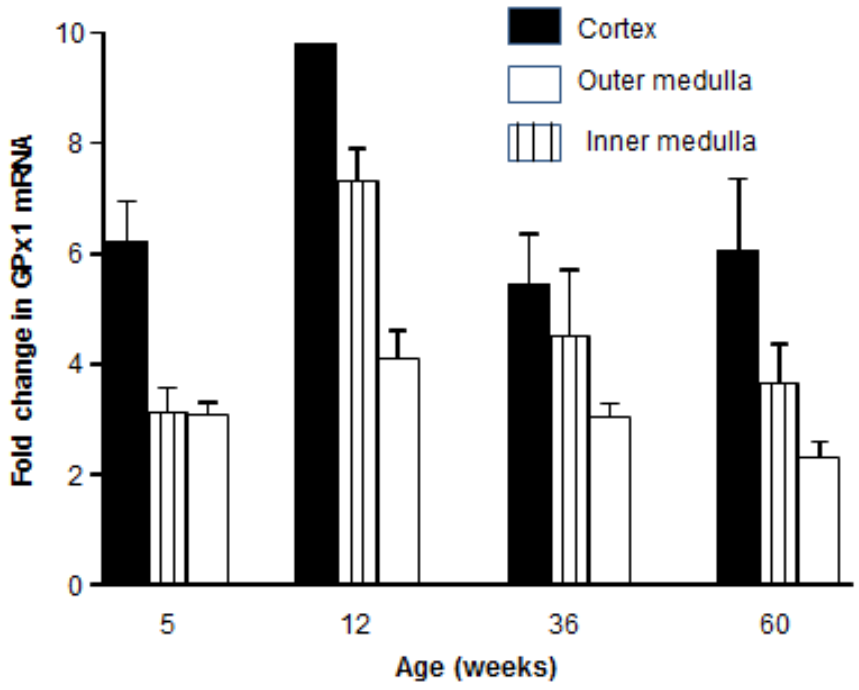

Figure 3a – GPx4 Expression in the Cortex

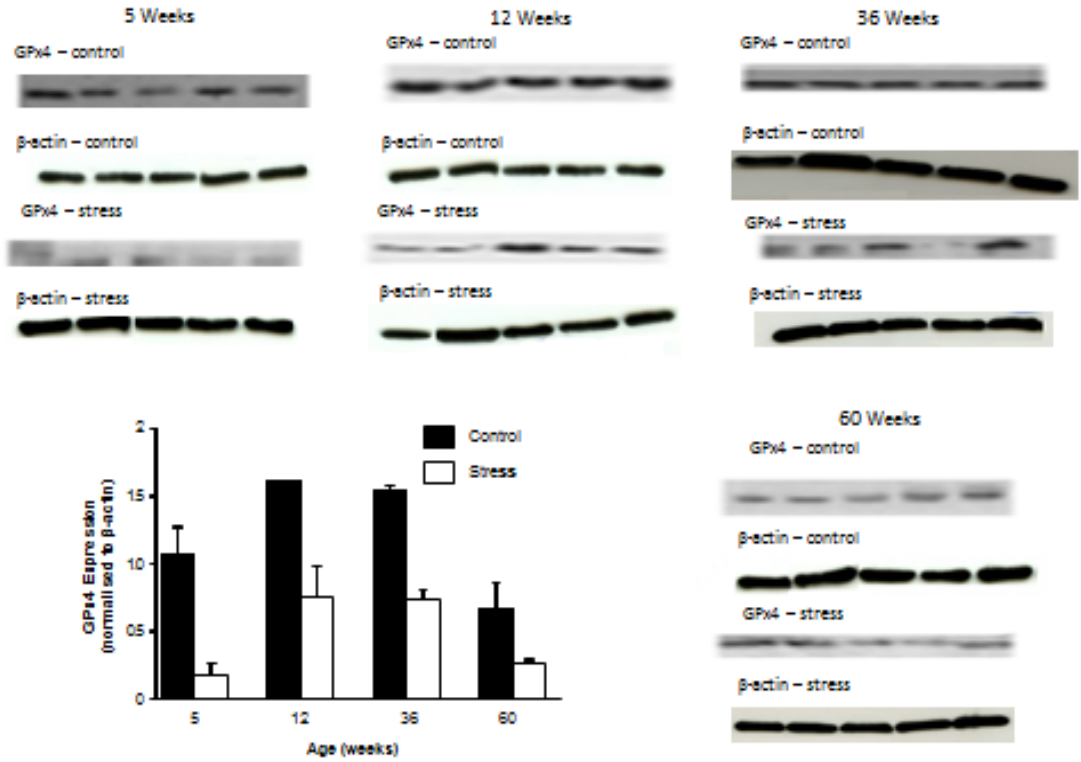

Figure 3b – GPx4 Expression in the Outer medulla

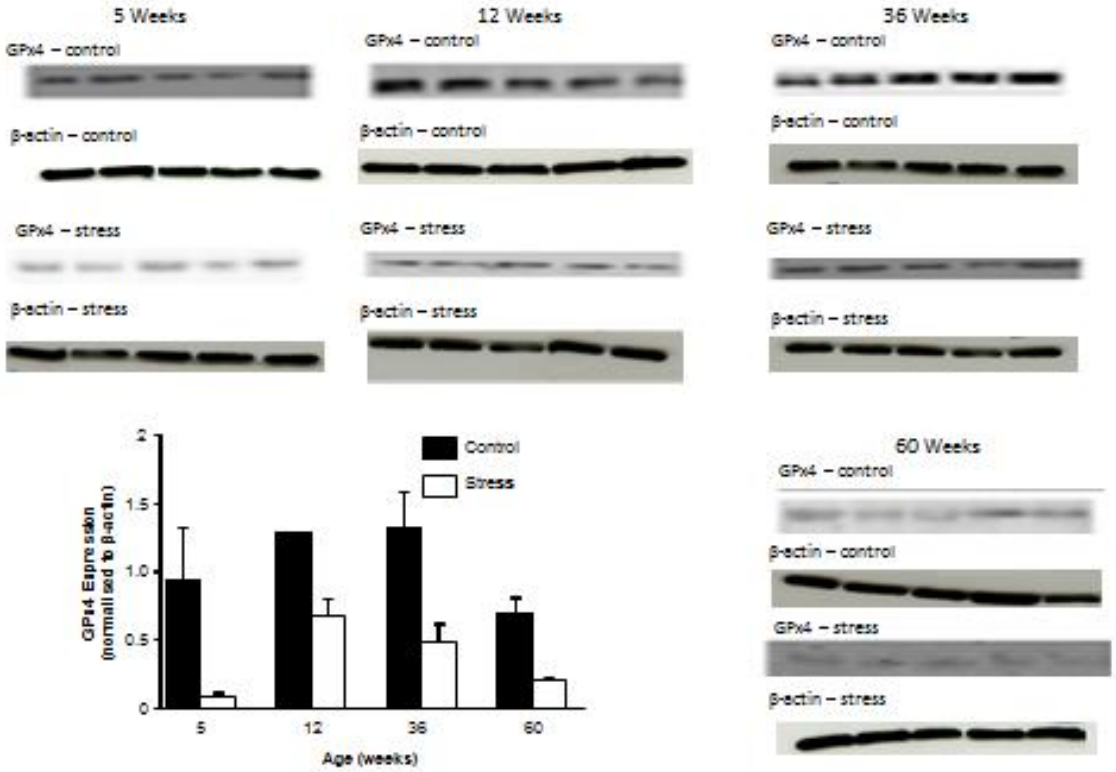

Figure 3c – GPx4 Expression in the Inner medulla

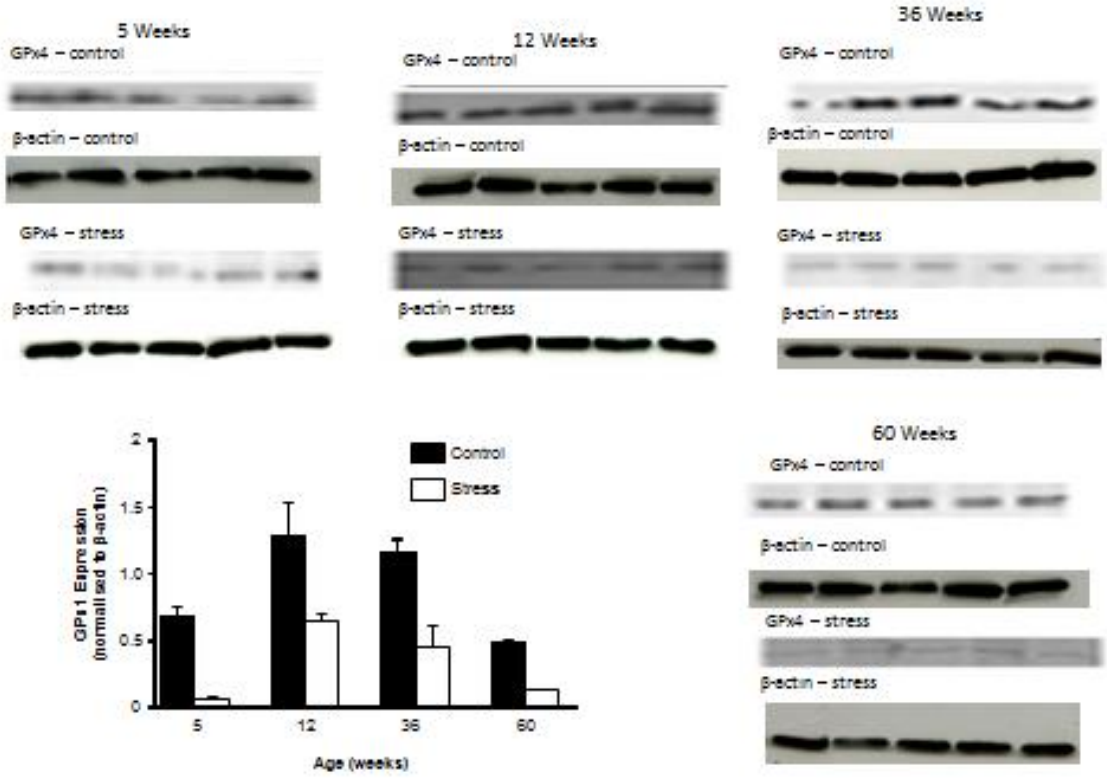

Figure 4 – GPx4 mRNA Expression

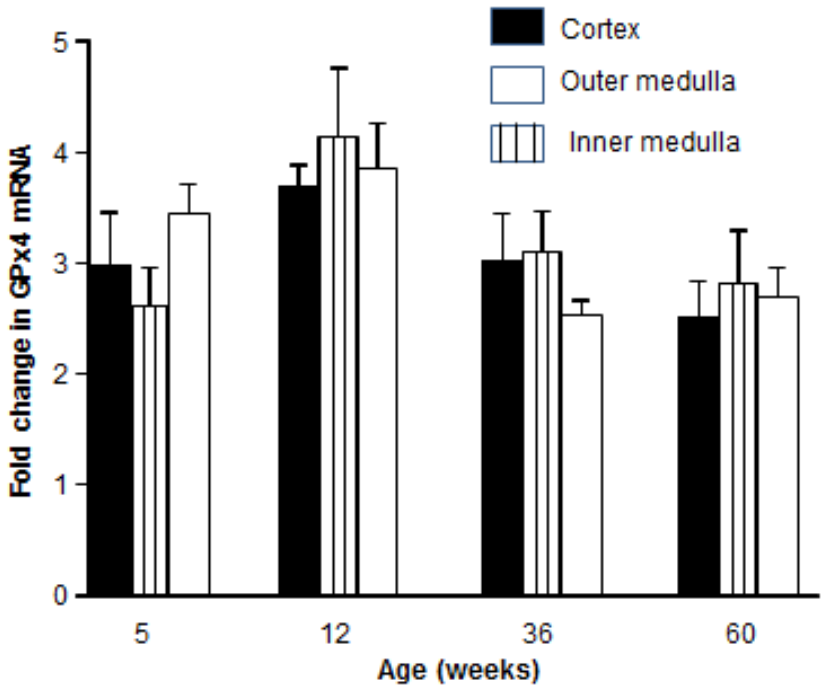

Figure 5 – HSP70 mRNA Expression