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A study of antioxidant protection in fluoride induced rat kidney lysosomal damage

A thesis submitted for the degree of
Doctor of Philosophy (Ph. D)

By

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at

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(2005)

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Declaration

I hereby declare that this thesis is entirely my own work, except where otherwise stated, and it has not been submitted for a degree at this or any other university. I agree that the library may lend or copy the thesis upon request.

Mahmud H Arhima

Dedicated

To

My parent, my wife, my daughter Sarah, and my son Abdul-raoof.

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List of Abbreviations and symbols

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BPB	Bromophenacyl bromide
COX	Cyclooxygenase
CR	Creatinine
DFO	Deferoxamine
EDTA	Ethylendiaminetetraacetic acid
GSH	Glutathione
HPLC	High-performance liquid chromatography
i.p	Intraperitoneal
LRF	lysosomal rich fraction
LOX	Lipooxygenase
LP	α-Lipoic acid
MDA	Malondialdehyde
ml	Millilitre
NaF	Sodium fluoride
NAG	N-acetyl-β- D-glucosamindase
NADPH-oxidases	Reduced nicotinamide dinucleotide phosphate oxidase
NDGA	Nordihydroguaiaretic acid
PUFAs	Polyunsaturated fatty acids
PYC	Pycnogenol
PLA_2	Phospholipase A_2

PLC	Phospholipase C
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric Acid Reactive Substances
µg	Microgram
µl	Microlitre
µ mol	Micromolar
v/v	Volume/volume
w/v	Weight/volume

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Publications and presentations arising from this thesis:

1. Fluoride causes release of renal lysosomal (NAG) isozyme with biphasic effects on MDA levels in vitro. **Br.J.Pharmacol** 2003; Suppl 138. P96. Presented at British Pharmacological Society Meeting. Brighton (UK) 7-10th Jan 2003. (Arhima MH and Sharma SC).

2. Pycnogenol ameliorates fluoride induced rat kidney lysosomal damage in vitro. **Ir J Med Sci.** 172: 24. Presented at Royal Academy of Medicine in Ireland (Section of Biomedical Sciences) meeting Dublin, 10th Jan 2003 (Arhima MH and Sharma SC)

3. Role of redox-active iron in fluoride induced release of NAG isozymes in vitro. **Ir J Med Sci.** 172:15-16. Presented at Royal Academy of Medicine in Ireland (Section of Biomedical Sciences) meeting Cork, 18th June 2003(Arhima MH and Sharma SC).

4. The effect of Pycnogenol on fluoride induced rat kidney lysosomal damage in vitro. **Phytotherapy Research.** 2004 Mar; 18 (3): 244-246.
(Arhima MH, Gulati, O.P and Sharma SC)

5. The effect of chronic high fluoride intake on gentamicin-induced acute nephrotoxicity. Royal Academy of Medicine in Ireland (Section of Biomedical Sciences) meeting Dublin 7th January 2005. (Arhima MH, Davey G and Sharma SC)

Summary.

Fluoride as an inorganic chemical species is ubiquitous in our environment. Fluorosis is a disease or state of chronic poisoning from long-term exposure to excessive quantities of inorganic compounds of fluorine and is a serious health problem in some countries, which is attracting the attention of the World Regulatory Agencies. Epidemiologically, the risk of fluorosis is now well recognized all over the world with greater emphasis on environmental fluoride and its misuse in consumer items, particularly oral hygiene products. Excessive fluoride ingestion over a prolonged period can adversely influence many tissues and organs. Kidney is one of the main target organs attacked by excessive amount of fluoride. Fluoride has been shown to affect human as well as animal kidney structures and functions and increase NAG isozyme release in human and animals urine after renal cell lysosomal damage. The lysosomal membrane damage and the consequent lysosomal acid hydrolase(s) release have been shown to jeopardize cellular integrity and to cause cell apoptosis or necrosis.

Increased generation of free radicals, enhanced lipid peroxidation and disturbed antioxidant defence systems have been proposed to mediate the pathogenesis of fluoride toxicity in tissues.

The results presented in this study have explored that fluoride under acute in vivo and ex vivo conditions has the ability to destabilise rat kidney cell lysosomes and increase NAG isozyme release. Whereas rats exposed to high fluoride concentrations in drinking water over a prolonged period of time did not show high enzymeuria at times tested. We have also shown that fluoride has the ability to increase free radical formation indicated by enhanced MDA level in both models (in vivo and ex vivo). This fluoride induced lysosomal enzyme release, NAG, and other altered biomarkers

were transient and reversible by withdrawal of fluoride administration. The results presented in this thesis have also revealed that fluoride induced lysosomal destabilization ex vivo is amenable to block by some free radical scavengers.

We have also shown that fluoride induced acute renal failure (indicated by high enzymeuria and decreased creatinine excretion) can be ameliorated by natural antioxidants (ascorbic acid and Pycnogenol). This fits well with the recently published studies, which show that ascorbic acid and some other antioxidants can attenuate fluoride induced toxic effects in other organs and tissues.

We have also shown that animals exposed to high fluoride concentrations in drinking water may develop cross-resistance against gentamicin-induced nephrotoxicity. The results presented in this study have further shown that fluoride induced nephrotoxicity is also reversible despite its continuous administration and that animals, which recovered from fluoride induced acute renal failure or animals exposed to high fluoride concentrations in drinking water also acquire resistance to the subsequent nephrotoxicity induced by gentamicin. The oxidative stress adaptation may play a role in this tolerance development. We concluded that fluoride is a pro-oxidant nephrotoxic agent and has the ability to destabilise renal lysosomes directly and liberate its enzymes content. The pro-oxidant activity of fluoride is involved both the enzymatic and the non-enzymatic lipid peroxidation pathways. Free radical scavengers can prevent the lysosomal damage as well as renal toxicity induced by fluoride.

CHAPTER ONE

GENERAL INTRODUCTION

SECTION 1: THE LYSOSOME

1.1. Introduction:

Lysosomes are defined morphologically and biochemically as cytoplasmic organelles 0.2-0.4 μm in diameter delimited by a delicate single lipoprotein membrane and containing a variety of hydrolytic enzymes (hydrolases) most of which have maximal activities at acidic pH with very high latency (Pitt, 1975). Lysosomal system is the main intracellular mechanism for turnover of endogenous and exogenous macromolecules. Endogenous macromolecules (proteins, membranes or intact organelles) which either surrounded by a pairs of smooth endoplasmic reticulum membrane fuses with the lysosome or a lysosome may invaginate and enclose a piece of cytoplasm in similar double bounded vacuole, this process is referred as ***autophagy***. While the extracellular macromolecules (e.g. bacteria) delivered to the lysosomes by fusion of a prelysosomal vacuole (endosome) formed after endocytosis with lysosomal compartment, this process is referred as ***heterophagy*** (Dean, 1977; Dunn, *et al.,* 1980; Klionsky and Emr, 2000).

1.2. Morphology:

The lysosome is a heterogeneous cytoplasmic vacuole surrounded by unilamellar membrane, which is clearly distinct from the mitochondria, and from those small vacuoles, which contain crystalline inclusions (peroxisomes, 0.5 μm in diameter). On the basis of electron microscopy ***primary*** and ***secondary lysosomes*** have been

tentatively distinguished. Primary lysosomes contain hydrolytic enzymes that have not yet participated in a digestive event. Secondary lysosomes are acid-phosphatase-positive vacuoles containing acid hydrolases that have participated in or are engaged in a digestive event (Glaumann and Ballard, 1987). These vacuoles are heterogeneous in shape and size, depending on the type of material harboured- endogenous cytoplasm or phagocytosed exogenous material- and the stage of degradation of the entrapped materials (Glaumann *et al.,* 1981).

1.3. Origin of the lysosomes:

Studies have suggested the existence of two main sub-cellular routes in the production of primary lysosomes. The first hypothesis is that after the formation of lysosomal proteins on ribososmes attached to the rough endoplasmic reticulum (RER), they are transported to the smooth endoplasmic reticulum (SER) to the cisternae of the Golgi body (dictysome). These cisternae are probably in direct continuity with each other, in the three-dimensional network, and the lysosomal enzymes can thus travel from one face of the stack to the other. At the mature face, varied Golgi vesicles are formed and these include primary lysosomes (Dean, 1978). Alternative pathway has been proposed by Novikoff, who has developed the concept of the Golgi-associated Endoplasmic Reticulum forming Lysosomes (GERL), in which he proposed direct formation of lysosomes from smooth endoplasmic reticulum close to the Golgi apparatus without Golgi involvement. Budding of endoplasmic regions rich in lysosomal enzymes is envisaged (Dean, 1978).

1.4. Lysosomes composition:

1.4.1. Lysosomal membrane:

The lysosomal membrane has a typical single phospholipid bilayer (Winchester, 2001), which forms a barrier between intracellular compartments of disparate composition. Unique features of lysosomal membrane include its apparent resistance to degradation by lysosomal hydrolase, its role in maintaining and generating an acidic intralysosomal environment, ability to transport selectively the products of lysosomal hydrolysis, and the specificity with which it interacts and fuses with other membrane organelles of the vacular system (Iveson *et al.,* 1989). The majority of the lysosomal membrane proteins have been found exposed to the cytosolic space (Schneider *et al.,* 1978). This could explain the finding that lysosomal membranes are rapidly digested when exposed to proteases from out side (Henell *et al.,* 1983).

Piqueras and co-workers (1994) have characterised the passive permeability of the lysosomal membrane, and they have demonstrated that lysosomes from renal proximal tubules have a membrane that is relatively leaky to water, protons, and small non-electrolytes and it is determined by the lipophilicity but not by molecular volume of the solutes as other biological membranes. However, most of the very small split products assumed to be permeate rapidly across the membrane either passively as mentioned above or through substrate –specific porters located on the lysosomal membrane (Forster and Lloyd, 1988). It is certain that most xenobiotics will cross the lysosome membrane only if they are able to do so by passive diffusion.

Whereas, the specificity of membrane porters make them unlikely to participate in xenobiotics translocation (Lloyd, 2000).

1.4.1.1. Lysosomal pH:

Since lysosomal hydrolases function best at low pH, the lysosomal interior would be expected to be acidic (pH around 5.0) (Zdolsek and Svensson, 1993; Zatta *et al.,* 2000). One of the most important functions of lysosomal membrane is the maintenance of an acidic pH in the lumen of the lysosomes. This is achieved by vacular proton pump (V-type H^+-ATPase) (fig. 1.1), which is couples hydrolysis of ATP (Adenosine triphosphate) in the presence of magnesium to the translocation of protons (Moriyama *et al.,* 1992; Winchester, 2001). Alternatively, Reijngoud and Tager (1977), have proposed that the acidic intralysosomal pH is maintained by a Donnan equilibrium in which negatively charged enzymes or non-diffusible groups derived from hydrolysis cause trapping of protons in the lysosomal matrix. However, since lysosomes have a low permeability to H^+ and to cations Na^+ and K^+ at physiologic temperatures, the Donnan equilibrium can only play a minor role in the acidification processes of the lysosomes (Glaumann and Ballard, 1987).

Lysosomes maintain an acidic interior pH, which while essential for the activity of the hydrolases inside, threatens the maintenance of the cytoplasmic pH if lysosomal acid hydrolases released (Lloyd and Forster, 1986).

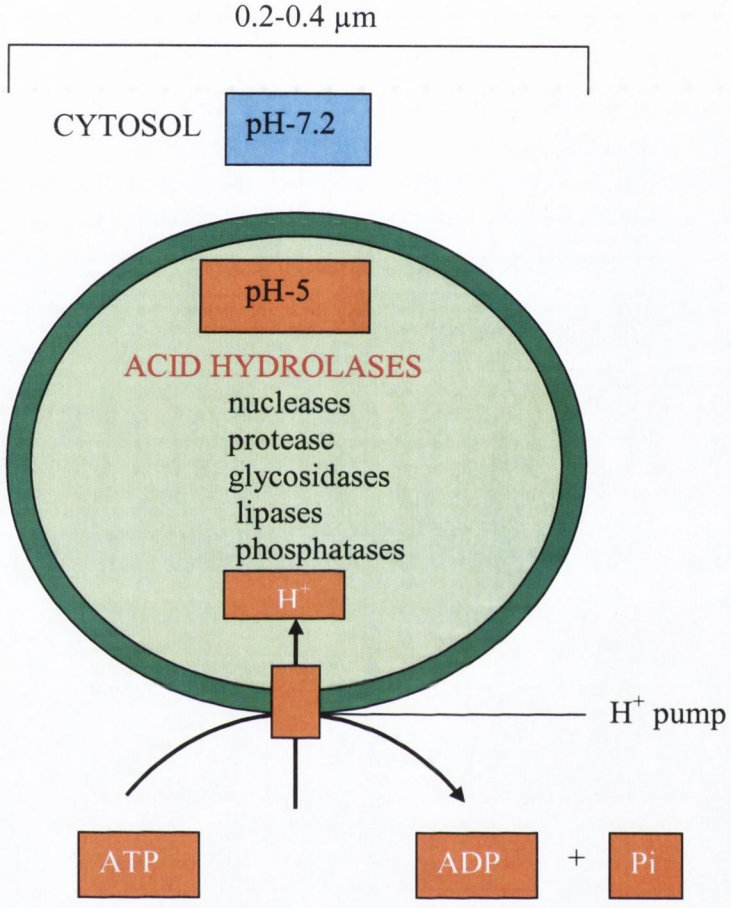


Fig. 1.1. Lysosomes. The acid hydrolases are hydrolytic enzymes that are active under acidic pH conditions. The lumen is maintained at an acidic pH by an H^+-ATPase in the membrane that pumps H^+ into the lysosome.

1.4.2. Contents Of Lysosomes:

Inside the lysosomal membrane two different types of component can be distinguished, the intrinsic components and the lysosomal food.

1.4.2.1.Intrinsic contents:

The hydrolytic enzymes are considered as the intrinsic lysosomal component. About 60 or more enzymes are known to be present in the lysosomes of one or more cell types: there are several proteinases, glycosidases, nucleases, phospholipases, phosphatases and sulphatases (table 1.1 shows the main classes of lysosomal enzymes with some examples). The pH optima of enzyme activities are normally in the acidic range. In general these enzyme armoury is sufficient to degrade most cellular macromolecules (Dean, 1977).

1.4.2.2 Lysosomal food:

All kinds of materials taken up by lysosomes (digestible or indigestible) can be considered as a part of the lysosomal content. As long as the food is digestible, not only the sequestered material is a part of the lysosome content, but also derivatives of digested substances must form a part of lysosomal content (Glaumann and Ballard, 1987). In addition, indigestible particles, as tracer substances like cytochrome C and this can be the case after heterophagic uptake of extracellular constituents, such as

collage may present in the lysosome. This shows that the lysosomal compartment cannot essentially distinguish between digestible and indigestible substrates (Kiesewetter and Kugler, 1985).

Table 1.1.

The main classes of lysosomal enzymes with some selected examples. (Adapted and modified from, Dean, 1977).

Class	Enzyme	Typical substrate	Reaction	Optimal pH
Oxidoreductases Acting on hydrogen peroxide as acceptor	Peroxidase	Benzidine, protein as donar: H_2O_2	Donor oxidized; H_2O_2 converted to H_2O	5.5
Hydrolases acting on ester bonds (esterases)	Cholesterol esterase	Cholesterol oleate	Liberate fatty acid and cholesterol	4.0
	Acid phospatase	p-nitrophenyl phosphatase	Liberate phosphosphoric acid.	3.0-6.0
	Deoxiribo-nuclease II	DNA	DNA split to 3-phosphololi-gonucleotides	4.5-5.5
Hydrolases acting on glycosyl bonds (glycosidases)	β-glucur-onidase	β-D-glucur-onic acid-phenol-phathalin	Liberate terminal residues of β-D-glucuronate	4.0-5.5
	N-acetyl-β- D-glucosamindase	glycoproteins	Carbohydrate and aminoacids	5.0
Hydrolases acting on peptide bond Peptidases& proteinases	Cathepsin D	Proteins	Liberates small Peptides	3.0-3.5

1.4.2.3. Metal ions:

Ions of several metals (iron, manganese, etc) are found normally in lysosomes, at concentrations higher than other organelles (Dean, 1977). This sequestration of metals might protect the cell from the deleterious effects of such ions. The ongoing decomposition of iron containing metalloproteins, within these acidic organelles is accompanied by the release of redox-active iron which upon export from the lysosome, may be a major intracellular source of free iron for the continued synthesis of new iron –containing proteins (Ollinger and Brunk, 1995; Persson *et al.,* 2001a). Furthermore, autophagy of cytosolic ferritin would promote the release of redox active iron within lysosomes due to the conjugate action of an acidic pH and the hydrolytic enzymes (Radisky and Kaplan, 1998; Klionsky and Emr, 2000). This system of iron recycling renders the lysosome as the most important pool of redox-active iron in the cell (Yu *et al.,* 2003).

1.5. Function of lysosomes:

The lysosomal system is the main intracellular mechanism for the turnover of endogenous and exogenous macromolecules, which are delivered to the lysosomes by the processes of autophagy and heterophagy (Stromhaug and Klionsky, 2001; Nicola and Straus, 2004). The digestion of the macromolecules occurs in the lumen of the lysosomes and is catalysed by a cocktail of predominantly hydrolytic enzymes with a characteristic acidic pH (Winchester, 2001). Proteins are broken down to dipeptides or free amino acids; complex carbohydrates, to lower oligosaccharides or

monosaccharides; nucleic acids, to nucleosides and phosphate; neutral fats or phospholipids, to free fatty acids and glycerol (Dean, 1977).

Subcellular structures such as mitochondria (Kissova, *et al.,* 2004) and peroxisomes (Sakai *et al.,* 1998) are attacked and digested to soluble, diffusible molecules, as are microorganisms engulfed by endocytosis (Nicola and Straus, 2004).

However, the normal fate of digestion products is diffusion through the lysosome membrane into the cytoplasm where further catabolism may occur.

1.6. Expansion of the lysosomal compartment:

In addition to the physiological fluctuations in size of the lysosomal compartment, enlargement of lysosomes and increase in lysosome volume fraction is a well-known phenomenon in a number of pathological and manipulated states: (1) Excess feeding by uptake of large amounts of proteins by kidney tubular cells suffering from proteinuria (Glaumann and Ballard, 1987), (2) Accumulation of indigestible materials (Pfeifer *et al.,* 1984), (3). Impairment of enzymatic degradation due to genetic deficiency of lysosomal enzymes like α-glucosidase (Hesselink *et al.,* 2003), some lysosomal storage diseases are listed in (table 1.2), or induced artificially by lysosomal inhibitors like, chloroquine (Kwok and Richardson, 2004), (4). Faulty back-transport of split products to the cytosol has been considered as causes of lysosomal expansion as in case of cystenosis in which there is deficiency in the amino acid transport (Strehle, 2003).

Table 1.2.

Selected lysosomal storage diseases and their characteristics. (Adapted and modified from Dean, 1977).

Disorder	Enzyme deficiency
Mucopolysaccharidoses - Hurler and Scheie syndromes - Sanfilippo syndrome	 α -Iduronidase N-Acetyl-α-glucosaminidase
Sphingolipidoses - Krabbe's disease - Tay-Sachs disease	 β-Galctosidase Hexoaminidase A
Disorders of glycoprotein metabolism - Fucosidosis - Mannosidosis	 α -L-Fucosidase α-Mannosidase
Other disorders with single enzyme defect - Pompe's disease - Wolman's disease	 α -Glucosidase Acid lipase
Multiple enzyme deficiency -Multiple sulphatase deficiency	 Arylsulphatase; steroid sulphatase; iduronate sulphatase; heparan sulphatase.
Disorder of unknown origin -Cystinosis	Accumulation of cystine in lysosomes

1.7. Kidney lysosomes:

Renal lysosomes are abundant in proximal tubular cells while less was found in the distal nephron structures (Pfaller, 1982; Usuda *et al.,* 1998). The physiological importance of kidney lysosomes in the catabolism of proteins has been clearly established (Peterson *et al.,* 1984). Filtered proteins are mainly reabsorbed in the proximal tubule while only minor proportion is reabsorbed in the lower parts of the nephron (Haga, 1989; Tojo *et al.,* 2001). Furthermore, experimentally induced proteinuria has been found to increase the activity of lysosomal enzymes in the kidney cortex, changing the lysosomal population, and inducing kidney cortex hypertrophy (Haga *et al.,* 1988). These features of protein reabsorption and catabolism have also been found to be of significant in regulation of haemostasis of body vitamins by reabsorption of protein bounded vitamins in the proximal tubule in which the protein is degraded in the lysosomes and the vitamin either stored or secreted at the basolateral plasma membrane (Raila and Schweigert, 2001). In contrast to the heterogeneity observed in the rat kidney lysosomes in terms of size, and morphology, only one single population is present in the tubules of the rabbit kidney (Hjelle *et al.,* 1981). This heterogeneity has been suggested to be due to functional differences of kidney lysosomes and possibly different cell origins (Andersen *et al.,* 1987).

1.7.1.Renal NAG isozymes:

Over the past 30 years, attention has been directed towards the evaluation of urinary enzymes as non-invasive markers of tubular damage. N-acetyl-β- D-glucosamindase

(NAG; EC 3.2.1.30), which is also named Hexoaminidase, is a lysosomal glycosidase enzyme found in high concentrations in renal proximal tubules (Hir *et al.,* 1979; Bourbouze *et al.,* 1984). It is one of the 60 or more enzymes that reside in lysosomes and participate in the degradation of glycoproteins, glycolipids, and glycosaminoglycans (Neufeld, 1989). Measurement of NAG isozyme activity in the human urine is widely used for assessment of renal damage resulting from various renal diseases (Garbin Fuentes *et al.,* 2000; Holdt-Lehmann *et al.,* 2000; Tylicki *et al.,* 2003; Laube *et al.,* 2004), and in case of nephrotoxic xenobiotic exposures (Tassi *et al.,* 2000; Ida *et al.,* 2001). NAG isozymes activity as well as other lysosomal enzymes like acid phosphatase, cathepsin D, ribonuclease II and deoxyribonuclease have also been used experimentally for assessment of lysosomal integrity by measuring the enzyme release in some *in vitro* models (Yao and Zhang, 1997; Acharya *et al.,* 2004). The major NAG isozymes normally excreted in human and animals urine are NAG-A (acidic) NAG- B (basic) and several minor intermediate forms (I_1, I_2, As and P), which are distinguished according to their different charge characteristics using DEAE-cellulose chromatography (Paraire *et al.,* 1983; Tassi *et al.,* 1992). The percentage of urinary NAG-A isoform is the greatest in normal urine and its excretion is related to various cell functions like exocytosis and is referred as ***functional enzymuria***, whereas the urinary NAG-B isoform, instead, is referred as ***lesional enzymuria*** as its presence, in urine, is correlated with tubular cell lysis (Bourbouze *et al.,* 1984; Gibey *et al.,* 1986; Costanzi *et al.,* 1996). The increase in urinary NAG-B isoform is due to a lesion of the proximal tubule (Tassi *et al.,* 2000). However, the determination of total NAG isozymes (A and B isoforms) in urine is predominant for the assessment of renal damage since NAG-A remained unchanged during by cell lysis so the percentage of lesional isoform B (NAG-B) increases with

the total NAG isozymes activity increased (Ellis *et al.,* 1975; Gibey *et al.,* 1986; Tassi *et al.,* 2000). The NAG isozymes are found to be resistant to mechanical inactivation by tissue homogenisation (Den Tandt *et al.,* 1991).

1.8. Role of lysosomes in cell and tissue injury:

Lysosomes contain many hydrolytic enzymes, which can degrade all cellular macromolecules. Moderate lysosomal rupture with consequent slow release of lysosomal hydrolytic enzymes may result in programmed cell death, ***apoptosis***, by activation of apoptotic-inducing factor (procaspases), which might be a direct effect of the released enzymes (cathepsins) and /or indirectly through attacking mitochondria and the release of cytochrome C. The early release of the lysosomal enzymes may activate feedback processes that cause further lysosomal rupture. Such feedback processes may be an attack from outside of the released lysosomal enzymes and /or activation of lytic cytosolic pro-enzymes (Antunes *et al.,* 2001; Brunk *et al.,* 2001), while complete release of lysosomal contents may result in a ***necrosis,*** complete cell death (Brunk *et al.,* 2001). Therefore liberation of lysosomal acid hydrolases would jeopardize cellular integrity which is considered as key factor in some pathological disorders like interstitial pulmonary diseases (Perez-Arellano *et al.,* 1996), in rheumatoid arthritis (Sohar et al 2002), or pyelonephritis related renal injury (Gupta *et al.,* 1996) and in some other pathological disorders (Poole and Mort, 1981). They are also incriminated in some xenobiotic induced cell damage (Li *et al.,* 2000; Yasuda *et al.,* 2000). Thus lysosomal integrity is of apparent importance to cellular pathology and to protect against xenobiotic induced cellular damage especially when oxidative stress is considered to play a major role.

1.9. Mechanisms of lysosomal enzymes release:

Latency of lysosomal enzymes is commonly used to define the fundamental property of lysosomes being, packages, of acid hydrolases enclosed by a membrane, which is impermeable to substrates and enzymes.

Since the lysosomes are considered as the most important pool of labile, redox-active, low molecular-weight iron in the cell because it is the organelle responsible for degradation of most cellular metalloproteins (Starke *et al.,* 1985; Ollinger and Brunk, 1995; Yu *et al.,* 2003) so these metabolic function makes these organelles vulnerable to oxidative stress and may burst due to formation of hydroxyl radical when the liberated redox-active iron exposed to oxidant via intralysosomal Fenton-like reaction (described in section 2) and associated peroxidative membrane destabilization (Brunk *et al.,* 2001). It has also been indicated that a regulatory mechanism between oxidative activity and lysosomal pH. Activation of lysosomal reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase may result in increase of intralysosomal superoxide and hydrogen peroxide concurrent with pH elevation (due to consumption of H^+ and generation of OH^-) a mechanism that destabilize lysosomes (Chen, 2002). Either the inhibition of intralysosomal degradation of metalloproteins by lysosomotropic alkalinising agent, ammonium chloride in which the consequent increase in lysosomal pH inhibits intralysosomal proteolysis, or chelation of intralysosomal reactive iron by deferoxamine dramatically decreased apoptotic death induced by subsequent exposure to H_2O_2 (Ollinger and Brunk, 1995; Yu *et al.,* 2003). Free radicals and the consequent membrane lipid peroxidation have been shown to increase lysophospholipids and free fatty acids in lysosomal membrane, which

sensitise lysosomal membrane to deleterious effects of phospholipases (Weglicki *et al.,* 1984). It is also observed that lysosomal membrane thiol groups afflicted by redox state of the cell in which oxidation of membrane thiol groups was found to enhance lysosomal proton leakage (Wan *et al.,* 2001), a mechanism would increase the lysosome pH and consequently decrease the lysosomal degradation capacity with consequent increase lysosomal enzymes release. The leakage of protons can also destabilize the lysosomal osmotic balance by H^+/K^+ exchange, since lysosomes *in vivo* are surrounded by a high concentration of cytoplasmic K^+. Also oxidation of thiol groups may sensitise the lysosomes to hypotonic osmotic lysis and modulation of water permeability (Wan *et al.,* 2002).

SECTION 2: FREE RADICALS AND LIPID PEROXIDATION

1.10. A brief history of free radicals:

It is now more than 100 years since Moses Gomberg discovered the first organic free radical. During this time, research has revealed that free radicals are present in the atmosphere, in our bodies and in some very important chemical reactions. Indeed, free radicals have an impact on all our lives. Examples range from the body's ageing process, to the large-scale preparation of plastics used in the household.

1.11. Definition of free radicals:

The term free radical means any species capable of independent existence that contains one or more unpaired electrons. All free radicals contain an odd number of electrons in which the unpaired electron occupies an atomic or molecular orbital itself. Nowadays the term "radical" is often used in place of "free radical", since radicals can exist in the free and bound state. A superscript dot ($^{\bullet}$) after the formula is usually used to donate free radical species (Halliwell and Gutteridge, 2001).

1.12. Chemistry of free radicals generation:

The fact that they are highly reactive (unstable) means that they have low chemical specificity; i.e. they can react with most molecules in their vicinity. This includes proteins, lipids, carbohydrates and DNA. It also means that in trying to gain stability

by capturing the needed electron, they don't survive in their original state for very long. When the "attacked" molecule loses its electron, it becomes a free radical itself, starting a chain reaction (Halliwell and Gutteridge, 2001).

Radicals can be formed by the loss of a single electron from non-radical (Eq.1),

$$X \longrightarrow e^{-} + X^{\bullet} \qquad \text{(Eq.1)}$$

or by the gain of a single electron by a non-radical(Eq.2).

$$Y + e^{-} \longrightarrow Y^{\bullet} \qquad \text{(Eq.2)}$$

Radicals also can be formed when a covalent bond is broken if one electron from each of the pair shared remains with each atom, a processes known as homolytic fission (Eq.3).

$$A{:}\,B \longrightarrow A^{\bullet} + B^{\bullet} \qquad \text{(Eq.3)}$$

Only when two free radicals meet, their unpaired electrons can form a shared electron pair in a covalent bond and both radicals are lost (Eq. 4a & b).

$$A^{\bullet} + B^{\bullet} \longrightarrow AB \qquad \text{(Eq.4a)}$$

$$A^{\bullet} + A^{\bullet} \longrightarrow A_2 \qquad \text{(Eq.4b)}$$

1.13. Free radical and reactive species:

Free radicals species are generally more reactive than non-radicals species due to their unpaired electron, but different types of free radicals vary widely in their reactivity (Slater, 1984; Halliwell and Chirico, 1993). The oxygen molecule (O_2) qualifies as a free radical because it contains two unpaired electrons, but is not particularly reactive due to a special electron arrangement that makes the reactions with oxygen spin restricted (Halliwell and Gutteridge, 1990). However, when oxygen is partly reduced, several different reactive oxygen species (ROS), both radicals and non-radicals may be produced (Eq 5a-d) (Slater, 1984; Halliwell and Chirico, 1993). Examples of ROS are superoxide anion radicals ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$). The hydroxyl radical is extremely reactive free radical with a very short half-life, 10^{-9} second, very unstable and can attack a large array of molecules of the nearby environment (Nelson and McCord, 1998). Reactive nitrogen species (RNS) like gaseous radicals nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) are examples of free radicals.

$O_2 + e^- \longrightarrow O_2^{-\bullet}$ (Superoxide). (Eq. 5a)

$O_2^{-\bullet} + e^- + 2H^+ \longrightarrow H_2O_2$ (Hydrogen peroxide). (Eq. 5b)

$H_2O_2 + e^- + H^+ \longrightarrow HO^{\bullet} + H_2O$ (Hydroxyl radical). (Eq. 5c)

$HO^{\bullet} + e^- + H^+ \longrightarrow H_2O$ (water). (Eq. 5d)

Hydrogen peroxide is a strong oxidizing agent but as is the case with dioxygen, its direct reaction with many organic compounds occurs only very slowly. It will rapidly react with transition metals to form an oxidant (Hydroxyl radical) capable of reacting with organic molecules (Eq. 6a-c). The most often cited of this reaction is Fenton's reaction (Eq. 6b). Fenton was the first to report oxidation of organic compound (tartaric acid) by this system (Aust *et al.,* 1985; Halliwell and Gutteridge, 2001). *In vivo* much of the hydroxyl radical production comes from the reduction of H_2O_2 by $O_2^{-\bullet}$ (Eq. 6c) (the Haber-Weiss reaction), which is in fact, a two step process catalysed by transition metals (Eq. 6a) and involving the Fenton reaction (Aust *et al.,* 1985; Halliwell and Gutteridge, 2001).

$$Fe^{III} + O_2^{-\bullet} \longleftrightarrow Fe^{II} + O_2 \qquad \text{(Eq.6a)}$$

$$Fe^{II} + H_2O_2 \longrightarrow Fe^{III} + HO^- + HO^\bullet \quad \text{(Fenton reaction)} \qquad \text{(Eq.6b)}$$

$$O_2^{-\bullet} + H_2O_2 \longrightarrow O_2 + HO^- + HO^\bullet \quad \text{(Haber-Weiss reaction)} \qquad \text{(Eq.6c)}$$

1.14. Sources of free radicals:

The main sources of free radicals are biological and environmental sources.

1.14.1. Environmental sources:

Free radicals are generated by a wide variety of sources. The external environment generates ROS from many sources including visible light, ultraviolet, X-ray, γ-radiation and ozone, metals and metalloids (Gracy *et al.,* 1999; Sugden, *et al.,* 2004). Man-made pollutants such as automobile emissions and chemical oxidants in air,

water, and the food chain also contribute to the antioxidant challenge. Behavioural activities (e.g., smoking,) also can contribute to oxidative damage (Gracy *et al.,* 1999).

1.14.2. Biological sources:

Free radicals are not only produced as unwanted products; they are also formed deliberately in the body for useful purposes and have important physiological functions, they are involved in the inactivation and killing of bacteria and viruses (Babior and Curnutte, 1987; Rice-Evans and Burdon 1993; Halliwell, 1994.).

Plasma membrane is a major source of ROS through NADPH oxidases (reduced nicotinamide dinucleotide phosphate oxidase) located on both side of the membrane (Moldovan and Moldovan, 2004). Mitochondrial respiration is one of the main sources of ROS in normal metabolism (Gredilla *et al.,* 2004; Ouyang and Giffard, 2004). Endoplasmic reticulum has also been shown to produce ROS, which is largely arises from the cytochrome P-450 system. The cell nucleus is also known to produce ROS through its electron transport chain, which can leak electrons to give $O_2^{-\bullet}$ (Moldovan and Moldovan, 2004). Recently there are growing evidences suggesting a potential role of lysosomes in generation of free radicals in which its metal ion content suggested to play a fundamental role through a Fenton like chemistry (Persson *et al.,* 2003).

Several cytoplasmic enzymes have been implicated in free radical generation like xanthine oxidase, which is one of the most extensively studied enzymes, that catalyse the conversion of hypoxanthine to xanthine to uric acid, during which $O_2^{-\bullet}$ is produced (Matsumoto *et al.,* 2003). Xanthine oxidase can also directly generate $HO^{\bullet}$

radicals via reduction of H_2O_2 (Kuppusamy and Zweier, 1989). Free radicals also generated during the actions of lipoxygenase (LOX) and cyclooxygenase (COX) in eicosanoid metabolism (Gutteridge, 1995; Armstead, 2003). Several other biologically important molecules oxidized in the presence of O_2 to yield $O_2^{-\bullet}$ (Halliwell and Gutteridge, 2001).

1.15. Free radical damage:

Free radicals are capable of oxidizing biomolecules such as DNA, proteins and lipids leading to cellular alteration and ultimately tissue damage (Aust *et al.,* 1985). Specifically, peroxidation of membrane lipids may cause impairment of membrane function, decreased fluidity, inactivation of membrane- bound receptors and enzymes, increased permeability to ions, and possibly eventually membrane rupture (Gutteridge and Halliwell, 1990; Gutteridge, 1995). If the oxidative stress is particularly severe, it can produce cell death (Halliwell, 1997; Gate *et al.,* 1999). Death can occur by necrosis, or by activation of suicide pathway present within all cells, apoptosis (Stoian *et al.,* 1996; Hampton and Orrenius 1997).

1.16. Lipid peroxidation:

Lipids are a heterogeneous group of compounds having several important functions in the body such as being an efficient source of energy, constituents in cell membranes and nerve tissues and many other physiological functions (Murray *et al.,* 2003).
When lipids are oxidised without release of energy, unsaturated lipids go rancid. This process is called lipid peroxidation in which the insertion of an oxygen molecule is

catalysed by free radicals (non-enzymatic lipid peroxidation) or enzymes (enzymatic lipid peroxidation) (Halliwell and Gutteridge, 1990; Gutteridge, 1995).

1.16.1. Non-enzymatic lipid peroxidation:

Lipid peroxidation- a free radical fingerprinting method

Lipid peroxidation is probably the most extensively investigated free radical-induced process. The number of double bonds in the fatty acid determines the susceptibility of a fatty acid to peroxidation. Polyunsaturated fatty acids (PUFAs) are readily attacked by free radicals and become oxidised into lipid hydroperoxides, whereas saturated fatty acids (SFAs) with no double bonds and monounsaturated fatty acids (MUFAs) with one double bond are more resistant to peroxidation. Adjacent double bonds weaken the energy of attachment of the hydrogen atoms present on the next carbon atom. Therefore, the greater the number of double bonds in the fatty acid chain, the easier the removal of hydrogen atom that is why PUFAs are more susceptible to lipid peroxidation (Wagner *et al.,* 1994; Porter *et al.,* 1995). Once the process is initiated, it proceeds as a free radical-mediated chain reaction.

1.16.1.1. Lipid peroxidation chain reactions:

Initiation of lipid peroxidation is caused by attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group upon a PUFA (Ahmed, 1995; Gutteridge, 1995) (fig. 1.2). Since a hydrogen atom in principle is a free radical with a single unpaired electron on carbon atom to which it was originally attached. The carbon-centred radical is established by molecular rearrangement to form a conjugated diene, followed by reaction with oxygen to give a peroxyl radical.

Peroxyl radicals are capable of abstracting a hydrogen atom from another adjacent fatty acid side-chain to form a lipid hydroperoxide, but can also combine with each other or attack membrane proteins. When the peroxyl radical abstracts a hydrogen atom from a fatty acid, the new carbon-centred radical can react with oxygen to form another peroxyl radical, and so the ***propagation*** of chain reaction of lipid peroxidation can continue (Fig. 1.2). A single substrate radical may result in conversion of multiple fatty acid side chains into lipid hydroperoxides (Gutteridge and Halliwell, 1990; Halliwell and Gutteridge, 2001). Decomposition of hydroperoxides generates a complex mixture of secondary lipid peroxidation products such as hydrocarbon gases (e.g. ethane and penthane) and aldehydes (e.g. malondialdehyde (MDA) and 4-hydroxynonenal (HNE)). Lipid peroxides are fairly stable molecules at physiological temperature, therefore the aldehydic by products are formed, *in vivo*, only in small amounts during the peroxidation of most lipids, which is catalysed by transition metals (like redox-active iron). Under physiological conditions, amino acids, proteins, and nucleic acids are more readily attacked by MDA and HNE (Lee *et al.,* 1992; Halliwell and Gutteridge, 2001) MDA and HNE arises largely from enzymatic and non enzymatic peroxidation of PUFAs with more than one double bond, such as linolenic, arachidonic and docosahexaenoic acids (Halliwell and Gutteridge, 2001) Because of the short half-life of free radicals, detection of these lipid peroxidation end products, MDA most common, after acid and heat hydrolysis of lipid hydroperoxides are generally used to indicate free radicals implication in tissue injury (finger print assay).

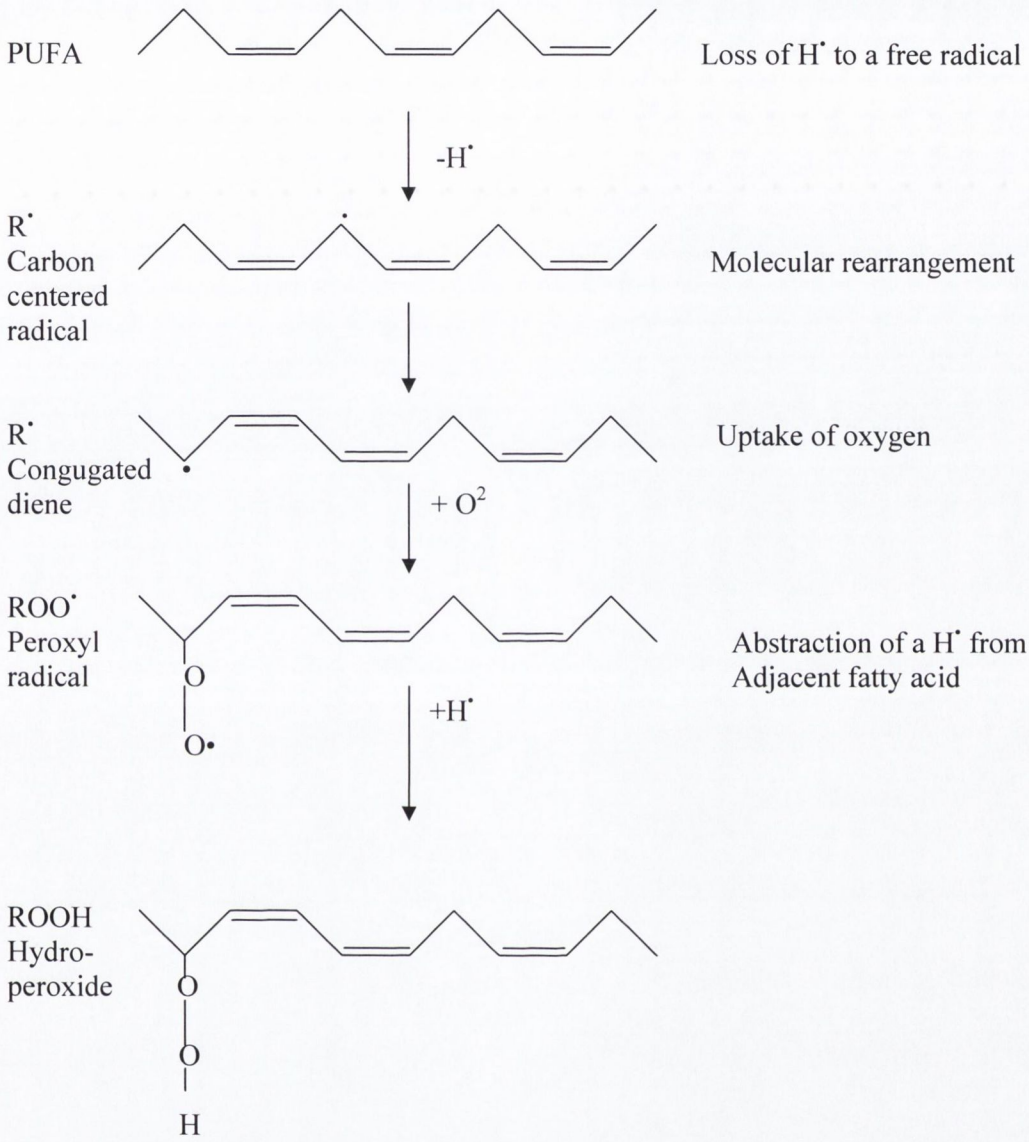


Fig. 1.2. Mechanism of non-enzymatic lipid peroxidation (Gutteridge 1995)

1.16.2.Enzymatic lipid peroxidation:

Cyclooxygenase and lipoxygenase catalyses lipid peroxidation.

The peroxidation of PUFAs can proceed not only through non-enzymatic free radical-induced pathways, but also through processes that are enzymatically catalysed. The enzymatic lipid peroxidation may be referred only to the generation of lipid hydroperoxides achieved by addition of an oxygen molecule to PUFAs catalysed by enzymes. Free radicals are probably important intermediates in enzymatically-catalysed reaction. COX and LOX fulfil the definition of enzymatic lipid peroxidation when they catalyse the controlled peroxidation of various fatty acid substrates. The endoperoxides and hydroperoxides produced by enzymatic lipid peroxidation become stereospecific and have important biological functions upon conversion to stable active compounds (Halliwell and Gutteridge, 1990; Yamamoto, 1991; Gutteridge, 1995; Wallace, 1997). Both enzymes are involved in the formation of eicosanoids, which comprise a large and complex family of biologically active lipids derived from PUFAs.

1.17. Oxidative stress, antioxidants, adaptation and repair systems:

1.17.1. Oxidative stress:

Oxidative stress has been defined as a disturbance in the balance between antioxidants and prooxidants (Free radicals and other reactive species), with increased levels of prooxidants leading to potential damage. This imbalance can be an effect of depletion of endogenous antioxidants, low dietary intake of antioxidants and/or

increased formation of free radicals and other species (Sies, 1991; Ahmed, 1995; Halliwell, 1997).

1.17.2. Antioxidants:

Because some free radical production can be very damaging to cells and tissues, organisms have evolved sophisticated antioxidant defence and repair systems for protection against free radicals and free radical damage at different sites.

Antioxidant is defined as any substance that, when present at low concentrations, compared with those of the oxidizable substrate considerably delays or inhibits oxidation of the substrate (Halliwell and Gutteridge, 1995; Gutteridge, 1995).

The antioxidants exist in both the aqueous and membrane compartments of cells and can be enzymes or non-enzymes and synthesized in the body, or provided as micronutrients. Antioxidant can act at several different stages in an oxidative sequence; removing key reactive oxygen species such as superoxide and hydrogen peroxide, removing catalytical metal ions, scavenging the initiating free radicals such as hydroxyl, alkoxyl, and peroxyl species and breaking the chain of an initiated sequence. Many antioxidants are capable of multiple mechanisms of action, like phenolic compounds, which can act by chain-breaking molecule, scavenge hydroxyl radical, and bind metal ions.

1.17.2.1. Enzymatic antioxidants:

Superoxide dismutase (SOD) is present in the cytosol (copper/zinc dependent enzyme) and mitochondria (manganese dependent enzymes) which is rapidly promote

the dismutation of superoxide into hydrogen peroxide and oxygen at a rate considerably faster than it occurs uncatalyzed (Eq.7) (Zelko *et al.,* 2002; Skrzycki and Czeczot, 2004). The hydrogen peroxide formed by the dismutation reaction can be destroyed by two enzymes, catalase (Eq.8) and glutathione peroxidase (GSHPx) (a selenium-containing enzyme). The GSHPx is requiring reduced glutathione (GSH) for its reaction as hydrogen donor in which the later oxidized to give the oxidized form of glutathione (GSSG) (Gutteridge, 1995). GSHPx also can act on peroxides other than H_2O_2. Thus it can catalyse GSH-dependent reduction of fatty acid hydroperoxides (e.g. linoleic and linolenic acid peroxidation products). GSHPx cannot act upon fatty acid peroxides esterified to lipid molecules in lipoproteins or membranes: they have to be first released by the action of lipase enzymes (Ahmed, 1995; Halliwell and Gutteridge, 2001).

$$2\ H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2H_2O \qquad \text{(Eq. 7)}$$

$$H_2O_2 \xrightarrow[\text{GSHPx}]{2\ GSH} GSSG + 2H_2O \qquad \text{(Eq.8)}$$

1.17.2.2. Non-enzymatic antioxidant:

As well as protein antioxidants, several compounds are thought to be important in the antioxidant defence mechanism. These can be divided into compounds synthesized by body, and compounds obtained from diet.

a. Compounds synthesized by body cells:

1)- Bilirubin:

Most of bilirubin produced in mammals results from the catabolism of haemoglobin (released from the aging red blood cells) in the spleen, liver and bone marrow (Maines, 1988). Bilirubin circulates bound to albumin. The antioxidant activity due to albumin- bound bilirubin includes the inhibition of peroxyl radical induced oxidation of fatty acids (Neuzil and Stocker, 1993).

2)-Uric acid:

Uric acid is produced by the oxidation of hypoxanthine and xanthine by xanthine oxidase and dehydrogenase enzymes. Uric acid is an efficient scavenger of activated oxygen species (Chamorro *et al.,* 2004) and can stabilises ascorbic acid in human serum by complexing with iron (Sevanian *et al.,* 1985).

3)-Coenzyme Q-10:

Coenzyme Q-10, also called ubiquinone (because it is ubiquitous to all biological systems) is a component of the respiratory chain, which resides in the inner membrane of the mitochondrion. This coenzyme Q-10 can scavenge ROS and thereby inhibit lipid peroxidation (Halliwell and Gutteridge, 2001).

4)-Glutathione:

Glutathione is a ubiquitous tripeptide molecule, consisting of three amino acids (cysteine, glutamic acid and glycine), has facile electron-donating capacity, linked to its sulphydryl (-SH) group (Sies, 1997). This potent electron-donating capacity renders it as both potent antioxidant per se (direct detoxification of free radicals) and a convenient cofactor for enzymatic reactions that require readily available electron pairs (Sies, 1997; Halliwell and Gutteridge, 2001).

Glutathione exists in two forms: The antioxidant "reduced glutathione" conventially called glutathione and abbreviated GSH; the oxidized form is sulphur-sulphur linked compound, known as glutathione disulphide or GSSG.

The antioxidant glutathione is also used by enzymes (e.g. GSH dehydrogenases) to convert dehydroascorbate to ascorbate (May *et al.,* 2003), ribonucleotides to deoxyribonucleotides, and a variety of –S-S-<-->-SH interconvention (Sies, 1997). Firstly GSH is oxidized to GSSG (oxidized form), this is then reduced to GSH, in order to keep cells GSH/GSSG ratio high, and is accomplished mainly by the enzyme glutathione reductase. This enzyme uses the coenzyme NADPH as source of electrons (Meister and Anderson, 1983) (Eq. 9).

$$GSSG + NADPH + H^{+} \longrightarrow 2GSH + NADP^{+} \qquad \text{(Eq.9)}$$

In addition to its antioxidant property GSH can protect against metal ion induced free radical generation due to its known copper chelation effect (Hanna and Mason, 1992).

5)-Alpha-lipoic acid:(Thiotic acid)

Alpha-lipoic acid is sulphur containing antioxidant and an essential cofactor in the multienzyme complex that catalyses the decarboxylation of α-keto acids and α-ketoglutarate. It exists in oxidized and reduced forms, both of which have antioxidant properties. Although the levels of lipoic acid in tissues and body fluids are very low, but its ability to reduce GSSG to GSH, dehydroascorbate to ascorbate and it can also regenerate α-tocopherol from α-tochopheryl (Packer *et al.,* 1995; Flohe, *et al.,* 1997) have provoked attempts to use it as therapeutic antioxidant, e.g. in the treatment of diabetes mellitus (Packer *et al.,* 1995).

b- Compounds derived from diet:

1)-Ascorbic acid:

Ascorbic acid (vitamin C) has an effective free radical scavenging power over a wide variety of free radicals (Frei *et al.,* 1989; Rose and Bode, 1993; Evans and Halliwell, 2001; Whiteman *et al.,* 2003), and considered as an extremely antioxidant (Frei, 1989) found at high levels in a variety of tissues in comparison to plasma level. It is involved in many physiological chemical reactions in which it is subsequently oxidized to a compound known as dehydroascorbic acid. Accumulation of dehydroascorbic acid at micromolar (Rose *et al.,* 1992) concentration has been suggested to disrupt cell membranes and act as a neurotoxin (Hisanaga *et al.,* 1992). In normal, healthy tissues, dehydroascorbic acid generally appears to be recycled

immediately back to ascorbic acid, catalysed by GSH as mentioned above, to maintain the high ratio of ascorbic acid to dehydroascorbic acid.

2)-Vitamin E:

Vitamin E is a lipid soluble antioxidant and is a collective name of eight different tocopherols and tocotrienols. The most effective form biologically is the RRR-α-tocopherol, formerly called d-α-tocopherol (Sies, 1997). Tocopherols and tocotrienols inhibit lipid peroxidation largely because they scavenge lipid peroxyl radicals much faster than these radicals can react with adjacent fatty acid side-chains or with membrane proteins (Burton et al 1983; Ingold *et al.,* 1987). In addition, tocopherols can both quench and react with ROS and might protect membranes against these species.

3)-Vitamin A and related compounds:

Vitamin A (retinol) is formed in the intestine from its precursor β-carotene. β-carotene and the carotenoids as whole are abundant in green plants, carrots and other vegetables. Vitamin A and other carotenoids (β-carotene, lutein and lycopene) are effective radical scavengers (Halliwell and Gutteridge, 2001).

4)-Plant phenols:

A phenol contains an –OH group attached to a benzene ring. Plants contain a huge range of phenols other than tocopherols and tocotrienols, which have a powerful

antioxidant effect (Salah *et al.,* 1995). Flavonoids are good examples of this antioxidant group, which are widely distributed in plants and their daily consumption in some countries exceed vitamin E daily intake (Keli *et al.,* 1996). Flavonoids consist of dozens of structurally similar compounds, typically only differing in the degree of ring substitution, the type of substitution (hydroxyls, methoxyls, etc.) and the type and the degree of glycosylation (Rice-Evans *et al.,* 1996). Flavonoids are powerful inhibitors of lipid peroxidation, ROS/RNS generation, lipoxygenase, and cyclooxygenase enzymes and have the ability to bind to various metal ions (Laughton *et al.,* 1991).

1.17.3. Adaptation and repair systems:

Oxidative stress can result in adaptation or cell injury. Cells can usually tolerate mild oxidative stress, which often results in up-regulation of the synthesis of antioxidant defence systems in an attempt to restore the oxidant/antioxidant balance (van der Valk *et al.,* 1985; Soejima *et al.,* 1998). However, it has been suggested that the adaptation to oxidative stress does not always involve increased antioxidant defences and this adaptive mechanism may be due to change in sensitivity of targets normally vulnerable to oxidative damage (Gille and Joenje, 1989).

When tissue has been injured due to oxidant challenge some enzymes will serve as a repair system to eliminate molecules or cell components that were damaged by oxidants or free radical reactions escaped the antioxidant defences. This group of enzymes may form a second line of defence mechanism and some times referred as secondary antioxidants (Davies, 1985; Ahmed, 1995). The lipid peroxides within membranes can be removed by phospholipid hydroperoxide glutathione peroxidase

enzymes (PHGPx) (Antunes *et al.,* 1995). Alternatively, they may be cleaved from membranes by the action of phospholipases whereupon the released free fatty acid peroxides can be acted upon by ordinary GSHPx (Miyamoto *et al.,* 2003). The preference of these phospholipases for oxidized fatty acids is still debatable (Halliwell and Gutteridge, 2001).

There also appears to be no limited mechanism for the repair of oxidatively damaged proteins, if repair entails a selective removal and replacement of the damaged amino acid (except for oxidized cysteinyl and methionyl residues which can be repaired via enzyme catalysed disulfide exchange, methionine sulfoxide reductase (Yermolaieva *et al.,* 2004). However, oxidatively damaged proteins possess an increased proteolytic susceptibility and, it follows, that its proteolytic degradation will increase the pool of free amino acids for the de novo synthesis of a new protein (Ahmed, 1995).

SECTION 3: FLUORIDE NEPHROTOXICITY AND ITS FREE RADICALS PROFILE.

1.18. Fluoride's chemical-physical properties & its availability:

Fluoride is the ionic form of fluorine (F), a halogen and the most reactive of all elements, which might be contributed to its high electronegativity. Fluorine has a strong irritating odour, it is pale yellow or colourless gas as hydrofluoric acid (HF), also occur as odourless colourless cubic or tetragonal crystals as in case of sodium fluoride (NaF) which has an atomic weight of 19.0, atomic number of 9.0 and 9 electrons distributed in the first and second energy levels (fig. 1.3). Fluorine is a natural component of the biosphere and the 13^{th} most abundant element in the crust of the earth. As such, it is not surprising that it has been found in a wide range of concentrations in virtually all inanimate and living things (Kono, 1994).

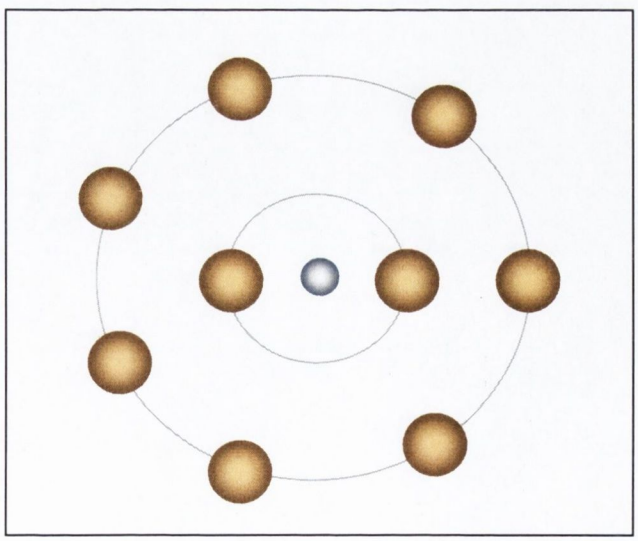


Fig. 1.3. Schematic representation of the shell structure of fluorine.

Fluoride is present in nearly all-fresh ground waters, though the concentration in some water supplies is very small and the range of fluoride levels in drinking water varies in different parts of the world. It reaches about 100 ppm (100 mg/L) in some areas in Africa and Asia whereas in USA and Europe is much lower. Fluoride is also found in seawater in concentrations ranging from 0.8-1.4 ppm. Additionally fluorides are widely distributed in the atmosphere and its organic form is the third most prevalent class of industrial air pollutants (Kennedy, 1990). Thus fluoride, in varying concentrations, is freely available in nature. Fluoride compounds are added in small amounts to water sources to prevent tooth decay. It is also a constituent of toothpaste for the same reason.

1.19. Fluoride intake:

The major sources of fluoride intake are water, beverages, food, and fluoride-containing dental products, where the fluoride content in food depends on the concentration of fluoride in soil and water. The fluoride concentrations in groundwater range from less than 0.1mg/L to more than 100 mg/l and depend mainly on the concentration and solubility of fluoride compounds in the soil (Whitford, 1996). A substantial amounts which may approach or exceed dietary fluoride intake, may come from inadvertent swallowing during and after the use of fluoride-containing products (Burt,1992), in which the fluoride concentrations range from 230 ppm (0.05% NaF mouth rinse) to over 12,000 ppm (1.23% acidulated phosphate fluoride gel) (Whitford,1996).

1.20. Absorption and plasma concentrations of fluoride:

When taken by mouth, the absorption of fluoride begins in the oral cavity. Fluoride can enter the body by passage through the oral mucosa, especially from acidic solutions, at a measurably but substantially lower rate than that which occurs after swallowing (Whitford *et al.,* 1982).

An appreciable amount of fluoride can be absorbed from the stomach (Whitford and Pashley, 1984) and this process occurs by diffusion (Villa *et al.,* 1993) and is inversely related to pH so that factors which promote the secretion of gastric acid increase the rate of fluoride absorption, which leads to earlier and higher peak plasma fluoride levels, and vice versa (Whitford and Pashley, 1984; Messer and Ophaug, 1993; He *et al.,* 1998). The pH dependence of absorption from oral cavity and the stomach is consistent with the hypothesis that HF but not the ionic fluoride, is the permeating moiety (Kawase and Suzuki, 1989; He *et al.,* 1998).

High dietary fat levels may increase the absorption of ingested fluoride, which might be a consequence of the reduced rate of gastric emptying and therefore, increasing the residence time of fluoride in stomach (McGown *et al.,* 1976). Divalent ions like calcium and magnesium may complex with fluoride in the stomach and therefore decrease its absorption. Most of the fluoride that fails to be absorbed from the stomach will be absorbed from the intestine and generally, less than 20% of the amount ingested each day is excreted in the faeces (Whitford, 1996).

A measurable increase in plasma fluoride occurs within the first few minutes after oral ingestion whereas the peak plasma concentration, which is followed by rapid decline, typically occurs at about 60-90 min (Cowell and Taylor, 1981).

The plasma fluoride level increases in proportion to the chronic level of fluoride intake (Ekstrand, 1978; Whitford 1990) suggesting that body fluid fluoride level is not homeostatically controlled, and therefore, plasma fluoride levels can be used as an index of previous exposure to the fluoride ion (Waterhouse, 1980).

1.21. Tissue distribution:

A steady-state relationship exists between plasma and soft tissue fluoride levels (Whitford *et al.,* 1979), this means the plasma and intracellular fluoride levels of any given tissue are not equal, although the ratio of the concentrations is constant, even when plasma fluoride levels are changing rapidly. In order for this type of distribution to exist, cell membranes must be readily permeated, and binding of fluoride by cellular components must be minimal or absent, which is consistent with the hypothesis that HF, not ionic fluoride, is in diffusion equilibrium across cell membranes (Gutknecht and Walter, 1981).

The various soft tissues are distinguishable by their tissue-to plasma (T/P) fluoride concentration ratios. Table 1.3 shows the T/P fluoride ratios of various rat tissues, which were obtained 60 min after the intravenous injection of the radio-isotope (^{18}F) (Whitford *et al.,* 1979). The T/P ratios for kidney and whole femur exceeded unity. These high values were explained by the facts that fluoride in the tubular fluid of the nephron of the rat is normally about 100 times more concentrated than that of plasma, and that fluoride is an avid bone-seeker (Whitford, 1990).

Table 1.3.

Tissue- to plasma18F concentrations of rats 60 minutes after intravenous injection. (adapted from Whitford et al.,1979).

Tissue	T/P Ratio	Tissue	T/P Ratio
Brain	0.084 ± 0.001	Tongue	0.685 ± 0.017
Fat	0.112 ± 0.014	Spleen	0.697 ± 0.010
Skin	0.433 ± 0.021	Lung	0.825 ± 0.018
Heart	0.462 ± 0.036	Liver	0.980 ± 0.036
Diaphragm	0.610 ± 0.039	Kidney	4.160 ± 0.340
Submandibular Gland	0.627 ± 0.014	Femur	7.520 ± 0.740
Abdominal wall Muscle	0.663 ± 0.028		

Data expressed as mean ± SE. Ratios for fat, skin, and femur expressed in terms of wet weight; all others expressed in terms of tissue water.

1.22. Renal handling of fluoride:

The rapid decline in plasma level of fluoride, and its efficient removal from plasma is due to renal excretion and uptake by bone (Kono *et al.,* 1986; Whitford, 1990).

Fluoride ion is basically freely filtered from plasma in the glomerular capillaries into the urinary space of Bowman's capsule, after which it undergoes a variable degree of tubular reabsorption (Whitford, 1990). The proposed mechanism for the reabsorption of fluoride from the renal tubules is similar to that of the gastric absorption of the ion,

in that it involves the diffusion of HF, therefore renal clearance of fluoride is dependent on urinary pH (Whitford and Pashley 1991). When the urine is relatively alkaline, all of the fluoride exists in the ionic form and thus remains within the tubule to be excreted. However when the urine is relatively acidic, proportionately more of the fluoride exists in the un-dissociated form; this would increase the rate of diffusion from the tubule into the interstitial fluid, thus leaving less fluoride to be excreted (fig. 1.4). In the interstitial fluid, where the pH of the tubular fluid is neutral, HF would dissociate, and the fluoride ion would diffuse into relatively leaky capillaries and be returned to the systemic circulation (Whitford, 1990).

1.23. Uptake by calcified tissues:

Fluoride is extensively taken up by bone and other calcified tissues (Giachini and Pierleoni, 2004). This has been suggested to be a second major mechanism by which fluoride is cleared from plasma and other body fluids. The clearance rate of fluoride from plasma is essentially equal to the sum of the renal and skeletal clearance rate (Ekstrand et al.1980; Whitford, 1999). Fluoride associated with bone is not irreversibly bound but rather may be readily mobilized (Grandjean, 1982; Grandjean and Thomsen, 1983). The major variable, which affects the rate of fluoride uptake by bone, is age or the stage of skeletal development. It has been indicated that the amount of fluoride taken up by bone and retained in the body is inversely related to age. The most plausible explanation for the age –dependency of fluoride retention is that the crystallites of younger bone are smaller, more numerous, and loosely organized. They are heavily hydrated, and, therefore, they offer a much larger surface area for the uptake of fluoride than does more mature bone (Whitford, 1999).

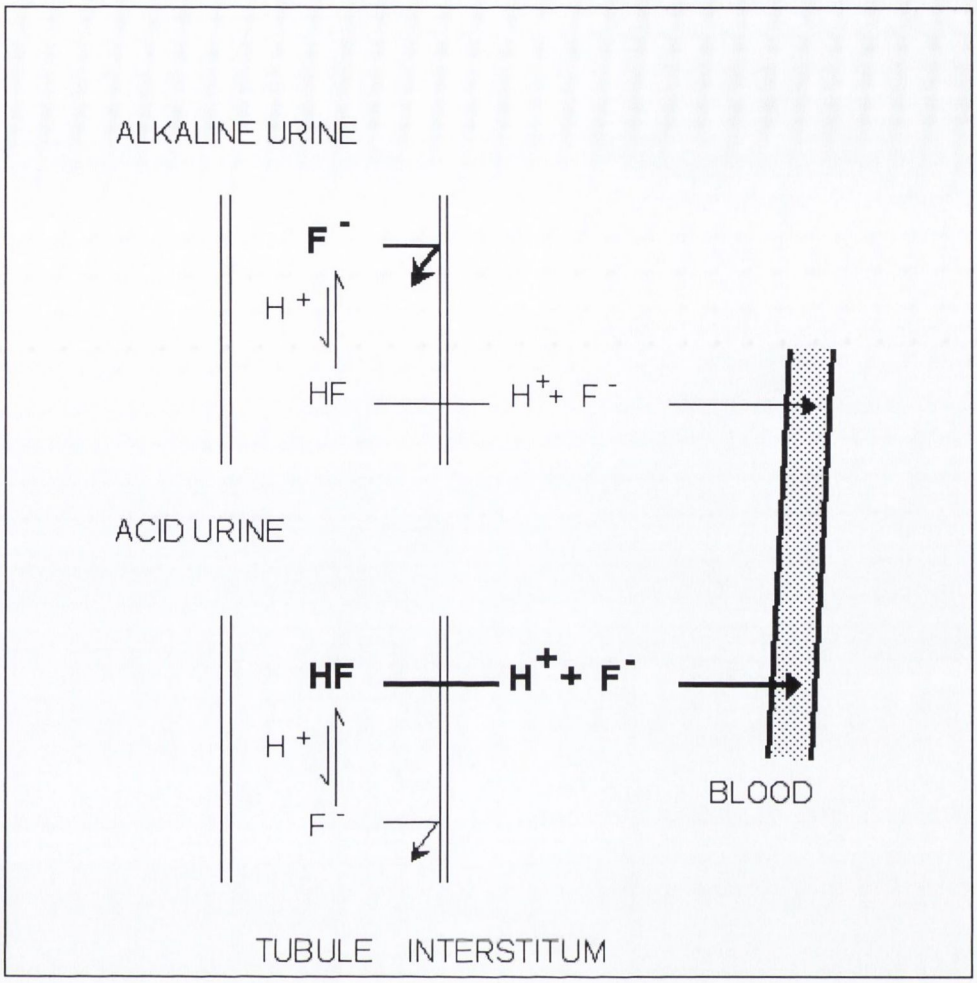


Fig. 1.4. The proposed mechanism for the re-absorption of fluoride, as hydrofluoric acid (HF), from the renal tubule (Adapted from Whitford, 1990).

1.24. The use of fluoride in bone disorders.

Fluoride is considered as bone forming agent as it increases bone volume, an effect due to increased osteoblastic activity (Briancon and Meunier, 1981; Kleerekoper, 1998; Schulz, 2000), and also decreased fracture risk (Mamelle *et al.,* 1988).

A combination of NaF, calcium and vitamin D supplemented diet has also been found successful in treating cases of osteoporosis (Kono, 1994; von Tirpitz, 2000).

1.25. Fluoride and dental caries.

Based on extensive epidemiological studies on the relationship between water fluoride concentrations and dental caries, dental fluorosis determined that 0.7mg /L was optimal because it provided a high degree of protection against dental caries and a low prevalence of milder forms of dental fluorosis (Hardman *et al.,* 1996).

Topically applied fluoride can also prevent caries development (Strohmenger and Brambilla, 2001). The cariostatic effect of systemic and topical fluoride agents are presumed to be due to: (1) reduced enamel solubility and therefore increased resistance to caries, (2) decreasing demineralisation as well as promote remineralization of incipient lesions, (3) in addition fluoride at sufficiently high concentrations can inhibit bacterial growth and reduces the rate of acid production by cariogenic microorganisms (Margolis and Moreno, 1990).

1.26. Fluoride intoxication:

1.26.1.Acute fluoride toxicity:

The certainly lethal dose (CLD) of NaF for human beings is 5-10 g (Whitford, 1990; Hardmann *et al.,* 1996). The CDL is equivalent to LD_{100}, i.e., it would be expected that no adult who ingested NaF in that dose range would survive (Whitford, 1990). Acute fluoride toxicity can happen by drinking water with high fluoride content (Gessner *et al.,* 1994; Penman et al 1997) or accidental ingestion of fluoride containing products like insecticides, or dental products (Fluoride tablets or tooth pastes and mouth washes) (Augenstein *et al.,* 1991). Death has been recorded in both cases (Augenstein *et al.,* 1991; Flanders and Marques, 1993; Gessner *et al.,* 1994). The initial symptoms of acute fluoride poisoning are salivation, nausea, abdominal pain, vomiting and diarrhoea, which might be secondary to the local action of fluoride on the intestinal mucosa (Vogt *et al.,* 1982; Gessner *et al.,* 1994; Penman et al 1997). Myopathological symptoms like numbness or tingling of extremities, tetany and convulsion may develope (Gessner *et al.,* 1994; Hardman *et al.,* 1996). Disturbance in electrolyte balance also occurs, particularly hypocalcaemia (complexation of calcium by fluoride ion) and hyperkalaemia (activation of K^+ channels)(Greco *et al.,* 1988; Augenstein *et al.,* 1991; Bradberry and Vale, 1995; Takase *et al.,* 2004). Depression of renal and respiratory functions is also seen (Whitford, 1996), while death usually results from respiration paralysis or cardiac failure (Hardman *et al.,* 1996).

1.26.2.Chronic fluoride toxicity:

Fluorosis is a disease or state of chronic poisoning from long-term exposure to excessive quantities of inorganic compounds of fluorine and is a serious health problem in some countries. An estimated 62 and 70 million people are afflicted with dental, skeletal, and/ or non-skeletal fluorosis in China and India (Susheela, 1999; Liu *et al.,* 2003). In endemic areas, both skeletal and dental fluorosis becomes prevalent. Mottled enamel or dental fluorosis is a specific disturbance of tooth formation caused by excessive fluoride intake (Frazao *et al.,* 2004). Endemic skeletal fluorosis is a chronic metabolic bone and joint disease caused by ingesting large amounts of fluoride either through water or rarely from foods of endemic areas (Teotia *et al.,* 1998; Choubisa *et al.,* 2001). It is also affects the homeostasis of bone mineral metabolism. The total quantity of ingested fluoride is the single most important factor, which determines the clinical course of the disease that is characterized by immobilization of joints of the axial skeleton and the major joints of the extremities (Krishnamachari, 1986).

Chronic fluorosis is not restricted to teeth and bones but also extends to affect most other body tissues and organs. Both in animals and human fluoride were found to afflict badly the structures and functions of many organs and tissues like brain (Bhatnagar *et al.,* 2002), liver (Kolodziejczyk *et al.,* 2000), pancreas (Matsuo *et al.,* 200), kidneys (Willinger *et al.,* 1995) and reproductive organs (Ghosh *et al.,* 2002). However the mechanism of its intoxication is still not clearly understood.

1.27. Fluoride targeting Kidneys:

The potential for health effects of fluoride exposures on renal function is enhanced because of selective absorption by the kidney and the kinetic of fluoride distribution and excretion. Furthermore, the tissue-to-plasma fluoride concentration ratios for soft tissues are highest in the kidneys (Table 1.3). Therefore kidney is considered as a target organ for any adverse effects of fluoride (Kennedy, 1990). Moreover, in conditions like renal insufficiency the risk of fluorosis is increased as a result of increased plasma fluoride level (Turner et al, 1996).

In experimental animals, transitory renal dysfunction has been observed such as polyuria, and proximal tubular necrosis following acute single dose exposure to fluoride in rats which has been suggested due its rapid elimination (Daston *et al.,* 1985; Usuda *et al.,* 1998; Dote *et al.,* 2000).

Histological studies have shown extensive necrosis and vacuolisation of the renal proximal tubules following acute single dose of fluoride (Takagi and Shiraki, 1982; Shashi *et al.,* 2002) in rats and rabbits. This tubular necrosis has been followed by complete regeneration almost at 7 days (Takagi and Shiraki, 1982) after fluoride exposure. It has been also observed that isolated perfused rat kidney exposed to high fluoride concentrations shows functional (in ability to concentrate urine with cessation of glomerular filtration) and morphological changes (widened interstitum and occurrence of vesicular material of podocytic origin in urinary space) (Rush and Willis, 1982; Willinger *et al.,* 1995). However, no signs of nephrotoxicity at caries prophylactic concentrations have been observed. Chronic fluoridation (NaF at 380 mg/L for 6 weeks) has also been demonstrated to produce necrosis of the proximal and distal tubules in rats (Lim *et al.,* 1978). In addition, Greenber (1986) has revealed

several changes in the renal system after chronic high fluoride ingestion in the mouse. These included edamatous swelling and degeneration of glomerular tufts and nephron.

It has also been reported that ingestion of excess fluoride facilitates calcium oxalate crystalluria and promotes the formation of bladder stones in rats (Anasuya, 1982). This inturn is believed to be due to increased parathyroid hormone secretion (Suketa et al, 1983). Changes in kidneys phospholipid content and the proportion of unsaturated fatty acids have been detected in rats fed high fluoride in drinking water (Guan *et al.,* 2000; Shao *et al.,* 2001).

There is also clinical evidence suggesting that exposure to high fluoride provokes nephrotoxic changes in human cells (Cittanova *et al.,* 1996) and may result in renal failure in patient with chronic fluorosis (Lantz *et al.,* 1987). The incidence of urolithiasis has been found to be 4.6 times higher in fluoride endemic area (3.5 to 4.9 ppm) than the non-endemic area (Singh *et al.,* 2001), in which they have suggested that fluoride may behave as mild promoter of urinary stone formation by excretion of insoluble calcium fluoride, increasing oxalate formation and increasing the oxidative burden.

To date numerous studies have examined the fluoride-related nephrotoxicity induced in patients under halogenated anaesthesia. After their inhalation, fluorinated anaesthetics are metabolised by the cytochrome P-450 system, producing inorganic fluoride (Kharasch and Thummel, 1993; Garton *et al.,* 1995; Kharasch *et al.,* 1999). Serum inorganic fluoride levels were elevated after the exposure to these anaesthetics (Wiesner *et al.,* 1996), and it was 5 times higher in renal cortex than serum (Kusume *et al.,* 1999). Urine fluoride concentrations can exceed those of plasma by ~50-fold (Mazze *et al.,* 1973). This is undoubtedly due to intraluminal fluoride concentration

and because these anaesthetics can be defluorinated directly within tubular cells by the cytochrome P-450 systems (Kharasch *et al.,* 1995). Thus both the filtered load and the intracellular fluoride generation may contribute to the tubular fluoride burden. Once a critical, but unknown, fluoride threshold occurs, proximal tubular necrosis and acute renal failure can develop which is manifested by lysosomal enzymes secretion (Matsumura *et al.,* 1994) and inability to concentrate urine (Jaramillo and Cummings, 1979; Higuchi *et al.,* 1995). However, the mechanism by which this injury occurs has not been clearly defined.

1.28. Effect of fluoride on NAG:

Urinary lysosomal enzymes are reliable biomarkers of nephrotoxicity and they are useful for early diagnosis of tubule damage induced by drugs and chemicals. From over 60 urinary enzymes, the lysosomal enzyme NAG is the best known and investigated (Khalil-Manesh *et al.,* 1992; Holdt-Lehmann *et al.,* 2000; Tassi *et al.,* 2000; Ida *et al.,* 2001).

Fluoride has been observed to target kidney lysosomes and to increase the release of NAG in urine in human following fluorinated anaesthetics (Hara *et al.,* 1998; Laisalmi *et al.,* 2001) and in experimental animals exposed either to acute dose of fluoride (Usuda *et al.,* 1998; Usuda *et al.,* 1999), or chronic high fluoride concentration in drinking water (Bai *et al.,* 1999). The increase in urinary NAG isozyme was of significant correlation with the dose of fluoride administered (Usuda *et al.,* 1999). The early changes of urinary NAG isozyme activity in the absence of other biochemical changes like blood urea nitrogen (BUN), creatinine (CR) and *CR* clearance (biomarkers of glomeruli function) (Higuchi *et al.,* 1995) or less

pronounced than changes in NAG activity (Usuda *et al.,* 1998) may reflect that the effect of fluoride on glomerular function is less severe than proximal tubules. The subcellular organelles, lysosomes, have been incriminated in many xenobiotic induced cytotoxicity because of the hydrolytic enzymes hosted by them.

1.29. Fluoride and oxidative stress:

Excessive fluoride ingestion over a prolonged period can adversely influence many tissue and organs characterized by a vast array of symptoms and pathological changes in addition to its well-known effects on the skeleton and teeth.

Increased generation of ROS, enhanced lipid peroxidation and disturbed antioxidant defence systems have been proposed to be an important mediating factor in the pathogenesis of several xenobiotics (table 1.4). However, the mechanism by which fluorosis produces its toxic effects on the whole body is still unclear.

Recently, more and more scientists have paid greater attention to the relationship between fluoride and free radicals (Rzeuski *et al.,* 1998). Fluoride is known to stimulate the so-called respiratory burst and the production of superoxide radicals of human and animals neutrophils (Toper, 1987; Della Bianca, 1988). This processes is associated with the activation of NADPH-dependent membrane oxidase appearing in these cells. Similar effects are exerted by fluoride stimulation in human polymorphonuclear leukocytes, whereas hydroxyl radical is also produced along with superoxide radical. Moreover, high fluoride concentrations are likely to inhibit SOD. Production of hydroxyl radicals and superoxide radicals are dependent on fluoride concentration; superoxide radicals prevail at higher fluoride concentration, whereas low ones there is dominance of hydroxyl radicals generated in the Haber-weiss

reaction (Zhao *et al.,* 1989). In rat liver macrophages fluoride ions elicit the release of arachidonic acid and prostaglandins (Schulze-Specking *et al.,* 1991; Shao *et al.,* 2001). Similar results have been observed with respect to leukotrienes in human neutrophils (Brom *et al.,* 1989). The cascade of arachidonic acid begins by lipoxygenation, while when the resulting endoperoxides of fatty acids in the reaction with H_2O_2 produce hydroxyl radicals (Nave *et al.,* 1991).

It has been also observed that fluoride enhanced the XOD activity in brain and gastromucnemius muscle (Lakshmi and Pratap, 2000). Serum NO level has been detected to be elevated during chronic high fluoridation in chicks which is suggested to be due to excessive expression of inducible NO synthase in the target tissue (Xu *et al.,* 2001; Liu *et al.,* 2003). Drinking highly fluorinated water (above the permissible limit) has been shown to induce oxidative stress manifested by high lipid peroxides and alters in various antioxidant systems in plasma and tissues in animals (Shivarajashankara *et al.,* 2001; Liu *et al.,* 2003; Shivarajashankara *et al.,* 2004). Acute dosing of fluoride has also shown to increase the level of lipid peroxides in liver, lung, testis, and kidney of the rat (Soni *et al.,* 1984).

Children and young people drinking water contaminated with fluoride in endemic areas were shown to have oxidative stress as evidenced by elevated levels of MDA in their blood, indicating increased lipid peroxidation. Also they have shown significant alterations in the antioxidant systems in the blood (SOD, GSHPx, GSH, ascorbic acid and uric acid) (Saralakumari and Rao, 1991; Li and Cao, 1994; Shivarajaashankara *et al.,* 2001; Singh *et al.,* 2001).

Table 1.4.

Some selected examples of xenobiotics and correlated oxidative stress induced damage (adapted from Wallace, 1997).

Tissue damage	**Xenobiotic**
Liver damage	-Carbon tetrachloride -Ethanol -Acetominophen
Kidney damage	-Haloakenyl cysteine -Acetominophen -Cisplatin -Aminoglycosides
Cardiomyopathies	-Catechilamines -Doxorubucin -Phenylenediamines
Nervous system injury	-Metals: Fe, Cu, Hg and Cd -Aluminum.
Haematopoiesis toxicity	-Gold salts -Chloramphenicol -Benzene
Lung injury	-Cigarate smoking -Bleomycin -Paraquat

1.30. Antioxidants in fluorosis:

Recently, some experimental and clinical studies have turned toward the use of small molecular weight antioxidants to ameliorate fluorosis toxic effects as well as the treatment of acute fluoride poisoning. Vitamin C and E are potent antidotes and function as therapeutic agents in several disease states (Rodriguez-Porcel *et al.,* 2004) and xenobiotics induced toxicity (Sen Gupta *et al.,* 2004) especially those involving in oxidative related events. These two antioxidants were described as potent antidotes either separately or combined with calcium phosphate in treatment of acute fluoride and arsenic kidney toxicity. Calcium phosphate reduces the risk of hypocalcaemia as well as precipitates fluoride and thereby decreases its absorption in stomach (Chinoy and Shah, 2001). Vitamin C and E have also to protect against testicular and ovarian toxicity (Chinoy *et al.,* 2001), gastrocnemius muscle (Chinoy and Memon 2001) and other pathological disorders induced by chronic fluorosis (Chinoy *et al.,* 1993) Induced by fluoride. β-carotene and SOD have been also shown to improve the weakened antioxidant systems induced by chronic fluorosis and efficiently counteracted the decreased growth rate in the rat (Sun *et al.,* 1998). A recent report by Susheela and Bhatnagar (2002) has shown that fluorosis in patients can be reversed after a year by supplementation of a diet rich in essential nutrients and antioxidants.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Collection of blood and urine samples and separation of kidney lysosomal rich fraction (LRF):

All the experiments undertaken in this study were performed in accordance with current legislation on animal experimentation in Ireland and approved by Bioresources Unit, Trinity College.

2.1.1. Blood collection:

One and half ml and 5 ml blood samples were collected from animals under halothane anaesthesia for recovered and unrecovered animals respectively by cardiac puncture in vacutainer containing ethylendiaminetetraacetic acid (EDTA). EDTA was selected as anticoagulant because it has the advantage of chelating heavy metals that catalyse the auto-oxidation of plasma and tissue homogenate.

Plasma was separated immediately after blood collection by centrifugation at 2500 rpm (690 g) for 10 minutes at 4 °C using Mikro 22 R centrifuge.

A 0.5 ml (in duplicate) of plasma sample was used for ascorbic acid determination immediately after the separation processes. The rest of plasma was divided into 0.3 ml fractions and stored at – 70 °C for determination of plasma MDA, glutathione, and CR.

2.1.2. Animals housing and urine samples collection:

Male Wistar rats were supplied by the Bioresourses unit, Trinity College. Rat was housed in a single stainless steel metabolic cage, which allowed the collection of urine free of faeces in a disposable plastic beaker. The animals were kept in a temperature-controlled room with illumination cycles of 12 hr/day and they had a free access to water and food. Twenty-four hourly urine samples were collected, starting on the day before drug treatment (for baseline determination) and continued along the study. The last urine sample was collected 24 hr after last dose of tested drug had been given. After urine volume determination the samples were centrifuged at 1000g at 4 °C for 10 min and the fresh supernatants were used for NAG, MDA and CR levels determination.

2.1.3. Isolation of kidney lysosomal rich fraction (LRF):

Based on the methods described by (Win-Aung, 1998), renal LRFs were isolated by homogenisation and differential centrifugation (fig. 2.1).

2.1.3.1. Preparation of homogenate:

Adult male Wistar rats (300-325 g) were killed by cervical dislocation. The kidneys were excised and the renal cortex, distinguished by its light brown colour, was cut with surgical blade from the outer zone of renal medulla, which has a yellow-brown colour. The cortical tissue was washed twice with ice cold sucrose (0.3 M), minced

with scissors and diluted 1:8 (w/v) with 0.3 M sucrose. Then homogenized by Silverson homogenizer for one minute at maximum speed.

2.1.3.2. Isolation of LRF:

The renal homogenate was first centrifuged at 140 g for 10 minutes to sediment nuclei and unbroken cell debris. The supernatant was decanted and centrifuged once again in the same way. The supernatant was pooled in previously weighed microtubes and centrifuged at 9000 g for 3 min excluding time for acceleration and deceleration. The supernatant formed during this centrifugation is the cytosol fraction. The pellet formed in this centrifugation is reconstituted with the same volume (1:8 w/v) of 0.3 M sucrose and is referred to as 'lysosome-enriched fraction' (LRF). This pellet consists of three differently colored layers. The bottom layer is dark brown and is clearly distinguished from the middle layer, which is yellow brown. The bottom layer is the semipurified lysosomal fraction and the middle layer is the mitochondrial fraction. The top layer, which is almost white, contains brush border fragments. A freshly prepared lysosome-enriched fraction was used through out the study.

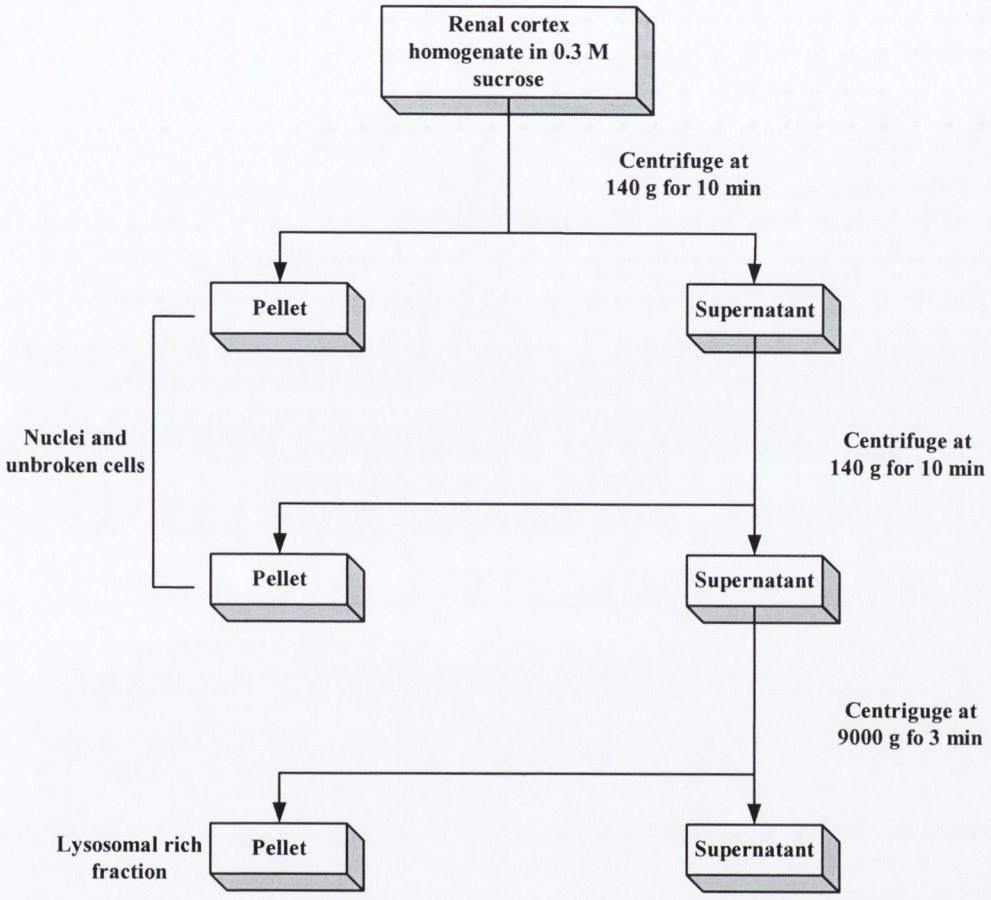


Fig. 2.1. Schematic representation of lysosomal rich fraction separation by differential centrifugation.

2.2. Assay of N-acetyl- β-D- glucosamindase isozymes activity:

2.2.1. Principle of assay:

The enzyme activity was estimated by simple and rapid colorimetric method (Xu *et al.,* 1999) based on the hydrolysis of substrate, p-nitrophenyl N-Acetyl β-D glucosaminide (Sigma), by NAG (Sigma) in an acidic pH (5.0). The amount liberated of p-nitrophenyl was estimated photometrically at 405 nm from the absorbance difference between test samples and reagent blank after termination of the reaction by alkalinization of the reaction mixtures.

2.2.2. Procedure:

A 100µl (in duplicate) of samples was mixed with 1.0ml buffer-substrate (3.0 mM substrate in acetate buffer (0.1 N sodium acetate and 0.1 N acetic acid, pH 5.0) for test and with 1.0 ml buffer only for reagent blank in ordinary test tubes and incubated for 15 min at 37°C. Then 2.0 ml of 1.0 M sodium carbonate pH (11.0) was added to all tubes to terminate the reaction and develop the yellow colour. The colour absorbance was measured at 405 nm wavelength in Shimadzu UV-spectrophotometer against reagent blank.

2.2.3. Calculation of NAG isozymes activity:

The above measures were converted to NAG isozymes units by direct calculation from standard curve prepared in the same way by using (0.00078- 0.0125 units) N-acetyl- β-D- glucosamindase (Sigma). (r = 0.999, fig. 2.2). NAG isozymes activity was expressed as enzyme units. One enzyme unit had the ability to hydrolyse 1 nmol of substrate/min/mg protein. The intra-assay variation of NAG isozymes activity was 3.8% and the inter-assay variation was 6.4%.

2.3. Protein assay:

2.3.1. Principle of assay:

Total protein content was measured by Hatree version of the Lorry assay (Hatrre, 1972). In this assay the divalent copper ion forms a complex with peptide bonds in which it is reduced to monovalent ion under alkaline condition. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue.

2.3.2.Reagents:

Three reagents as described below were prepared.

Reagent A: Consisted of 2 g sodium potassium tartrate. 4 H_2O, 100 g sodium carbonate (Sigma), 500 ml 1 N NaOH (Sigma) and H_2O to one litre.

Reagent B: 2 g sodium potassium tartrate. 4 H_2O (BDH laboratory), 1 g copper sulphate 5 H_2O (Analar), 90 ml H_2O and 10 ml 1 N NaOH. Reagent A & B stable for 2-3 months.

Reagent C: Consisted of 1 volume of Folin-Ciocalteau reagent (Sigma) diluted with 15 volumes H_2O.

2.3.3.Procedure:

Serial dilutions of bovine serum albumin (Sigma) to give a concentration range of 0.03 to 0.3 mg/ml for the standard curve were prepared in 1 ml acetate buffer (pH 5.0) (fig. 2.3). Unknown samples were also prepared in 1 ml volume of this buffer. A buffer blank of 1 ml volume in duplicate was also simultaneously prepared. The kidney tissue homogenate samples were diluted in acetate buffer (1:60 v/v). A 0.9 ml of the reagent A was added to each tube and after brief mixing, they were incubated at 50 °C for 10 minutes in water bath. At the end of this period the tubes were taken out of the water bath and left on the bench for further 10 minutes. Then 0.1 ml of reagent B was added to each tube and following a brief mixing left on the bench for another 10 minutes. Then 3.0 ml of reagent C was added rapidly to each tube mixed and incubated at 50°C for 10 minutes in water bath. Cool to room temperature and

chromatogens. The MDA-TBA adduct was eluted from the column with methanol/phosphate buffer 40:60 (v/v) with a flow rat of 1 ml/min and quantified spectrophotometrically at 532 nm with an average retention time of 5 min and recorder chart-speed of 3 cm/min and attenuation of 1. MDA concentrations were calculated by reference to calibration curve prepared by assays of tetraethoxypropane (TEP) (Sigma), which undergo hydrolysis to liberate stoichiometric amounts of MDA ($r = 0.998$, fig. 2.4).

2.4.1.2. Preparation of solutions:

TBA solution, 42 m mol/l: A 0.6 g of 2-thiobarbituric acid was dissolved in approximately 80 ml of water, with stirring on hot-plate (50-55 °C). Then cooled to room temperature and diluted to 100 ml with water. Stored at room temperature, this reagent stable for two weeks.

TEP standard solutions: In 25-ml volumetric flask 50 µl of 1,1,3,3-tetraethoxypropane reagents diluted to the mark with 40% ethanol solution and stored at 4 °C prepared freshly each month and. For an intermediate standard 0.5 ml was pipetted of this TEP stock standard solution into a 100-ml volumetric flask and diluted to the mark with 40% ethanol solution. This intermediate standard is prepared freshly each fortnight and stored at 4 °C. To prepare TEP working standards solutions (0.61,1.22,2.43,4.86, 9.72 and 19.44 µmol/L), 0.375, 0.75,1.5, 3.0, 6.0 and 12 ml were pipetted into six 25-ml volumetric flasks, respectively, and diluted the contents to the mark with water. These TEP working standard solutions were prepared weekly and stored at 4 °C.

Mobile phase: The HPLC mobile phase was prepared just before use by mixing 400 ml of HPLC-grade methanol (Sigma) and 600 ml of potassium phosphate buffer solution (50 mmol/l, pH 6.8, Merck) in a side-arm suction flask, and then de-gased by reducing the pressure and using Millipore membrane filters (0.45 μm) contains nitrocellulose.

2.4.1.3. Method:

In each analytical run, an assay reagent blank, TEP working standards solutions (0.63, 1.22, 2.43, 4.86, 9.72 and 19.44 μmol/l) and plasma or supernatant of kidney homogenate specimens in duplicate were measured. Polypropylene caped tubes of 15 ml capacity marked and 0.75 ml of 0.44-mol/l phosphoric acid solution, 50 μl of TEP standard and plasma or kidney homogenate specimens were pipetted into respective tubes and vortex mixed. Then 0.25 ml of 0.6% TBA solution, and distilled water (0.5 ml for reagent blank, 0.45 ml for TEP standards and test samples) were added to adjust the final volume to 1.5 ml. The tubes were capped and placed in a boiling water bath for 60 min, then transferred into an ice-water bath until the HPLC analysis are performed.

A 0.5ml of each boiled sample was transferred into a polypropylene microtube containing 0.5ml of methanol-NaOH solution (a mixture of 4.5ml of 1 mol/l NaOH solution and 45.5 ml of HPLC grade methanol, Sigma) and vortex mixed. Each tube was centrifuged for 5 minutes at 9500 g to sediment the precipitated plasma or tissue proteins.

Equilibrate the HPLC apparatus by pumping mobile phase at 1ml/min for at least 2 hr, until the recorder baseline is stable then sequentially 50 μl of each blank, TEP

standard, and protein-free samples was injected into the HPLC system and the absorbance recorded at 532 nm. The average retention time is about 5.0 minutes. A calibration curve was prepared by plotting the peak area of the blank and TEP standards sample. The concentrations of the lipid peroxide in the plasma and kidney was determined from the calibration curve and expressed as MDA equivalent. Plate 1- shows the representative chromatograms of TEP standards and test samples MDA measurement. The percentage recovery of internal standard (TEP) was 98%. The intra-assay variation of MDA estimation was 4.9% and the inter-assay variation was 6.2%.

2.5. Urinary Thiobarbituric Acid Reactive Substances (TBARS) Assay:

2.5.1.Principle:

The urinary response to *in vivo* lipid peroxidation was measured by TBARS (Lee *et al.,* 1992) which is still the most widely employed assay used to determine lipid peroxidation (Armstrong and Browne, 1994). In TBARS assay after lipoproteins acid precipitation, one molecule of MDA is reacted with two molecules of thiobarbituric acid with the production of a pink pigment adduct MDA $(TBA)_{2,}$ which having absorption maximum at 532 nm.

2.5.2.Method:

Samples of 0.5 ml of urine were mixed thoroughly with 3.0 ml of 5% Trichloroacetic acid TCA (Sigma) mixed and centrifuged at 1360 g for 15 min at 4 °C to remove a

fine precipitate. One ml of the supernatant was pipetted into screw capped polypropylene tubes and mixed with 1 ml of saturated thiobarbituric acid (0.6%). The mixture was heated in 80 ºC water bath for 90 minutes and the cooled in an ice-water bath for at least 20 minutes. The absorbance of the tested sample was read at 532 nm in Shimadzu UV-spectrophotometer against blank taken from the supernatant and mixed with water instead of TBA and treated in the same way. TBRAS are expressed in terms of malondialdehyde (MDA) equivalents. In this assay TEP (2.65- 85 nmol/L) is used to construct MDA standard curve against which unknown samples can be plotted (r = 0.99, fig. 2.5). The intra-assay of TBARS assay was 5.8% and the inter-assay was 9.2%

2.6. Ascorbic acid analysis:

2.6.1. Principle:

Total ascorbic acid measured in plasma and tissue homogenate colorimetrically by derivatization with 2,4-dinitrophenylhydrazine after oxidation (Omaye *et al.,* 1979). In which ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketogulonic acid. These products in the presence of 2,4-dinitrophenylhydrazine (DNPH) (Merck) form the derivative bis-2, 4-dinitrophenylhydrazone. This compound, in concentrated sulphuric acid, undergoes a rearrangement to form a product with an absorption band at 520 nm (orange red colour). The oxidation and derivatization reactions are run in the presence of thiourea (Sigma) to provide a mildly reducing medium.

2.6.2. Reagents:

2,4-dinitrophenylhydrazine (Merck)/thiourea (Sigma)/copper (DTC) solution consists of 0.0125 % thiourea, 0.03% $CuSO_4$. $5H_2O$, 2.2% of 2,4-dinitrophenylhydrazine and bring to a total volume of 100 ml with 10 N H_2SO_4.

2.6.3. Procedure:

A 0.5 ml of fresh plasma or supernatant of kidney homogenate was added to 0.8 ml of cold 10% trichloroacetic acid (TCA), mixed and centrifuged at 9500 g for 5 min to precipitate proteins. Then 0.5 ml of the supernatant was transferred to a polypropylene tube with screw cap contains 0.2 ml of DTC, vortex mixed and incubated at 37 °C for 4 hours and then cooled in ice for 10 minutes. 0.5 ml of TCA (reagent blank) and a serial dilution from stock L-ascorbic acid (Sigma) to form (0.7-22.7 m mol/L) (Standard) were also treated in the same way. A 0.8 ml of 65% sulphuric acid was added to each ice-cold tube, well mixed and read at 520 nm against reagent blank. The ascorbic acid content was calculated directly from standard curve prepared from L-ascorbic acid (r = 0.991, fig. 2.6).

2.7. Determination of glutathione (GSH):

The non-protein free glutathione concentration was measured using spectrophotometric assay (Wan *et al.,* 2001) by 5,5-Dithio-bis (2-nitrobenzoic acid)

(DTNB), Elman's reagent (Sigma). The absorbance of the yellow colour developed was measured calorimetrically at 412nm wavelength.

2.7.1. Method:

To a 0.1 ml of plasma or supernatant of kidney homogenate 0.1 ml of 5% TCA was added, mixed and allowed to stand at room temperature for 5 min. Then 0.1ml of phosphate buffer (pH 7.0) was added, vortex mixed and centrifuge at 9500 g for 10 min. A 0.1 ml of the supernatant was taken, mixed with 1.0 ml of 1.0 mM DTNB and Incubated for 15 min at 37 °C and read against reagent blank containing 1.0 ml DTNB and 0.1 ml phosphate buffer. The concentration of GSH was obtained directly from reference curve prepared from GSH (0.05-0.2 m mol/L, Sigma) (r = 0.998, fig.2.7). The within assay variation was 4.1% and the inter-assay variation was 5.8%.

2.8. Determination of plasma and urine creatinine (CR):

2.8.1. Principle:

The methods for CR determination most widely used today are based on Jaffe reaction (Varley *et al.,* 1980), the reaction occurs between CR and picrate ion formed in alkaline medium; an orange-red adduct develops which is followed photometrically at 500 nm. Plasma CR was determined by the same principle after protein precipitation.

2.8.2. Methods:

(A)- Urinary CR:

Thirty micro litre of urine was diluted to 3.0 ml of water (1:100 v/v), vortex mixed with 1.0 ml picric acid of 40 mmol/l (Sigma) and followed by 1.0 ml of 750 mM NaOH. A 3.0 ml of standard solution (1.13 mg/100ml creatinine sulphate, Sigma) and 3.0 ml H_2O (reagent blank) were treated in the same way, then allowed to stand for 15 minutes and read at 500 nm. The intra-assay variation of CR assay was 3.1% and the intra-assay variation was 7.3%.

$$\text{Urinary CR (g/l)} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 1.13$$

(B)-Plasma CR:

A 0.2 ml of plasma was mixed with 0.3 ml of H_2O and then protein was precipitated by adding 0.1 ml of 10% sodium tungestate and 0.2 ml of 0.33 mol/l sulphuric acid, vortex mixed and allowed to stand for 10 minutes. The mixture was centrifuged for 5 min at 9500 g and 0.6 ml of the supernatant was taken and 0.2ml of 40 mmol/l picric acid and 0.2 ml of 750 mmol/l of NaOH were added, mixed and allow to stand at room temperature for 15 min. A 0.2ml of working standard CR solution (2.26 mg/

100 ml) and 0.2 ml H_2O as reagent blank were treated in the same way. Then read at 500nm.

$$\text{Plasma CR, (mg/100 ml)} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 2.26$$

2.9. Histological study:

Small pieces of kidney tissue were fixed in 10% formalin for 48 hrs, embedded in paraffin wax and cut in fine sections of 5µm thickness using microtome. These sections were then fixed on poly-L-lysine coated slides and left overnight at 37 °C. The slides were then deparaffinized in xylene and rehydrated in graded series of absolute alcohol. The slides were counterstained with hematoxylin for 3 minutes and rinsed once again with lukewarm running tap water for blue colour development. Each slide was immersed in Eosin for 10 seconds and rinsed with tap water. The slides were finally dehydrated through alcohol and xylene and mounted in DPX (containing xylene mixture of isomers, dibutyl phothalate, Sigma). A quantitative cellular histological damage was determined with Olympus CH2 light microscope X 20. One section from each slide was selected randomly before microscopic examination and histologically examined by a histologist unaware of the treatments. Six fields were used from each section and the damaged and undamaged renal tubular epithelial cells (indicated by cell membrane damage and cytoplasm shrinkage) were counted. The percent of the damaged cells was determined and compared with control group.

2.10. Statistical analysis:

Results throughout were expressed as mean ± SD (standard deviation). All statistical analyses of the results achieved in this thesis were carried out on a PC computer using Prism 4.0 software. Linear regression analysis was used to examine the correlation coefficients, and Analysis of Variance (ANOVA) was employed to determine the effect of variables on the dependent factor studied.

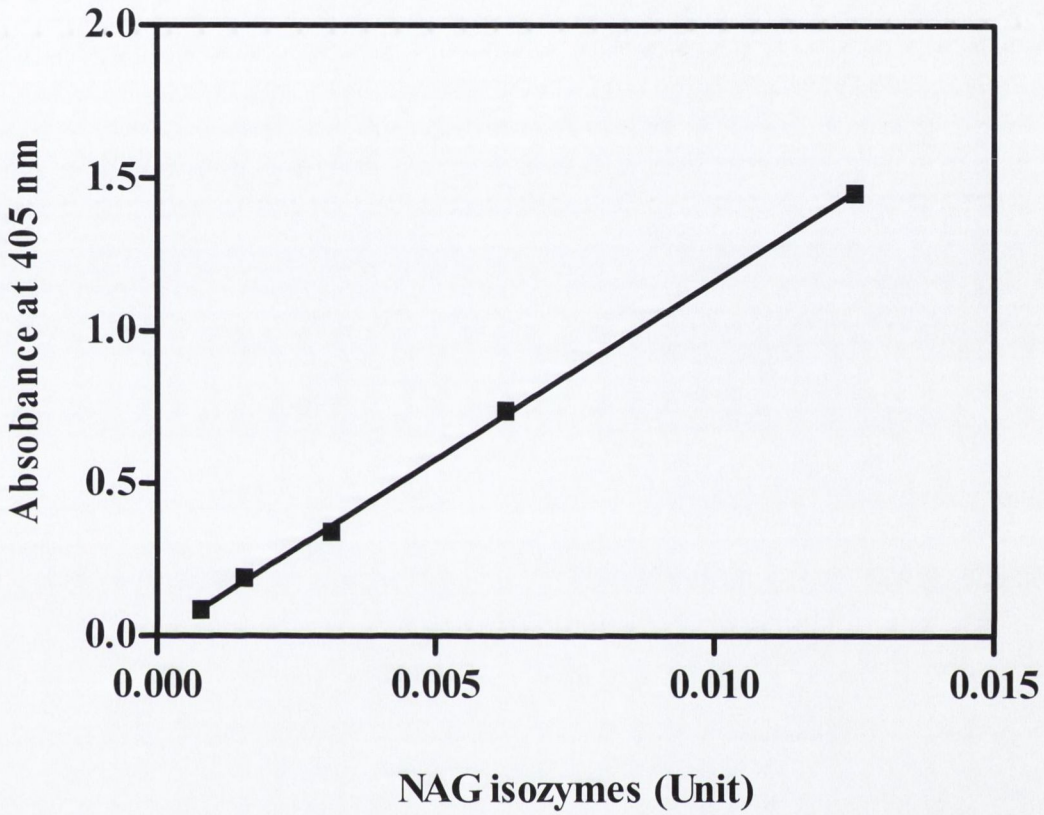


Fig. 2.2. Standard curve for NAG isozymes activity (r = 0.999). One enzyme unit had the ability to hydrolysel nmol of substrate/min/mg protein

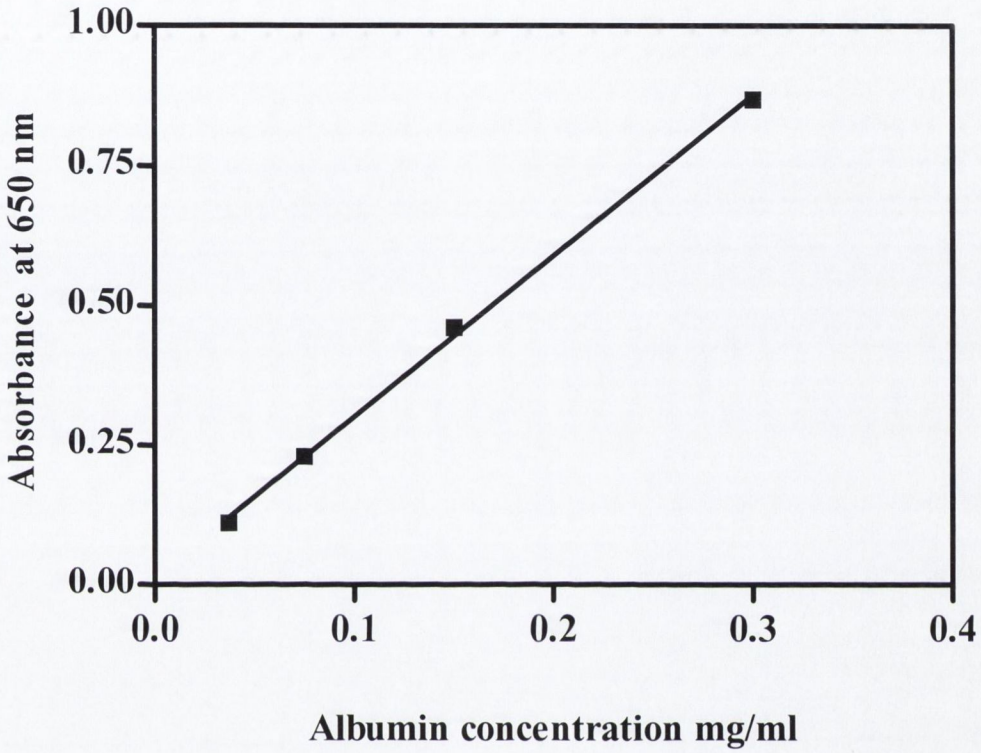


Fig. 2.3. Standard curve generated from albumin for measuring tissue protein ($r = 0.998$).

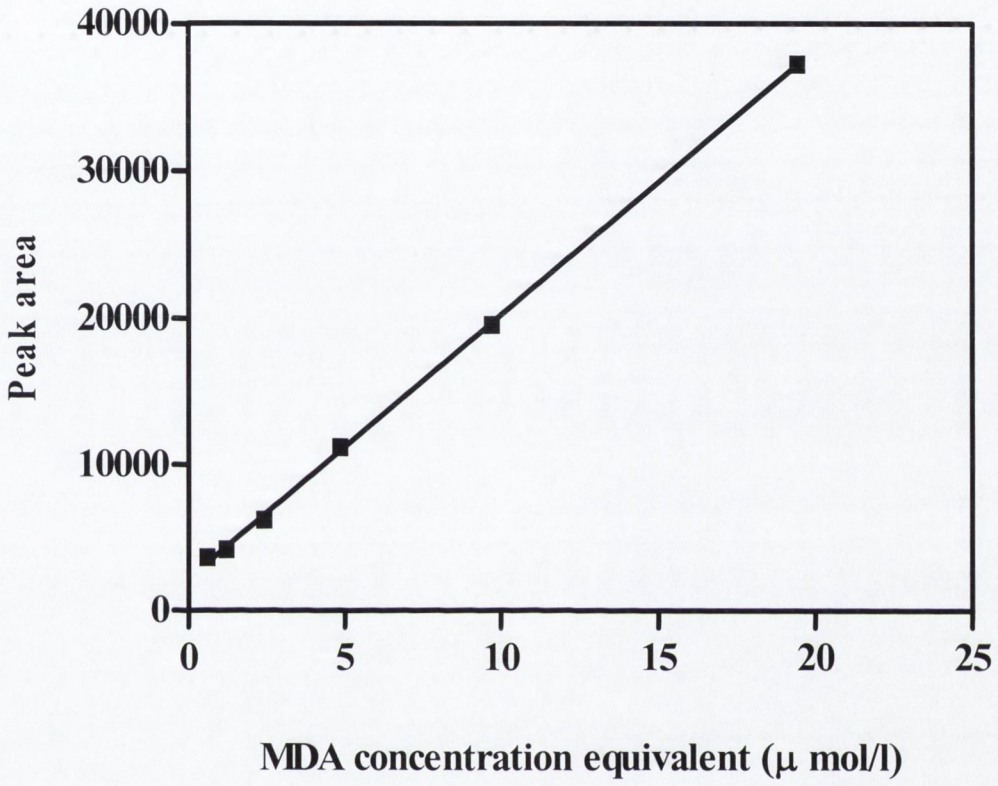


Fig. 2.4. Standard curve obtained from TEP for measuring plasma and tissue lipid peroxides as MDA equivalent by HPLC technique (r = 0.998).

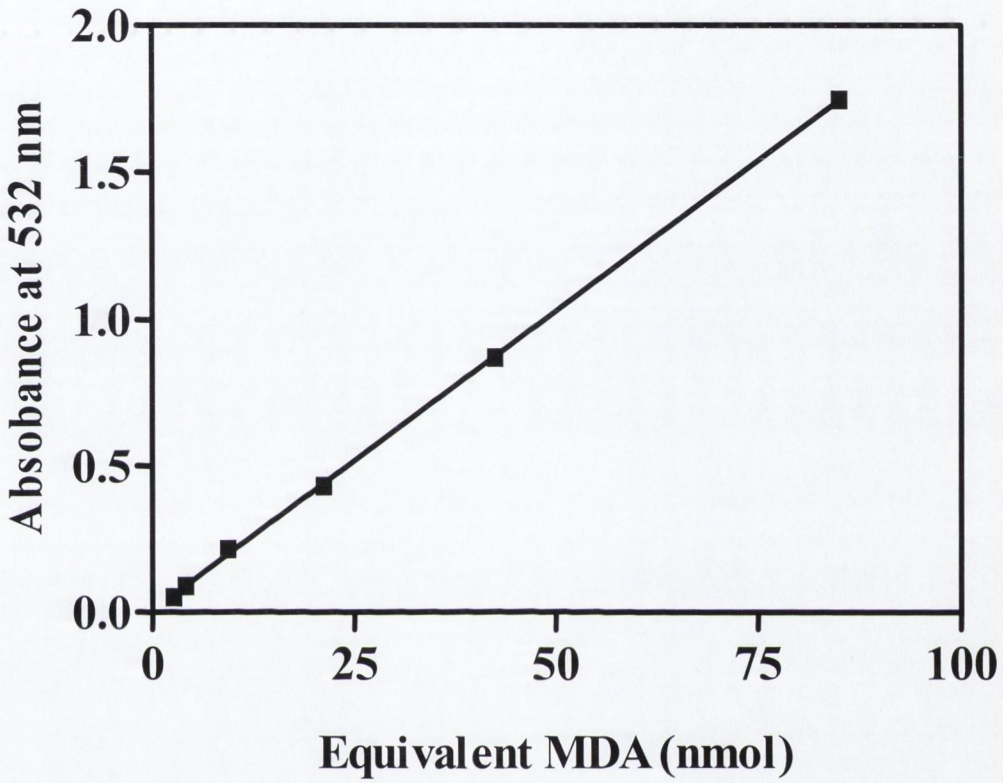


Fig. 2.5. Standard curve obtained from TEP for measuring urinary lipid peroxides as MDA by TBRS technique (r =0.99).

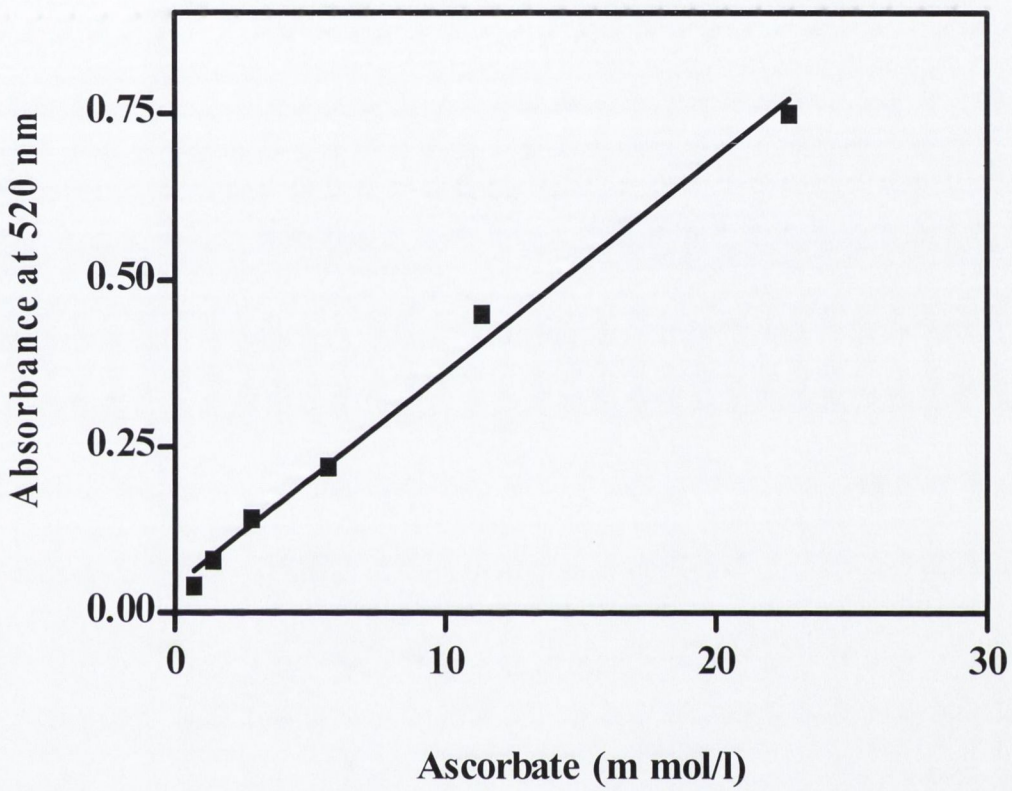


Fig. 2.6. A standard curve for measuring plasma and tissue ascorbic acid concentrations (r = 0.991).

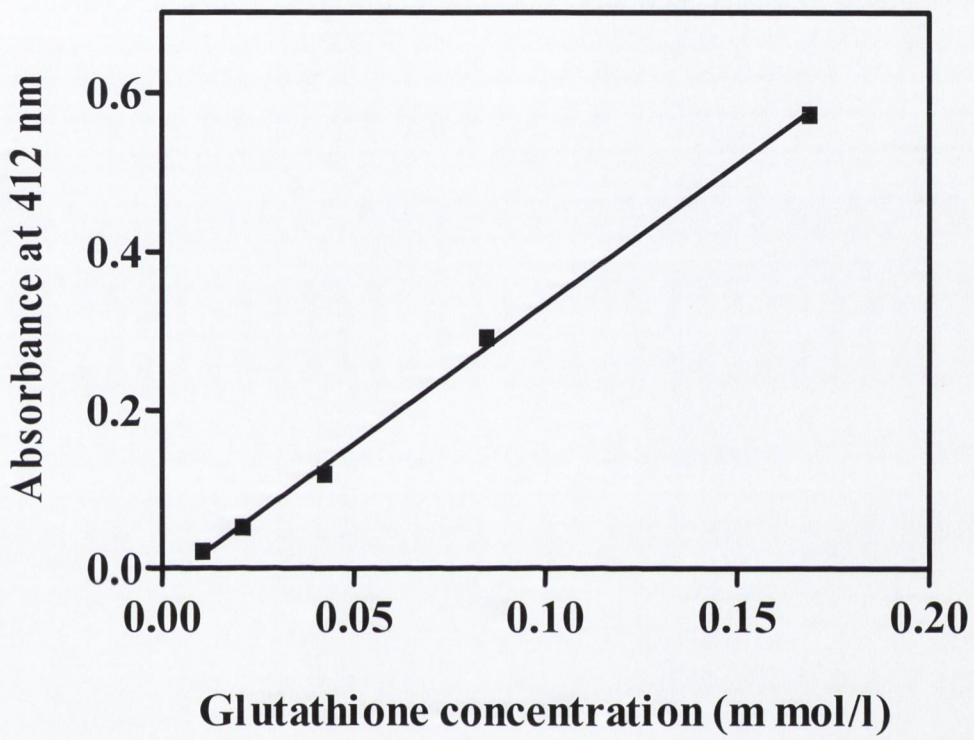
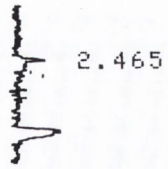


Fig. 2.7. Standard curve for measurement of plasma and tissue GSH (r=0.998).



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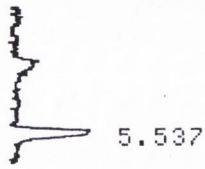

0.61 µ mol/l TEP

CHROMATOPAC C-R3A FILE 3
SAMPLE NO 0 METHOD 41
REPORT NO 7146

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.537	3654			100	
	TOTAL	3654			100	

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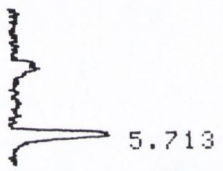

1.22 µ mol/l TEP

CHROMATOPAC C-R3A FILE 3
SAMPLE NO 0 METHOD 41
REPORT NO 7147

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.713	4177			100	
	TOTAL	4177			100	

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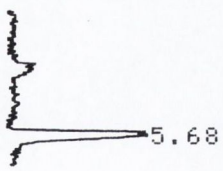

2.43 µmol/l TEP

CHROMATOPAC C-R3A FILE 3
SAMPLE NO 0 METHOD 41
REPORT NO 7148

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.68	6202			100	
		6202			100	

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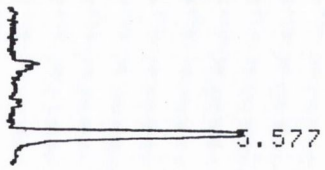

4.86 µ mol/l TEP

CHROMATOPAC C-R3A FILE 3
SAMPLE NO 0 METHOD 41
REPORT NO 7149

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.577	11185			100	
	TOTAL	11185			100	

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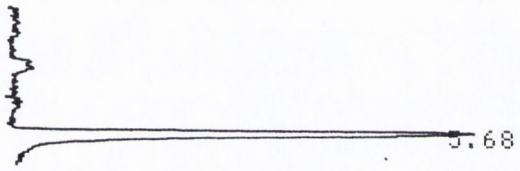
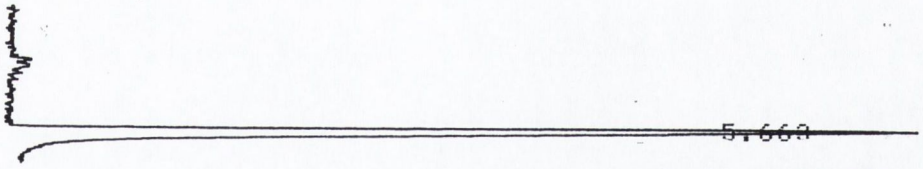


9.72 µ mol/l TEP

CHROMATOPAC C-R3A FILE 3
SAMPLE NO 0 METHOD 41
REPORT NO 7150

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.68	19534			100	
	TOTAL	19534			100	

START



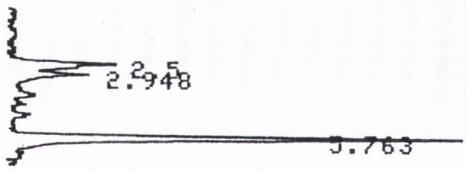
19.44 µmol/l TEP

CHROMATOPAC C-R3A FILE
SAMPLE NO 0 METHOD 41
REPORT NO 7151

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.663	37262			100	
	TOTAL	37262			100	

START

START

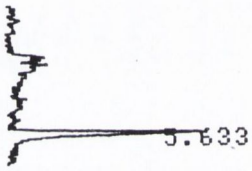


Kidney homogenate MDA

CHROMATOPAC C-R3A FILE 3
SAMPLE NO 0 METHOD 41
REPORT NO 6874

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.5	3119			18.1017	
2	2.948	1533	V		8.8957	
3	5.763	12580			73.0026	
	TOTAL	17233			100	

START



Plasma MDA

CHROMATOPAC C-R3A FILE 3
SAMPLE NO 0 METHOD 41
REPORT NO 6753

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.633	4911			100	
	TOTAL	4911			100	

START

Plate 2.1. Representative chromatogram of TEP standard, kidney homogenate, and plasma MDA.

CHAPTER THREE

THE EFFFECT OF FLUORIDE ON RENAL LYSOSOMAL INTEGRITY

3.1. Introduction:

High concentration of fluoride is noxious to the health of humans and animals. There are many reported patterns of fluoride toxicity in the world. These include endemic fluorosis that is related to high concentration of fluoride in drinking water (Li and Cao, 1994) reaching as high as 100 ppm in some countries, industry related air pollution (Kono *et al.,* 1987), clinically used fluorinated anaesthetics (Kusume, 1999), and the misuse of fluoride containing consumer items, particularly the oral hygiene products (Bottenberg *et al.,* 2001). Death has been also recorded due to excessive fluoride intake either from drinking highly fluorinated water (Gessner *et al.,* 1994) or in the industrial workplace (Takase *et al.,* 2004).

The kidneys are primary organs concerned with excretion and retention of fluoride (Kono *et al.,* 1986; Whitford, 1996) and thus are generally involved in fluoride intoxication. This toxicity can vary from sub-clinical to overt clinical impairment (Partanen, 2002) and there is now growing evidence to suggest that fluoride intake provokes nephrotoxic changes in the human (Kennedy, 1990; Singh *et al.,* 2001) and animals (Dote *et al.,* 2001; Cittanova *et al.,* 2002; Shashi *et al.,* 2002). Histopathological studies have revealed that acute and chronic fluorosis does lead to subtle renal damage, which is manifested by degeneration of tubular epithelia, extensive vacuolisation and necrosis in renal tubules, hypertrophy of glumeruli and interstitial nephritis (Takagi and Shiraki, 1982; Shashi *et al.,* 2002).

Free radicals contribute to cell inflammatory changes (Virgili *et al.,* 1998) and also in xenobiotics induced nephrotoxicity (Cuzzocrea *et al.,* 2002). Fluoride has the ability to generate ROS (Elferink, 1981; Zhao *et al.,* 1989), which has been thought mediated through its known activation of NADPH-oxidase (Toper *et al.,* 1987; Della

Bianca *et al.,* 1988; Hartfield and Robinson, 1990). NADPH-oxidase catalyses one-electron reduction of oxygen to produce superoxide anion using NADPH as substrate. This on further dismutation produces other ROS (Bokoch and Knaus, 2003). Fluoride enhanced NADPH-oxidase activity is thought to be initiated by stimulation of G-proteins systems (Gabig *et al.,* 1987; Toper *et al.,* 1987), and/or inhibition of GTPase activating proteins (GAPs), which are thought to play a crucial role in shutting off G-proteins-mediated responses, therefore resulting in prolonged prevalence of proteins in the GTP-bound state. This as a consequence, activates NADPH-oxidase (Kanaho *et al.,* 1985; Szaszi *et al.,* 1999). Fluoride also has been reported to increase renal and other tissues lipid peroxide levels in animal (Guan *et al.,* 2000; Wang *et al.,* 2000) and human serum (Singh *et al.,* 2001).

Fluoride has been observed to disrupt kidney lysosomes and cause the release of NAG isozymes in urine after acute and chronic exposure to high fluoride concentration in animals (Cittanova *et al.,* 1996; Usuda *et al.,* 1998; Bai *et al.,* 1999) and in patients operated under fluorinated anaesthetics (Hara *et al.,* 1998; Laisalmi *et al.,* 2001) even in the absence of changes in other renal damage biomarkers like CR and BUN (Higuchi *et al.,* 1995). The lysosomes, which are more abundant in renal cortex than other parts of the nephron, are considered as very sensitive organelles to free radicals and oxidative stress perturbations because they host the highest concentration of redox-active iron in cell (Yu *et al.,* 2003). Moreover some studies have indicated that cellular injury induced by free radicals may be mediated through lysosomal damage (Antunes *et al.,* 2001; Brunk *et al.,* 2001). Neither the mechanism of fluoride induced nephrotoxicity or the mechanism by which fluoride increase the lysosomal enzymes release is clearly understood.

3.2. Aim of study:

An understanding of the mechanism of fluoride induced noxious effects should facilitate the prevention and cure of fluoride related deleterious effects and with this in mind, we took the advantage to investigate for the first time the direct effect of fluoride on renal lysosomes integrity in cell free system (LRFs) and the possible association between the loss of lysosomal membrane integrity and free radical formation.

Considering the results of previous researchers we have also investigated the effect of fluoride on kidney lysosomal enzyme latency after its long time exposure in drinking water, following an acute exposure in rats and its association with lipid peroxidation. Also we have investigated some biomarkers (urine volume, urinary CR), which are known to change when renal cells are exposed to a noxious fluoride doses. This could give indication to the site and possible mechanism of fluoride induced renal dysfunction. We have also attempted to provide an easy and applicable method on human for detection of free radicals by measuring urinary MDA level under acute fluoride intoxication and the possible dietary interference on MDA level.

3.3. Experimental protocols:

3.3.1. Selection of optimum medium pH for the study:

LRFs obtained from renal cortices of freshly killed male Wistar rats (300-325 g) by the technique of Win-Aung *et al.,* (1998) (described in methods, section 2.1.3) were

resuspended in 0.3 M ice-cold sucrose (1: 8 w/v). They were gently mixed with acetate buffer pH (5.0, 6.6 and 7.4) in 1:1 v/v ratio in microcentrifuge tubes set in duplicate. They were then incubated at 37 °C, for 1 hr followed by centrifugation for 20 min at 9000 g to sediment the un-lysed lysosomes (Win-Aung *et al.,* 1998). A 100 µl of the supernatant was used for the assessment of free NAG isozymes activity as described in chapter 2 (section 2.1.3) and expressed as enzyme units, one enzyme unit will hydrolyse 1 nmole substrate/ min/mg protein.

3.3.2. Effects of NaF on kidney lysosome integrity and MDA formation *in vitro*:

LRFs after being reconstituted with 0.03 M sucrose were mixed with acetate buffer (pH 5.0)(1:1 v/v) and incubated (in duplicate) with and without NaF varying final concentrations (0.75-192 mM) for 30, 60, or 120 min at 37 °C. LRFs preparations were centrifuged again as mentioned above and the lysosomal integrity was evaluated by measuring NAG isozymes activity in the supernatant as described in methods (section 2.2). MDA level was also measured in the supernatant by HPLC technique described in methods (section 2.4) and expresses as nmol/mg protein. Results are presented as mean value ± SD and statistically analysed by Analysis of variance (ANOVA) and followed by Bonferroni post test. The correlation between NAG isozyme release and MDA level was studied by non-linear, polynomial; second order regression. The limit of significance was established as $P < 0.05$

3.3.3. Effects of acute NaF *in vivo*:

3.3.3.1. Effects on urinary NAG isozymes, CR and MDA and food consumption:

Three groups of six male Wistar rats in each group, weighing 200-250 g were isolated in metabolic cages and kept under the conditions mentioned in the methods section. A 24 hr urine sample was collected and 24 hr food intake was determined before NaF injection (to provide various biomarkers baselines values). Rats of the first group were injected with single intraperitoneal, *i.p*, NaF 15-mg/kg in 0.5 ml saline (162 mM), whereas the second and the third groups were injected with 25 (270 mM) and 35-mg/kg (378) *i.p* respectively in the same manner. Twenty-four hourly urine sample collection and food consumptions were continuously determined for 3 days. Urine volume, NAG isozymes, MDA, and CR were measured in the supernatant as explained in methods (section 2.2, 2.5 and 2.8). The results were statistically analysed by repeated measures ANOVA with Bonferroni post test and the limit of significance was established as $P < 0.05$.

3.3.3.2. Effect of NaF on kidney tissue lipid peroxides level:

The animals were killed by cervical dislocation after 1, 3, 6, 12 and 24 hr of treatment with 25-mg/kg *i.p* NaF (test group). Another group of animals, which received only equivolume normal saline, was also killed also by the same way (control group).

Kidneys were, excised by scissors and washed twice in 0.3 M ice-cold sucrose solution containing 1 mM EDTA. This was chopped by scissors and homogenised in the same solution (1: 5 w/v). This homogenate was spun at 4 °C at 9000 g for 15 min. Lipid peroxide was measured in the supernatant by the HPLC technique described methods (section 2.4). The results statistically evaluated by one-way ANOVA with Bonferroni correction.

3.3.4. Effect of chronic high fluoride in drinking water on urinary NAG isozymes activity:

Eighteen male Wistar rats (4 weeks old) weighing 100-120g has been supplied by the Bioresourses unit, Trinity College. They were randomly allocated to three groups of 6 animals each (one control + 2 experimental). The control group was given deionised water while the two experimental groups received 30 and 100 ppm NaF (0.72 mM and 2.4 mM) in drinking water respectively. All the animals were fed a standard pellet diet, drinking water ad libitum and kept under the same conditions described in methods (section 2.1.2) for 10 months. Animals were housed in stainless steel metabolic cages for 24 hr, in which the animals had free access to water and diet. Their urine samples were collected in disposable beakers and urine volume and NAG isozymes content were determined as described in methods (section 2.2). This NAG isozymes and urine volume determination was repeated twice weekly for three months and twice monthly for the rest period. Data was analysed by one-way ANOVA and Bonferroni post test.

3.4. Results:

Our results have shown that the lysosomal enzyme release is affected by the pH of incubating medium (fig.3.1). The lowest lysosomal enzymes release, indicated by NAG isozymes, is observed by incubating LRFs at pH 5.0 (0.89 ± 0.10 units). This lysosomal enzyme release is increased at pH 6.6 and 7.4 (1.44 ± 0.17 and 2.6 ± 0.3 $P<0.05$ respectively). The results in table 3.1 show that the release of NAG isozymes from renal lysosomes is increased with the amount of fluoride present in the incubating medium. In contrast the results in table.3.2 show that a wide range of fluoride concentrations (capable of releasing NAG isozymes from lysosomes) has inhibitory effect on MDA formation and its level in the supernatant. However it emerges that fluoride has a biphasic effect on MDA formation (table 3.2 and fig. 3.2). In low concentrations (3 and 6 mM NaF) it has stimulant effect without releasing NAG isozymes but at concentrations, 24 mM or higher, it produces a marked reduction in the formation of MDA with concomitant increase in NAG isozyme release from lysosomes. Furthermore the level of MDA has a strong inverse non-linear relationship with lysosomal damage indicated by NAG isozyme activity in the supernatant ($MDA = 7.3 - 2.94\ NAG + 0.327\ NAG^2$, $r = -0.736$. fig. 3.3).

During the *in vivo* studies death was not recorded at any tested dose of NaF. Tables 3.4 to 3.8 show dose-dependent variation of the measured parameters after single-dose administration of NaF. Table 3.3 show that the urine volume is significantly increased (approximately 2 fold of the control value) only on the first day after 35 mg/kg NaF and then gradually decreases to levels near the control value by day 3. CR

excretion (table 3.4) shows significant transient decline after 24 hr of both 25 and 35 mg/kg NaF administration ($P<0.05$) and then reverses to nearly normal value. The NAG isozyme activity recovered in urine is increased after 24 hr of NaF (25 and 35 mg/kg) administration by approximately 2.5 folds and 3 folds of the baseline values respectively (table 3.5). This dramatic increase in NAG isozyme activity was decreased on day 2 after 25 mg/kg and after 35 mg/kg. In both cases the NAG activity returns to nearly the baseline values on day 3. In the groups given 25 and 35 mg NaF, the urinary MDA was almost remained unchanged whereas food consumption was significantly reduced on day 1 in-group treated with 25-mg/kg ($P<0.05$) and maintained until the second day in-group treated with higher dose. In both groups the effect on food consumption was reversed to non-significant value by day 3 (table.3.6 & 3.7). The results in fig. 3.4 show that renal tissue MDA level is enhanced after NaF (25 mg/kg *i.p*), which reaches peak level after 6 hr of its administration ($P<0.01$) and returns to baseline value after 12 hr.

Animals exposed to high fluoride in drinking water for 10 months did not show any significant differences between group values either for the urine volume or its content of NAG isozymes at various time tested (table 3.8).

3.5. Discussion:

Effect of NaF on renal NAG isozymes release and MDA level *in vitro*:

The lysosomal membrane prevents acid hydrolases associated with lysosomes from having accesses to molecules present in the surrounding medium. Its deterioration

could be responsible for the degradation processes that take place in cells under various pathological and toxicological conditions (Li *et al.,* 2000; Sohar et al 2002). Clinical (Hara *et al.,* 1998; Laisalmi *et al.,* 2001; Trevisan *et al.,* 2003) as well as experimental studies using different animal species (rat is the most widely species used) (Usuda *et al.,* 1998; Usuda *et al.,* 1999; Bai *et al.,* 1999; Cittanova *et al.,* 2002) have shown that fluoride targets kidney lysosomes and causes lysosomal membrane rupture that release acid hydrolase enzymes, but the mechanism of this lysosomal rupture is still not defined. This lysosomal damage induced by NaF occurs in some cases before other changes in kidney functions like glumeruli function (Higuchi *et al.,* 1995), which might indicate that the lysosomal effect may be the initiator of kidney dysfunction induced by fluoride.

LRF has been selected as an appropriate model by several workers to test the effect of some agents on the lysosomal integrity (Powell and Reidenberg, 1984; Win-Aung, 1998). In our *in vitro* model, we have used LRF of rat renal cortex suspended in iso-osmotic acetate buffered sucrose to pursue the direct effect of NaF on lysosomal membrane integrity. The pH used in all vitro experiments was 5.0. The acetate buffer pH 5.0 was shown to release the minimum amount of NAG isozymes under basal conditions (fig. 3.1). Stirling (1972) has shown that the optimal pH for NAG isozyme activity is 5.0 while the increase in NAG isozymes release by more neutralized incubating media (pH 6.6 and 7.4) is consistent with previous reports (Buckmaster *et al.,* 1988) which might reflect the loss of lysosomal integrity by pH gradient effect and enhanced ions permeability to achieve acid-base balance between the acidic lysosomal milieu (pH around 5.0) and the surrounding medium (Henning, 1975; Reijngoud and Tager, 1977). Furthermore the lysosome has been found to be less

permeable to ions when incubated at 37 °C in isotonic acetate buffered sucrose (Davidson and Song, 1975).

The data presented in this study has shown that fluoride induced NAG isozyme release from renal lysosomes occurs in a concentration and time dependent manners *in vitro* (table 3.1). Higher concentrations (24 mM and more) of NaF only had the ability to release NAG isozymes in the supernatant. The early release of lysosomal enzymes may activate feedback processes that cause further lysosomal rupture and might explain the time dependent increase in NaF treated as well as the non-treated control fractions. Such feedback processes may be an attack from outside of the released lysosomal enzyme (Zhao *et al.,* 2000; Brunk *et al.,* 2001; Antunes *et al.,* 2001). The possibility that fluoride may enhance NAG isozymes activity was ruled out by measuring NAG isozymes activity in the presence and absences of different concentrations of NaF, and in both cases the activity was the same. The free NAG isozyme activity recovered in control fractions, untreated (table 3.1) might be a consequence of the homogenisation, fractionation and/or shaking (agitation) during separation and re-suspension of lysosomes after their pelleting (Haga *et al.,* 1987; Kalra *et al.,* 1989).

Biological membranes, including lysosomal membrane, are rich in PUFAs, and thus would be targets for oxidative damage, moreover the lysosomes have been suggested as very vulnerable organelles to free radical induced damage because of the high content of metal ions (especially low molecular weight redox active iron) that would initiate the oxidative damage through Fenton like chemistry (Persson *et al.,* 2003; Yu *et al.,* 2003).

Free radical formation, the consequent of phospholipids peroxidation and altered membrane phospholipids structure have been implicated in fluoride provoked renal

dysfunction (Sharma and Chinoy, 1998; Guan *et al.,* 2000; Shao *et al.,* 2001) and other tissues deleterious effects (Wang *et al.,* 2000; Ghosh *et al.,* 2002; Shivashankara *et al.,* 2002), a processes which is known to change membrane fluidity, permeability with final loss of the membrane integrity (Slater, 1984; Housset, 1987; Halliwell and Gutteridge, 2001).

NaF concentrations of 3 and 6 mM, which released significantly MDA, failed to release NAG isozymes from the lysosome (see table 3.1 and 3.2). The kidney and its vasculature are rich sources of NADPH-oxidase, which is though to provoke several renovasculature injures by the excessive formation of ROS under stimulatory conditions (Touyz, 2004). Therefore NaF increased MDA formation in our study may be related to its known activation of NADPH-oxidase, generation of ROS and consequently the increase in lipid peroxides level. However since the low concentrations (3 and 6 mM) of NaF did not show any significant changes in NAG isozyme activity, the loss of lysosomal membrane integrity by higher NaF concentrations (24 mM and more) was significantly correlated ($r = -0.736$, $P<0.001$) with the fall in MDA level (fig. 3.2 and 3.3). Phospholipases A_2 (PL A_2) and phospholipase C (PLC) and several other lipases have been detected and separated from lysosomal compartments (van Kuijk *et al.,* 1987; Sevanian *et al.,* 1988; Gamache *et al.,* 1988). These phospholipases have been shown to hydrolyse membranes peroxidized PUFAs as well as non-peroxidized phospholipids (Beckman *et al.,* 1981; Dickens *et al.,* 1988). Hydrolysis of peroxidized PUFAs by lysosomal lipases like PLA_2 produce the reactive fatty acid hydroperoxides, which are subsequently reduced by other antioxidant enzymes like peroxidase released from lysosomes, a mechanism has been shown to decreases lipid peroxide level and thereby leaving less lipid peroxides in the supernatant of our system (Antunes *et al.,*

1995). Furthermore fluoride, per se (Jeremy and Dandona, 1987; Murao *et al.,* 2000) and altered membrane phospholipid composition (Weglicki *et al.,* 1984; Rossi *et al.,* 2001) has been reported to enhance PL A_2 and PLC activities. Therefore, depending on the above mentioned factors, we suggest that the NAG isozymes released by higher concentrations may be preceded by the formation of free radicals and a consequent lipid peroxidation, while the fall in MDA level corresponding to NAG release may indicate the antioxidant potential of some of the liberated and/or enhanced lysosomal enzymes as a part of their defence mechanism for eliminating lipid hydroperoxides and free radicals. Further work needed to be carried to assess the antioxidant potential of lysosomal enzymes whether the decreased MDA level was associated with a decrease in free radicals formation or it is a direct effect on lipid peroxides only and how phospholipases are implicated in this processes.

Effect of acute fluoride intoxication on kidney NAG isozymes and other biochemical changes in the rat:

Histopathological and biochemical investigations in renal fluoride studies have revealed that renal failure may develop after exposure to acute fluoride intoxication and it is more pronounced in proximal tubules than other parts of the nephron (Takagi and Shiraki, 1982; Usuda *et al.,* 1999). Considering the results of these researchers, we have investigated some biomarkers (like urine volume, its CR and NAG isozyme content) and some other biomarkers, which expected to be changed (like food consumption, urinary and kidney tissue MDA levels) after acute fluoride intoxication. Based on our preliminary experiments we subjected rats to single different doses of NaF (15, 25 and 35 mg/kg *i.p*). The results in this study (table 3.3 to 3.7) show that

the urinary volume was transiently increased after fluoride administration (polyuria) in a dose dependent fashion, which was significant ($P < 0.05$) at the highest dose used (35 mg/ kg). This reversible polyuria phenomenon was in line with previous reports (Suketa and Mikami, 1977; Usuda *et al.,* 1998). The polyuria after administration of fluoride to laboratory animals is suggested to be due to the inhibition of salts and water resumption and a refractoriness to vasopressin, (Wallin and Kaplan 1977; Bosch, 1996) and by increased renal blood flow to medulla (Frascio, 1972). In this study the observed increase in urine volume after fluoride administration may be the result of some of these features. Fluoride has also been found to afflict Glomerular filtration rate (GFR) and to alter urine and serum CR levels in some renal fluoride toxicity studies (Goldberg et al 1996; Usuda *et al.,* 1998) but its effect on renal tubules is more pronounced (Usuda *et al.,* 1998). The 24-hr urinary excretion of CR was adopted for the assessment of GFR based on the assumption that rats used in this study having close range of body weight (200-250), had similar plasma CR concentration (Willis *et al.,* 1976).

In our observations (table 3.4), glomerular function was decreased indicated by significant reduction in urinary CR excretion on the first day following treatment with 25 and 35 mg/kg NaF ($P<0.05$). This glomerular damage was less pronounced than the proximal tubules damage indicated by NAG enzymatic activity recovered in urine (table 3.5, $P<0.01$ in both doses) on day one and returned to normal values on day 2. This reversible renal damage caused by single dose of NaF is in agreement with the previous clinical (Hara *et al.,* 1998; Hase *et al.,* 2000) and experimental (Usuda *et al.,* 1998; Usuda *et al.,* 1999) studies, which might be due to the rapid fluoride clearance (Whitford, 1990).

Free radicals and the consequent lipid peroxidation have been incriminated in fluoride nephrotoxicity (Soni *et al.,* 1984; Guan *et al.,* 2000; Singh *et al.,* 2001).

A dose of 25 mg/kg NaF has been chosen to assess renal tissue MDA level because it is the first dose significantly induced acute renal toxicity manifested by altered urinary NAG isozyme as well as CR levels (table 3.4 and 3.5). Our results have revealed that acute administration of NaF (25 mg/kg) temporarily increases lipid peroxide levels in the kidney tissue (fig. 3.4). This temporary formation of MDA, which reaches a maximum value after 6 hr of fluoride administration, may be implicated in the nephrotoxic changes that were observed in this study. The rapid decline of tissue MDA after 12 hr is again may be due to the rapid elimination of fluoride (being a small molecule is rapidly filtered through the glomerulus) and/or reduction of oxidised lipids by the released lysosomal enzymes.

MDA-generating substances are normal constituent of rat and human urine (Dhanakoti and Draper, 1987) and it has been suggested as biomarker for *in vivo* lipid peroxidation (Pryor and Godber, 1991). However, measurement of urinary MDA also includes the contribution from dietary sources and may reflect the amount of exogenous MDA-generating substances consumed in diet (Brown *et al.,* 1995). We fed all rats the same standard pellet diet, and we measured their 24-hr urinary MDA output (expressed as MDA/CR ratio) and their food consumption after fluoride administration. Urinary MDA level remained almost unchanged (table 3.6). On the other hand the food consumed by animals was significantly reduced (table 3.7). This reduction in food consumption caused by fluoride is in agreement with previous reports (Pillai *et al.,* 1988; van den Broek *et al.,* 2000) which has been suggested is due to loss of appetite (Pillai *et al.,* 1988). Therefore our result support the contention

that urinary aldehydic products of lipid peroxidation (MDA) might be an unreliable indicator of the general state of peroxidative stress *in vivo* (Draper *et al.,* 2000).

Effect of long-term high fluoride intake on kidney lysosomes in the rat:

Long-term drinking high fluoride (30 and 100 ppm NaF) has been shown to afflict kidney and other tissue structure (Guan *et al.,* 2000; Wang *et al.,* 2000) and would produce plasma concentrations of fluoride that is attainable by humans from environmental exposure (Borke and Whitford, 1999). Therefore considering these reports we have selected the above-mentioned concentrations to study the effect of fluoride on renal NAG isozymes release.

In our results chronic high fluoride intake (30 ppm and 100 ppm NaF) did not alter the urine volume or its NAG isozyme contents in 10 months period at the time tested in both groups compared to control group (0.0 ppm NaF). The NAG isozymes values remain almost unchanged by time and among different groups, whereas urine volume shows some non significant changes by time (6-8 months) in all groups, which might be due to increased water intake, as the animals get older. Our finding was inconsistent with a previous observation reported by Bai et al (1999) in which they have observed that fluoride at comparable concentration of our highest concentration used (50 mg/L fluoride ~110 ppm NaF) has increased NAG isozymes release in urine only after one week exposure and continued for one month. This discrepancy may be due to difference in some of experimental conditions, which include urine sample collection intervals. In our study urine samples were collected only twice a week, which is approved by the current legislation on animals experimentation in Ireland. Also the age of animals used may have influence on fluoride pharmacokinetics. We

have conducted our experiment on four weeks old rats, at the beginning, and it has been suggested that the rate at which fluoride is cleared from plasma by calcified tissues is substantially higher than that by kidney in younger subjects than older and this is due to the higher surface area of the loosely organized crystallites in the developing calcified tissues during growth (Whitford, 1999), therefore leaving less fluoride to pass through kidneys. It has also been reported that prolonged continuous exposure to non-toxic concentrations of fluoride may develop resistance to higher concentrations (Hongslo *et al.,* 1980; Sato *et al.,* 1986). This continued kidney cells insensitivity to fluoride (for 10 months) even when the animals get older (bone become more mature) and the uptake of fluoride by calcified tissue is diminished (Whitford, 1990), might be due tolerance development. Therefore age related changes in skeletal tissue and tolerance development may explain some of the discrepancies between our results and of Bai et al (1999). In our support recent studies have shown that rats exposed to high fluoride concentrations (30 and 100 ppm) in drinking water show adaptation to some antioxidants (Shivarajashankara *et al.,* 2003) that might counteracts free radical induced deleterious effects, a mechanism that believed to play an important role in fluoride intoxication. Other factors like strain difference (Everett *et al.,* 2002), and differences in ambient temperature, which might alter water consumption, and thereby fluoride intake might have an impact on its toxic effects (Brouwer *et al.,* 1988; Lima and Cury, 2003).

3.6. Conclusions:

Our data from *in vitro* and acute *in vivo* studies support the recently held view that free radicals play an essential role in fluoride related changes in renal cell function.

Moreover our *in vitro* (cell free system) study provided a novel insight regarding the capability of fluoride to induce renal lysosomal damage directly in dose and time dependent manner and it is accompanied with dose dependent dual effect on MDA level. The inversely association of the inhibitory effect of NaF on MDA level with the lost of lysosomal membrane integrity and liberation of lysosomal enzymes may indicate the antioxidant potential of these enzymes as a part of their defence mechanism for elimination of free radicals. We have also shown that drinking high fluoride over a prolonged time was unable to alter both urine volume and its NAG isozyme content at least at the time tested. Our results have also shown that, assessment of urinary MDA is not a reliable marker at least under fluoride intoxication to reflect body's general oxidative stress.

Table 3.1. The effect of different concentrations and incubation time of NaF on the release of NAG isozyme in vitro.

Concentrations of NaF mM	NAG isozymes (units)		
	30 min (n=6)	60 mim (n=6)	120 min (n=6)
0.00	0.65 ± 0.04	0.84 ± 0.13	0.90 ± 0.09
0.75	0.64 ± 0.03	0.73 ± 0.12	0.88 ± 0.10
1.50	0.66 ± 0.04	0.82 ± 0.04	0.95 ± 0.17
3.00	0.63 ± 0.03	0.91 ± 0.11	0.97 ± 0.11
6.00	0.70 ± 0.10	0.94 ± 0.09	1.00 ± 0.13
12.0	0.75 ± 0.08	0.98 ± 0.07	1.11 ± 0.14
24.0	0.81 ± 0.15	1.22 ± 0.11 *	1.52 ± 0.11 **
48.0	1.20 ± 0.15 **	1.40 ± 0.20**	1.93 ± 0.17 **
96.0	1.71 ± 0.11 **	2.65 ± 0.25**	3.20 ± 0.18 **
192	2.25 ± 0.26 **	4.10 ± 0.56**	5.30 ± 0.38 **

Mean ± SD, * $P<0.05$ and ** $P<0.01$ compared to the control (0.0 NaF).

One NAG isozyme unit will hydrolyse 1nmol substrate/min/mg protein.

Table 3.2. The effect of different concentrations and incubation time of NaF on MDA level in the supernatant of rat renal LRFs in vitro.

Concentrations of NaF mM	MDA (nmole/ mg protein)		
	30 min (n=6)	60 min (n=6)	120 min (n=6)
0.00	2.30 ± 0.22	2.90 ± 0.26	4.40 ± 0.27
0.75	2.13 ± 0.31	3.00 ± 0.36	4.60 ± 0.41
1.50	2.22 ± 0.23	3.10 ± 0.45	4.90 ± 0.35
3.00	2.30 ± 0.35	3.30 ± 0.40	5.10 ± 0.52 *
6.00	2.43 ± 0.40	3.34 ± 0.50	5.40 ± 0.70 **
12.0	2.20 ± 0.30	2.70 ± 0.32	4.90 ± 0.31
24.0	2.10 ± 0.24	2.30 ± 0.30 *	3.70 ± 0.30 *
48.0	1.60 ± 0.18 **	1.43 ± 0.25 **	2.00 ± 0.17 **
96.0	1.39 ± 0.14 **	1.30 ± 0.23 **	1.35 ± 0.20 **
192	1.10 ± 0.16 **	1.00 ± 0.45 **	0.84 ± 0.14 **

Mean ± SD, * $P<0.05$ and ** $P<0.01$ compared to control (0.0 NaF).

Table 3.3. Effect of different doses of NaF on 24 hr urine volume (ml).

NaF (mg/ kg *i.p*)	Time			
	Day 0 (Baseline)	**Day 1**	**Day 2**	**Day 3**
15 mg	9.5 ± 1.5	10.8 ± 2.0	8.75 ± 1.5	9.75 ± 1.8
25 mg	8.0 ± 2.5	11.0 ± 3.7	9.25 ± 1.5	9.50 ± 2.6
35 mg	9.25 ± 1.3	17.8 ± 6.8 *	13.8 ± 3.2	10.5 ± 1.4

Mean ± SD, n=6. *P<0.05 compared to baseline value.

Table 3.4. Effect of different doses of NaF on 24 h urine CR excretion (mg/24hr).

NaF (mg/ kg *i.p*)	Time			
	Day 0 (Baseline)	**Day 1**	**Day 2**	**Day 3**
15 mg	9.35 ± 1.4	8.67 ± 1.3	9.42 ± 3.2	10.4 ± 2.7
25 mg	9.80 ± 1.9	6.53 ± 2.1 *	8.15 ± 1.9	8.81 ± 2.0
35 mg	10.6 ± 2.2	6.15 ± 1.7 *	8.00 ± 1.8	9.22 ± 1.7

Mean ± SD, n=6. * P<0.05 compared to baseline value.

Table 3.5. Effect of different doses of NaF on urinary NAG isozymes activity (Units).

NaF (mg/ kg *i.p*)	Time			
	Day 0 (Baseline)	**Day 1**	**Day 2**	**Day 3**
15 mg	0.82 ± 0.25	0.98 ± 0.4	0.76± 0.26	0.90 ± 0.25
25 mg	0.80 ± 0.23	2.10 ± 1.4 **	1.2 ± 0.45	0.77 ± 0.234
35 mg	0.93 ± 0.25	2.9 ± 1.85 **	1.5 ± 0.418	1.165 ± 0.37

Mean ± SD, n=6. **P<0.01 compared to baseline value.

One NAG isozyme unit will hydrolyse 1µmol substrate/min/litre urine.

Table 3.6. Effect of NaF on urinary MDA level (nmol/mg CR/day).

NaF (mg/ kg *i.p*)	Time			
	Day 0 (Baseline)	**Day 1**	**Day 2**	**Day 3**
15 mg	12.9 ± 4.8	12.1 ± 2.8	13.7 ± 3.1	14.0 ± 4.6
25 mg	13.6 ± 5.0	10.6 ± 6.5	14.3 ± 3.6	16.5 ± 6.3
35 mg	15.3 ± 4.9	11.5 ± 4.8	11.4 ± 3.4	10.3 ± 2.7

Mean ± SD, n=6.

Table 3.7. Effect of NaF on food consumption (g/day).

NaF (mg/ kg *i.p*)	Time			
	Day 0 (Baseline)	**Day 1**	**Day 2**	**Day 3**
15 mg	31.2 ± 1.2	30.1 ± 2.1	30.5 ± 1.5	30.8 ± 1.6
25 mg	28.8 ± 2.0	25.3 ± 2.0*	27.7 ± 2.1	28.0 ± 2.2
35 mg	31.3 ± 1.3	17.3 ± 10 **	23.7 ± 6.6*	26.3 ± 2.6

Mean ± SD, n= 6. * $P<0.05$ and ** $P<0.01$ compared to baseline value.

Table 3.8. Monthly observation of urine volume and its NAG isozymes content of rats exposed to different concentrations of NaF in drinking water for 10 months. (Mean ± SD, n=6)

Treatment						
Month	**0.0 ppm NaF**		**30 ppm NaF**		**100 ppm NaF**	
	Urine vol. (ml)	**NAG (U/l)**	**Urine vol. (ml)**	**NAG. (U/l)**	**Urine vol. (ml)**	**NAG (U/l)**
0.0	5.7 ± 0.99	0.76 ± 0.05	6.5 ± 1.2	0.69± 0.1	5.5 ± 0.5	0.8 ± 0.13
1	7.8 ± 1.5	0.85 ± 0.17	8.0 ± 1.9	0.84 ± 0.3	7.2 ± 2.1	0.72± 0.12
2	8.5 ± 2.2	0.94 ± 0.25	7.4 ± 2.1	0.73 ±0.19	7.6 ± 1.6	0.79 ±0.18
3	8.1 ± 2.5	0.88 ± 0.16	6.8 ± 2.5	0.85 ±0.25	8.9 ± 2.8	0.91 ±0.25
4	9.7 ±2.7	0.68 ± 0.09	10.9± 1.5	0.76 ±0.20	8.4 ±1.5	0.95 ±0.13
5	8.9 ± 2.1	0.99 ± 0.15	11.1 ±2.3	1.01 ±0.3	7.8± 2.5	0.88 ±0.10
6	12.5 ± 2.7	0.84 ± 0.19	12.9±1.8	0.75 ±0.22	9.5 ±2.2	0.65± 0.24
7	13.1 ± 1.8	1.05 ± 0.27	12.1 ± 2.3	0.87 ±0.34	11.2 ± 3.1	0.72 ±0.35
8	12.8 ±2.4	0.72± 0.15	13.4 ± 2.4	0.92 ± 0.3	12.9±2.2	0.81±0.24
9	11.8±1.9	0.94 ± 0.09	12.8 ±2.1	0.83 ±0.17	10.8±3.2	0.77±0.32
10	9.7 ± 2.5	0.92 ±0.14	9.4 ± 2.9	0.85 ±0.13	9.6 ± 3.7	0.89±0.08

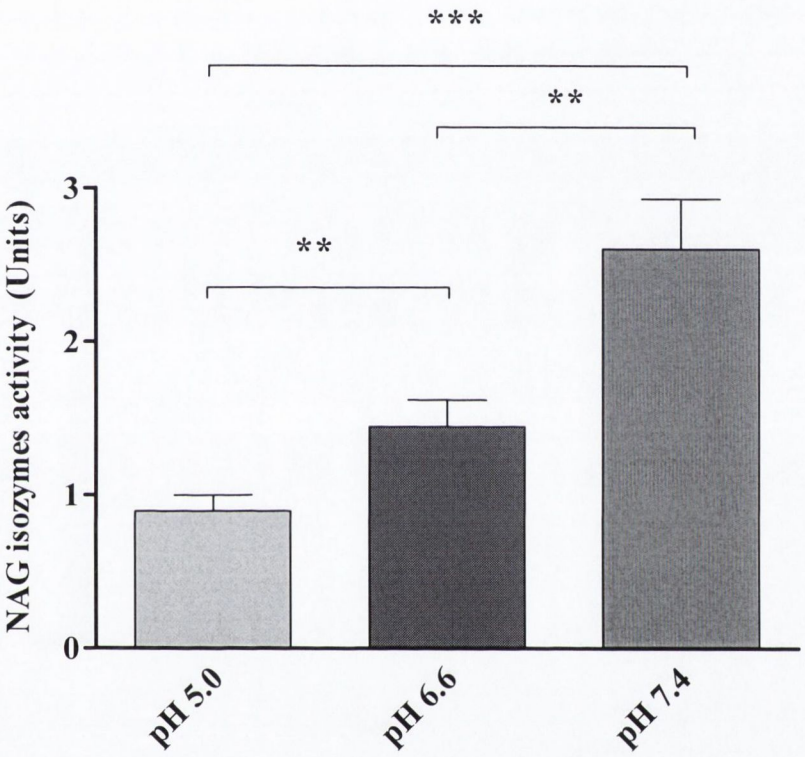


Fig. 3.1. Effect of pH on lysosomal integrity manifested by NAG isozyme release (Mean ± SD, n= 6. ** P<0.01, *** P<0.001).

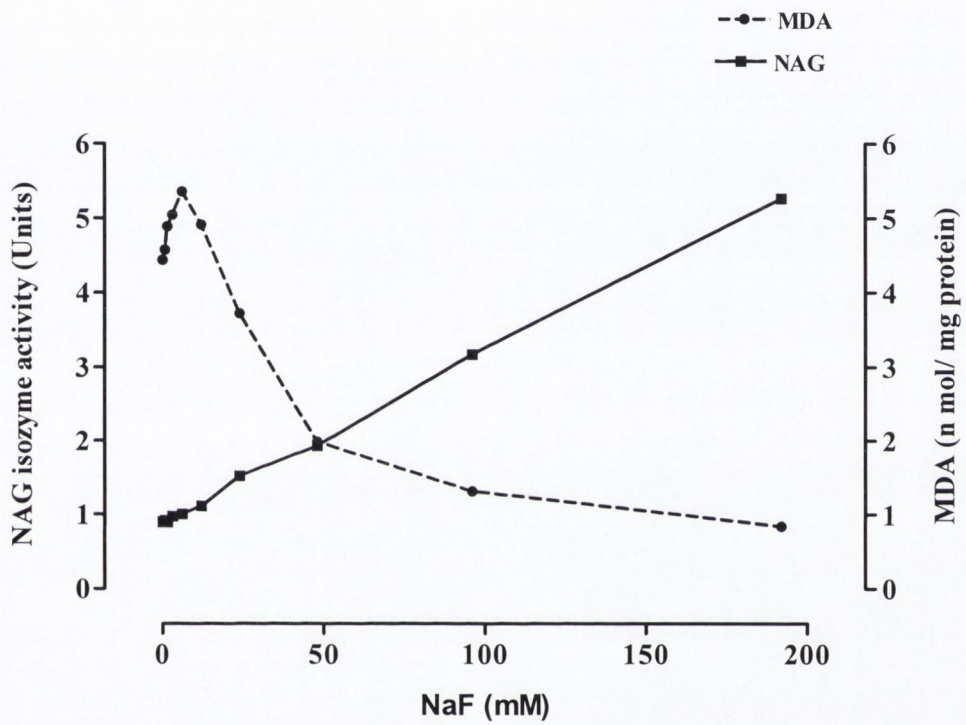


Fig. 3.2. Relationship between lysosomal damage, manifested by NAG isozymes release, and MDA level in the supernatant of renal lysosomes incubated with NaF (0.75 to 192 mM) at 37 °C for 2 hr. For clarity mean values without SD are shown.

$$MDA = 7.3 - 2.94\ NAG + 0.327\ NAG^2$$

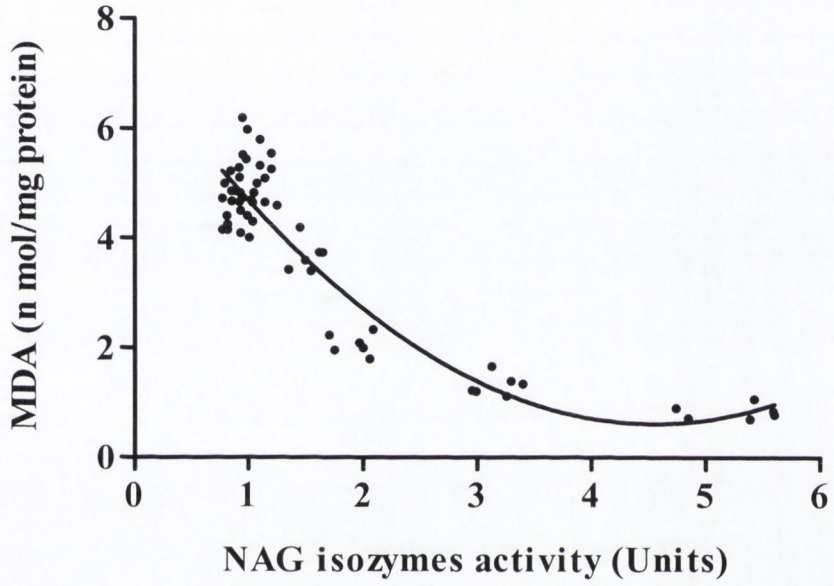


Fig. 3.3. The association between NAG isozyme and MDA levels in the supernatant of rat kidney LRFs incubated with NaF (0.75 to 192 mM) for 2 hr at 37 °C ($r = -736$. $P<0.001$).

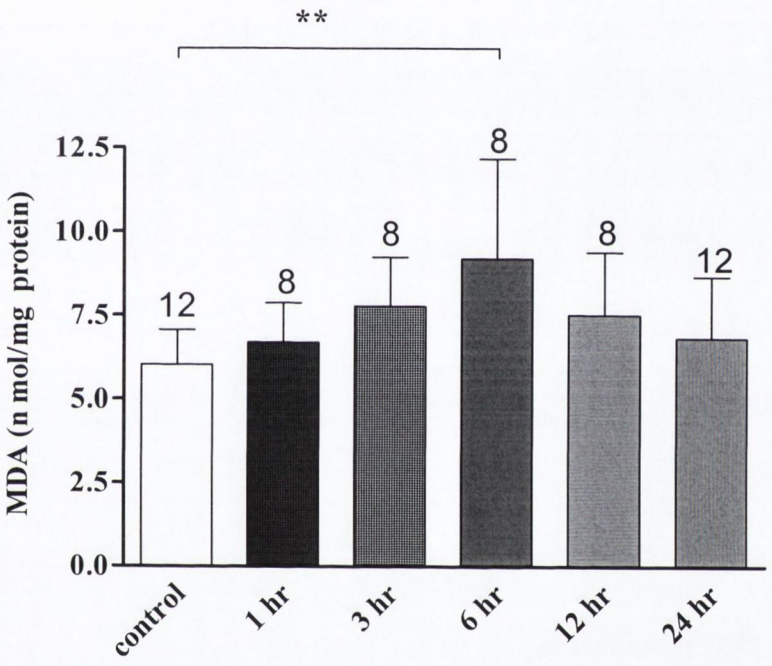


Fig. 3.4. The level of MDA in rat kidney tissue 1, 3,6,12, and 24 hr following NaF 25 mg/kg *i.p.* Mean ± SD. ***n*** for each group is printed above each respective bar; **P<0.01compared to control (non-treated group).

CHAPTER FOUR

EFFECTS OF SOME ANTIOXIDANTS ON FLUORIDE INDUCED RAT KIDNEY LYSOSOMAL DAMAGE

41. Introduction:

Increased oxygen radical generation, lipid peroxidation and altered antioxidant defence systems are considered to play an important role in the pathogenesis of many diseases and toxic actions of a wide range of compounds (Gracy *et al.,* 1999). These processes have even been proposed to be an important mediating factor in the causation of detrimental effect of fluoride (Shivarajaashankara *et al.,* 2001; Singh *et al.,* 2001). In the previous chapter we have shown that fluoride has the ability to release NAG isozymes and increase MDA level after *in vitro* and *in vivo* exposure to noxious fluoride concentrations. Lipid peroxidation (a mechanism proposed to prime the toxic effect of fluoride) has been described to be initiated through both non-enzymatic and enzymatic pathways. The superoxide an ion is the initiator of non-enzymatic lipid peroxidation either by the causing direct lipid peroxidation of PUFAs or through the formation of other more reactive oxygen species like hydrogen peroxide and hydroxyl radical by self and/or enzymatic (SOD) dismutation (Hallwell and Gutteridge, 2001). The redox active metal ions (iron and copper) are believed to play a crucial role in induction of PUFAs oxidation especially in lysosomal phospholipids peroxidation, which is considered as the cell's largest pool of reactive iron (Ollinger and Brunk, 1995; Persson *et al.,* 2001a). The ROS may also mediate lipid peroxidation though activation of arachidonic acid metabolism pathways, which include COX and LOX pathways (Kanner *et al.,* 1987). This arachidonic acid metabolism may further liberate free radicals, lipid peroxides and other physiological active compounds, all collectively known as eicosanoids (Kanner *et al.,* 1987). Moreover, fluoride has been suggested to enhance PLA_2 and C activities (Stasi *et al.,*

1992; Murao *et al.,* 2000) and thereafter release arachidonic acid (Wessel *et al.,* 1989). It has also been observed that fluoride stimulates formation of prostaglandins and leukotrienes, which are metabolites of COX and LOX pathways respectively (Brom *et al.,* 1989; Schulze-Specking *et al.,* 1991). Therefore, from these previous reports, both enzymatic and non-enzymatic pathways may be implicated in the lipid peroxidation processes induced by fluoride. Recently, some researchers have reported that low molecular weight antioxidants, β-carotene, vitamin C and E, GSH and, SOD, have the ability to inhibit the lipid pro-oxidant activity of fluoride (Sharma and Chinoy, 1998; Sun *et al.,* 1998; Sun *et al.,* 2001). It has also been reported that free radical scavengers like vitamins C and E can efficiently prevent as well as reverse the toxic effects of fluoride in mice liver and gastrocnemius muscle (Chinoy *et al.,* 1993), as well as the testicular and ovarian toxicity (Chinoy *et al.,* 2001) and ameliorate embriotoxicity induced by fluoride in pregnant rats (Verma and Sherlin, 2002). Under clinical conditions, diet rich in essential nutrients and antioxidants have also been shown to reverse fluorosis (Susheela and Bhatnagar 2002).

4.2. Aims of study:

Considering the above-mentioned reports and our results of chapter 3 we have conducted this study to:

(1)- Extend our previous suggestion that free radicals play a major role in fluoride induced lysosomal damage by assessment the effects of a variety of antioxidants acting on different substrates in the free radical cascade reaction and to clarify the possible implication of some lysosomal enzymes like PLA_2 and PLC in the positive feed back mechanism of fluoride induced rat kidney lysosomal damage *in vitro*:

a- Antioxidants which inhibit free radical catalysed lipid peroxidation:

Mannitol, Pycnogenol (PYC), Deferoxamine (DFO), α-Lipoic acid (LP).

b-Antioxidants which inhibit enzymatic catalysed lipid peroxidation:

b1-PLA_2 and PLC inhibitors; Bromophenacyl bromide (BPB), Mepacrine and U71223.

b2-COX and LOX inhibitor; Indomethacin and Nordihydroguaiaretic acid (NDGA),

(2)- We have also assessed the *in vivo* effects of the natural antioxidants (ascorbic acid and PYC) in the prevention of fluoride induced acute renal toxicity in the rat.

4.3. Experimental protocols:

4.3.1. Assessment of the effect of free radical scavengers against fluoride induced renal lysosomal damage *in vitro*:

Freshly obtained LRFs from the renal cortex of male Wistar rats (300-325 g) were incubated without (control) and with different scavengers at different final concentrations. All the scavengers used in this study were purchased from Sigma except PYC was provided by Horphage Research, Geneva, Switzerland and U73122 was purchased from Tocris The concentration selection was based on our preliminary experiments and was comparable to the previously published work (shown in table 4.1), in a medium containing 0.3 M sucrose and 0.06 M acetate buffer pH 5.0 for 15 min at 37°C. Scavengers sparingly soluble in water were dissolved in methanol and then diluted in buffer to provide a final concentration of 1.25% final concentration. After 15 min all samples were challenged with NaF (48 mM) and incubated for

further 60 min. NAG isozymes release in the supernatant and protein content were estimated as described in chapter 3 (section 2.2 and 2.3). The vehicle and /or scavengers were also incubated with commercial NAG isozyme for the same incubation period for interference assessment.

The data obtained from this study has been expressed as mean ± SD and evaluated by one way ANOVA and Bonferroni post test. A *P* value less than 0.05 was considered statistically significant.

4.3.2. Effect ascorbic acid and PYC on fluoride induced renal toxicity:

Forty-four male Wistar rats (200-250 g) were obtained from Bioresourses unit, TCD. Four groups (8 in each group), separately housed in metabolic cages under the same conditions described in methods (section 2.1.2) and provided free access to standard rat chow and water. A 24-h urine samples were collected for providing baseline values. The first group received single dose of NaF (25-mg/kg *i.p*), the second and third groups were given ascorbic acid (250-mg/kg) and Pycnogenol (25-mg/kg) *i.p* three times daily. Then these groups were challenged with 25-mg/ kg NaF one hour after the first dose of scavengers has been injected. The last group served as a control and received only equivalent volume of saline. All the doses were given in 0.5 ml saline. Twenty-four hour urine samples were collected following the drug administrations and processed as previously described. Urinary creatinine and NAG isozyme were assessed as described in methods. The two other groups (6 rats in each) were given only equivalent doses of ascorbic acid and Pycnogenol.

The data are expressed as the mean ± SD and the results were analysed by ANOVA for multiple comparison with Bonferroni post test. A *P* value less than 0.05 was considered statistically significant.

Table 4.1. Antioxidants concentration and end points summary.

Scavenger	Concentrations	Solvent	End point	Reference
Mannitol	25, 50, 100 mM	Acetate buffer	$OH^{\bullet}$	Zhu *et al.*, 2002
Pycnogenol	40, 80, 160 µg/ml	Acetate buffer	ROS, RNS, Fe chelation	Packer *et al.*, 1999
Deferoxamine	1, 5, 10 mM	Acetate buffer	Fe chelation	Niihara *et al.*, 2002
α-Lipoic acid	0.25,0.5,1.0 mM	Methanol	Fe chelation	Persson *et al.*, 2001
BPB	1.25, 2.5, 5 µM	Methanol	PLA_2	Sandler *et al.*, 1989
U-73122*	5.0, 10 µM	Methanol	PLC	Aspinwall *et al.*, 2000
U-73343**	5.0& 10 µM	Methanol	PLC	Aspinwall *et al.*, 2000
Indomethacin	1.75, 3.5, 7 µM	Methanol	COX	Zhang *et al.*, 2005
NDGA	0.4, 0.8, 1.6µM	Methanol	LOX	Zhang *et al.*, 2005
Mepacrine	10 ,20, 40 µM	Acetate buffer	PLA_2	Tepperman, 1999

***U-73122** is 1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione.

****U-73343** is 1-[6-[((17b)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-2,5-pyrrolidinedione (the in active analog of U-73122).

4.4. Results:

In vitro experiments:

The concentrations of all the antioxidants and vehicles used in this study are shown in table 4.1. None of the antioxidant at concentrations used in this study had any impact on the commercial NAG isozymes activity. The selection of a final concentration of 48 mM NaF, 15 min preincubation and one-hour incubation time to test the ability of various antioxidants to inhibit lysosomal NAG isozyme release was based on our preliminary experiments.

The hydroxyl radical scavenger, mannitol attenuates NaF induced NAG isozyme release. As shown in fig. 4.1 pretreatment of incubating medium with mannitol (50 &100 mM) significantly reduces lysosomal enzyme release enhanced by NaF (1.36 vs. 1.16 and 1.06, $P<0.05$ and $P<0.01$ respectively). The results in fig. 4.2 clearly indicate that significant inhibition of NAG isozymes release from renal lysosomes occurs when 40 μg/ml PYC is added to the incubating medium (1.36 vs. 1.18, $P<0.05$) and further inhibition occurs when higher concentrations of PYC is present in the incubating medium. The presence of iron chelator, DFO did not prevent the lysosomal destabilization induced by NaF at any of the tested concentrations, whereas LP (also iron chelator agent) attenuates lysosomal NAG isozymes release at 1 mM concentration ($P<0.05$ fig. 4.4). The PLC inhibitor (U-73122) at 10 μM, reduces NAG isozyme release induced by NaF (1.36 ± 0.19 vs. 1.07 ± 0.18, $P<0.05$ fig. 4.5), and the inactive form (U-73343) at same concentrations does not show any protective activity against the effect of NaF effect. In contrast BPB (PLA_2 inhibitor) enhances

the NaF induced lysosomal enzyme release in a dose dependent manner, which is significant at 5.0 μM (1.36 ± 0.19 vs. 1.77 ± 0.15, $P<0.01$ fig. 4.6). Mepacrine (another PLA2 inhibitor) fails to alter fluoride induced NAG isozymes level in the supernatant (fig. 4.7).

Data shown in fig. 4.8 and 4.9 show that the presence of COX inhibitor, indomethacin, and LOX inhibitor, NDGA, in the incubating medium significantly ($P<0.05$) stabilize the lysosomes against the fluoride insult.

***In vivo* experiments:**

A significant impairment of glomerular function and renal tubules damage was observed by 25-mg / kg NaF which is manifested by decreased urinary CR excretion (9.35 ± 1.4 vs. 6.08 ± 1.24, $P<0.05$) and increased NAG isozyme activity (0.87 ± 0.2 vs. 2.08 ± 0.8, $P<0.01$). This renal tubules damage as well as the glomerular dysfunction effects of NaF was prevented by either PYC or ascorbic acid (fig. 4.10 and 4.11).

4.5. Discussion:

***In vitro* studies:**

In model systems of cultured cells the intracellular release of lysosomal hydrolytic enzymes occurs through damaged lysosomal membranes, and results in the cellular degeneration and death (Brunk and Sevensson, 1999; Li *et al.,* 2000; Brunk *et al.,* 2001; Kurz *et al.,* 2004) is supporting the earlier concept made by de Duve in lat

1960s who has suggested that the release of lysosomal acid haydrolases may jeopardize cellular integrity and has nicknamed lysosomes "suicide bags". So stability of these acidic vacular compartments is very important in maintaining cellular integrity and the search for substances that could protect the membrane of lysosomes against agent causing its alteration is interesting from a physiopathological and pharmacological point of view.

Mannitol, a sugar alcohol, is a specific hydroxyl radical scavenger sugar alcohol, osmotic diuretic agent, which has been shown to reduce the extent of ischaemic injury and improve the function of the myocardium. Mannitol is used to investigate the implication of hydroxyl radicals under pathological and xenobiotic induced oxidative stress (Ambrosio and Flaherty, 1992; Desesso *et al.,* 1994). The hydroxyl radical is the most reactive ROS known and it can react very quickly with almost every type of molecule found in the living cells: sugars, amino acids, phospholipids, DNA bases and organic acids (Halliwell and Gutteridge, 2001). Moreover it has been suggested that neither superoxide radicals nor hydrogen peroxide are by themselves damaging to lysosomes, but the hydroxyl radical (formed by Fenton chemistry) is the damaging radical of lysosomal membrane under peroxidative conditions (Zdolsek and Svensson, 1993). Recently mannitol has been shown to inhibit hepatocyte lysosomal membrane disruptive effect of nitrofurantoin, a free radicals initiator (Pourahmad *et al.,*2001).

In our results shown in fig. 4.1, mannitol was able to protect lysosomes against fluoride induced lysosomal membrane damage and this could be due to its known inhibition of hydroxyl radical formation and the consequent oxidation of various membrane components. Therefore hydroxyl radicals may have a crucial role in fluoride induced lysosomal damage.

Pycnogenol® (PYC), a blend of flavonoids, has been recognised as a potent scavenger of ROS (superoxide, hydroxyl and peroxyl radicals) and RNS (nitric oxide and peroxynitrite radicals) which are the most important free radicals in biologic environment (Packer *et al.,* 1999). Recent animals studies have shown that PYC can inhibit histamine release from mast cells induced by free radical initiators (Sharma *et al.,* 2003a). It has also been suggested that PYC can reduce cell toxicity caused by antitumor drugs (Feng *et al.,* 2002) and protect G6PD (glucose 6 phosphate dehydrogenase) deficient human erythrocyte against haemolytic injury caused by ROS forming agents (Sharma *et al.,* 2003b).

Several plant flavonoids have been indicated to protect lysosomes against oxidative damage by their free radical scavenging activity as well as by a direct action on the lysosomal membrane making it more resistant to oxidative attack (Decharneux *et al.,* 1992). The ability of PYC to form inert complex with redox-active iron may also take part in its protective effect against fluoride induced lysosomal damage (Morel *et al.,* 1993; Yoshino *et al.,* 1998).

Lipids are considered as significant targets of oxidative damage, which consequently lead to the release of cytotoxic lipid peroxidation by product aldehydes, like MDA. Apart from free radicals formation, PYC can also protect biomembranes from the damage caused by MDA (Kim *et al.,* 2000). The plant flavonoids (PYC) have also been observed to inhibit LOX (Packer *et al.,* 1999) and thereby it is possible that PYC may inhibit non-enzymatic lipid peroxidation pathway in our model.

Our results have shown that PYC at concentrations (40-160 µg/ml) efficiently stabilize lysosomal membrane against fluoride insult. This protective ability of PYC may be mediated by one or more of the above discussed mechanisms.

Deferoxamine (DFO). The synergistic damage to cells and tissues, which can be caused by a combination of ROS and redox-active iron, is well appreciated. It has been shown that lysosomes are very vulnerable to oxidative stress due to its high content of redox-active iron and may be ruptured by intralysosomal Fenton-reaction and the associated membrane peroxidation (Persson *et al.,* 2001; Yu *et al.,* 2003).

DFO is an important iron-chelating agent as it has the ability to form inert complex with free iron (ferrioxamine)(Ollinger and Brunk, 1995). Its antioxidant potential is thought to be due to iron chelation and direct ROS scavenging activities (Hoe *et al.,* 1982; Sinaceur *et al.,* 1984; Niihara *et al.,* 2002). DFO has been observed to protect cells from oxidant induced death by chelating intralysosomal iron under *in vitro* conditions (Pourahmad *et al.,* 2002; Yu *et al.,* 2003) whereas its short plasma half life (5-10 min following intravenous injection) and low LD_{50} (300 mg/kg in mice) limited its *in vivo* use (Persson *et al.,* 2003). DFO failed to show any protective activity against fluoride induced lysosomal damage in the present study (fig. 4.3). DFO is a hydrophilic compound and its translocation across plasma membrane to localize exclusively in lysosomes has been strongly suggested to be solely through fluid phase endocytosis and it cannot penetrate the membranes by simple diffusion (Lloyd *et al.,* 1991; Ollinger and Brunk, 1995; Cable *et al.,* 1999; Persson *et al.,* 2003). Therefore the failure of DFO to provide protection against fluoride induced lysosomal damage may be related to its inability to cross-lysosomal membrane in the cell-free system model used in this study.

α-Lipoic (Thioctic) acid (LP) is used as therapeutic agent in a variety of diseases and xenobiotic induced toxicity (Sandhya *et al.,* 1997; Packer *et al.,* 1995) where enhanced free radical peroxidation of membrane phospholipids (at least partly) play an important role in the injury cascade. It has been reported that the antioxidant LP

inhibits lysosomal destabilization induced by oxidants, an effect suggested to be mediated through its iron chelating activity (Persson *et al.,* 2001b). In our study LP acid attenuated the lysosomal destabilization induced by fluoride indicating that phenomenon of lysosomal rupture may be (at least in part) mediated through intralysosomal iron driven Fenton chemistry and the consequent peroxidative membrane damage.

Lysosomes have been identified to contain a variety of lipolytic enzymes, which exhibit optimal activity at acidic pH range (4-5). This includes PLA_1 and PLA_2, PLC, neutral lipids lipases and several other lipases (Beckman *et al.,* 1981). The membrane phospholipids have been shown to hydrolyse by various lysosomal lipases and result in accumulation of lysophospholipids and free fatty acids, which in turn afflict the membrane integrity and permeability properties (Weglicki *et al.,* 1984; Dickens *et al.,* 1988). In addition to their potential role in the modification of membrane phospholipids composition, phospholipases also govern the availability of arachidonic acid, which is released from membrane phospholipids and is utilized for the biosynthesis of highly active oxygenated derivatives by cyclooxygenase and lipoxygenase metabolites. An association between lipid peroxidation and enhanced PLA_2 activity has been demonstrated in various membranes including lysosomal membrane (Madesh and Balasubramanian, 1997; Zhao *et al.,* 2001). This association has suggested to be mediated by direct ROS activation of PLA_2 (Madesh and Balasubramanian, 1997; Zhao *et al.,* 2001). Alternatively, free radical induced membrane lipid physical property changes may enhance lipase activity and consequently increased lytic effect of these radicals (positive feedback) (Weglicki *et al.,* 1984).

While these observations have stressed the detrimental effects of radical-mediated lipid catabolism, others have proposed that PLA_2 and PLC might serve as a repair process by hydrolysing the peroxidized phospholipids and restore the structural and functional integrity to the phospholipid bilayer (van Kuijk *et al.,* 1987; Gamache *et al.,* 1988; Antunes *et al.,* 1995). However the preference of these phospholipases toward the peroxidized phospholipids is still debatable (Halliwell and Gutteridge, 2001; Miyamoto *et al.,* 2003).

Both PLA_2 and PLC activities have reported to be enhanced by fluoride (Wessel *et al.,* 1989; Murao *et al.,* 2000). It has also been observed that fluoride increases the release of arachidonic acid, a substrate of enzymatic lipid peroxidation, from membrane phospholipids (Wessel *et al.,* 1989).

U 73122 is widely used antagonist of phosphoinositide- specific PLC (Smallridge *et al.,* 1992; Feisst *et al.,* 2005); which decreases arachidonic acid release and leukotriene formation (Wang *et al.,* 1984; Salari *et al.,* 1993). In this study U 73122 significantly protected fluoride induced lysosomal damage in a dose-response pattern, whereas no change was observed with equivalent concentrations of the inactive analogue, U 73343. Therefore our results suggest that lysosomal PLC participates in fluoride induced lysosomal damage which might be through direct attack on membrane phospholipids (feedback mechanism) with the consequent increase in lysophospholipids and free fatty acid levels, this may also initiating arachidonate metabolism, enzymatic lipid oxidation, and thereby changing membrane physical properties like membrane integrity and permeability.

The PLA_2 inhibitors, **bromophenacyl bromide (BPB)** and **mepacrine** did not show any protective effect against fluoride induced lysosomal enzyme release in this study.

In contrast, BPB was found to increase fluoride induced lysosomal damage in a dose dependent manner.

It has been reported that **BPB,** at comparable concentrations used in this study, suppressed superoxide generation and inhibits lysosomal enzyme release elicited by chemotatic peptide N-formylmethionylleucylphenylalanine (Smolen and Weissmann, 1980). PLA_2 has been reported completely inhibited by BPB by alkaylation of the Histidine 48 residue of the enzyme. This residue modification has been shown to affect the PLA_2 structure, its membrane binding affinity, and the effects of PLA_2 on the membrane structure (Verheij *et al.,* 1980; Fujii *et al.,* 1998; Tatulian, 2003). The most interesting, is that the BPB induced modification of the enzyme structure is a pH dependent reaction, in which it is almost absent at acidic pH (5.0) and reach its highest activity at ~ pH 7.5. This pH dependent inactivation of PLA_2 has been related to the pKa value of the amino acid histidine (7.53), which participates in the BPB reaction, and the BPB can react with the deprotonated histidine but not the protonated (Miyake *et al.,* 1989; Fujii *et al.,* 1998). However, this reagent has been found to destroy the membrane sulphydryl groups, interact with other amino acid residues and has the ability to disrupt platelets (Hofmann *et al.,* 1982; Kyger and Franson, 1984; Pintado *et al.,* 1995). Therefore, the possibility of PLA_2 inhibition by BPB may be not feasible in our model (pH 5.0) and may explain the non-effectiveness of BPB. Since fluoride has been suggested as a membrane-breaking agent and inhibiting Ser/Thr phosphatase (Wang *et al.,* 2001), it is likely that the non-specific interactions of BPB with amino acids and its deleterious effect on membrane sulphydryl groups might sensitise the lysosomal membrane and thereby potentiated fluoride induced lysosomal damage. Moreover, supporting this hypothesis, we have found in preliminary exploratory that BPB at higher than concentrations has the ability to induce lysosomal

damage. Mepacrine is a well-known inhibitor of PLA_2 and has been used to assess the physiological and pharmacological importance of this enzyme. Mepacrine has also been reported to have both inhibitory effect (Hofmann *et al.,* 1982) and biphasic effects (Authi and Traynor, 1982; Chan *et al.,* 1982) on PLA_2. Therefore, the inability of mepacrine to modulate fluoride effects in our study may be due to its dual effect on PLA_2 activity.

The results in this study show a potential role for PLC in fluoride induced lysosomal damage while further work is needed to explore the role of PLA_2 by measuring the enzyme activity, modifying some of the experimental conditions like media pH or the use of other enzyme inhibitors.

Oxygenases-catalysed PUFAs peroxidation and their eicosanoid products, have potential roles in some pathological conditions (Janero *et al.,* 1989). It has also been reported that the arachidonic acid mobilization in the activated neutrophil is associated with phospholipid degradation, and the consequent lysophospholipid formation might mediate lysosomal enzyme release (Lindahl *et al.,* 1988). Fluoride has been shown to elicit the arachidonic acid and eicosanoid formation (Brom *et al.,* 1989 Schulze-Specking *et al.,* 1991).

Compounds, which inhibit these oxygenases, and consequently attenuate production of fatty-peroxide eicosanoid precursors, have a wide pharmacological interest. **Indomethacin** a specific COX_1 and COX_2 inhibitor (Coceani *et al.,* 2005) and **Nordihydroguaiaretic acid (NDGA)** a general LOX inhibitor (Tang and Honn, 1997) have the ability to intervene in the arachidonic acid metabolism, inhibiting formation of eicosanoids and suppressing ROS generation (Smolen and Weissmann, 1980; Kanner *et al.,* 1987; Bell *et al.,* 1991).

The data in this study indicate that pre incubation of renal lysosomes with indomethacin or NDGA resulted in a dose dependent diminution of lysosomal NAG, release induced by fluoride.

These results give further evidence to previous reports which have indicated that both indomethacin and NDGA, can stabilize the lysosomes under conditions in which arachidonic acid metabolism, eicosanoid formation and ROS are implicated in lysosome destabilization (Smolen and Weissmann, 1980; Lindahl *et al.,* 1988; Agha and Gad, 1995). Therefore our findings suggested that arachidonate metabolism *via* LOX and COX pathways are implicated in fluoride induced NAG isozyme release.

***In vivo* studies:**

Antioxidants and antioxidant enzymes protect living organisms against the attack from ROS. An adequate daily intake of the individual antioxidants is therefore important to protect the cells against oxidative damage (Nagyova *et al.,* 2004). Ascorbic acid is a potent reducing agent and has the ability to scavenge free radicals (Evans and Halliwell, 2001; Whiteman *et al.,* 2003).

Ascorbic acid, similar to the dose that we used (or higher) has been found to reduce resuscitation fluid volume requirements as well as severity of respiratory dysfunction in thermally injured patients (Tanaka *et al.,* 2000) and has also been found to reduce kidney enlargement, reduce glomerular volume and reduce albumin clearance in diabetic rats (Craven *et al.,* 1997) and many other pathological as well as xenobiotic induced toxic effects in animals in which free radicals has been incriminated (Rabl *et al.,* 1993; Wang and Salahudeen, 1995). Ascorbate has been shown to have a pro-oxidant activity in the presence of transition metal ions *in vitro*, in which it has been

shown to reduce metal ions (like ferric to ferrous) which drives the production of the most serious ROS ($OH^{\bullet}$) *via* the so-called Fenton reaction and formation of dehydroascobate, lipid pro-oxidant (Miller and Aust, 1989; Song*et al.,* 2001). However, the *in vitro* pro-oxidant activity of ascorbate is unlikely relevant to the *in vivo* situations, even in the presence of iron overload (Chen *et al.,* 2000; Proteggente *et al.,* 2000). Ascorbate has also been reported to augment the pro-oxidant activity of fluoride under *in vitro* conditions (Kundu and Hallinan 1995). Based on these reports, we assessed the protective ability of ascorbate against fluoride induced renal failure *in vivo* along with PYC. Since both ascorbic acid and PYC are natural antioxidants and can be derived from food, therefore it is worthwhile evaluating the potential for ascorbic acid and PYC to prevent fluoride induced renal toxicity. The present study clearly shows that administration of PYC or ascorbic acid with NaF significantly ameliorated fluoride induced renal tubular damage; decreased urinary NAG isozyme level, and improved glomerular function (increased urinary CR excretion). This withstanding ability shown in animals treated with ascorbic acid or PYC may be related to their free radical scavenging activity and inhibition of oxidative stress perturbation is consistent with our *in vitro* studies, which have shown that PYC and other antioxidants can stabilize renal lysosomes against fluoride insult. It is also noteworthy that the capability of ascorbic acid to resist fluoride induced renal toxicity is consistent with recent reports that have observed that ascorbic acid can ameliorate fluoride induced embryotoxicity (Verma and Sherlin, 2002) and can reverse fluoride induced damage to reproductive organs (Chinoy *et al.,* 2001) and also, liver and gastrocnemius muscle toxicity (Chinoy *et al.,* 1993). The present study strongly suggests that ascorbic acid and PYC may have a potential as therapeutic agents to protect against fluoride induced renal toxicity.

4.6. Conclusions:

The results of this study indicate that both enzymatic and non-enzymatic lipid peroxidation pathways are implicated in fluoride induced lysosomal damage and also explored that at least lysosomal PLC is participated in fluoride lysosomal damage through a feed back mechanism, while the role of PLA_2 need further work to be confirmed or ruled out. We concluded that fluoride induced renal lysosomal damage is amenable to a blockade by free radical scavengers (PYC, mannitol), iron sequestering agent (LP) and enzymatic lipid peroxidation inhibitors (indomethacin and NDGA). This protective activity of antioxidants against fluoride induced lysosomal destabilization effect gives further evidence and support our previous suggestion that free radicals play a major in the development of fluoride induced lysosomal destabilization.

We have also explored in the *in vivo* study that natural antioxidants (PYC and ascorbic acid) efficiently attenuated fluoride induced acute nephrotoxicity, which raises the significant role of dietary antioxidants in the amelioration of general fluorosis in people continuously exposed to high fluoride concentrations.

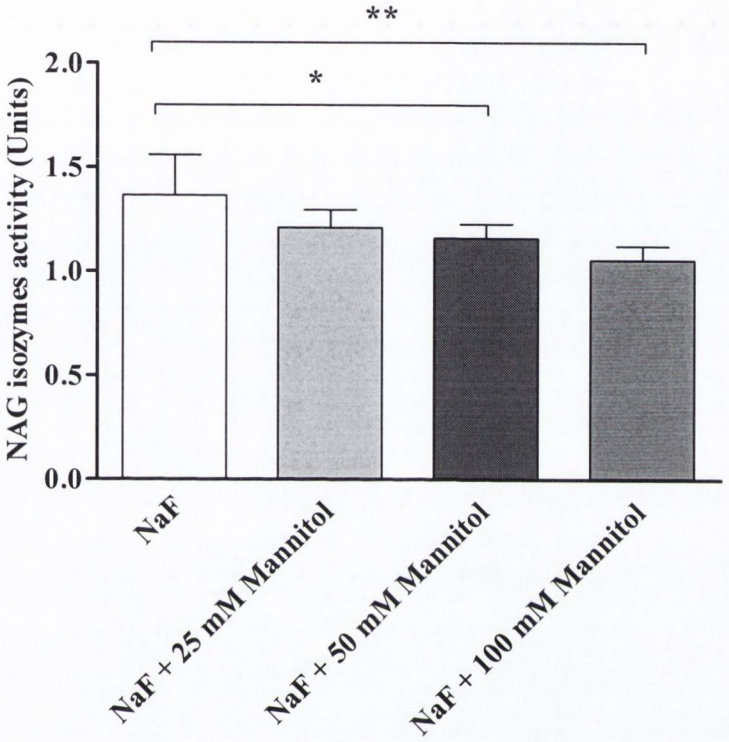


Fig. 4.1. The effect of Mannitol on NaF (48 mM) induced NAG isozymes release from rat kidney lysosomes in vitro. Mean ± SD, n= 6 * $P<0.05$, ** $P<0.01$ compared to 48 mM NaF.

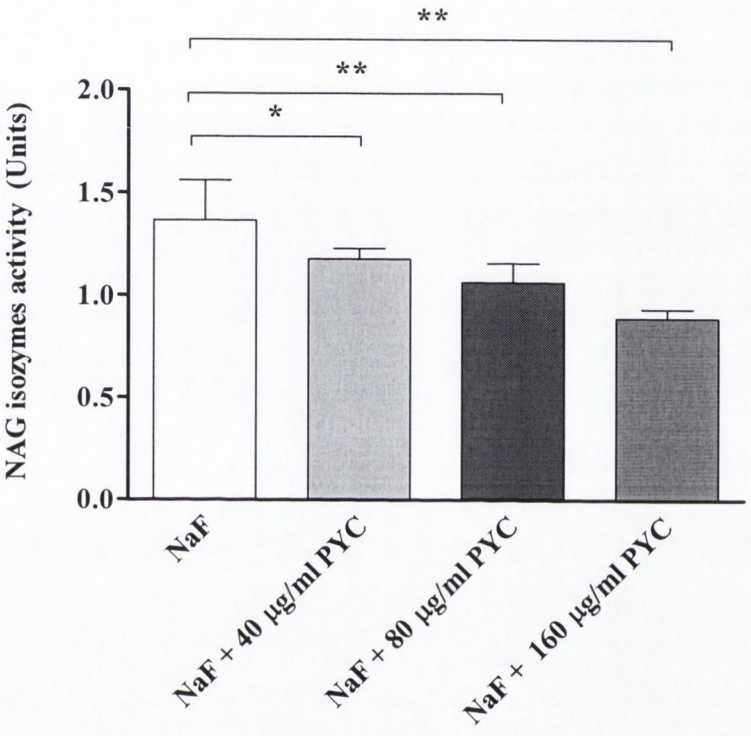


Fig. 4.2. The effect of Pycnogenol® (PYC) on NaF (48 mM) induced NAG isozymes release from rat kidney lysosomes in vitro. Mean ± SD, n= 6, *P<0.05, **P<0.01, compared to 48 mM NaF.

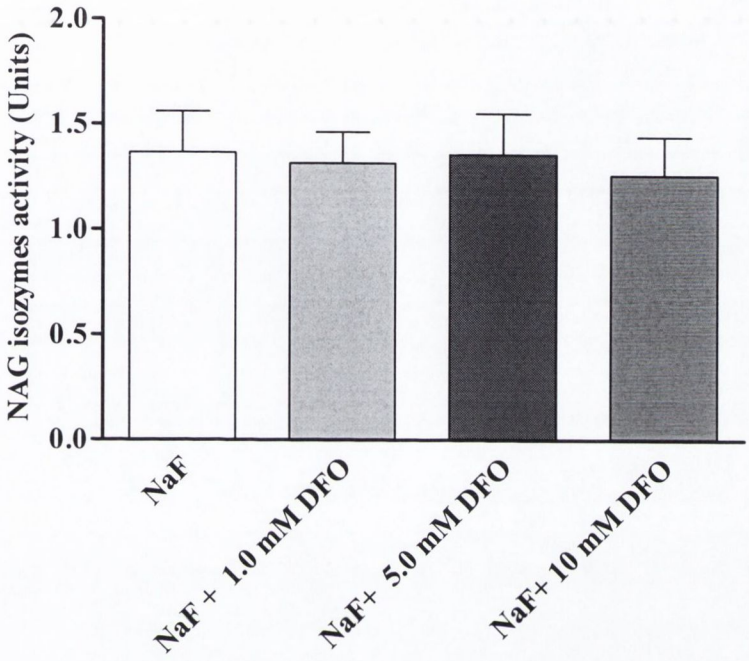


Fig. 4.3. The effect of deferoxamine (DFO) on NaF (48 mM) induced NAG isozymes release from rat kidney lysosomes in vitro. Mean ± SD, n= 6.

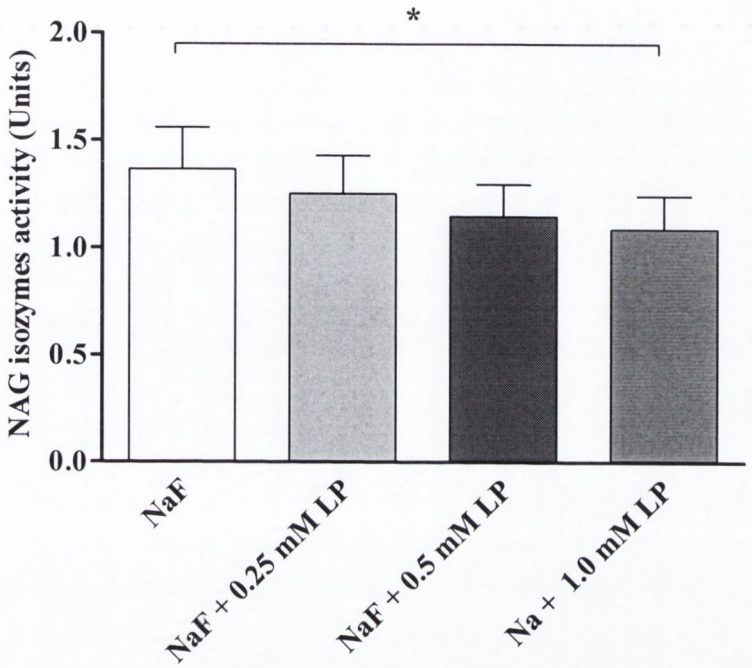


Fig. 4.4. The effect of α-Lipoic acid (LP) on NaF (48 mM) induced NAG isozymes release from rat kidney lysosomes in vitro. Mean ± SD, n= 6, * $P<0.05$ compared to 48 mM NaF.

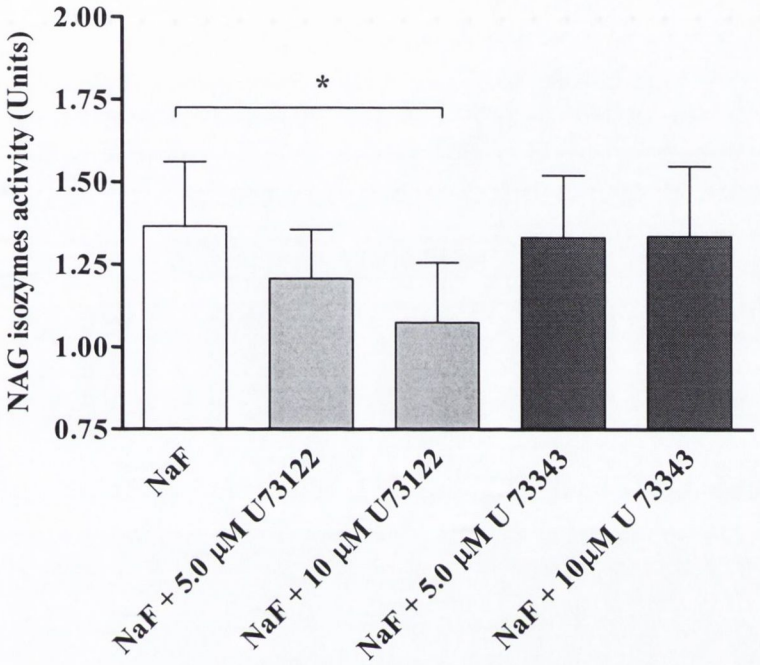


Fig. 4.5. The effect of PL C inhibitor (U 73122) and its inactive analog (U 73343) on NaF (48 mM) induced NAG isozymes release from rat kidney lysosomes in vitro. Mean ± SD, n= 6, * $P<0.05$ compared to 48 mM NaF.

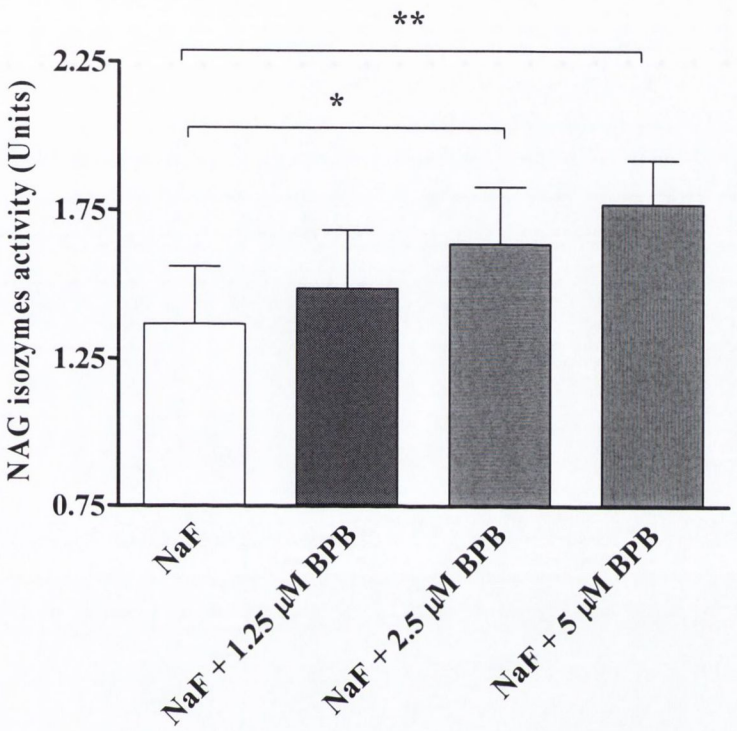


Fig. 4.6. The effect of bromophenacyl bromide(BPB) on NaF (48 mM) induced NAG isozymes release from rat kidney lysosomes in vitro. Mean ± SD n=6, * $P<0.05$, ** $P<0.01$ compared to 48 mM NaF.

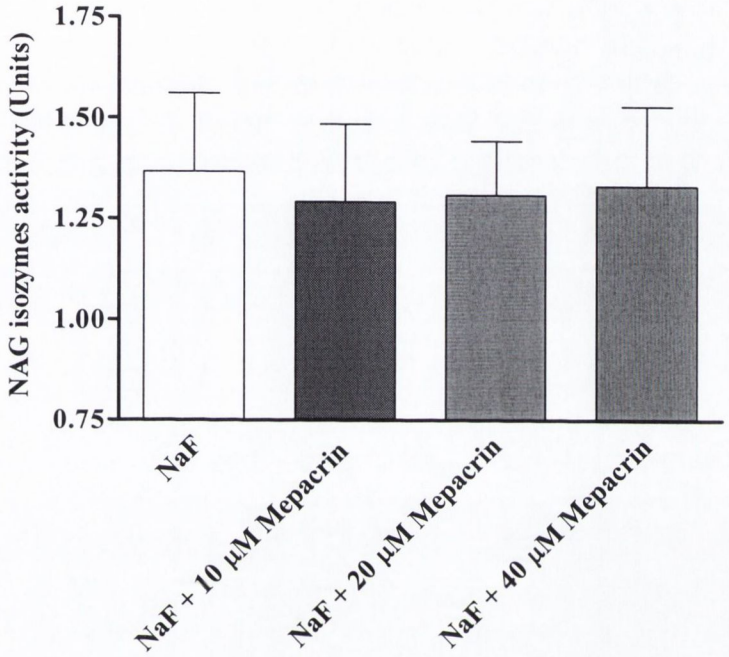


Fig. 4.7. The effect of mepacrine on NaF (48 mM) induced NAG isozymes release from rat kidney lysosomes in vitro. Mean ± SD, n=6.

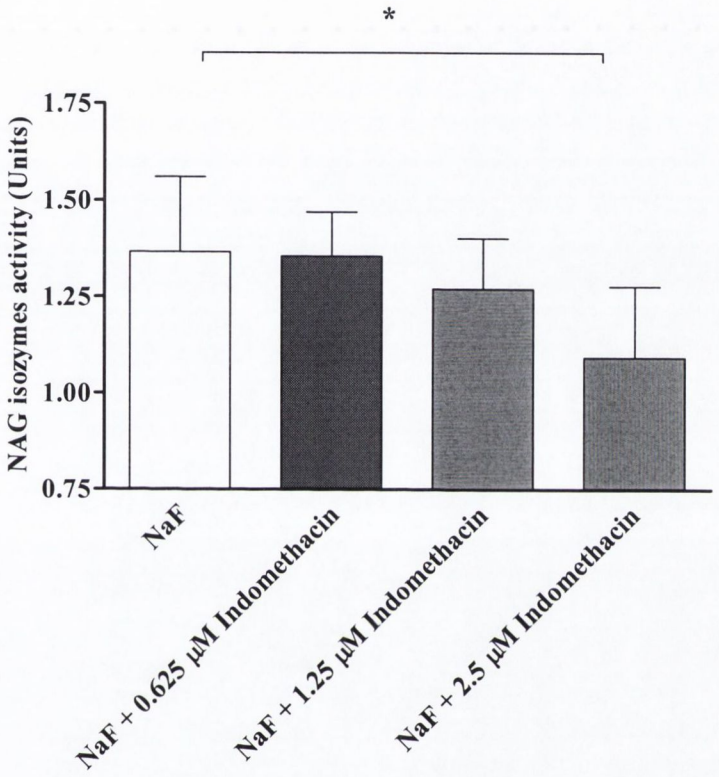


Fig. 4.8. The effect of indomethacin on NaF (48 mM) induced NAG isozymes release from rat kidney lysosomes in vitro. Mean ± SD, n=6, * $P<0.05$ compared to 48 mM NaF.

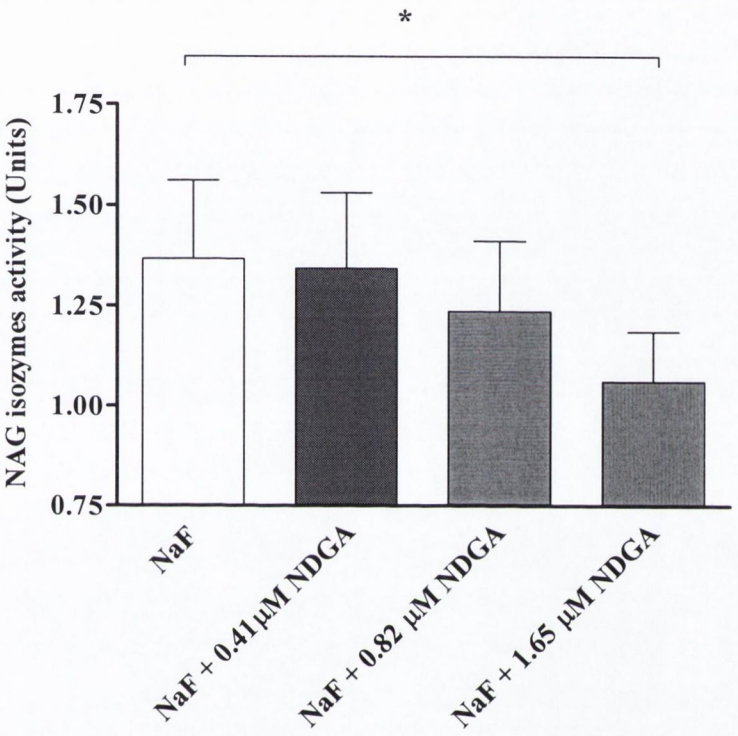


Fig. 4.9. The effect of NDGA on NaF (48 mM) induced NAG isozymes release from rat kidney lysosomes in vitro. Mean ± SD, n=6, * $P<0.05$ compared with 48 mM NaF.

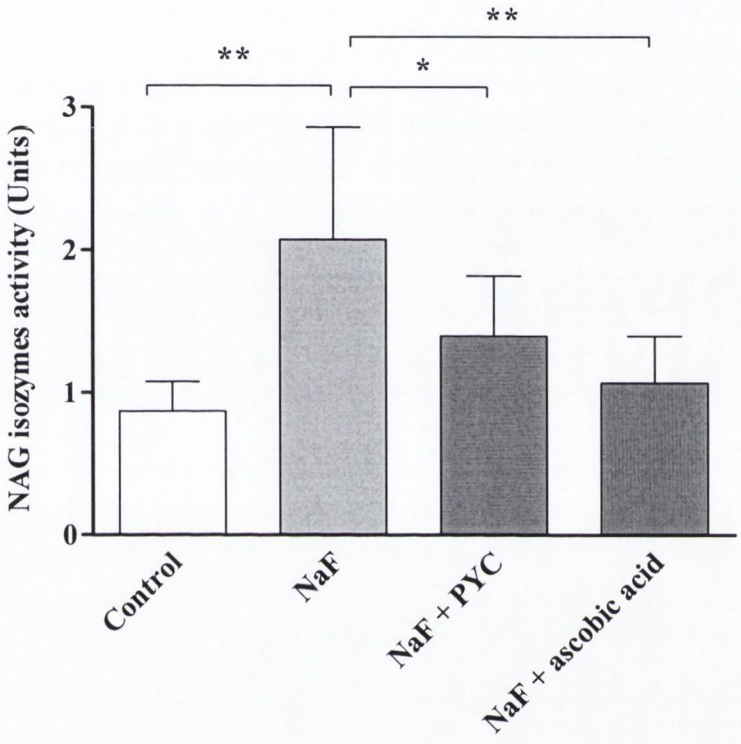


Fig. 4.10. Urinary NAG isozymes level 24 hr following NaF (25-mg/kg i.p) with and with out PYC (25 mg/kg three times) or ascorbic acid (250 mg/kg three times). Mean ± SD, n= 8, *P<0.05, **P<0.01 compared to NaF treated group.

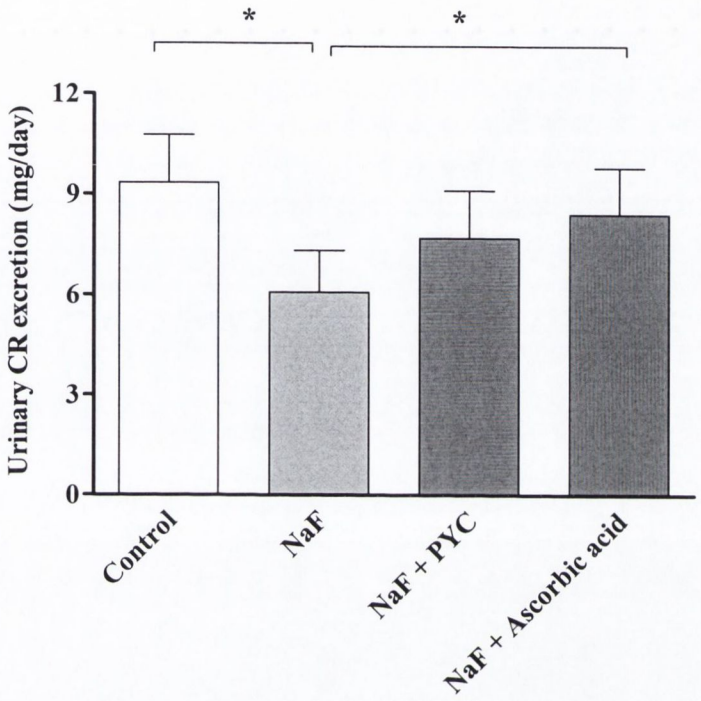


Fig. 4.11. A 24 hr urinary CR excretion following NaF (25-mg/kg i.p) with and with out PYC (25 mg/kg three times) or ascorbic acid (250 mg/kg three times). Mean ± SD, n= 8, *P<0.05 compared to NaF treated group.

CHAPTER FIVE

THE EFFECT OF PROLONGED HIGH FLUORIDE INTAKE ON KIDNEY CELLS SENSITIVITY

5.1. Introduction:

Fluoride is cytotoxic to cells *in vitro*, causing inhibition of cell growth and cell death (Holland *et al.,* 1980). However stepwise increases in fluoride concentration, can adapt cells to grow at concentrations, which kill normal cells (Holland and Hongslo, 1978a & b; Mankovitz *et al.,* 1978). This fluoride resistance persists even after removal of fluoride (Hongslo *et al.,* 1974). Furthermore Sato *et al.,* (1986) have observed that cells incubated in the presence of increasing fluoride concentration gradually to a final concentration that completely inhibits growth, has some degree of fluoride resistance. Decreased fluoride sensitivity was also observed in liver cells isolated from rats chronically treated with high fluoride in their drinking water (Hongslo *et al.,* 1980).

It has also been reported that human proximal tubular cells when cultured with sublethal concentrations of NaF can acutely increase cell resistance to superimposed nephrotoxic attack by myoglobin (Zager and Iwata, 1997). Moreover, data from *in vivo* studies have shown that isoflurane (a fluorinated anaesthetic) acts during early reperfusion after prolonged ischemia to salvage myocardium from infarction and reduces the threshold of ischemic postconditioning (Ludwig *et al.,* 2004; Chiari *et al.,* 2005). The *in vivo* relevance of such fluoride induced cell insensitivity has not been shown. Gentamicin is polycation and highly polar aminoglycoside antibiotic that is still commonly used in the treatment of life-threatening infections. The broad-spectrum activity of aminoglycosides against aerobic gram +ve and gram –ve organisms, chemical stability, and rapid bactericidal action has often made them first-line drugs in a variety of clinical situations (Siegenthaler *et al.,* 1986; Appel, 1990). However, its clinical use is limited by its nephrotoxicity. In some cases this side

effect is so severe that the use of the drug must be discontinued. It has been estimated that up to 30% patients treated with aminoglycosides for more than 7 days show some signs of nephrotoxicity (Mathew, 1992). Acute renal failure due to gentamicin is accompanied either with oliguria (Gordillo *et al.,* 1981) or polyuria (Erden *et al.,* 2000).

Gentamicin is incorporated and accumulated in proximal tubule lysosomes, and has the ability to release NAG isozymes from renal cells (Pedraza *et al.,* 2000).

Fluoride given to mammals is mostly concentrated in two organ and tissues: in the kidneys for excretion and in mineralised tissues where it is bound to the mineral phase (Whitford, 1996). The kidney tissue-to-plasma fluoride ratio, which is well above unity, represents a major exception to most soft tissue-plasma ratio (Whitford *et al.,* 1979).

5.2. Aim of study:

We have undertaken this study to assess if rat kidney cell sensitivity to superimposed nephrotoxin, gentamicin, is modulated by chronic high fluoride intake ***in vivo***. This study is also designed to investigate whether fluoride induced renal toxicity is reversible under continuous exposure to high fluoride. We have also investigated the mechanism of how kidney cells acquired resistance from the perspective of lipid peroxidation and altered body's antioxidants level.

5.3. Experimental protocols:

Protocol 1:

Three groups of male Wistar rats, 6 in each group were used, in which the first group drank deionised distilled water (control group) and the second and third groups received 30 and 100 ppm NaF respectively in their drinking water for 10 months. All the animals were fed a standard pellet diet and given drinking water ad libitum. Animals were separated in metabolic cages and challenged with 50 -mg/kg *i.p*, gentamicin (Franklin Pharmaceuticals) for 10 consecutive days. A 24-hourly urine samples were collected starting a day before the first gentamicin dose to obtain urine volume and NAG isozymes baseline values, and continued for a period of 10 days. The last urine sample was collected 24 hr after last dose of gentamicin. After urine volume determination these sample were analysed for NAG isozymes as describe in methods (section 2.2).

The results were statistically analysed by two way repeated measures analysis of variance (ANOVA) and for multiple comparisons with Bonferroni correction. The level of significant difference was taken as $P < 0.05$.

Protocol 2.

Preliminary experiments revealed that 25 mg/kg of NaF given *i.p* was well tolerated by rats (no death has been recorded and rapid recovery from enzymuria) and gentamicin 50 mg/kg *i.p* (used in the first protocol) did not show enzymeuria during

12 days of continuous administration. The dose of gentamicin in this protocol was therefore doubled to100 mg/kg *i.p.*

Thirty male Wistar rats (three months old weighing 220-275 g) were randomly allocated into five groups (one control + 4 experimental) of six rats each. The animals were fed a routine solid diet and had tap water ad libidum. All animals were separated in metabolic cages and 24-hr urine samples were collected to obtain baseline value of urine volume and its content of NAG isozymes and then subjected to different treatment (summarised in table 5.1) as follow:

First stage: Three groups (group A, B, and C) of the test groups were given single injection of NaF (25 mg /kg *i.p*) while group D and control group (E) received only equivalent volume of saline (0.5 ml) for 15 consecutive days. Twenty-four hourly urine samples were collected on every alternative day and urine volume and its NAG isozyme content were determined. At the end of this stage blood samples (1.5 ml) were collected under general anaesthesia and processed as mentioned before. Plasma MDA GSH, and creatinine level were determined by the methods described in chapter three (sections 2.4, 2.7 and 2.8).

Second stage: during the second stage, test groups were subjected to different treatments as following:

Group A; continuously received the same dose of NaF for further 12 days and at the same time challenged with daily100 mg /kg gentamicin *i.p* for the same period given one hour after NaF dose.

Group B; This group continuously received the same dose of NaF for further 12 days with out gentamicin challenge.

Group C; Daily NaF dosing was discontinued and substituted with gentamicin 100 mg / kg *i.p* daily for 12 consecutive days.

Group D; The animals of this group were challenged with daily gentamicin (100 mg/kg *i.p*) for the same period.

Group E (control); The daily saline was continued for further 12 days.

Table 5.1. The duration and type of treatment of different animal groups in experimental protocol 2. The doses of NaF and gentamicin are (25 mg/kg *i.p*) and (100 mg/kg *i.p*) respectively.

Group	First stage (day 0 - 15)	Second stage (day 15 - 27)
A	NaF	NaF + gentamicin
B	NaF	NaF
C	NaF	Gentamicin
D	Saline	Gentamicin
E	Saline	Saline

Twenty-four hour urine samples were collected in every alternative day, during the first and the second stages of this experiment, so that rats spent one day in the metabolic cage and one day in the collection cage (6 in each cage). Urine volume and NAG isozyme activity were determined in fresh samples each time as described in methods (section 2.2).

After the last urine sample had been collected, blood sample was collected and plasma MDA, ascorbic acid, GSH, and CR, were determined as mentioned in methods (sections 2.4, 2.6, 2.7 and 2.8). The animals were then killed by cervical dislocation and both kidneys were resected. The left kidney was fixed in 10% formalin for histological examination (Hematoxylin and Eosin staining) described in methods (section 2.9), and the right kidney homogenised in 0.3 M ice-cold sucrose (1: 5 w/v) containing 1 mM EDTA and centrifuged at 9000 g for 15 min at 4 °C. The supernatant was used for the determination of ascorbic acid, GSH, and MDA as described in the methods section (2.4, 2.6 and 2.7). All the tissue measurements were expressed in terms of the protein concentration.

Data were statistically evaluated by two way repeated measures ANOVA for multiple comparisons with Bonferroni correction. One-way ANOVA was also used to evaluate the effect of fluoride and gentamicin on blood and tissue biochemical and histological markers. The level of significant difference was taken as $P < 0.05$.

5.4. Results:

First protocol: The results in table 5.2 show that, there is no significant change in urine volume in all groups after gentamicin treatment. NAG isozymes responses to gentamicin in the groups treated with fluoride were markedly different from the animals in the control group (fig. 5.1). There is rise in NAG isozyme values within the first two days in the first and third groups (control and 100 ppm NaF) and it was more sever in the control group. It reached the peak levels on day 7 and 8 in the control group (4.4 ± 3.6 and 5.2 ± 3.4 units), whereas the third group peak level was on day 6 (3.2 ± 2.3 units) for the 100 ppm NaF. Urinary NAG isozymes level of the

second group (30 ppm NaF) was remained unchanged. This rise in the NAG isozymes level return back to normal despite the continuous administration of gentamicin.

Second protocol: The results in fig. 5.2 to 5.6 show that the effect of NaF and gentamicin on urine volume and its NAG isozymes content. **In the first stage**, there is significant rise in urinary NAG isozymes in all groups treated with NaF (group A, B and C, $P<0.01$, fig. 5.3, 5.4 and 5.5) on day one, which is returned to normal values by day 5 of continuous NaF treatment. There is also rise in urine volume (polyuria) on the first day of NaF treatment and it was significant on day 3 ($P<0.05$). This polyuria was also reversed to non-significant level by day 5 despite the continuous administration of NaF.

At the end of the first stage (day 15), there is approximately 50% increase in plasma GSH level in groups treated with NaF (table 5.3, $P<0.01$) compared to the control group while the plasma MDA and creatinine level remained unchanged. **In the second stage,** the response to gentamicin treatment for 12 days was markedly different between groups pre-treated with NaF and non-treated groups. There is significant increase in urinary NAG isozymes activity in the group A and D after day 7 of the second stage (fig. 5.3 and 5.6, $P<0.05$ and $P<0.01$). The NAG isozyme activity and urine volume in the control group (E) and other groups (B and C) were remained unchanged (fig. 5.2, 5.4 and 5.5). Plasma CR level is significantly increased only in-group A and D and the other groups CR level was normal in comparison to the control group (table 5.4). Plasma as well as renal tissue GSH and MDA concentrations after 27 days of various treatments did not show significant changes. Plasma and kidney tissue ascorbic acid, was increased only in-group A and B (table 5.3 and 5.4, $P<0.01$). This enhanced ascorbic acid level was observed only in animals

with continuous administration with NaF (group A and B). Histological analysis (fig. 5.7 and plates 5.1 to 5.5) of renal tubular cells shows significant increase in the percentage of tubular epithelial cells in groups treated with either gentamicin only (group D) and group-A, which received concomitant treatments of fluoride and gentamicin (36.7 ± 3.6 and 39.1± 3.6 respectively, $P<0.001$) compared to control. While the other groups, which either received, a continuous administration of NaF (group B) and group C, which treated with NaF for two weeks and then followed with gentamicin further 12 days did not show changes compared the control (group E).

5.5. Discussion:

In the first protocol of this study, 50-mg/kg gentamicin caused a successful induction of renal damage as manifested by high enzymeuria in animals of nearly one year old. However in the preliminary experiments of second protocol conducted on animals of the same species and sex and of about 3 months age, this dose of gentamicin failed to induce enzymeuria after twelve days of continuous administration. It is generally accepted that advanced age is associated with significantly higher incidence of gentamicin and other aminoglycosides induced nephrotoxicity in both man and animals (Bauer and Blouin, 1983; Kojima *et al.,* 1984; Provoost *et al.,* 1985; Ali, 1995). However the mechanism for this age increase in nephrotoxicity is not completely understood. In addition to gentamicin, a decreased susceptibility of kidney of the young to drug induced nephrotoxicity has been reported with several other drugs like salicylates, acetaminophen and cephalosporins (*reviewed by* Beauchamp *et al.,* 1992). The lower susceptibility of young animals to gentamicin nephrotoxicity has been suggested due to its lower accumulation in renal cortex

compared with aged animals (Provoost *et al.,* 1985). In contrast, it has been shown that the elimination of gentamicin is independent on age (El-Sayed and Islam, 1989) and also the kidney cortical concentration of the antibiotic may not always be relevant to nephrotoxicity, as some nephroprotectant agents. For instance some polyanions and fish oil have been shown not to affect, or (sometimes) even increase the accumulation of gentamicin in the renal cortex (Ali, 1995). Moreover, Valdivielso and co-worker (1996) have observed that glomerular nitrite production is higher in the young than aging animals whereas no differences in cortical gentamicin concentration were observed between young and old animals. They have also suggested that the higher nitrite production in young animals may exert a protective role against gentamicin induced renal failure by compensating the vasoconstrictor substances released during an acute renal failure. Therefore the difference in animal age might explain the reduced sensitivity of animals in second protocol (as aged animals were used in the first protocol) and thereby we have doubled the dose of gentamicin (100 mg/kg) in the second protocol of this study.

Our results presented in chapter 1 have shown that animals given high fluoride concentrations (30 ppm and 100 ppm) for 10 months did not show any changes in both urine volume and its NAG isozymes content at time tested. Moreover the results in this study revealed that kidney cells of this chronically high fluoride fed animals developed resistance against gentamicin nephrotoxicity manifested by less enzymuria in comparison with animals had no fluoride (0.0 ppm) in drinking water (protocol 1). Furthermore the results of this study (second protocol) show that pretreatment of animals with acute NaF (25 mg/kg) for 15 days were more resistant to the subsequent gentamicin challenge (indicated by less enzymuria and renal histology) than control animals, which had saline for the same period of time. However the manifestation of

nephrotoxicity (in both protocols) caused by gentamicin was transient and their urine volume generally remained unchanged. The concomitant administration of both gentamicin and fluoride (protocol 2) did not alter gentamicin nephrotoxicity (no protection) and this might be related to the inability of kidney cells to withstand high dose of two nephrotoxins given simultaneously.

All the earlier reports that have shown cellular resistance development to high fluoride concentrations or to other insults (like myoglobin) were came from *in vitro* observations (Holland and Hongslo, 1978a & b; Hongslo *et al.,* 1980; Zager and Iwata, 1997). Of greater importance are our observations of the apparent cross-resistance of fluoride-adapted rats (protocol 1 and 2) to the nephrotoxic effect of gentamicin ***in vivo***.

The hypothesis made by Holland and Hongslo, (1978a) that fluoride resistant cells have the ability to exclude fluoride from intracellular milieu to maintain their vital functions, e.g. glycolysis, does not explain our present findings. Gentamicin is a polyamino-antibiotic of high molecular weight and fluoride is a simple halogenated compound of low molecular weight (FW 18.99) and there is no similarity in structure of these compounds to account for the mechanism of cross-resistance. Moreover, Decorti and his colleagues (1999) have observed that fluoride increases gentamicin endocytosis. Furthermore, the suggestion made by Zager and Iwata, (1997) that fluoride protects proximal tubular cells against myoglobin, *in vitro*, may be mediated by depletion of cytosolic PLA_2, after initial increase, this also does not explain our findings, since gentamicin induced nephrotoxicity is believed (at least in part) mediated by inhibition of PLA_2 and PLC and the consequent lysosomal phospholipidosis (Kacew, 1987; Soejima *et al.,* 1998; Carrier *et al.,* 1998), a mechanism that might enhance (and not reduce) gentamicin nephrotoxicity.

Our previous results (chapter 1) and of others (Elfrink, 1981; Guan *et al.,* 2000; Liu *et al.,* 2003) have shown that fluoride has the ability to increase the ROS generation and induce lipid peroxidation cascad. Gentamicin induced nephrotoxicity has been also attributed to the excessive production of ROS (Walker and Shah, 1988; Sha and Schacht, 1999; Cuzzocrea *et al.,* 2002). Thus ROS and the consequent lipid peroxidation is implicated in both fluoride and gentamicin deleterious effects. It has been reported that cells when exposed oxidant insult can up regulate the synthesis of antioxidant defence systems to restore oxidant/antioxidant balance (van der valk *et al.,* 1985; Soejima *et al.,* 1998). Our results of this study have revealed that antioxidants like ascorbic acid and GSH level in plasma and kidney tissue was elevated following fluoride prolonged treatment (protocol 2). This adapted antioxidant systems observed in our study was consistent with previous reports (Shivarajashankara *et al.,* 2001a & b; Shivashankara, *et al.,* 2002) who have observed that prolonged fluoride ingestion can adapt some antioxidant defence systems (GSH, GSHPx and ascorbic acid), while others have shown that fluoride has the ability to increase ascorbic acid synthesis and mobilization in animals (Chinoy *et al.,* 1993). Therefore we suggest that this enhanced antioxidants defence systems in fluoride adapted rats may counteract the subsequent gentamicin induced ROS challenge and thereby develop such kidney cells resistance to gentamicin nephrotoxicity. This suggestion is consistent with previous reports, indicated that recovered animals from prior acute renal failure induced by gentamicin or other nephrotoxins of known pro-oxidant profile (spermine and gossypol) develop cross-resistance against the subsequent gentamicin induced-nephrotoxicity (Elliott *et al.,* 1982; Jennings and Reidenberg, 1988; Soejima *et al.,* 1998), so that the acquired kidney cells insensitivity, observed in our study is not a unique feature of fluoride. Earlier reports

and our results presented in the first chapter of this thesis have shown that fluoride renal toxicity is transient and reversible by fluoride withdraw. The results of this study have explored that fluoride induced renal toxicity is also transient and reversible despite the continuous administration of fluoride (protocol 2) and this renal toxicity reversibility may be also related to the ability of fluoride to adapt body's antioxidant defence systems.

In addition, to our suggestion that adaptation of antioxidant defence system after the fluoride exposure may have a potential role in the development of such acquired insensitivity against gentamicin nephrotoxicity, other possible mechanisms that might be participated cannot be ruled out. Although the observations came from previous *in vitro* study have shown that fluoride did not inhibit gentamicin cell-delivery, which is taken up by adsorptive endocytosis, but rather fluoride may enhance gentamicin endocytosis (Decorti *et al.,* 1999), the *in vivo* relevance of this finding is still un known. Nevertheless, this observation may not rule out the possibility of kinetic interaction between fluoride and gentamicin *in vivo*. The adsorptive endocytosis of gentamicin is not inhibited by fluoride but the fusion of the endocytosed vesicle, endosome, with the primary lysosomes is ATP-dependent process (Schmid *et al.,* 1998) and fluoride has been shown to deplete cellular ATP (Lochhead *et al.,* 1997). Therefore it is possible that fluoride did not inhibit gentamicin endocytosis but it might inhibit its accumulation in the lysosome. Moreover, addition of fluoride to cells has been shown to cause a prompt increase in cytosolic Ca^{+2} concentration that has been suggested due to an increase in intracellular Ca^{+2} release, and trans-membrane Ca^{+2} influx (Dominguez *et al.,* 1991; Murao *et al.,* 2000). Furthermore chronic high fluoridation has been shown to decease renal cells Ca^{+2} efflux as well as intracellular organelles, endoplasmic reticulum, sequestration of calcium by inhibition

of Ca^{+2} pump proteins (Borke and Whitford 1999). Recent reports have indicated that Ca^{+2} loading attenuate renal histological as well as biochemical disorders induced by gentamicin and this nephroprotectant effect suggested to be mediated by competitive displacement of the antibiotic from binding sites on plasma and organelle membranes (Humes *et al.,* 1984; Ali *et al.,* 2002).

The opposing effects of fluoride and gentamicin on PLC and PLA_2 may form the basis of another mechanism by which fluoride counteracts gentamicin-induced nephrotoxicity. Many researchers have suggested that (at least in part) gentamicin nephrotoxicity might be due to its initial inhibitory action on lysosomal PLC and PLA (Hostetler and Hall, 1982; Chatterjee, 1987; Soejima *et al.,* 1998). Such an action will result in the accumulation of phospholipids (phospholipidosis) in lysosomes. In contrast however fluoride has the ability to activate these two phospholipase enzymes (Stasi *et al.,* 1992; Murao *et al.,* 2000), which may be due to activation of G- proteins (Jeremy and Dandona, 1987), since these proteins have the ability to enhance PLC activity (Hodson *et al.,* 1999). Alternatively it might be a consequence of the lipid peroxidation suggested mechanism that leads to the formation of lipid peroxide by-products, which have the ability to increase PL C and PLA_2 activities (Rossi *et al.,* 2001).

Further work is needed to explore permanence and other tissue response to such an adaptive mechanism induced by fluoride. Clinical investigation also needed to clarify the effect of such an adaptive mechanism on the antibacterial activity of gentamicin.

5.6. Conclusions:

We concluded that the decreased gentamicin toxicity observed in rat chronically exposed to high fluoride concentrations (30 ppm and 100 ppm) in drinking water for 10 months; indicate that kidney cells *in vivo* are affected by this fluoride dosage, necessitating such an adjusted insensitivity.

We have also concluded that fluoride induced nephrotoxicity is reversible despite the continuous exposure to fluoride and kidney cells of animals recovered from acute renal failure induced by fluoride may acquire insensitivity toward superimposed nephrotoxic drug, gentamicin. Adaptation of body's antioxidant systems and other discussed possible mechanisms may play an important role in fluoride-developed kidney cells insensitivity.

Table 5.2. Daily excretion of urine in untreated and fluoride (30 and 100 ppm) treated rats for 10 months following a challenge with gentamicin (50 mg/kg i.p) for ten consecutive days.

Treatment	**NaF (ppm)**		
Day	**0.00**	**30**	**100**
0	9.67 ± 2.5	9.42 ± 2.9	9.58 ± 3.7
1	5.17 ± 1.0	8.83 ± 2.5	7.50 ± 3.2
2	3.58 ± 2.3	7.08 ± 2.3	6.25 ± 3.9
3	6.67 ± 3.4	7.16 ± 4.8	5.25 ± 4.5
4	5.58 ± 3.3	6.00 ± 3.4	10.5 ± 5.5
5	5.80 ± 4.2	5.91 ± 3.2	7.28 ± 4.2
6	4.90 ± 4.3	6.83 ± 4.8	7.00 ± 4.4
7	7.16 ± 4.9	5.66 ± 2.0	9.50 ± 2.0
8	6.96 ± 4.8	6.92 ± 2.8	11.0 ± 5.1
9	9.50 ± 5.3	8.17 ± 2.7	13.4 ± 5.9
10	10.7 ± 5.1	7.50 ± 2.9	12.3 ± 6.2

Mean ± SD, n=6.

Table 5.3. The levels of plasma CR, MDA and GSH after 15 days of NaF (25-mg/kg i.p) daily dose administration.

Subjects	Plasma CR (mg/dl)	Plasma MDA (µmol/l)	Plasma GSH (µmol/l)
Control groups (n=12)	0.56 ± 0.11	1.48 ± 0.33	15.0 ± 3.3
Fluoride treated groups (n=18)	0.63 ± 0.15	1.73 ± 0.40	22.9 ± 6.90 **

Mean ± SD, ** $P<0.01$ compared to the control group.

Table 5.4. The plasma level of CR, MDA and antioxidants (ascorbic acid and GSH) after 27 days of various treatments.

Subjects	CR (mg/dl)	Ascorbic acid (mg/dl)	MDA (µmol/l)	GSH (µmol/l)
Group A	0.80 ± 0.17 *	1.58 ± 0.27 **	1.42 ± 0.34	17.9 ± 2.47
Group B	0.70 ± 0.11	1.69 ± 0.38 **	1.50 ± 0.30	16.8 ± 1.73
Group C	0.67 ± 0.05	1.3 ± 0.33	1.37 ± 0.25	15.6 ± 1.73
Group D	0.82 ± 0.09 *	0.91 ± 0.23	1.25 ± 0.35	15.07 ±1.9
Group E	0.61 ± 0.03	1.07 ± 0.22	1.30 ± 0.24	15.75 ± 1.50

(Mean ± SD, n = 6 *P<0.05, **P<0.01) compared to the control group (E).

Group A; continuously received NaF for 15 days followed by NaF + gentamicin for the next 12 days.

Group B; continuously received of NaF alone for 27 days.

Group C; received NaF for 15 days followed by gentamicin alone for the next 12 days.

Group D; given saline for 12 days followed by gentamicin alone for the next 12 days

Group E (control); given daily saline injection for 27days.

Table 5.5. Kidney tissue antioxidants (GSH and ascorbic acid) and MDA level after 27 days of various treatments.

Subjects	Ascorbic acid (µmol/g protein)	MDA (nmol/mg protein)	GSH (µmol/g protein)
Group A	41.2 ± 2.8 **	4.75 ± 0.30	7.0 ± 0.90
Group B	39.7 ± 4.1 **	5.42 ± 0.80	8.3 ± 0.70
Group C	32.0 ± 5.6	5.55 ± 1.3	9.1 ± 1.0
Group D	28.6 ± 3.7	4.85 ± 0.50	9.2. ± 0.50
Group E	27.0 ± 3.0	5.9 ± 0.55	8.0 ± 0.55

(Mean ± SD, n = 6 **P<0.01) compared to the control group (E).

Group A; continuously received NaF for 15 days followed by NaF + gentamicin for the next 12 days.

Group B; continuously received of NaF alone for 27 days.

Group C; received NaF for 15 days followed by gentamicin alone for the next 12 days.

Group D; given saline for 12 days followed by gentamicin alone for the next 12 days

Group E (control); given daily saline injection for 27days.

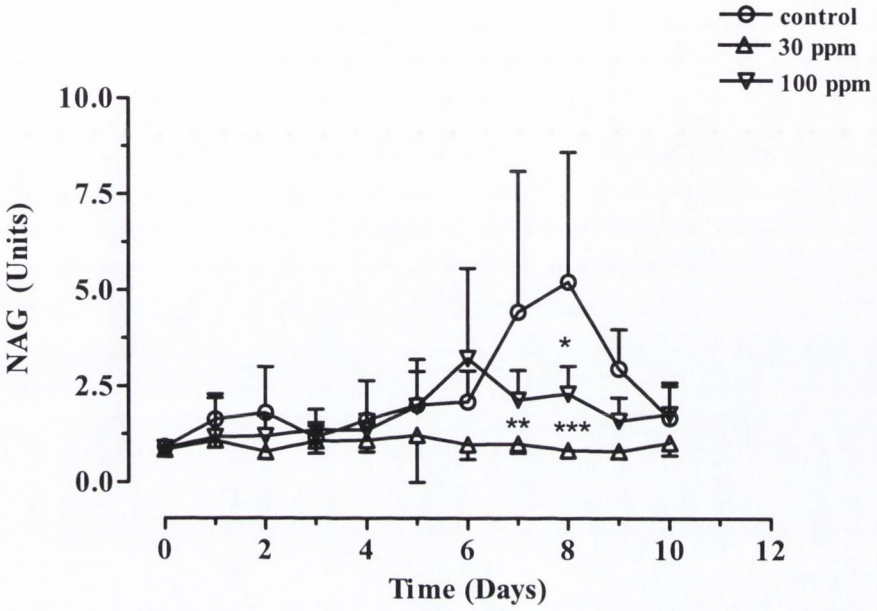


Fig. 5.1. The effect of 10 months sodium fluoride administration (0.0, 30 and 100 ppm) in drinking water on urinary NAG isozymes values in rats challenged with gentamicin (50 mg/kg i.p) for 10 consecutive days. Results are expressed as mean ± SD, n=6, $P<0.05$*, $P<0.01$ ** compared to the control values (0.0 ppm NaF).

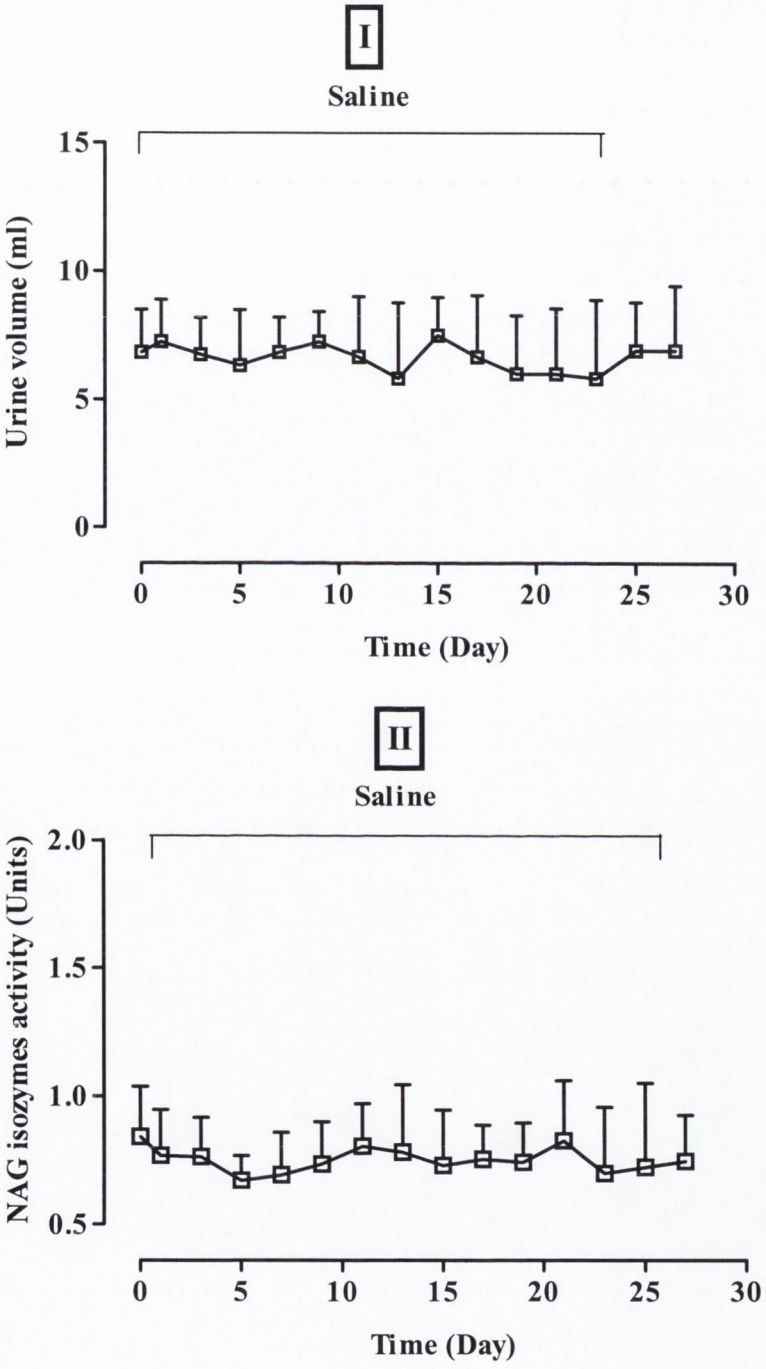


Fig. 5.2. The daily urine volume (I) and NAG isozyme activity (II) of the control animals (received daily 0.5 ml saline i.p) for 27 days. (Mean ± SD, n = 6).

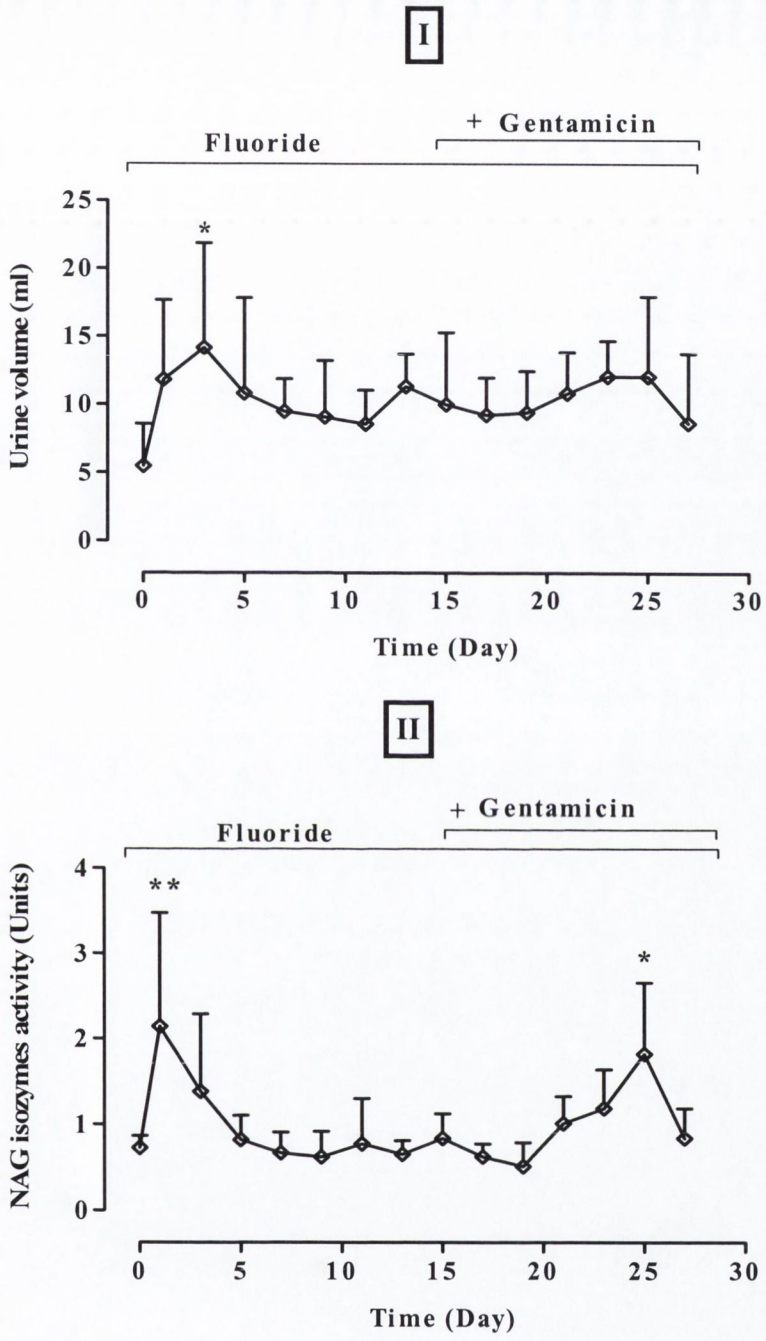


Fig. 5.3. The effect of daily NaF administration (25 mg/Kg) alone for 15 days and with gentamicin (100 mg/kg) for the next 12 days (group A) on urine volume (I) and its NAG isozyme activity (II). (Mean ± SD, n = 6

* $P<0.05$, ** $P<0.01$ compared to control group.

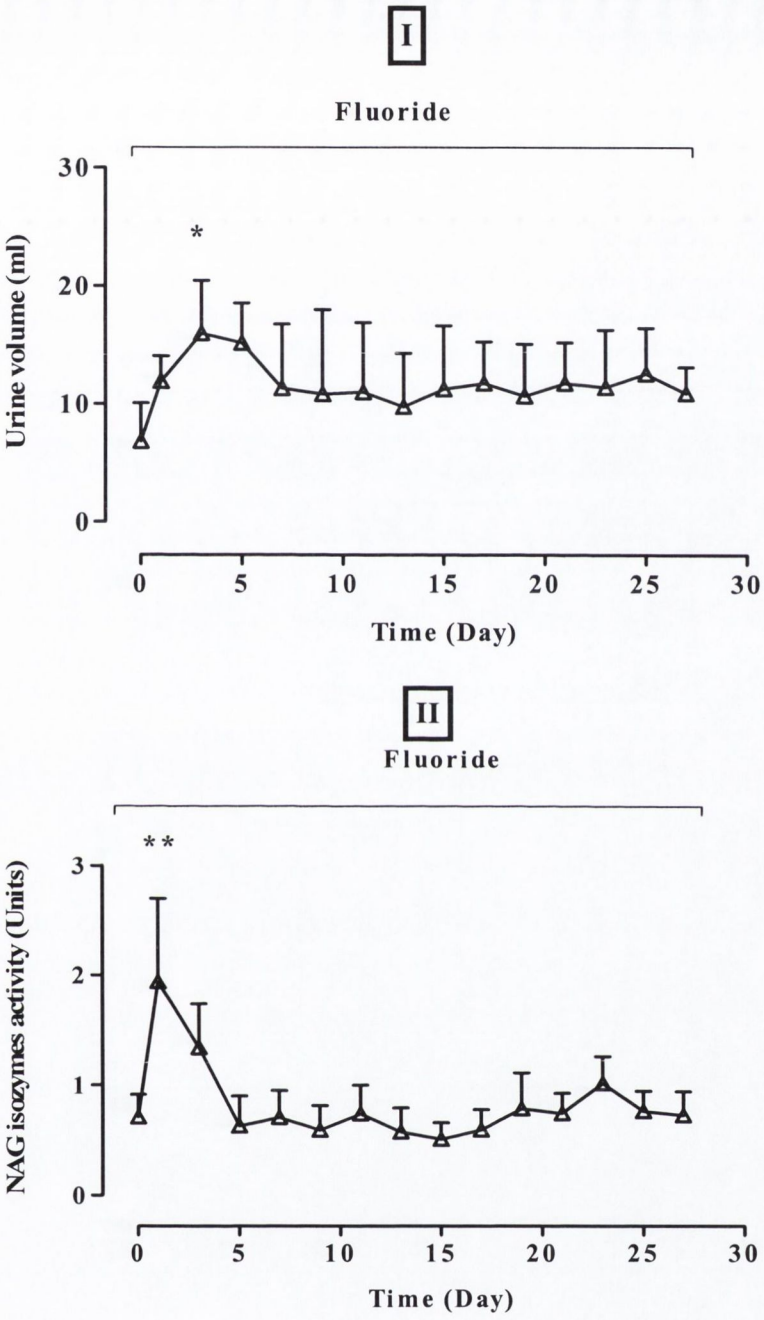


Fig. 5.4. The effect of daily NaF administration (25 mg/Kg) for 27 days on urine volume (I) and its NAG isozyme activity (II). (Mean ± SD, n = 6 * $P<0.05$, ** $P<0.01$) compared to the control group.

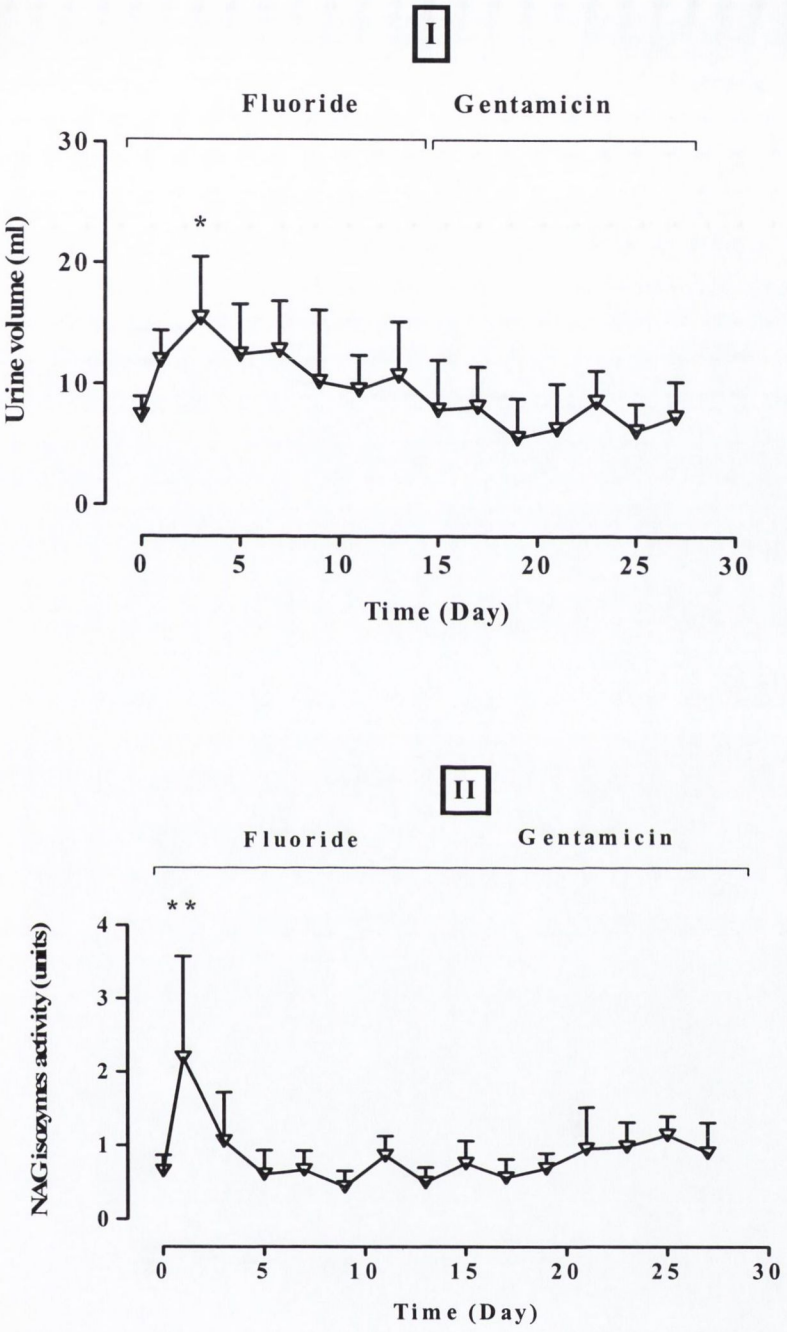


Fig. 5.5. The effect of daily NaF administration (25 mg/Kg) for 15 days followed by gentamicin (100 mg/kg) alone for next 12 days on urine volume (I) and its NAG isozyme activity (II). (Mean ± SD, * $P<0.05$, ** $P<0.01$) compared to control group.

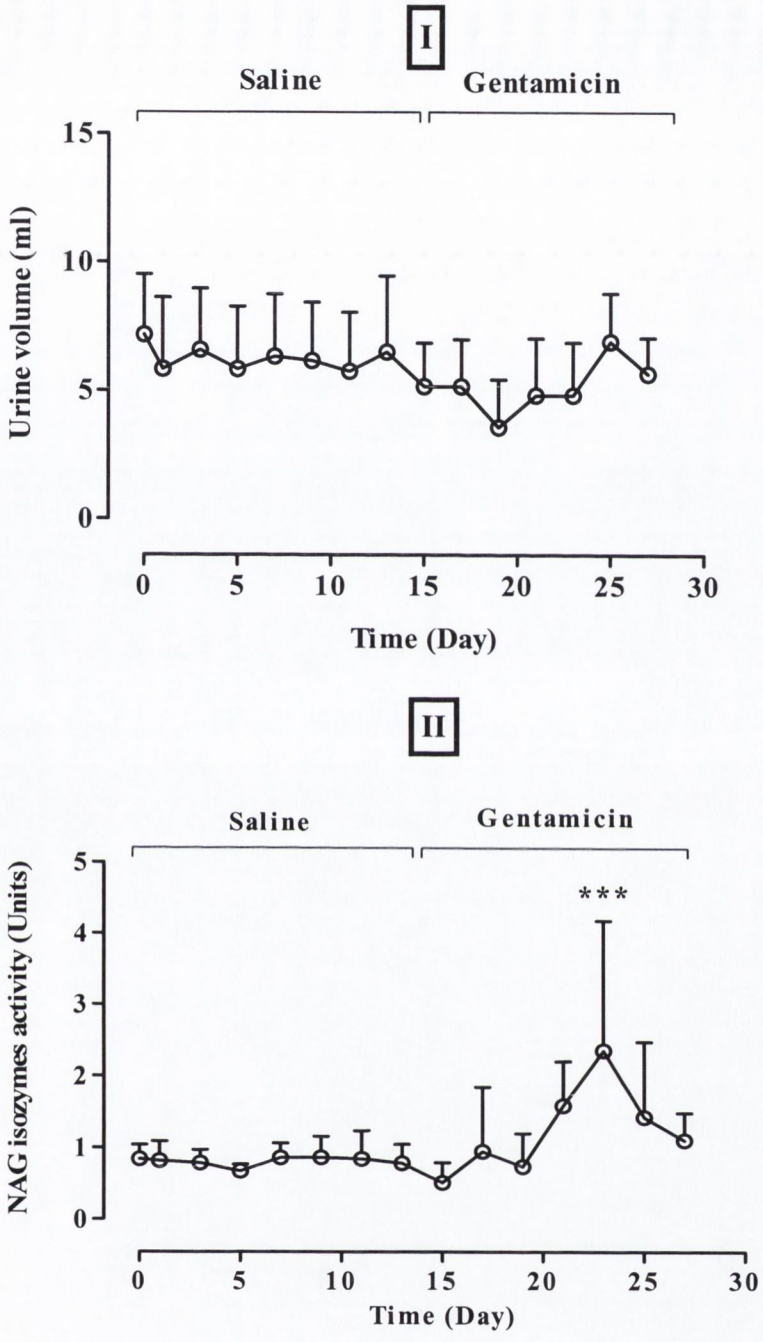


Fig. 5.6. The effect daily gentamicin (100 mg/kg) for the last 12 days of the experiment on urine volume (I) and its NAG isozyme activity (II). (Mean ± SD, ***P<0.001) compared to control.

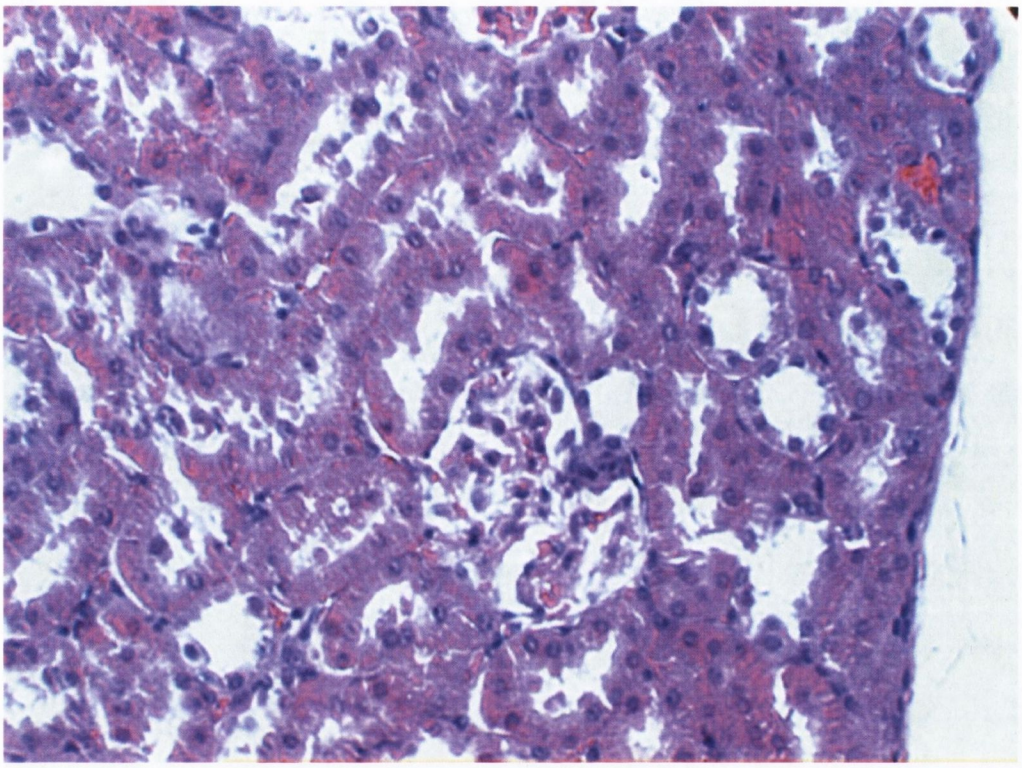


Plate 5.1. Histological study made on renal tubules of rat given saline alone for 27 days (group control, E). H&E. X20.

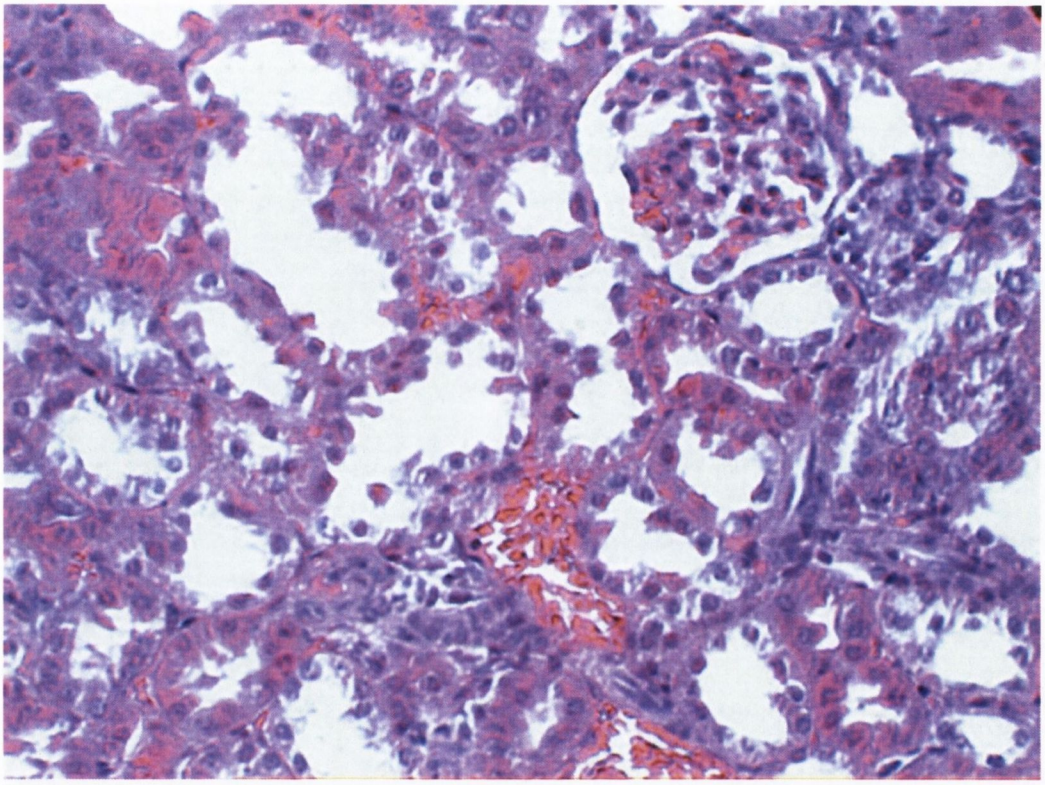


Plate 5.2. Histological study made of renal tubules of rat given NaF (25 mg/kg *i.p*) for 15 days followed with NaF and gentamicin (100 mg/kg *i.p*) for the next 12 days (group A). H&E. X20.

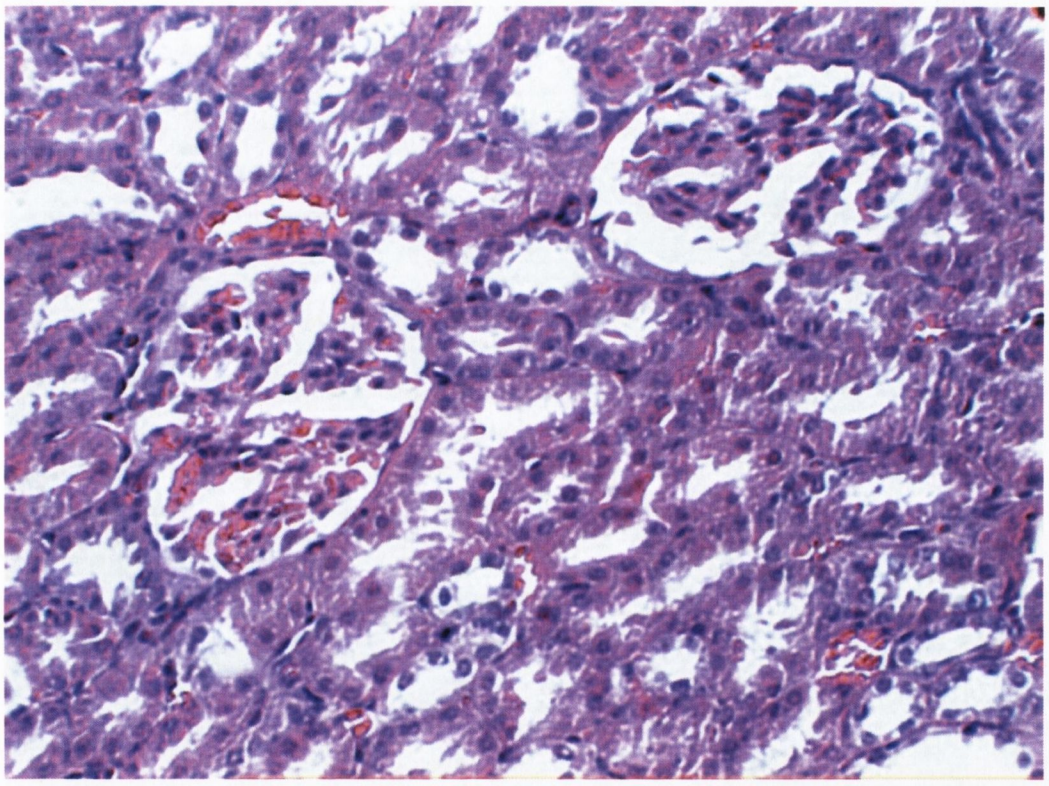


Plate 5.3. Histological study made on renal tubules of rat given NaF (25 mg/kg *i.p*) for 27 days (group B). H&E. X20.

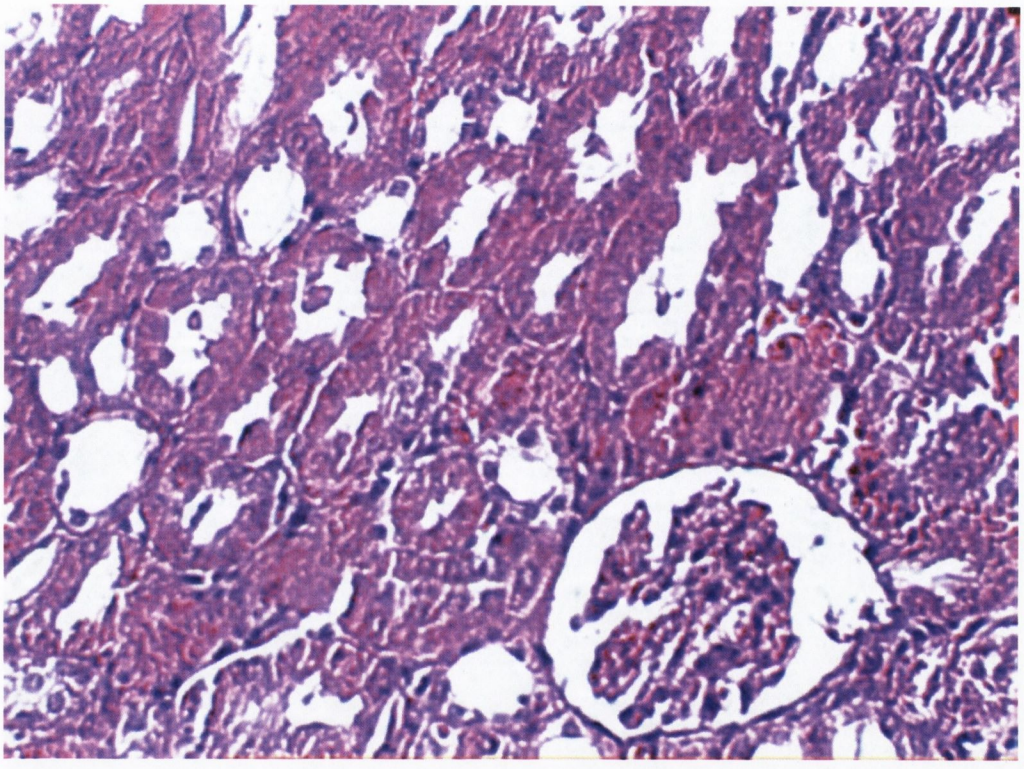


Plate 5.4. Histological study made on renal tubules of rat given NaF (25 mg/kg *i.p*) for 15 days followed with gentamicin (100 mg/kg *i.p*) alone for the next 12 days (group C). H&E. X20

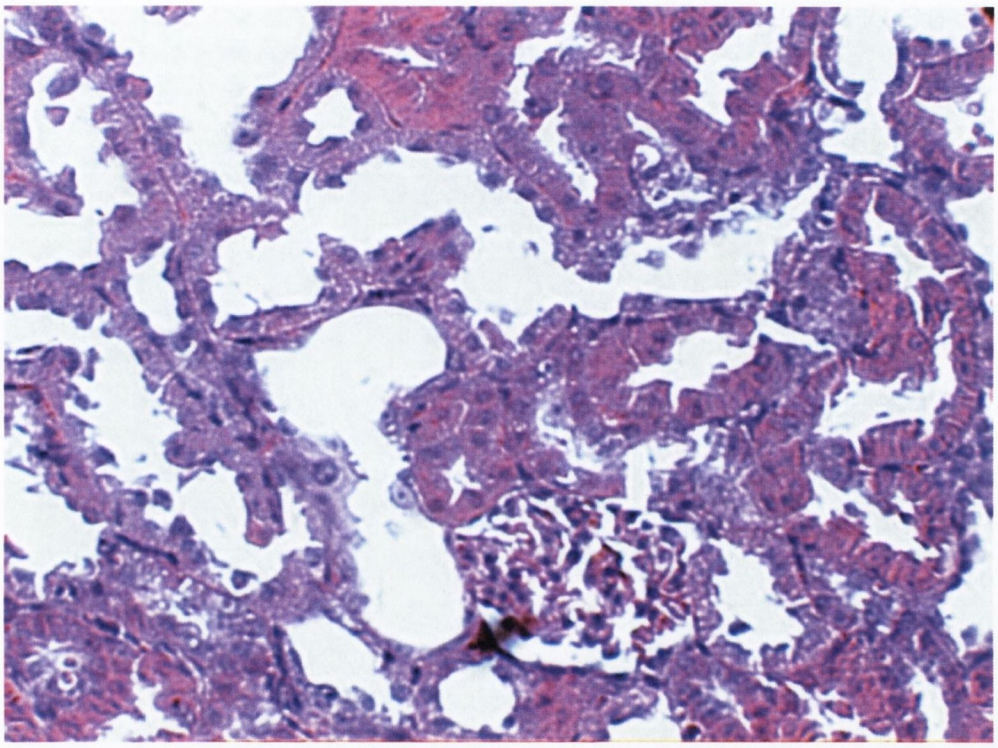


Plate 5.5. Histological study made on renal tubules of rat given saline for 15 days followed with gentamicin (100 mg/kg *i.p*) alone for the next 12 days (group D). H&E. X20

CHAPTER SIX

GENERAL DISCUSSION

It would appear from the literature cited in this study, that interest in fluorosis is still thriving. However, despite extensive studies, the mechanism (s) of its toxicity is still uncertain.

Endemic fluorosis has induced a severe hazard to human health in some developing countries. The sequential adverse health effects following drinking water containing high fluoride concentrations and out-of-control exposure to fluoride in some advanced countries have also been noted. High fluoride exposure also occurs during therapeutic exposure to fluoride for the treatment of osteoporosis (Pak *et al.*, 1997). Excessive fluoride ingestion over a prolonged period can adversely influence many tissues and organs characterized by a vast array of symptoms and pathological changes. Death has also been reported due to exposure to high fluoride concentrations in drinking water and in fluoride exposed work place environments.

The kidneys are among the most sensitive body organs in their histopathological and functional responses to excessive amounts of fluoride. Recent research have shown that fluoride destabilizes kidney cell lysosomes and release, NAG isozymes in human and animal urine. The mechanism by which fluoride exerts this destabilizing effect is still undefined. However, in the past decade a series of remarkable studies have suggested that free radicals, with much focusing on ROS, and the consequent oxidative stress are implicated in fluoride induced toxicity.

Our results from acute fluoride intoxication *in vivo* explored that fluoride afflict kidney structure and functions, which are manifested by renal lysosomes damage (increase urinary NAG activity), in ability of kidney to concentrate urine (polyuria) and decrease glomerulus function (decrease urinary CR excretion) and the effect on renal lysosomes is the most significant. These fluoride-induced renal changes were transient and reversible after fluoride withdrawal. An observation in this study, while

supporting earlier findings gives further evidence that the lysosomal enzyme (NAG) is a very sensitive and early indicator in fluoride induced renal toxicity and fluoride effect on kidney is more pronounced in proximal tubules than other parts of the nephron. This may be due to the large abundance of lysosomes in proximal tubules than other parts of the nephron. The results presented in chapter 3 have also shown that the level of kidney tissue MDA increases following acute fluoride intoxication, which might indicate that the observed kidney damage induced by fluoride may be related to its free radical profile.

We have also designed an *in vitro* cell free system (LRFs) model to explore the mechanism by which fluoride induced renal lysosomal damage. This study has been the first attempt to examine the direct effect of fluoride on renal lysosomes integrity and its association with free radicals. The results of this study revealed that fluoride has the ability to destabilize renal lysosomes directly and increase NAG isozymes release in dose and time dependent manners. The result also shows that fluoride has the ability to increase MDA formation under *in vitro* condition (at low concentrations) and to decrease MDA formation (at high concentrations). Our findings in this study demonstrated that this lysosomal destabilization under *in vitro* conditions, increased NAG isozyme release, have a strong inverse relationship with the decrease in MDA level ($r = - 0.736$) which might reflect the ability of some of the released lysosomal enzymes like (phospholipases and peroxidases) to detoxify lipid peroxides and this may form a part of a defence mechanism of these organelles against free radical induced deleterious effects.

The results in chapter 3 clearly show that fluoride can directly destabilize kidney lysosomes and gives new evidence that free radicals may play a major role in fluoride induced renal lysosomal damage and advanced the knowledge of understanding the mechanism, nature and primary target of fluoride toxicity in the nephron. Our findings in this chapter also provide a novel insight on the biphasic effect of fluoride on MDA level, the possible antioxidant potential of some lysosomal enzymes, their role in lipid peroxide detoxification may explain some of the controversies about fluoride induced oxidative stress.

Lysosomes contain many hydrolytic enzymes, which can catabolise all cellular macromolecules. Lysosomal rupture and the consequent release of lysosomal hydrolytic enzymes may result in programmed cell death, ***apoptosis*** or complete cell death, ***necrosis*** depending on the severity of lysosomes rupture (Brunk *et al.*, 2001) and this has been incriminated in many pathological and xenobiotic induced tissue disorders. Therefore, based on the nature of lysosomal enzymes and on the assumption that stabilizing these organelles may protect cells from the harm effects of these hydrolytic enzymes and thereby protecting the whole tissue. We have attempted to stabilize renal lysosomes against fluoride insult by using several free radical scavengers acting at different pathways in the radicals cascade event. Antioxidant acting on different substrates like PYC (inhibit ROS, RNS, break down chain reaction and inhibit LOX mediated lipid peroxidation) significantly stabilizes the lysosome against fluoride insult under *in vitro* conditions. We have also explored that inhibition of hydroxyl radical by specific inhibitors (mannitol) protect the lysosomes against fluoride induced damage. Our results also revealed that inhibition of Fenton like reaction by redox-active iron chelator (LP) significantly inhibits fluoride induced lysosomal damage. Furthermore, data from this study also indicated that fluoride

induced lysosomal damage can be attenuated by inhibition of the enzymatic pathway of the peroxidation cascade by using either LOX inhibitor (NDGA), COX inhibitor (indomethacin) or PLC inhibitor (U 73122), whereas the latter enzyme has also been incriminated in the feedback mechanism of lysosomal membrane damage.

Therefore our findings in this study indicated that fluoride induced lysosomal damage is at least amenable to a blockade by antioxidants acting on different substrate in the lipid peroxidation chain reaction. This protective ability would be of great value not only in the possible therapeutic effect of these agents in amelioration of the fluoride induced nephrotoxicity but also further advancing our understanding of more possible substrate(s) involved in fluoride mediated oxidative stress.

The results from the *in vivo* section of this chapter have elucidated the potential effect of the natural antioxidants, PYC and ascorbic acid, in amelioration of fluoride induced renal toxicity *in vivo* (decrease urinary NAG isozymes release and increase CR excretion), which also indicate a close consistency between our *in vitro* and *in vivo* results. This has supported the recently held contention that fluorosis can be prevented and ameliorated by natural antioxidants. Therefore PYC and ascorbic acid could be used for preventing and/or combating (at least) renal fluorosis in endemic areas of the world. Further studies may be needed to evaluate the effect of PYC and ascorbic acid in the amelioration of other tissues disorder induced by fluoride.

Several reports from *ex vivo* studies have observed that incubation of cells with stepwise increase of fluoride concentration or sublethal concentration alter the sensitivity of these cells whereby they can withstand lethal concentrations of fluoride and other toxic agents.

A greater importance is our observation presented in chapter 5 that such acquired insensitivity develops after a long-term exposure to high fluoride concentrations *in*

vivo. Our results from chapter 3 have shown that ingestion of high fluoride concentration (30 and 100 ppm) for 10 months failed to alter either urinary volume and its NAG isozymes content at time tested compared to non-fluorinated animals. These fluorinated animals appeared more resistant to a superimposed nephrotoxin with known free radical profile (gentamicin) than non-fluorinated, indicated by less enzymuria during gentamicin course. Although no changes have been detected during the 10 months fluoride course, the development of such cross-resistance against gentamicin may indicate that kidney cells are affected by chronic ingestion of fluoride, necessitating such an adjusted insensitivity. We have shown in chapter 3 that the acute fluoride renal toxicity was transient and reversible by drug withdrawal. In the second section of chapter 5 we have explored that fluoride induced renal toxicity was reversible also despite the continuous fluoride administration and animals recovered from fluoride induced renal failure are more resistant to a subsequent renal intoxication by gentamicin than control group. The results presented in this study have also revealed that prolonged ingestion of high fluoride concentration could adapt body's antioxidants like ascorbic acid and GSH. Therefore, we suggest that, these adapted antioxidants may play a major role in counteracting the subsequent free radicals attack induced by gentamicin and develop such cross-resistance. Further work is needed to clarify more the mechanism (s) of fluoride induced kidney cell insensitivity, whether other cells can develop such resistance and clinical significance of this insensitivity.

The important outcomes of this study are summarised as follow:

1- Fluoride is a renal lysosomal targeting agent and has the ability to destabilize this organelle directly and releases its enzymes content and its pro-oxidant activity may have a potential role. This lysosomal targeting effect of fluoride may explain its pronounced effect in proximal tubule (lysosomes most abundant part) than other parts of the nephron. The dual effect of fluoride on MDA level seen in this study may explain also some of the controversies about fluoride pro-oxidant activity.

2- The results of this thesis have also advanced our understanding regarding fluoride pro-oxidant profile and explored that both the enzymatic (oxygenases stimulation) and the non-enzymatic (ROS stimulation) lipid peroxidation pathways are implicated in its lysosomal damage effect. Moreover our results have also explored the potential role of antioxidants in stabilization of lysosomes against fluoride induced lysosomal damage and explored the therapeutic effect dietary antioxidants (PYC and ascorbic acid) in the prevention of fluoride renal toxicity.

3- Chronic exposure to high fluoride concentration can adapt the body's antioxidant level (ascorbic acid and GSH). The findings of this study have revealed kidney cells of animals exposed to high fluoride concentrations in drinking water over a prolonged time as well as animals recovered from fluoride induced renal failure can withstand gentamicin induced renal toxicity. This acquired cross-resistance developed in fluorinated animals may be due to antioxidants adaptation.

Recommendation for further research:

On the basis of the results obtained in this thesis, it is suggested that the following areas of research maybe worthwhile investigating in the future:

1- Identification of the lysosomal enzyme (s) that possess antioxidant activity.

2- The effects of antioxidant lysosomal enzyme(s) on protein oxidation and free radical formation.

3- The role of lysosomal PLA_2 in fluoride induced lysosomal damage (feedback mechanism).

4- The clinical value of PYC and ascorbic acid in prevention and treatment of fluorosis.

5- The permanence of fluorosis induced cell insensitivity and other possible mechanisms implicated in such insensitivity development.

6- The effect of high fluoride intake on the clinical significance of the aminoglycoside antibiotic (gentamicin), does fluorosis affect the antibacterial activity of gentamicin?

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