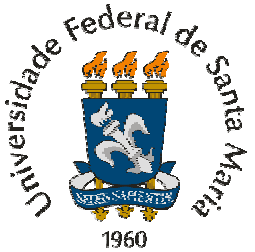

UFSM

Doctor of Philosophy (PhD) Thesis

Hepato- and Reno-Protective Effects of Synthetic Organoselenium compound, binaphthyl diselenide, against Chemical-Induced Oxidative Stress

By

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Supervisor

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PPGBT- CCNE-UFSM
Santa Maria, RS-Brazil
2010

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Federal University of Santa Maria
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The undersigned examining committee, approved the thesis entitled

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to

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ABSTRACT

Thesis of Doctor's Degree

Federal University of Santa Maria, RS, Brazil

Hepato- and Reno-Protective Effects of Synthetic Organoselenium compound, binaphthyl diselenide, against Chemical-Induced Oxidative Stress

AUTHOR: Mohammad Ibrahim

ADVISOR: Prof. Joao Batista Teixeira da Rocha

Co- Supervisor : Cristina Wayne Nogueira

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The liver presents extraordinary functional diversity, particularly in the control of energy production, immune defense and volemic reserve. The human being is exposed occupationally and in the environment to a variety of hepatotoxic compounds, such as the use of paints and their derivatives (2-nitropropane, 2-NP), chemical reagents (glycerol) and exposure to cigarette (2-NP). Therefore, it is interesting, the study of therapies to prevent or even reverse the poisoning caused by these compounds. Considering, that reactive oxygen species (ROS) have an important role in various diseases, especially in liver and kidneys diseases, the use of antioxidant therapies should be considered. The interest in organoselenium biochemistry and pharmacology has increased in the last two decades due to a variety of organoselenium compounds that possess biological activity. Previously our research group, have demonstrated that diphenyl diselenide had hepatoprotective effects against 2-NP. However, no studies are available regarding the effect of the size of the organic moiety of diselenides on the hepatotoxicity of 2-NP. Consequently, in view of the literature data indicating the strong influence of the organic moiety of diselenides on their

pharmacological, toxicological and biological activities and the limited data about the effect of the aromatic ring on the biological activities of diselenides, therefore in this study we investigated the antioxidant activity of binaphthyl diselenide in models of oxidative damage *in vivo* in rats liver and kidneys. Our results shows that the potential antioxidant activity of binaphthyl diselenide ((NapSe)2; 50 mg/kg, p.o.) against the 2-NP-induced hepatoxicity in rats, using different end points of toxicity (liver histopathology, plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine). In addition, in view of the association of oxidative stress with 2-NP exposure, hepatic lipid peroxidation, ascorbic acid levels, δ-aminolevulinate dehydratase (δ-ALA-D) and catalase (CAT) activities were evaluated. 2-NP caused an increase of AST, ALT and hepatic lipid peroxidation, also caused hepatic histopathological alterations and δ-ALA-D inhibition. (NapSe)2 (50 mgkg⁻1) prevented 2-NP-induced changes in plasmatic ALT and AST activities and also prevented changes in hepatic histology, δ-ALA-D and lipid peroxidation. Results presented here indicate that the protective mechanism of (NapSe)2 against 2-NP hepatotoxicity is possibly linked to its antioxidant activity. Similarly binaphthyl diselenide protected against oxidative damage on glycerol-induced renal damage in rats. So Adult male Wistar rats were treated with $(NapSe)_2$ (50 mgkg⁻1, p.o) or vehicle. After 24 h $(NapSe)_2$ treatment, the animals received an intramuscular injection of glycerol (8ml/kg, dissolved in saline) or vehicle as a divided dose into the hind limbs. Twenty-four hours afterwards, rats were euthanized and the levels of urea and creatinine were measured in plasma. Non-protein thiol (NPSH) levels and catalase (CAT) activity were evaluated in renal homogenates. Histopathological evaluations were also performed in kidneys of rats. The rats exposed to glycerol presented swelling of the proximal and distal tubules with

evidence of cell damage and death. Glycerol-exposed rats presented an increase in renal failure markers (plasmatic urea and creatinine levels) and a reduction in renal CAT activity. No change was observed in NPSH levels in kidneys of rats exposed to glycerol. $(NapSe)_2$ protected against the alterations caused by glycerol in rats. $(NapSe)_2$ increased per se NPSH levels (33%) in kidneys of rats. The results demonstrated that treatment with $(NapSe)_2$ protected against renal damage induced by glycerol in rats, probably due to its antioxidant effect. Based on these results, we can conclude that, the binaphthyl diselenide administered orally at a dose of (50 mgkg⁻1) did not cause toxicity in rats.The binaphthyl diselenide was effective in protecting against liver damage induced by 2-NP in rats. The binaphthyl diselenide was effective in protecting against renal damage induced by glycerol in rats. The precise mechanisms that may be involved in protection and the pharmacological action of organoselenium against 2-NP induced hepatotoxicity and glycerol-induced renal demage are yet to be fully understood. However the present study holds great promise that organoselenium compounds are first line candidates in the management of these diseases.

Keywords: liver damage, selenium, binaphthyl diselnide, glycerol, 2-nitropropane

Abbreviation list

AST- aspartate aminotransferase

ARF- acute renal failure

ATN -acute tubular necrosis

ALT- alanine aminotransferase

CAT- Catalase

Cu- Copper

CCl_4 – carbon tetrachloride

$CCl_3^{\bullet}$ -trichloromethyl radical

DNA- Deoxyribonucleic Acid

DPDS-Diphenyl diselenide

H_2O_2- Hydrogen peroxide

GSH- Glutathione

GPx- glutathione peroxidase

GFR- glomerular filtration rate

(HO-1)-heme oxygenase-1

ID - iodothyronine deiodinase

SOD- Superoxide dismutase

LPO- lipid peroxidation

ROS - Reactive oxygen species

($O2^{\bullet-}$)- Superoxide radical

Zn-SOD- Zinc Superoxide dismutase

Mn-SOD- Manganese - Superoxide dismutase

XO- Xanthine oxidase

RNS -Reactive nitrogen species

NO• - Nitric oxide

NOSs - Nitric oxide synthases

$ONOO^-$ -Peroxynitrite anion

sGC- Guanylate cyclase

GSSG- Oxidised glutathione

IMPDH- inosine monophosphate dehydrogenase

LOX -lipoxygenases

UrdPase-uridine phosphorylase

TMS- thymidylate synthase

TK- tyrosine kinase

PKC-Protein kinase C

GST-glutathione-Stransferase

δ-ALA-D - Delta-aminolevulinate dehydratase

PhSeSePh- diphenyl diselenide

Se- Selenium

TrxR- thioredoxin reductase

ROOH-hydroperoxides

PhSH- benzylic thiols

ROO•-peroxyl radicals

MDA- malondialdehyde

$^{\bullet}OOCCl_3$ -peroxy trichloromethyl radical

$R^{\bullet}$- alkoxy

$ROO^{\bullet}$ peroxy radicals

2-NP -2-Nitropropane

8-OHG - 8-hydroxyguanine

NF-κB -factor-kappa B

$(NapSe)_2$ – binapthyl diselenide

NPSH – Non protein thiol

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Thank you.

Mohammad Ibrahim

Organization of the thesis

The results that make up this thesis are presented in the form of Published manuscripts and are found under the scientific articles.

The sections **Material & Method, Results, Discussion of results, and References** are found in the articles itself and present an integral part of this study.

The item, Discussion is for the purpose of brief and short interpretation on scientific articles presented

The references refer only to citations that appear in the items, Introduction and discussion on results.

1 Introduction:

1.1 Selenium (Se) Based Anti-Oxidants:

Selenium (Se) belongs chemically to the 16 group of element and possesses remarkably chemical properties. Se represents an essential element of fundamental importance to human health. Selenium is an essential trace element that has provoked considerable interest owing to the recent identification of prokaryotic and eukaryotic enzymes containing the 21st amino acid, selenocysteine (Moller et al., 1984).

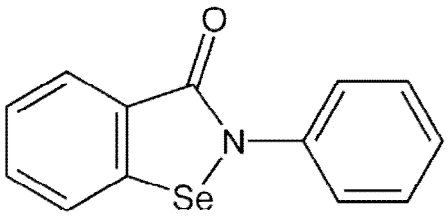
Because of the specific redox properties of selenium, the presence of a selenol group, instead of a thiol, at the active site of an enzyme confers a dramatic catalytic advantage. In mammals, selenium exerts its biological effect mainly in selenoenzymes that include glutathione peroxidase (GPx), iodothyronine deiodinase (ID), and thioredoxin reductase (TrxR). Glutathione peroxidase is an antioxidant enzyme that protects biomembranes and other cellular components from oxidative damage by catalyzing the reduction of a variety of hydroperoxides (ROOH); it uses GSH as the reducing substrate (Galet et al., 1994 Phadnis et al. 2005). Since the discovery that ebselen (2-phenyl-1,2-benzoisoselenazol-3-(2H)-one, 1) (Fig.1.A) exhibits significant antioxidant activity by mimicking the active site of GPx (Brown et al., 2001) several groups have pursued the design and synthesis of low-molecular-weight GPx mimics, either by modifying the basic structure of ebselen, or by incorporating some structural features of the native enzyme. The synthetic GPx mimics reported in the literature include benzoselenazolinones (Schrauzer et al., 2000) selenenamide , diaryl selenide , various diselenides , hydroxyalkyl selenides , a selenocysteine derivative (Schrauzer et al., 2000). Although several mechanisms have been proposed to account for the GPx-like behavior of ebselen, the available data suggest that

ebselen and its related compounds express their GPx activity mainly by the generation of catalytically active selenols. The formation of a reactive selenol species is also required for diselenides to exhibit antioxidant activities. Back et al. have shown that the hydroxyalkyl selenides do not produce any selenol, but that they undergo facile oxidation with organic peroxides to produce cyclic seleninates as the catalytically active species (Stumm and Lee, et al., 1961). The selenocysteine derivative, on the other hand, has been shown to undergo oxidation followed by elimination reactions to produce a selenenic acid, which in turn reacts with thiols to generate the corresponding selenol (Schrauzer et al., 2000). Although ebselen exhibits interesting therapeutic properties, including anti-inflammatory activity, it is a relatively inefficient catalyst for the in vitro reduction of hydroperoxides with aryl and benzylic thiols (such as PhSH and BnSH) as cosubstrates (Back and Moussa, 2002; Sarma and Mugesh et al., 2001). The relatively poor GPx-like antioxidant activity has been ascribed to undesired thiol exchange reactions that take place at the selenium center in the selenenyl sulfide intermediate (Back and Moussa, 2002). However, ebselen has been shown to be a good antioxidant in vivo, as it exhibits significant GPx activity in the presence of natural thiols such as GSH. It has important health effects related to immune response and cancer prevention.

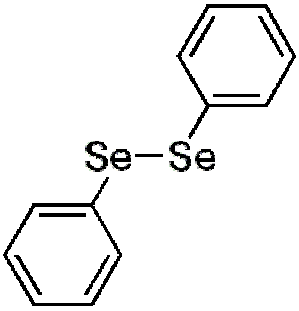
Diphenyl diselenide (DPDS) (Fig.1.B), the simplest diaryl diselenide, is particularly important as a potential antioxidant drug in view to the fact that it has been shown to be more active as a glutathione peroxidase mimic, less toxic to rodents than ebselen and has also low toxicity for non-rodent mammals after long term exposure (Nogueira et al., 2004).

It is curious to note that despite the interesting properties and applications of naphthyl chalcogens, their chemistry remains largely unexplored as compared to the phenyl analogue.

Naphthyl moiety belongs to a class of linear acene and consists of a planar and rigid aromatic ring system with 10πp electrons. Not surprisingly, presence of naphthyl ring in many selenium/tellurium containing compounds has been associated with their greater stability (Menon et al., 1996) antioxidant properties (Mugesh et al., 2001a; 2001b; Engman et al.,1995),.Among the naphthyl chalcogens we studied binaphthyl diselenide ,it has already been reported that (NapSe)2 has thiol peroxidase-like activity in vitro (Mugesh et al., 2001b).



A.



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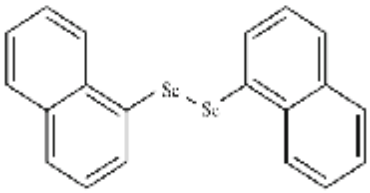


C.

Fig.1. Chemical structure of ebselene (A), diphenyl dislenide (B) and binaphthyl diselenide (C).

1.2 Toxicology of Organoselenium Compounds

Selenium containing compounds are toxic and the molecular mechanism underlining this toxicity is still not completely understood. However, Painter proposed that the toxicity of inorganic selenium could be related to the oxidation of thiols of biological importance. The fact that selenite was a good catalyst for the oxidation of a variety of biologically significant thiols, including glutathione (GSH), cysteine, dihydrolipoic acid, and coenzyme A, helped to explain, at least in part, the biochemistry of selenite poisoning (Nogueira et al., 2004). Therefore, these studies confirmed the early proposition of Painter and established that selenite exerts at least part of its toxic action by catalyzing the oxidation of biologically significant sulfhydryl containing molecules. However, considering excellent reviews on selenium toxicity, we can conclude that in view of toxicological considerations, toxicity of selenium compounds not only depends on the chemical form and the quantity of the element consumed, but also on a variety of other factors including species, age, physiological state, nutrition and dietary interactions, and the route of administration (Tinggi et al., 2003). However, the ability of organoselenium compounds to interact with thiol of biological significance is of prime interest to our study.

Organoselenium compounds are known to inhibit a variety of enzymes such as nitric oxide synthase (NOS), inosine monophosphate dehydrogenase (IMPDH), lipoxygenases (LOX), uridine phosphorylase (UrdPase), thymidylate synthase (TMS), tyrosine kinase (TK), and iodothyronine deiodinase (ID). In addition to these enzymes, some other enzymes such as NADPH oxidase, protein kinase C (PKC), glutathione-Stransferase (GST), NADPH-cytochrome reductase, and papain are inhibited by organoselenium compounds. Some of

these enzymes are implicated in inflammatory processes, and therefore, the inhibitory effects are expected to contribute to the anti inflammatory actions of the organoselenium compounds in vivo. It should be noted that organoselenium blocks the activity of several enzymes by reacting with the critical –SH groups of the enzymes (Oshita et al., 1994). The interest of the present study would centre on three thiol containing enzymes: Na+ K+ - ATPase, Lactate dehydrogenase and delta amino levulinic acid dehydratase. Delta-aminolevulinate dehydratase or porphibilinogen synthase (δ-ALA-D) is a sulfhydrylcontaining enzyme that is extremely sensitive to oxidizing agents (Rocha, et al., 1995). δ-ALA-D catalyzes the asymmetrical condensation of two molecules of 5-aminolevulinic acid (ALA) molecules to form porphobilinogen. The inhibitory effect of organoselenium compounds on the activity of the enzyme seems to be related to PhSeSePh formation. The mechanism of porphibilinogen synthesis is similar in animals and plants; however, the enzyme obtained from these sources exhibits subtle structural diversity (Jaffe, 1995) Aminolevulinate dehydratase from plants, in marked contrast to the enzyme from rats, was not inhibited by diphenyl diselenide (Barbosa et al., 1998). The divergent response of the plant enzyme to diphenyl diselenide (and to diphenyl ditelluride and other simple organoselenium and organotellurium compunds) is presumably related to differences in the quantity and spatial proximity of cysteinyl residues in the three dimensional structure of the enzyme within the plant and the mammal (Markham et al., 1993). In fact, the plant enzyme has no cysteinyl residues in close spatial proximity as observed in the active site of the mammal enzyme (Jaffe et al., 1995). In view of this, proposed mechanism shown in figure 4 has been used to explain why the mammal enzyme is inhibited by diphenyl diselenide (Farina et al., 2002) whereas the plant enzyme is not

affected by these compounds (Nogueira et al., 2004).

1.3 Reactive Oxygen Species (ROS)

The generation of reactive oxygen species (ROS) is considered to be a primary event under a variety of stress conditions. The consequences of ROS formation depend on the intensity of the stress and on the physicochemical conditions in the cell (i.e. antioxidant status, redox state it has been generally accepted that active oxygen produced under stress is a detrimental factor, which causes lipid peroxidation, enzyme inactivation, and oxidative damage to DNA (Noctor and Foyer, 1988; Valko et al., 2004).

ROS can be produced from both endogenous and exogenous substances. Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation (Poli et al., 2004). Mitochondria have long been known to generate significant quantities of hydrogen peroxide. The hydrogen peroxide molecule does not contain an unpaired electron and thus is not a radical species. Under physiological conditions, the production of hydrogen peroxide is estimated to account for about 2% of the total oxygen uptake by the organism (Loschen and Flohe, 1971). However, it is difficult to detect the occurrence of the superoxide radical in intact mitochondria, most probably in consequence of the presence of high superoxide dismutase (SOD) activity therein. Generation of the superoxide radical ($O_2^{\bullet -}$) by mitochondria was first reported more than three decades ago by (Loschen and Flohe, 1971). After the determination of the ratios of the mitochondrial generation of superoxide to that of hydrogen peroxide, the former was considered as the stoichiometric precursor for the latter. Ubisemiquinone has been proposed as the main reductant of oxygen in mitochondrial membranes (Inoue et al., 2003).

Mitochondria generate approximately 2–3 nmol of superoxide/min per mg of protein, the ubiquitous presence of which indicates it to be the most important physiological source of this radical in living organisms (Inoue et al., 2003). Since mitochondria are the major site of free radical generation, they are highly enriched with antioxidants including GSH and enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), which are present on both sides of their membranes in order to minimise oxidative stress in the organelle (Cadenas and Davies, 2000).

Superoxide radicals formed on both sides of mitochondrial inner membranes are efficiently detoxified initially to hydrogen peroxide and then to water by Cu, Zn-SOD (SOD1, localized in the inter membrane space) and Mn-SOD (SOD2, localized in the matrix). Besides mitochondria, there are other cellular sources of superoxide radical, for example xanthine oxidase (XO), a highly versatile enzyme that is widely distributed among species (from bacteria to man) and within the various tissues of mammals (Valko et al., 2004). Xanthine oxidase is an important source of oxygen-free radicals. It is a member of a group of enzymes known as molybdenum iron–sulphur flavin hydroxylases and catalyzes the hydroxylation of purines. In particular, XO catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming the superoxide anion in the first step and hydrogen peroxide in the second (Gupta et al., 1997).

Additional endogenous sources of cellular reactive oxygen species are neutrophils, eosinophils and macrophages. Activated macrophages initiate an increase in oxygen uptake that gives rise to a variety of reactive oxygen species, including superoxide anion, nitric oxide and hydrogen peroxide (Cadenas and Davies, 2000). Cytochrome P450 has also been proposed as a source of reactive oxygen species. Through the induction of cytochrome

P450 enzymes, the possibility for the production of reactive oxygen species, in particular, superoxide anion and hydrogen peroxide, emerges following the break down or uncoupling of the P450 catalytic cycle. In addition, microsomes and peroxisomes are sources of ROS. Microsomes are responsible for the 80% H_2O_2 concentration produced *in vivo* at hyperoxia sites (Li and Jackson, 2002; Klaunig and Kamendulis, 2004). Peroxisomes are known to produce H_2O_2, but not $O^{2}\bullet-$, under physiologic conditions. Although the liver is the primary organ where peroxisomal contribution to the overall H_2O_2 production is significant, other organs that contain peroxisomes are also exposed to these H_2O_2 generating mechanisms. Peroxisomal oxidation of fatty acids has recently been recognised as a potentially important source of H_2O_2 production as a result of malnourishment. The release of the biologically active molecules such as cytokines and others, from activated Kupffer cells (the resident macrophage of the liver) has been implicated in hepatotoxicological and hepatocarcinogenic events. Recent results indicate that there is a close link between products released form activated Kupffer cells and the tumour promotion stage of the carcinogenesis process (Li and Jackson, 2002; Klaunig and Kamendulis, 2004).

Reactive oxygen species can be produced by a host of exogenous processes. Environmental agents including non-genotoxic carcinogens can directly generate or indirectly induce reactive oxygen species in cells. The induction of oxidative stress and damage has been observed following exposure to various xenobiotics. These involve chlorinated compounds, metal (redox and non-redox) ions, radiation and barbiturates. For example 2-butoxyethanol is known to produce ROS indirectly, which causes cancer in mice (Cadenas, 1997).

ROS seem to affect the cell through a combination of the following factors: the amount of ROS produced (correlates with the severity of the stress) and biochemical status of the cell

(i.e. activity of antioxidative and other enzymes, antioxidant content, pH, energy resources, integrity of membranes, redox characteristics etc.). The particular mechanisms and the place of ROS in the signal transduction cascade are not yet known.

1.3 Reactive nitrogen species (RNS)

NO• is a small molecule that contains one unpaired electron on the antibonding orbital and is, therefore, a radical. NO• is generated in biological tissues by specific nitric oxide synthases (NOSs), which metabolise arginine to citrulline with the formation of NO• *via* a five electron oxidative reaction (Ghafourifar & Cadenas, 2005). Nitric oxide (NO•) is an abundant reactive radical that acts as an import ant oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulation (Bergendi et al.,1999). Due to its extraordinary properties, in 1992 was NO• acclaimed as the "molecule of the year" in *Science Magazine* (Koshland, 1992). NO• has a half-life of only a few seconds in an aqueous environment. NO• has greater stability in an environment with a lower oxygen concentration (half life>15 s). However, since it is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes (Chiueh, 1999). NO• has effects on neuronal transmission as well as on synaptic plasticity in the central nervous system. In the extracellular milieu, NO• reacts with oxygen and water to form nitrate and nitrite anions. Over production of reactive nitrogen species is called nitrosative stress (Klatt and Lamas, 2000). This may occur when the generation of reactive nitrogen species in a system exceeds the system's ability to neutralise and eliminate them. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function. Cells of the immune system produce both the

superoxide anion and nitric oxide during the oxidative burst triggered during inflammatory processes. Under these conditions, nitric oxide and the superoxide anion may react together to produce significant amounts of a much more oxidatively active molecule, peroxynitrite anion (ONOO−), which is a potent oxidising agent that can cause DNA fragmentation and lipid oxidation (Carr and McCall, 2000):

$$NO\bullet + O_2^{\bullet -} \rightarrow ONOO^- \quad (1)$$

Reaction (1) has one of the highest rate constants known for reactions of NO•, 7.0×109M−1 s−1. Thus NO• toxicity is predominantly linked to its ability to combine with superoxide anions. Nitric oxide readily binds certain transition metal ions; in fact many physiological effects of NO• are exerted as a result of its initial binding to Fe2+-Haem groups in the enzyme soluble guanylate cyclase (sGC) (Archer, 1993)

$$Fe^{2+}\{sGC\} + NO\bullet \rightarrow Fe^{2+}\{sGC\}–NO \quad (2)$$

The product is represented here as {Fe2+–NO•}, however, *{*Fe3+–NO−*}* is also commonly seen. The convention *{*FeNO*}*7, where the superscript is the sum of the metal d electron count (here 6 or 5) and the occupancy of the relevant $NO^{\bullet}$* orbital (here 1 or 2), is often employed to avoid specific assignment of oxidation states.

1.5 Free Radical and Antioxidant defense mechanisms

Defense mechanisms against free radical induced oxidative stress involve: (i) preventative mechanisms, (ii) repair mechanisms, (iii) physical defenses, and (iv) antioxidant defenses. Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT). Nonenzymatic antioxidants are represented by ascorbic acid (Vitamin C), δ-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants (Shen et al., 2005).

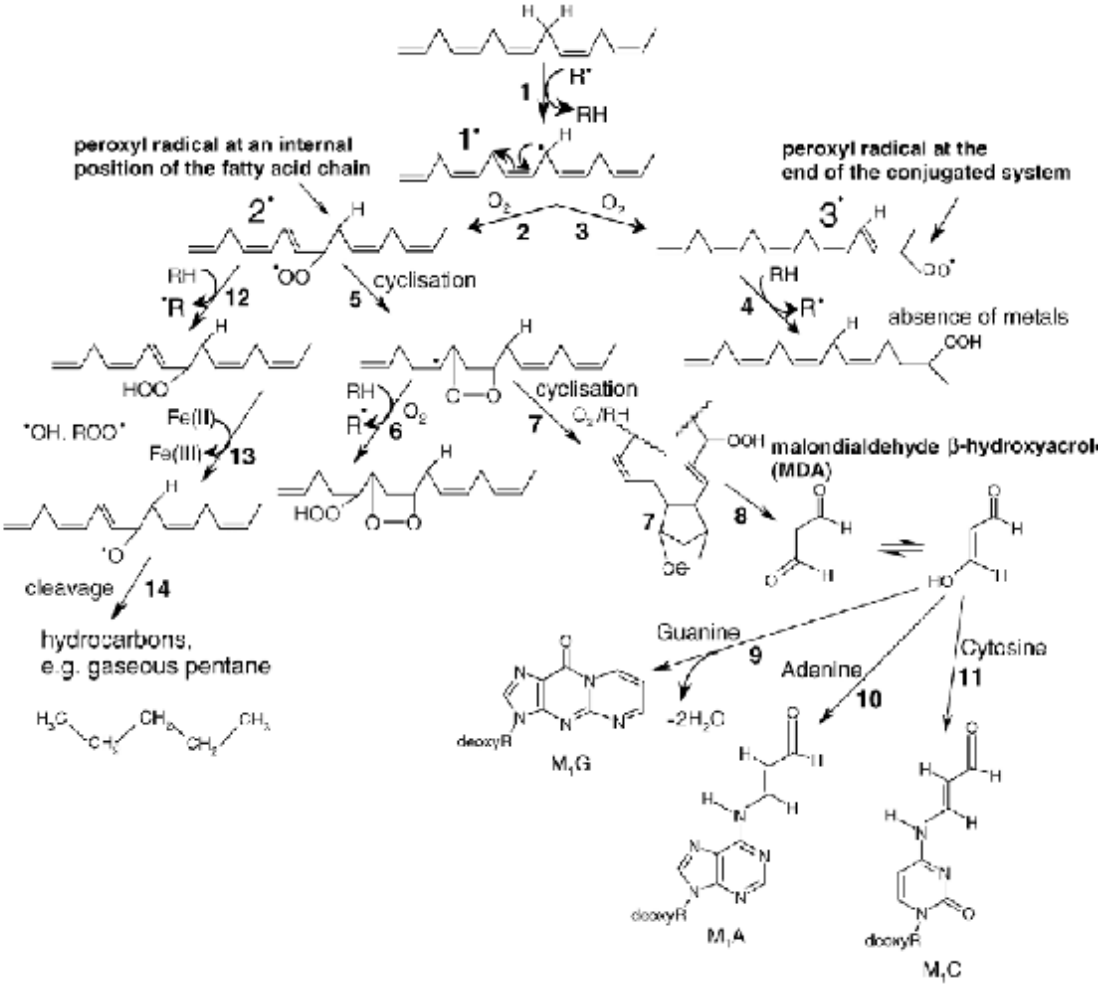
Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health. Recently, it has been shown that externally added GSH is readily taken up by mitochondria, despite the ~8mM. GSH present in the mitochondrial matrix (Shen et al., 2005).It therefore appears that GSH is taken up against a concentration gradient. GSH in the nucleus maintains the redox state of critical protein sulphydryls that are necessary for DNA repair and expression. Oxidised glutathione is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Jones et al., 2000; Pastor at el., 2000; El-Agamey et al., 2004): Too high concentration of GSSG may damage many enzymes oxidatively. The main protective roles of glutathione against oxidative stress are (Masella et al., 2005). (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathione transferase and others; (ii) GSH participates in amino acid transport through the plasma membrane (iii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase; (iv)

glutathione is able to regenerate the most important antioxidants, Vitamins C and E, back to their active forms; glutathione can reduce the tocopherol radical of Vitamin E directly, or indirectly, via reduction of semi dehydroascorbate to ascorbate. The capacity of glutathione to regenerate the most important antioxidants is linked with the redox state of the glutathione disulphide-glutathione couple (GSSG/2GSH) (El-Agamey et al., 2004). The various roles of enzymatic antioxidants (SOD, catalase, glutathione peroxidase) and non-enzymatic antioxidants (Vitamin C, Vitamin E, carotenoids, lipoic acid and others) in the protection against oxidative stress can be found in literature (Kojo, 2004; Miller et al., 2005; Smith et al., 2004; Stadtman, 1996).

1.6 Lipid Peroxidation

Reactive oxygen species can react with all biological macromolecules (lipids, proteins, nucleic acids, and carbohydrates). Polyunsaturated fatty acids are particularly susceptible targets of ROS attack. The overall process of lipid peroxidation consists of three stages: initiation, propagation and termination and is presented in scheme. 3 (Pinchuk et al.,1998; Nyska and Kohen, 2002). Once formed, peroxyl radicals (ROO•) can be rearranged via a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation process being malondialdehyde (MDA) (Shceme 1) (Nyska and Kohen., 2002). The major aldehyde product of lipid peroxidation other than malondialdehyde is 4-hydroxy-2-nonenal (HNE) (Shceme 1). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. Hydroxynonenal is weakly mutagenic but appears to be the major toxic product of lipid peroxidation. Numerous lipid peroxidation products are formed that can react with sulfhydryl (cysteine) or basic amino acids (histidine, lysine) (Sies and Cadenas, 1985). Organoselenides have been documented

to prevent lipid peroxidation brought about by Fenton-type reaction which may be related to their GPx-like activity and their ability to reduce hydroperxides (Anderson et al., 1994). Noteworthy diorganyl diselenides have also been reported to effectively diminish the effect of oxidative stress induced by various prooxidant in different tissues and under different conditions (Nogueira et al., 2004).



Scheme. 1. Various pathways of lipid peroxidation. The methylene groups of polyunsaturated fatty acids are highly susceptible to oxidation and their hydrogen atoms, after the interaction with radical R•, are removed to form carbon-centred radicals 1• (reaction (1)). Carbon-centred radicals react with molecular dioxygen to form peroxyl radicals (reactions (2) and (3)). If the peroxyl radical is located at one of the ends of the double bond (3•) it is reduced to a hydroperoxide which is relatively stable in the absence of metals (reaction (4)). A peroxyl radical located in the internal position of the fatty acid (2•) can react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centred radical (reaction (5)). This can then eitherbe reduced to form a hydroperoxide (reaction (6))

or through reaction (7) it can undergo a second cyclisation to form a bicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide. Compound 7 is an intermediate product for the production of malondialdehyde (reaction (8)). Malondialdehyde can react with DNA bases G, A, and C to form adducts M1G, M1A andM1C (reactions (9)–(11)). Peroxyl radicals located in the internal position of the fatty acid (2•) can, besides cyclisation reactions, (reaction (5)) also abstract hydrogen from the neighbouring fatty acid molecule, creating thus lipid hydroperoxides (reaction (12)). They can further react with redox metals (e.g. iron) to produce reactive alkoxyl radicals (RO•) (reaction (13)) which, after cleavage (reaction (14)) may form, e.g. gaseous pentane; a good marker of lipid peroxidation.

1.7 Hepatotoxicity and Oxidative stress:

Lethal cell injury is ultimately an irreversible process that occurs by either necrosis or apoptosis (Scheme. 2) Cell necrosis (Greek *nekrosis,* "deadness") is defined as injury causing irreversible loss of metabolic functions (e.g., ion gradients and adenosine triphosphate [ATP] generation) and structural integrity of the plasma membrane. Indeed, loss of plasma membrane integrity (cytolysis) is the hallmark of cell necrosis (Searle et al., 1982; Popper et al., 1986; Fawthrop et al., 1991). Apoptosis (Greek *apo,* "off"; *ptosis,* "falling") is best defined morphologically by nuclear and cell fragmentation resulting in the formation of membrane-bound fragments containing structurally intact, viable organelles referred to as apoptotic bodies (Scheme. 2) (Gerschenson and Rotello, 1992; Kerr and Searle, 1982).

In apoptosis, the plasma membrane remains intact and a characteristic pattern of DNA hydrolysis often occurs (ladder like pattern). In apoptosis, the cell actively participates in its own death by the purposeful activation of a specific program of events (cell suicide) (Searle et al., 1982; Goldstein et al., 1991; Raff, 1992; Vaux, 1993). ATP generation and protein synthesis are inhibited in necrosis but are preserved in the "active" process of apoptosis. Although both apoptosis and necrosis play important roles in cell pathophysiology, sublethal injury (reversible disruption of cell function without loss of structural integrity) is increasingly recognized as a key factor in long-term pathophysiological processes (e.g., disturbed signal transduction and tumorigenesis). All forms of cell injury occur in liver diseases and are of interest to the clinician.

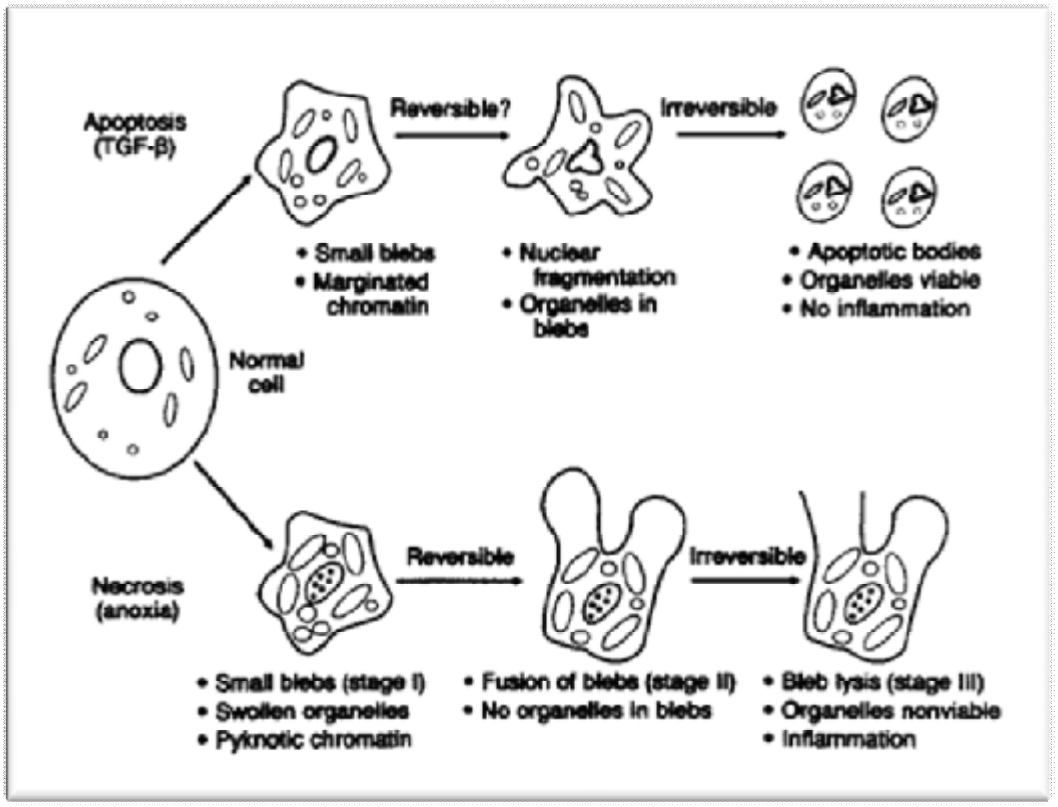
Several mechanisms initiate liver cell damage and aggravate ongoing injury processes. Mitochondria are prominent targets for the hepatotoxicity of many drugs. Dysfunction of

these vital cell organelles results in impairment of energy metabolism and an intracellular oxidant stress with excessive formation of reactive oxygen species and peroxynitrite. In addition to mitochondria, induction of cytochrome P450 isoenzymes such as CYP2E1 also promotes oxidant stress and cell injury. Once hepatocellular function is impaired, accumulation of bile acids causes additional stress and cytotoxicity (Goldstein et al., 1991). Cell injury, gut-derived endotoxin or a combination of both also activate Kupffer cells and recruit neutrophils into the liver. Although responsible for removal of cell debris and part of the host-defense system, under certain circumstances these inflammatory cells initiate additional liver injury (Searle et al., 1982; Goldstein et al., 1991). However, cell injury and death is not only determined by the nature and dose of a particular drug but also by factors such as an individual's gene expression profile, antioxidant status, and capacity for regeneration. It is evident from the literature that there are so many hepatotoxin that can cause liver demage some of them are as below.

CCl_4 is a classical hepatotoxicant that causes rapid liver damage progressing from steatosis to centrilobular necrosis. CCl_4 requires bioactivation by phase I cytochrome P450 system in liver to form reactive metabolic trichloromethyl radical ($CCl_3^{\bullet}$) and peroxy trichloromethyl radical ($^{\bullet}OOCCl_3$). These free radicals can bind with polyunsaturated fatty acid (PUFA) to produce alkoxy ($R^{\bullet}$) and peroxy radicals ($ROO^{\bullet}$) (Hung et al., 2006). These free radicals can react with sulfhydryl groups, such as glutathione (GSH) and protein thiols. The covalent binding of trichloromethyl free radicals to cellular protein is considered to be the initial step in a chain of events, eventually leading to membrane lipid peroxidation and finally cell necrosis (Brattin et al., 1985). Consequently, CCl_4 is known to induce reactive oxygen species (ROS) formation, deplete GSH of phase II enzyme, and reduce antioxidant

enzymes and substrates to induce oxidative stress that is an important factor in acute and chronic liver injury (Brattin et al., 1985).

Cadmium is one of the most important toxic chemicals due to its increasing level in the environment as a result of tobacco smoking, industrial and agricultural practices(Goering et al., 1995) . It has a very long biological half-life (10–30 years) in humans and its toxicity is dependent on the route, dose and duration of exposure (Satarug et al., 2003). Acute cadmium intoxication induced primarily hepatic and testicular damage whereas, chronic exposure resulted in renal injury and osteotoxicity (Rikans and Yamano et al.,2000). Parenteral administration of cadmium in rats caused a severe hepatic injury in the form of hepatocellular necrosis (Dudley., et al., 1986). The molecular mechanism that may be responsible for the toxicity of cadmium involves oxidative stress by disturbing the antioxidant defense systems and by producing reactive oxygen species (Thijssen et al., 2007). In viewof the fact that cadmium is a non-redox metal, it always adopts a single oxidation state and is not a strong inducer of reactive oxygen species. The major mechanism behind the case is the disruption of electron transfer chain and the induction of mitochondrial reactive oxygen species (ROS). Therefore, some authors have postulated that antioxidants should be one of the important components of an effective treatment of cadmium poisoning (El-Demerdash et al., 2004).



(Scheme. 2). Schematic representation of the morphological and ultrastructural changes occurring during cell death by necrosis and apoptosis. The cellular changes observed during apoptosis are contrasted with those associated with necrosis. Cell death by anoxia is chosen as a representative example of the environmental catrastrophes leading to necrosis, whereas apotosis by transforming growth factor 13 (TGF-I3) illustrates an example of the physiological stimuli inducing apoptosis.

1.8 Hepatotoxic inducer:

1.8.1 2-Nitropropane:

2-Nitropropane, a nitroalkane, is used as a constituent of paints and inks, in the manufacture of chemicals as industrial solvent and can be found in cigarette smoke. This compound is known to be an acute hepatotoxicant (Zitting et al., 1981) and a potent hepatocarcinogen in rodents when administered either by inhalation (Lewis et al., 1979) or orally (Fiala et al., 1987) and also a genotoxicant (Faila et al., 1987; Dayal et al., 1989). Epidemiological studies have revealed leukemia and non-Hodgkin's lymphoma among farmers exposed to solvents, including 2-NP although 2-NP has not been identified as a defined human carcinogen (World Health Organization, 1992). Most studies on 2-NP have been concerned with its ability to damage hepatic DNA although there are a few reports of its effects on other organs including the kidney and bone marrow, and a few studies related to cellular lipids and proteins. A number of investigators showed that 2-NP causes base modifications such as 8-hydroxyguanine (8-OHG) and 8-aminoguanine as well as DNA fragmentation in the liver and they considered these changes to be related to its carcinogenecity. Recent studies suggest that 2-NP generates reactive oxygen species which may account for the carcinogenic effects of this molecule in the liver (Kohl et al., 1995; Kohl and Gescher, 1997). The relevance of reactive oxygen species to the toxicity of 2-NP suggest that the resulting lipid peroxidation (LPO) may also be related to the carcinogenicity of the compound. The mechanism by which 2-NP exerts hepatotoxicity is not clearly understood, but many authors suggested that 2-NP metabolism may increase ROS levels and cause cellular damage (Roscher et al., 1990; Halliwell and Gutteridge, 1990).

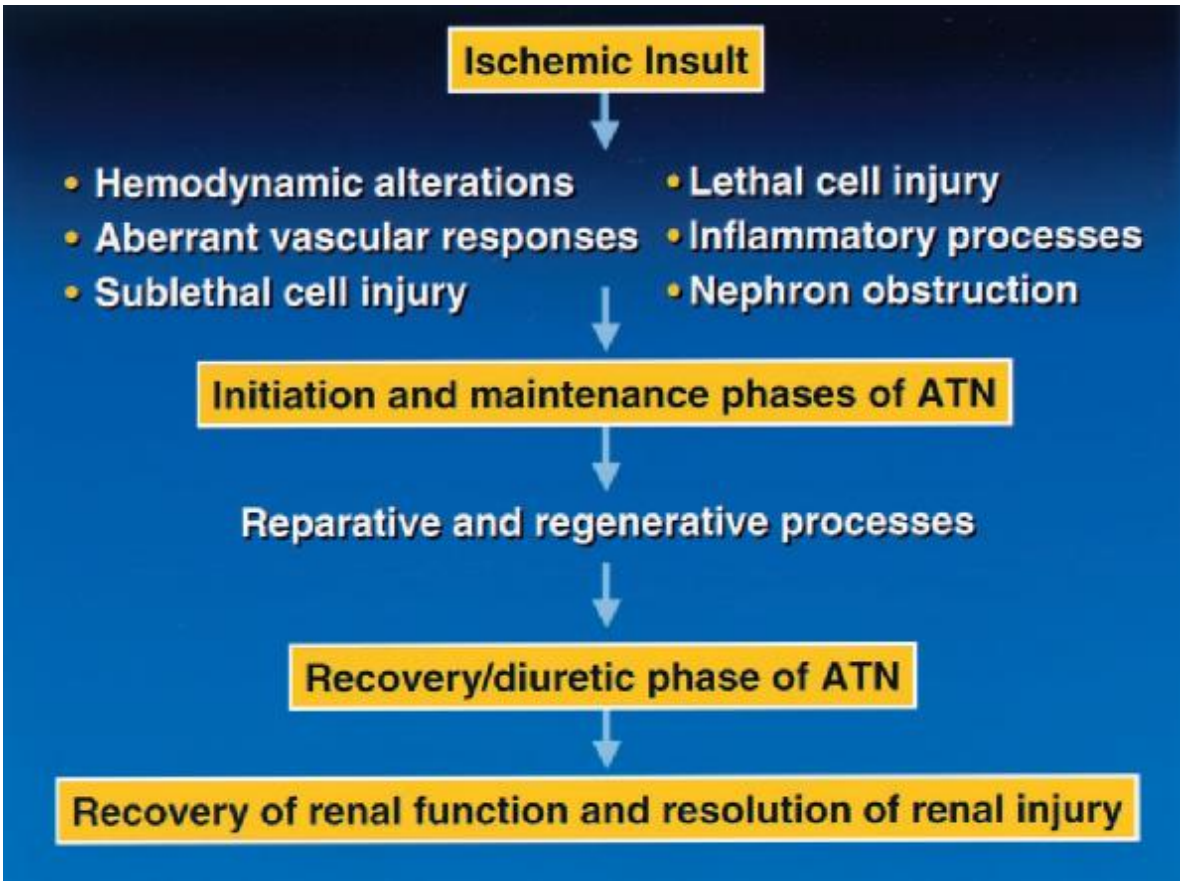
1.8.2 Reactive Oxygen Species and Acute Renal Failure:

Acute renal failure is commonly due to acute tubular necrosis (ATN), the latter representing an acute, usually reversible loss of renal function incurred from ischemic or nephrotoxic insults occurring singly or in combination. Such insults instigate a number of processes hemodynamic alterations, aberrant vascular responses, sublethal and lethal cell damage, inflammatory responses, and nephron obstruction that initiate and maintain ATN. Eventually, reparative and regenerative processes facilitate the resolution of renal injury and the recovery of renal function. Inordinate or aberrant generation of reactive oxygen species (ROS) is widely incriminated in the pathogenesis of tissue injury (McCord, 1985; Halliwell and Gutteridge, 1999).This ubiquitous involvement of ROS in disease processes reflects the far-ranging pathobiologic effects of ROS: actions of ROS that affect cellular vitality and its determinants, cellular growth and proliferation, tissue repair and regeneration, inflammatory and immune processes, and the regulation of various hemodynamic, hemostatic, and vascular systems (Dalton, 1999; Allen and Tresini, 2000).

The kidney has long been studied as an organ that can generate ROS (Rondoni and Cudkowicz, 1953; Guidet and Sudhir, 1989) and is vulnerable to the damaging effects of ROS. The intact, disease-free kidney generates small amounts of ROS in the course of renal oxidative metabolism. Healthy kidneys, organs that comprise less than 1% of body weight, consume relatively large amounts of oxygen, accounting for some 10% of total consumption of oxygen by the body; oxygen is used in such heightened amounts by healthy kidneys to sustain oxidative phosphorylation and synthesis of ATP, the latter needed in copious quantities for renal tubular transport processes (Gullans and Hebert, 1996). In health, the relatively low amounts of ROS generated by the kidneys are tolerated without

any apparent adverse effects. However, ROS, produced excessively or aberrantly by endogenous or infiltrating cells in the injured kidney, can contribute to, depending on the circumstance, acute renal injury or progressive renal damage. ROS have been invoked as a pathway for renal injury. ATN represents an acute, usually reversible, loss of renal function incurred by ischemic or nephrotoxic insults, the latter occurring singly or in combination (Thadhani et al., 1996; Lieberthal, 1997). The course of ATN is triphasic. The initiation phase of ATN is characterized by a precipitous decrease in glomerular filtration rate (GFR), triggered by ischemia and attendant hemodynamic alterations, and accompanied by evolving sublethal and lethal tubular epithelial injury. The established or maintenance phase is characterized by persistent reduction in GFR and is sustained by the aberrant hemodynamic responses and sublethal and lethal cell injury. Histologic features in established ATN are generally mild and more commonly involve sublethal rather lethal cell injury; sublethal alterations include brush border loss, vacuolization and flattening of proximal tubules, and dilation of tubules. Cellular necrosis is uncommon, involving few cells, and mainly those of the S3 segment of the proximal tubule (Racusen et al., 1997).

The distal tubular lumen may be occluded by casts, and distal, rather than proximal, tubular epithelial cells are more likely involved by apoptosis. Such structural and functional derangements resolve during the recovery or diuretic phase wherein reparative and regenerative responses restore renal architecture and normalize GFR. (Scheme. 3) summarizes the processes involved during the triphasic course of ATN.



(Scheme. 3). The processes involved during the triphasic course of acute tubular necrosis (ATN).

1.9 Acute renal Damage Inducer:

1.9.1 Glycerol:

Glycerol is a well-known material for the induction of acute renal failure (ARF) *in vivo*. ARF is a syndrome characterized by an acute loss of renal function. The most widely used animal model of myoglobinuric ARF involves subcutaneous or intramuscular injection of hypertonic glycerol which causes myolysis, hemolysis and intravascular volume depletion (Paller, 1988; Singh et al., 2003; Chander et al., 2006). The earliest changes in renal function include a pronounced fall in renal blood flow and a fall in glomerular function. Several studies have shown that when an acute volume load is administered within 6h of glycerol injection, the changes produced are reversible. However, when the same volume load is given 18–20 h after glycerol injection, the fall in glomerular filtration rate does not improve even though the renal blood flow is returned to normal (Chander et al., 2006).

The pathogenesis of glycerol-induced acute renal failure can involve decreased renal blood flow, as well as myoglobin release from damaged muscle and increased reactive oxygen species (ROS). Iron released from heme pigment myoglobin can promote ROS production, lipid peroxidation and acute tubular necrosis (ATN) (Baliga et al., 1997; Nath et al., 1992).

The results of *in vitro* studies have suggested that myoglobin can play the role of a Fenton's reagent, promoting hydroxyl radical formation. The ROS commonly involved in the pathogenesis of renal injury include the superoxide anion, hydrogen peroxide, hydroxyl radical and peroxynitrite anion (Baliga et al., 1997; Nath and Norby, 2000).The peroxynitrite anion is produced in the reaction between the superoxide anion and nitric oxide neutrophils and macrophages produce significant quantities of superoxide anion and nitric oxide during the inflammatory response. In addition, oxidative stress can also induce

the expression of heme oxygenase-1 (HO-1), a cytoprotective enzyme that is active during inflammatory responses (Maines, 1997; Morse and Choim, 2002) The nuclear factor-kappa B (NF-κB) system can be activated by the oxidative stress and inflammatory process seen in glycerol treated rats (Soares et al., 2006). It is known that NF-κB is present, albeit in an inactive form, in the cytoplasm of all cells, regardless of cell type. Upon stimulation, NF-κB is released from an inhibitory subunit (IκB) and translocates in to the nucleus, where it promotes the transcriptional activation of target genes. It has been suggested that, in many types of kidney diseases, NF-κB activation plays an important role by inducing the synthesis of inflammatory substances that provoke kidney damage (cytokines, growth factors and enzymes, as well as factors chemotactic for macrophages and monocytes) . The inflammatory process also contributes to glycerol-induced alterations in renal function and structure (Soares et al., 2002).

1.9.2 Selenium and Hepatotoxicity and Acute renal failure (ARF):

Several research groups have demonstrated that organoselenium compounds have hepatoprotective and reno-protective effects. Wendel and Tiegs reported that ebselen is an effective inhibitor of galactosamine/endotoxin-induced hepatitis, a model in which inhibition of lipoxygenases is considered to be involved. Similarly ebselen show protective effect against different heptotoxin agents like, paracetamol, CCl4, lipopolysaccharide and *Propionibacterium acnes*, alcohol, ethanol-induced hepatic vasoconstriction, and ischemia-reperfusion injury (Wendel and Tiegs, 1986; Wasser et al., 2001; Kono et al., 2001; Oshita et al., 1994; Ozaki et al., 1997; Li et al., 1994). This pharmacological profile suggests that ebselen an organoselenium compound has a promising potential in the therapy of diseases that are characterized by an initial over activation of the immune system. Moreover the

Reno-protective effect of selenium have been reported from our lab and other labs.(Brandão et al.,2009; El-Sharaky et al., 2007).

Our group has also investigated the protective effect of organoselenium compounds against the liver demage induced by different hepatotoxicant (Borges et al., 2005, 2006; 2008; Wilhelm et al., 2009, 2010) in rats.

2 General Objective

The main objective of this work was to know the protective effect of binaphthyl diselenide, against the chemical induce hepato- and renal damage in rats.

2.1 Specific Objectives

- To investigate the acute toxicity of binaphthyl diselenide in rats.
- If this Synthetic Organoselenium compound , binaphthyl diselenide, protects the 2-NP induced hepatotoxicity in rats.
- To see the effect of binaphthyl diselenide, on glycerol induce renal damage in rats *in vivo.*

3. First Manuscripts

Protective effect of binaphthyl diselenide, a synthetic organoselenium compound, on 2-nitropropane-induced hepatotoxicity in rats

Mohammad Ibrahim, Marina Prigol, Waseem Hassan, Cristina W. Nogueira and Joao B.T. Rocha

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Protective effect of binaphthyl diselenide, a synthetic organoselenium compound, on 2-nitropropane-induced hepatotoxicity in rats

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Organoselenides have been documented as promising pharmacological agents against a number of diseases associated with oxidative stress. Here we have investigated, for the first time, the potential antioxidant activity of binaphthyl diselenide ($(NapSe)_2$; 50 mg kg^{-1}, p.o.) against the 2-nitropropane (2-NP)-induced hepatoxicity in rats, using different end points of toxicity (liver histopathology, plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine). In addition, in view of the association of oxidative stress with 2-NP exposure, hepatic lipid peroxidation, ascorbic acid levels, δ-aminolevulinate dehydratase (δ-ALA-D) and catalase (CAT) activities were evaluated. 2-NP caused an increase of AST, ALT and hepatic lipid peroxidation. 2-NP also caused hepatic histopathological alterations and δ-ALA-D inhibition. $(NapSe)_2$ (50 mg kg^{-1}) prevented 2-NP-induced changes in plasmatic ALT and AST activities and also prevented changes in hepatic histology, δ-ALA-D and lipid peroxidation. Results presented here indicate that the protective mechanism of $(NapSe)_2$ against 2-NP hepatotoxicity is possibly linked to its antioxidant activity. Copyright © 2010 John Wiley & Sons, Ltd.

KEY WORDS — binaphthyl diselenide; selenium; 2-NP; antioxidant; hepatic damage; liver

INTRODUCTION

Selenium is an essential biological trace element that plays a crucial role as an integral component of several enzymes with antioxidant properties, including gluthathione peroxidase (GPx) and several other selenoproteins.[1] In the last two decades, the interest in organoselenium chemistry and biochemistry has increased, mainly due to the fact that these compounds possess very interesting biological activities.[1–5] The concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than classical antioxidant has led to the design of synthetic organoselenium compounds with potential therapeutic applications.[6]

In line with this, El-Bayoumy was the first to pioneer the research of aromatic selenium compounds in cancer chemoprevention in the 1980s.[7] Moreover, clinical trials in humans revealed beneficial effects of organoselenium compounds, such as ebselen, in pathological situations.[3] Similarly diphenyl diselenide has also been reported as a good GPx mimic and a good antioxidant in different experimental models of oxidative damage.[8–13]

2-Nitropropane (2-NP) has been widely used as a chemical intermediate, a solvent and a component of inks, paints, varnishes and other coatings.[14] This compound is known to be an acute hepatotoxicant and a potent hepatocarcinogen in rodents when administered either by inhalation or orally.[15] The exact mechanism by which 2-NP exerts hepatotoxicity is not clearly understood, but many authors suggested that 2-NP metabolism may increase reactive oxygen species (ROS) levels which can cause cellular damage.[16–18]

There are strong points of evidence suggesting that modification of the organic moiety of organoselenium compounds can have a profound effect on their biological and chemical activities.[19] In this vein, we have recently demonstrated that dicholesteroyl diselenide was a weaker antioxidant than diphenyl diselenide[19] possibly as consequence of a steric effect of the bulky organic moiety of the cholesteroyl group. Interestingly, this Se-containing cholesterol analogue had also weaker inhibitory effect on two sulphhydryl containing enzymes, indicating a possible advantageous effect of a bulky organic moiety in modulating the undesirable strong thiol oxidizing

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properties of diselenides. Previously, we have demonstrated that diphenyl diselenide had hepatoprotective effects against 2-NP.[11,16] However, no studies are available regarding the effect of the size of the organic moiety of diselenides on the hepatotoxicity of 2-NP. Consequently, in view of the literature data indicating the strong influence of the organic moiety of diselenides on their pharmacological, toxicological and biological activities[20] and the limited data about the effect of the aromatic ring on the biological activities of diselenides, it becomes important to investigate whether binaphthyl diselenide ($(NapSe)_2$) has protective effect against the liver damage induced by 2-NP in rats. Furthermore, as a first step of our investigation, we determined the potential acute toxicity of $(NapSe)_2$ which is not available in the literature.

EXPERIMENTAL PROCEDURE

Chemicals

$(NapSe)_2$ (Figure 1) was synthesized according to Kozlov and Suvorova[21] dissolved in canola oil. Analysis of the ^1H NMR and ^{13}C NMR spectra showed that $(NapSe)_2$ presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(NapSe)_2$ (99.9%) was determined by GC/HPLC. 2-NP was obtained from Sigma. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Animals

Male adult Wistar rats (200–250 g) from our own breeding colony were used. The animals were kept in a separate animal room, on a 12 h light/dark cycle, at a room temperature of 22 ± 2°C and with free access to food and water (Guabi, RS, Brazil). The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, The Federal University of Santa Maria, Brazil.

Experimental procedure

Acute toxicity. In the first set of experiments, $(NapSe)_2$ was administered at different doses to rats to find a dose which does not induce toxicity. For these experiments, rats were randomly divided into four groups consisting of seven animals each (control canola oil, 1 ml kg^{-1} and $(NapSe)_2$ at the doses of 10, 50 or 300 mg kg^{-1} of body weight). $(NapSe)_2$ was administered by intragastric gavage as a single oral dose.

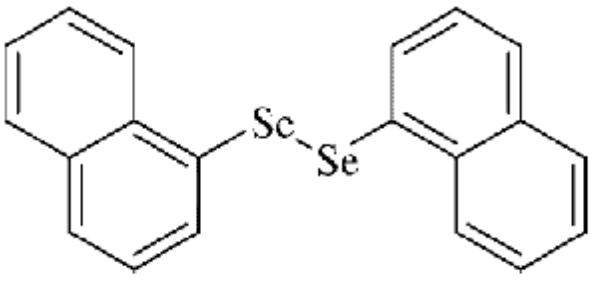

Figure 1. Chemical structure of binaphthyl diselenide

2-NP × (NapSe)$_2$. In the second set of experiments, the dose of 50 mg kg^{-1} of $(NapSe)_2$ was selected for the potential hepatoprotective effects against 2-NP because it caused no sign of toxicity in rats; 2-NP, a nitroalkane, is known to be an acute hepatotoxicant[17] and a potent hepatocarcinogen in rodents.[15,22]

Rats were randomly divided into four groups consisting of seven animals each as follow: group 1 (rats received two oral doses of canola oil; 1 ml kg^{-1}); group 2 (rats received orally canola oil and 24 h later 120 mg kg^{-1} 2-NP); group 3 (rats received orally 50 mg kg^{-1} $(NapSe)_2$ and canola oil 24 h later); group 4 (rats received orally 50 mg kg^{-1} $(NapSe)_2$ and 120 mg kg^{-1} 2-NP 24 h later).

Seventy-two hours after $(NapSe)_2$ administration (acute toxicity) or 24 h after 2-NP administration (2-NP × $(NapSe)_2$), all rats were anaesthetized for blood collection by heart puncture (haemolysed plasma was discharged). After this procedure, rats were euthanized and the liver or kidney of animals were removed, dissected and kept on ice until the time of assays.

The samples of liver and kidney were homogenized in 50 mM Tris-HCl, pH 7.4 (1/10, w/v), centrifuged at 2400 × *g* for 10 min. The low-speed supernatants (S1) were separated and used for biochemical assays.

Lipid peroxidation. Lipid peroxidation was performed by the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction as previously described by Ohkawa *et al.*[23] Briefly, the S1 were mixed with 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid; subsequently, they were heated in a boiling water bath for 15 min. TBARS were determined by the absorbance at 532 nm and were expressed as nmol MDA mg^{-1} protein.

Non-protein thiols (NPSH) determination. NPSH levels were determined by the method of Ellman.[24] To determine NPSH, S1 was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded and free −SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB. The colour reaction was measured at 412 nm. NPSH levels were expressed as μmol g^{-1} tissue.

Ascorbic acid determination. Ascorbic acid determination was performed as described by Jacques-Silva *et al.*[25] Proteins were precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of homogenate at a final volume of 1 ml of the solution was incubated for 3 h at 38°C then 1 ml H_2SO_4 65% (v/v) was added to the medium.

The reaction product was determined using a colour reagent containing 4.5 mg ml^{-1} dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg ml^{-1}) at 520 nm.

Catalase activity. CAT activity was assayed spectrophotometrically by the method of Aebi *et al.*[26] by monitoring the disappearance of H_2O_2 in the homogenate at 240 nm. Enzymatic reaction was initiated by adding an aliquot of 20 µl of S1 and the substrate (H_2O_2) to a concentration of 30 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in Units (one Unit decomposes 1 µmol of H_2O_2 per min at pH 7 at 25°C).

δ-ALA-D activity. Hepatic and renal δ-aminolevulinate dehydratase (δ-ALA-D) activity was assayed according to the method described by Sassa[27] by measuring the rate of product porphobilinogen (PBG) formation. An aliquot of 200 µl of S1 was pre-incubated for 10 min at 37°C. The enzymatic reaction was initiated by adding the substrate (δ-ALA) to a final concentration of 2.4 mM in a medium containing 100 mM phosphate buffer, pH 6.8 and incubated for 1 h at 37°C. Incubation was stopped by adding 250 µl trichloroacetic acid solution (10% TCA) with 10 mM $HgCl_2$. The reaction product was determined using modified Erlich's reagent at 555 nm.

Hepatic profile. Plasma enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used as the biochemical markers for the early acute hepatic damage as described by Reitman and Frankel[28] using a commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

Renal profile. Renal function was analysed using a commercial Kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil) by determining plasma urea by the method of Mackay and Mackay[29] and creatinine as described by Jaffe.[30]

Protein quantification. Protein concentration was measured by the method of Bradford[31] using bovine serum albumin as the standard.

Histopathology. After euthanasia, rats were subjected to a detailed necropsy evaluation. Organ weight for liver was recorded, and segment of the hepatic lobe was dissected and fixed in 10% buffered formalin solution for 24 h. The fixed tissues were dehydrated and then embedded in paraffin, sectioned at 4 µm thickness, deparaffinized, rehydrated and stained with haematoxylin and eosin (H&E), using standard techniques.

Liver sections were evaluated to signs of toxicity characterized by the damage of several liver cells around the central vein, cell infiltration, eosinophilic cells, tumefation of hepatocytes, sinusoidal dilatation and cellular vacuolization of liver cells. Pathological alterations were graded on a numeric score according to their severity. The severity was graded from score 0 to 3, with 0 indicating absent, 1 indicating low injury, 2 indicating moderate injury and 3 indicating intense liver injury.

Statistical analysis. Data were analysed by a one-way analysis of variance (ANOVA) for $(NapSe)_2$ toxicicological experiments. Two-way ANOVA [2 (control-$(NapSe)_2$) × (2 control-2-NP)] was used for the experiments on 2-NP-induced damage. Duncan's multiple range test was used when appropriate. Main effects are presented only when the second order interaction was non-significant. Correlation between plasmatic and hepatic biochemical determinations was done by Spearman Correlation. All data were expressed as means ± S.D. Values of $p < 0.05$ were considered statistically significant. The LD50 (lethal dose) and 95%, 99% confidence interval were calculated by the probit method using computer software SAS/STAT.

RESULTS

Acute toxicity

No mortality was observed in rats after exposure to $(NapSe)_2$.

Lipid peroxidation

Hepatic and renal TBARS levels were not changed in groups treated with 10, 50 or 300 mg kg^{-1} of $(NapSe)_2$, when compared to the control group (data not shown).

δ-ALA-D activity

Hepatic and renal δ-ALA-D activity was not affected by the acute treatment with $(NapSe)_2$ (data not shown).

Hepatic and renal profile

AST and ALT plasmatic activities were increased after exposure to the dose of 300 mg kg^{-1} $(NapSe)_2$ while at the doses of 10 and 50 mg kg^{-1} no increase were observed (Table 1).

The levels of creatinine were not altered by the treatment with $(NapSe)_2$ (data not shown). 2-NP × $(NapSe)_2$.

Lipid peroxidation

Two-way ANOVA of lipid peroxidation levels yielded a significant 2-NP × $(NapSe)_2$ interaction ($F_{1,27} = 9.030$; $p < 0.005$). *Post hoc* comparisons showed that 2-NP increased TBARS levels (~60%; $p < 0.05$) when compared to the control group and $(NapSe)_2$ significantly decreased these levels when compared to the 2-NP group (Table 2).

Table 1. Effect of binaphthyl diselenide on biochemical parameters in rats

Dose (mg kg^{-1})	AST	ALT
0	60.4 ± 8.7	30.6 ± 5.3
10	80.4 ± 13.1	32.0 ± 10.1
50	84.0 ± 6.1	37.0 ± 6.3
300	170.0 ± 24.4*	47.2 ± 8.9*

Data are reported as mean ± S.D. Data are reported as mean ± SD of seven animals per group.
*Denoted $p < 0.05$ as compared to all groups (one-way ANOVA/Duncan). AST and ALT are expressed as U ml^{-1}.

NPSH levels

A significant 2-NP × $(NapSe)_2$ interaction was found in hepatic NPSH levels ($F_{1,27} = 8.40$; $p < 0.007$). *Post hoc* comparisons demonstrated that rats exposed to 2-NP, $(NapSe)_2$ or to 2-NP + $(NapSe)_2$ presented an increase in hepatic NPSH levels of about 50, 25 and 50%, respectively ($p < 0.001$), when compared to the control group (Table 2).

Ascorbic acid

Two-way ANOVA did not demonstrate any modification in hepatic ascorbic acid levels after treatment with 2-NP plus 50 mg kg^{-1} $(NapSe)_2$ when compared to the control group (data not shown).

Catalase activity

Two-way ANOVA of CAT activity showed a significant main effect of 2-NP ($F_{1,26} = 7.284$; $p < 0.01$) and $(NapSe)_2$ ($F_{1,26} = 5.1$; $p < 0.03$). Isolated exposure to 2-NP tended to decrease CAT activity ($p < 0.07$), whereas $(NapSe)_2$ caused a significant increase in CAT activity ($p < 0.05$). Furthermore, the group treated simultaneously with 2-NP + $(NapSe)_2$ had a significant reduction in CAT activity, when compared to the $(NapSe)_2$ group (Table 2); however, the enzyme activity was similar to non-treated control group.

Table 2. Effect of binaphthyl diselenide on hepatic TBARS, NPSH, δ-ALA-D and catalase of 2-NP-exposed rats

Groups	TBARS**	NPSH†	δ-ALA-D‡	CAT¶
Control	31.4 ± 3.6	6.8 ± 0.7	21.6 ± 2.3	41.0 ± 4.7
2-NP	50.1 ± 13.9*	10.2 ± 1.0*	17.4 ± 1.4*	35.8 ± 6.9
$(NapSe)_2$	30.1 ± 4.8	8.5 ± 0.9*	22.5 ± 2.9	47.1 ± 7.8*
$(NapSe)_2$ + 2-NP	31.4 ± 4.5#	10.0 ± 0.9*	21.3 ± 3.8#	40.0 ± 3.1$

Data are reported as mean ± S.D. Data are reported as mean ± SD of seven animals per group. *Abbreviations*: C—control; 2-NP—2-nitropropane (120 mg kg^{-1}); $(NapSe)_2$—binaphthyl diselenide (50 mg kg^{-1}) and $(NapSe)_2$ + 2-NP—binaphthyl diselenide (50 mg kg^{-1}) plus 2-nitropropane (120 mg kg^{-1}).
*Denoted $p < 0.05$ as compared to the control group (canola oil); #denoted $p < 0.05$ as compared to the 2-NP group; $$p < 0.05$ when compared to $(NapSe)_2$ group (two-way ANOVA/Duncan); **Data are expressed as nmol equivalents MDA (malondialdehyde) mg−1 protein; †μg ascorbic acid g−1 tissue; ‡U mg−1 protein and nmol of porphobilinogen mg−1 protein h−1; ¶CAT (Catalase) was expressed in U mg−1 protein. One unit will decompose 1 μmol of H2O2 per min at pH 7 at 25°C.

δ-ALA-D activity

Two-way ANOVA of δ-ALA-D activity revealed a significant $(NapSe)_2$ × 2-NP interaction ($F_{1,26} = 7.686$; $p < 0.02$). *Post hoc* comparisons demonstrated that 2-NP inhibited δ-ALA-D activity (~19%; $p < 0.05$) and $(NapSe)_2$ completely prevented δ-ALA-D inhibition induced by 2-NP ($p < 0.05$; Table 2).

Hepatic profile

A significant 2-NP × $(NapSe)_2$ interaction for AST ($F_{1,27} = 9.930$, $p < 0.004$) and ALT ($F_{1,27} = 20.83$, $p < 0.0001$) activities was demonstrated (Table 3). *Post hoc* comparisons demonstrated that $(NapSe)_2$ administration was effective in ameliorating AST and ALT activities increased by 2-NP exposure.

Renal profile

Two-way ANOVA did not demonstrate any modification in creatinine levels when compared to the control group (data not shown).

Histopathology

The results of hepatic histopathological examination are shown in Table 4. When compared with the normal liver tissues of the control group (Figures 2A and 2B), liver tissues from rats exposed to 2-NP revealed extensive liver injuries, characterized by intense cells infiltration in the sinusoids capillaries (SC) and surround centrolobular vein (Figures 2C and 2D), sinusoidal dilatation, cellular vacuolization, cell infiltration, eosinophilic cells and loss of cellular architecture associated with cell pathology. Liver of rats exposed to 2-NP + $(NapSe)_2$ presented reduced inflammatory cells in the hepatic tissue and cellular

Table 3. Effect of binaphthyl diselenide on biochemical parameters of 2-NP exposed rats

Groups	AST*	ALT*
Control	65.7 ± 10.7	35.7 ± 7.4
2-NP	122.6 ± 50.4*	73.1 ± 17.6*
$(NapSe)_2$	57.2 ± 10.3	35.6 ± 7.3
$(NapSe)_2$ + 2-NP	53.0 ± 8.9#	36.5 ± 4.0#

Data are reported as mean ± S.D. Data are reported as mean ± SD of seven animals per group. *Abbreviations*: C—control; 2-NP—2-nitropropane (120 mg kg^{-1}); $(NapSe)_2$—binaphthyl diselenide (50 mg kg^{-1}) and $(NapSe)_2$ + 2-NP—binaphthyl diselenide (50 mg kg^{-1}) plus 2-nitropropane (120 mg kg^{-1}).
*Denoted $p < 0.05$ as compared to the control group (canola oil) (two-way ANOVA/Duncan); #denoted $p < 0.05$ as compared to the 2-NP group (two-way ANOVA/Duncan).

Table 4. Effect of binaphthyl diselenide at the dose of 50 mg kg^{-1} on histopathological scores in hepatic injury induced by 2-NP in rats

		Groups			
Parameters	Score	Control	$(NapSe)_2$	2-NP	$(NapSe)_2 + 2$-NP
Sinusoidal dilatation	0	0	0	0	0
	1	3	1	3	2
	2	2	3	0	3
	3	0	0	0	0
Cellular Vacuolization	0	0	0	0	0
	1	0	0	2	2
	2	0	0	0	2
	3	0	0	0	0
Cell infiltration	0	0	0	0	0
	1	5	5	1	5
	2	0	0	0	0
	3	0	0	4	0
Eosinophilic cells	0	0	0	0	0
	1	0	0	0	3
	2	0	0	1	2
	3	0	0	0	0
Loss of cellular architecture	0	0	0	0	0
	1	0	1	2	0
	2	0	1	1	0
	3	0	1	0	0

Grade designation of the histological findings: (0) absent, (1) low, (2) moderate, (3) intense. Each value is the number of animals with grading changes. Each group consists of five rats.

regeneration associated with intense mitosis, that characterize the normal aspect of tissue (Figures 2G and 2H).

Correlation between hepatic biochemical determinations

Spearman Correlation of biochemical hepatic determinations indicated a positive correlation between δ-ALA-D and CAT ($r = 0.62$; $p < 0.001$) and between TBARS and NPSH ($r = 0.39$; $p < 0.05$; Table 2). These results indicated that δ-ALA-D activity is positively associated with an antioxidant activity (CAT), which is in accordance with the sensitivity of δ-ALA-D to oxidative stress.[32,33] TBARS (a marker of oxidative stress) was associated with an increase in NPSH (a putative antioxidant activity), indicating that the increase in NPSH might be a compensatory response associated with a primary increase in oxidative stress (Table 2). In line with this, the negative correlation between NPSH and δ-ALA-D ($r = -0.48$; $p < 0.01$), further supports the notion that an increase in NPSH might be associated with an initial increase in hepatic oxidative stress (Table 2).

DISCUSSION

The increase in AST and ALT activities, hepatic lipid peroxidation levels, hepatic histological alterations and inhibition of δ-ALA-D activity can be attributed to hepatotoxicity induced by 2-NP in rats. Here we have

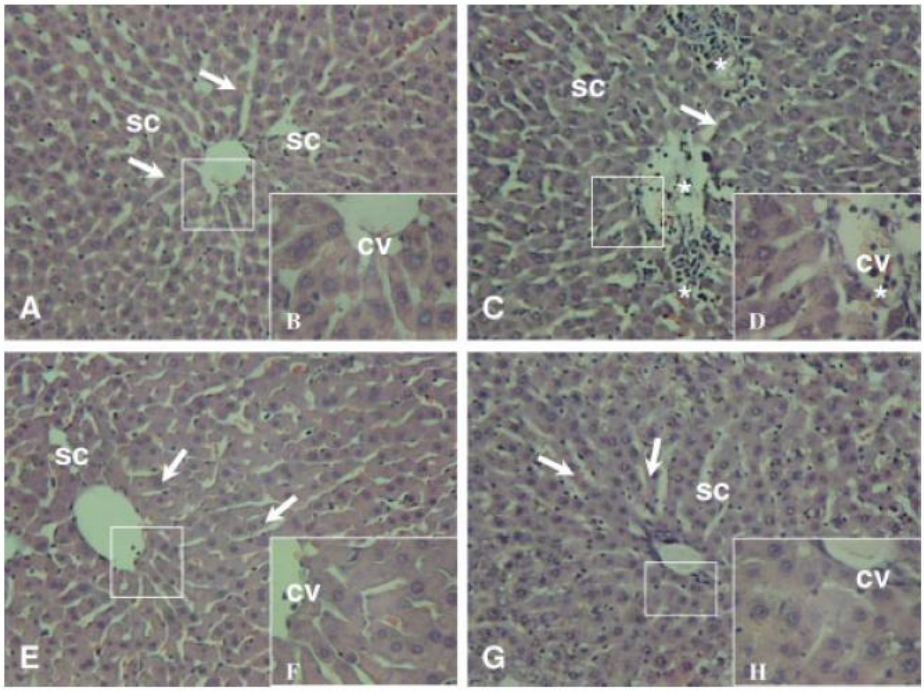

Figure 2. Photomicrography of the hepatic lobe segment (A) and (B) of a control animal, note the presence of sinusoid capillaries (SC) and hepatocyte strings around the centrilobular vein (CV) showing normal aspect; (C) and (D) of an 2-NP-exposed animal, note extensive liver injuries, characterized by intense cells infiltration in the SC and surround CV; (E) and (F) of a $(NapSe)_2$-treated animal, note the cellular architecture with normal aspect; (G) and (H) of 2-NP + $(NapSe)_2$ animal presented reduced inflammatory cells in the hepatic tissue and cellular regeneration associated with intense mitosis, that characterize the normal aspect of tissue, cells infiltration (*), HE 100× and 400×

demonstrated for the first time that $(NapSe)_2$ was effective against 2-NP poisoning, preventing changes in plasmatic AST and ALT activities and in hepatic lipid peroxidation levels, δ-ALA-D activity and histopathology induced by 2-NP. $(NapSe)_2$ has been reported in the literature as an important *in vitro* antioxidant compound.[34] However, literature data about its *in vivo* antioxidant activity are not available. Here, in the first set of experiments, $(NapSe)_2$ was administered at different doses to rats in order to find an oral dose which does not induce acute toxicity. The results clearly demonstrate that the parameters like hepatic and renal lipid peroxidation, δ-ALA-D activity and creatinine levels were not changed by $(NapSe)_2$ acute treatment. Only plasmatic AST and ALT activities were increased after exposure to the dose of $300\,mg\,kg^{-1}$ $(NapSe)_2$. Consequently, we concluded that $(NapSe)_2$ at a dose of $50\,mg\,kg^{-1}$ could be considered safe and caused no hepatic damage in rats. Therefore, this dose was selected for studying the potential $(NapSe)_2$ hepatoprotective action against 2-NP damage in rats.

Hepatic damage induced by 2-NP was evidenced by an increase in plasmatic AST and ALT activities, which is in accordance with our previous results.[11,16] Administration of $(NapSe)_2$ at the dose of $50\,mg\,kg^{-1}$ prevented 2-NP-induced hepatotoxicity as indicated by the restoration of ALT and AST activities to normal levels. The present study is in line with the findings from our research group in which $(PhSe)_2$ has been reported to protect 2-NP-induced hepatotoxicity via its antioxidant capacity.[11,16]

Histopathological investigations of the rat livers were carried out in order to improve our understanding of biochemical parameters. The most evident change in the liver damage induced by 2-NP was an intense cellular infiltration and the loss of cellular architecture associated with a disturbance of the hepatocyte structure predominantly in the centrilobular region. In agreement with biochemical markers of hepatic function, $(NapSe)_2$ treatment prevented histophatological modification caused by 2-NP exposure.

Liver has been implicated as the principal site of 2-NP metabolism which generates acetone and nitrite by the microsomal cytochrome P-450 system,[35] NO radicals and increases the peroxidative damage[14] which probably induces the hepatic lesion. Indeed, redox disturbances are known to negatively impact the body system through ROS generation, which can destroy, proteins, lipids and DNA by oxidation.[17,36–39]

An important finding of this study is that $(NapSe)_2$ decreased lipid peroxidation levels demonstrating the antioxidant potential of this compound. Possibly, the protective effect of $(NapSe)_2$ on hepatic damage induced by 2-NP seems to be related to the antioxidant potential of this compound. It is well documented that $(NapSe)_2$ effectively quenches free radicals because of the antioxidant potential of this compound,[34] which thereby prevents the membrane from free radical attack and thus protects the membrane and inhibits the lipid peroxidation. In addition, synthetic organoselenium compounds like $(NapSe)_2$ provided protection against free radicals and lipid peroxidation in different tissues, the results of this study are parallel with literatures[7,40–44]

The present study, also demonstrated that $(NapSe)_2$ was effective in protecting against inhibition of δ-ALA-D activity induced by 2-NP. The inhibition of δ-ALA-D by 2-NP is in agreement with previous data from our laboratory.[16] δ-ALA-D is a sulphhydryl enzyme very sensitive to situations in which oxidative stress plays a role.[11,32,33] In the current study, liver of rats showed marked oxidative stress, which could be linked to δ-ALA-D inhibition. In accordance, literature data have demonstrated that the inhibition of δ-ALA-D activity can be caused by the oxidation of −SH groups located at its active center[45] beside that, the results on hepatic NPSH levels demonstrated that 2-NP exposure causes an increase in NPSH levels that can be considered a compensatory response to the oxidative stress caused by 2-NP. We also observed an increase in hepatic NPSH levels in rats treated with $(NapSe)_2$. These results are similar to previous results from our research group, demonstrating an increase in the NPSH level after administration of an organoselenium compound.[9,46–48]

Another finding of the present study is that 2-NP exposure increased lipid peroxidation, without any alteration in ascorbic acid levels. A possible explanation for this fact is that ascorbic acids are not involved in protecting the oxidative damage induced by 2-NP, which is in accordance with previous published literature data.[16] Regarding renal function, our results are in accordance with those reported in the literature indicating no toxicity of 2-NP to the kidneys.[11,16]

Accordingly, the present study demonstrated tendency to an inhibition of CAT activity after 2-NP exposure. In fact, 2-NP decreased CAT activity and $(NapSe)_2$ was effective in restoring the enzyme activity to normal levels, suggesting that 2-NP may promote lipid peroxidation via a decrease in CAT activity.[11]

CONCLUSION

In conclusion, this study demonstrates that $(NapSe)_2$ has a protective effect against 2-NP-induced oxidative damage in the liver of rats. The presence of naphthyl group bonded to the selenium atom of $(NapSe)_2$ and the great potential of the selenium atom to stabilize radicals can be responsible for the quenching of free radicals, and antioxidant ability of $(NapSe)_2$ to protect against 2-NP in this study. These data may provide practical indications about the benefits of this organoselenium compound. However, additional research will be necessary to explain the exact mechanism underlying $(NapSe)_2$ exerts antioxidant effect.

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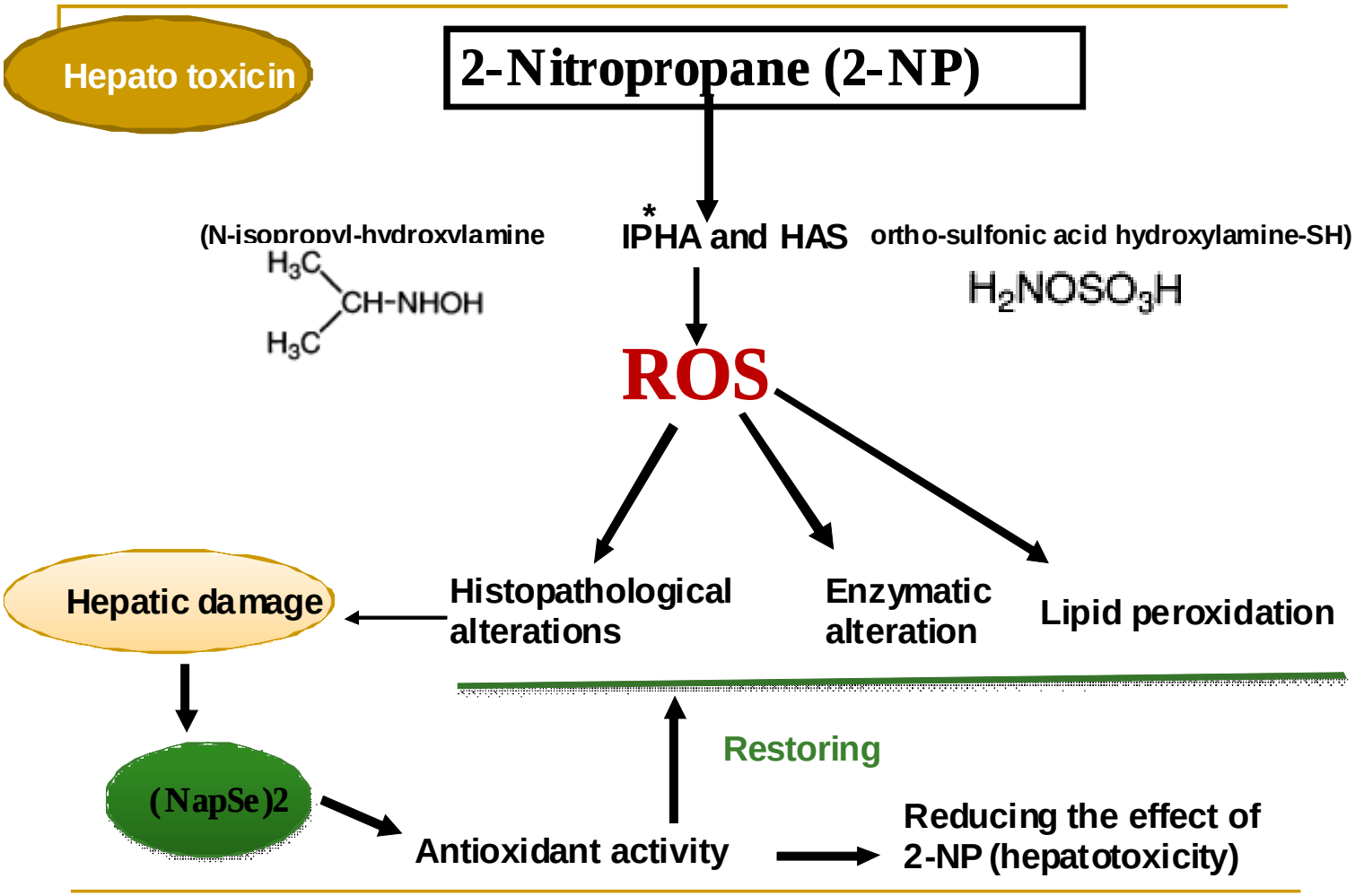

*(Kawanishi et al., 2002)

Scheme. 4. Binaphthyl diselenide Vs 2-Nitropropane

4. 2nd Manuscript

Involvement of catalase in the protective effect of binaphthyl diselenide against renal damage induced by glycerol

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Involvement of catalase in the protective effect of binaphthyl diselenide against renal damage induced by glycerol

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ABSTRACT

In the present study, the protective effect of binapthyl diselenide [$(NapSe)_2$] was investigated on glycerol-induced renal damage in rats. Adult male Wistar rats were treated with $(NapSe)_2$ (50 mg/kg, orally) or vehicle. After 24 h $(NapSe)_2$ treatment, the animals received an intramuscular injection of glycerol (8 ml/kg, dissolved in saline) or vehicle as a divided dose into the hind limbs. Twenty-four hours afterwards, rats were euthanized and the levels of urea and creatinine were measured in plasma. Non-protein thiol (NPSH) levels and catalase (CAT) activity were evaluated in renal homogenates. Histopathological evaluations were also performed in kidneys of rats. The rats exposed to glycerol presented swelling of the proximal and distal tubules with evidence of cell damage and death. Glycerol-exposed rats presented an increase in renal failure markers (plasmatic urea and creatinine levels) and a reduction in renal CAT activity. No change was observed in NPSH levels in kidneys of rats exposed to glycerol. $(NapSe)_2$ protected against the alterations caused by glycerol in rats. $(NapSe)_2$ increased per se NPSH levels (33%) in kidneys of rats. In conclusion, the results demonstrated that treatment with $(NapSe)_2$ protected against renal damage induced by glycerol in rats, probably due to its antioxidant effect.

1. Introduction

Almost 10–15% of all cases of acute renal failure (ARF) are caused by traumatic and nontraumatic clinical conditions manifested with muscle fiber disintegration or rhabdomyolysis. Dissolution of skeletal muscle content results in the release of iron from heme pigments hemoglobin and myoglobin into the systemic circulation. Progression of myoglobinuric renal injury is mediated through heme iron-mediated lipid peroxidation, tubular obstruction and promotion of free radical formation (Zager, 1996).

The most widely used animal model of myoglobinuric ARF involves subcutaneous or intramuscular injection of hypertonic glycerol which causes myolysis, hemolysis and intravascular volume depletion (Liano and Pascual, 1996; Chander et al., 2003). Several studies suggest that after glycerol injection, iron is released from heme pigments hemoglobin and myoglobin, and it promotes free radical formation, lipid peroxidation and renal dysfunction (Abassi et al., 1998; Singh et al., 2003).

The oxidative effects of reactive oxygen species (ROS) are controlled by non-enzymatic antioxidant defenses, such as ascorbic acid, vitamin E and glutathione (GSH), and also by enzymatic antioxidant defenses, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Halliwell and Gutteridge 1990; Davies, 2000).

Selenium is a structural component of several enzymes with physiological antioxidant properties, including GPx and thioredoxin reductase (Nogueira et al., 2004). In the last two decades the interest in organoselenium chemistry and biochemistry has increased, mainly due to the fact that these compounds have been described to possess very interesting biological activities (Nogueira et al., 2004; Savegnago et al., 2006a, 2006b). The concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than classical antioxidants have led to the design of synthetic organoselenium compounds (Arteel and Sies, 2001). In fact, studies have demonstrated that organoselenium compounds, such as diphenyl diselenide [$(PhSe)_2$] and ebselen, presented pharmacological properties (Nogueira et al., 2004, 2003a; Savegnago et al., 2006a).

The modification of the organic moiety of selenium compounds can have a profound effect on their biological activity (Nogueira et al., 2004, 2003b; Wilhelm et al., 2009). Recently, we have demonstrated that binaphtyl diselenide [$(NapSe)_2$] protected

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against hepatic damage induced by 2-nitropropane in rats (Ibrahim et al., 2010). Based on these reports, this study was designed to determine the involvement of a non-enzymatic (NPSH levels) and an enzymatic (CAT activity) antioxidant defenses in the protective effect of $(NapSe)_2$ on renal damage induced by glycerol in rats.

2. Materials and methods

2.1. Chemicals

Glycerol and p-dimethylaminobenzaldehyde were purchased from Sigma (St. Louis, MO, USA). $(NapSe)_2$ (Fig. 1) was synthesized according to Kozlov and Suvorova (1961) and was dissolved in canola oil. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that $(NapSe)_2$ presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(NapSe)_2$ (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Male adult Wistar rats (200–250 g) from our own breeding colony were used. The animals were kept in a separate animal room, on a 12 h light/dark cycle, at a room temperature of 22 ± 2 °C and with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil.

2.3. Experimental procedure

The animals were randomly divided into four groups consisting of six animals each. Rats were orally administered (by gavage) with $(NapSe)_2$ (50 mg/kg, dissolved in canola oil) or vehicle. After 24 h, the rats received an intramuscular injection of glycerol (8 ml/kg, dissolved in saline) or vehicle as a divided dose into the hind limbs (Soares et al., 2007). The dose (50 mg/kg) of $(NapSe)_2$ was chosen based on our previous studies (Ibrahim et al., 2010).

The protocol of rat treatment is given below:

Group 1: Canola oil+saline
Group 2: $(NapSe)_2$+saline
Group 3: Canola oil+glycerol
Group 4: $(NapSe)_2$+glycerol

At 24 h after the glycerol injection, the blood samples were collected directly from the ventricle of the heart in anesthetized animals. Subsequently, rats were euthanized by decapitation, left kidney was removed and rapidly homogenized in 50 mM Tris–HCl, pH 7.4 (1/10, w/v) and centrifuged at 2500g for 10 min. The low-speed supernatant (S_1) was separated and used for biochemical assays.

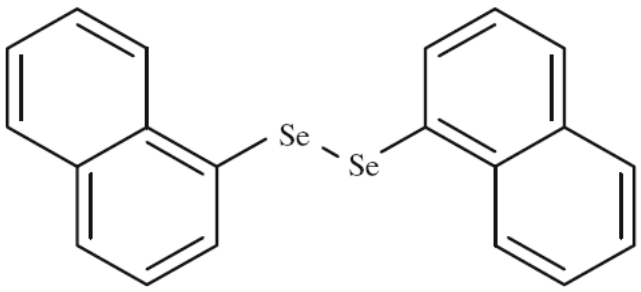

Fig. 1. Binapthyl diselenide chemical structure.

2.4. Renal markers of damage

Renal function was analyzed using a commercial Kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil) by spectophotometrical assay of urea (Mackay and Mackay, 1927) and creatinine (Jaffe, 1886) levels in plasma.

2.5. Histopathology

Renal morphology was assessed by light microscopy. The specimens of kidney were sliced and fixed in 10% buffered neutral formalin. After that, the samples were dehydrated in ethanol, cleared in xylene and embedded in paraffin. Transverse sections of 4 μm in thickness were subjected to hematoxylin and eosin (H&E) staining before examinations. The sections were analyzed by a histologist who was not aware of sample assignment to experimental groups. Each group consisted of five rats. A minimum of 10 fields was scored per kidney slice to obtain the mean value.

The histological features in renal injury were: distortion in the glomerular morphology, tubular necrosis, hyaline casts within the tubular lumen and dilatation in the tubular structure. The parameters were graded from score 0 to 3, with 0 indicating absent, 1 indicating low injury, 2 indicating moderate injury and 3 indicating intense liver injury.

2.6. Catalase (cat) activity

Renal CAT activity was determined in S_1 by the decomposition of H_2O_2 at 240 nm according to (Aebi, 1984). The enzymatic activity was expressed in Units/mg protein (one Unit decomposes 1 μmole of H_2O_2 per min at pH 7 at 25 °C).

2.7. Non-protein thiols (NPSH) determination

NPSH levels were determined by the method of (Ellman, 1959). To determine NPSH, S_1 was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded and free –SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB. The color reaction was measured at 412 nm. NPSH levels were expressed as μmol/g tissue.

2.8. Protein determination

Protein was measured in S_1 according to the method reported in a previous paper (Bradford, 1976), using bovine serum albumin as standard.

2.9. Statistical analysis

Data are expressed as means ± S.D. Statistical analysis was performed to compare treated groups to respective control groups using a two-way analysis of variance (ANOVA), followed by the Duncan's multiple range test when appropriate. Main effects are presented only when the higher second order interaction was non-significant. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Renal markers of damage

Two-way ANOVA of plasmatic urea levels revealed a significant glycerol × $(NapSe)_2$ interaction ($F_{1,27}$=15.303, $p < 0.001$). Post-hoc comparisons demonstrated that urea levels increased (around 2.8 times) in rats exposed to glycerol, when compared to the control group. Treatment with $(NapSe)_2$ was effective in protecting against the increase in urea levels (Fig. 2).

Two-way ANOVA of plasmatic creatinine levels demonstrated a significant glycerol × $(NapSe)_2$ interaction ($F_{1,22}$=9.774, $p < 0.01$). The rats exposed to glycerol presented an increase in creatinine levels (around 3.4 times) when compared to the control group. $(NapSe)_2$ was effective in protecting against this increase (Fig. 3).

3.2. CAT activity

Two-way ANOVA of renal CAT activity yielded a significant glycerol × $(NapSe)_2$ interaction ($F_{1,27}$=7.745, $p < 0.01$). Post-hoc comparisons showed that glycerol exposure significantly inhibited the enzyme activity (43%) in kidneys of rats. $(NapSe)_2$ treatment protected against the reduction of CAT activity caused by glycerol exposure in kidneys of rats (Table 1).

3.3. NPSH levels

Two-way ANOVA of renal NPSH levels demonstrated a glycerol × $(NapSe)_2$ interaction ($F_{1,24}$=19.132; $p < 0.001$). Post-hoc comparisons showed that glycerol exposure did not change the NPSH levels in kidneys of rats. $(NapSe)_2$ increased per se NPSH levels (33%) in rat kidney (Table 1).

3.4. Histopathology

The severity of the renal morphological changes induced by glycerol exposure was scored and summarized in Table 2. The control and $(NapSe)_2$ groups did not show any morphological change (Fig. 4 a, b, e, f and Table 2). By contrast, the kidneys of rats exposed to glycerol showed marked histopathological changes such as swelling of the proximal and distal tubules with evidence of cell damage and death. It was observed that the tubular nucleus

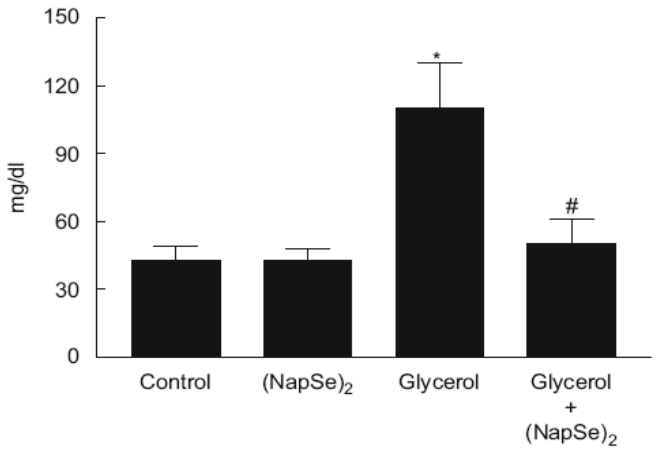

Fig. 2. Effect of $(NapSe)_2$ on plasmatic urea levels of rats exposed to glycerol. Data are reported as means ± S.D. of six animals per group. (*) Denotes $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan). (#) Denotes $p < 0.05$ as compared to the glycerol group (two-way ANOVA/Duncan).

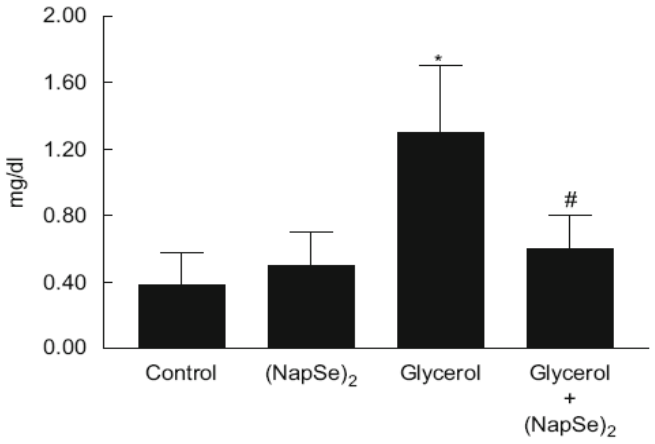

Fig. 3. Effect of $(NapSe)_2$ on plasmatic creatinine levels of rats exposed to glycerol. Data are reported as means ± S.D. of six animals per group. (*) Denotes $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan). (#) Denotes $p < 0.05$ as compared to the glycerol group (two-way ANOVA/Duncan).

Table 1
Effect of $(NapSe)_2$ on renal NPSH levels and CAT activity of rats exposed to glycerol.

	NPSH levels (μmol NPSH/g tissue)	CAT (U/mg protein)
Control	3.3 ± 0.3	34.96 ± 9.7
$(NapSe)_2$	4.4 ± 0.2*	35.46 ± 5.5
Glycerol	3.4 ± 0.4	20.11 ± 5.1*
$(NapSe)_2$ +glycerol	3.4 ± 0.3	35.21 ± 5.1

Data are reported as means ± S.D. from six rats in each group.

* Denotes $p < 0.05$ as compared to the control group (two-way ANOVA/Duncan).

Table 2
Effect of $(NapSe)_2$ on histopathological scores in renal injury induced by glycerol in rats.

Parameters	Score	Groups			
		Control	$(NapSe)_2$	Glycerol	Glycerol + $(NapSe)_2$
Distortion in the glomerular morphology	**0**	5	5	4	4
	1	0	0	0	1
	2	0	0	1	0
	3	0	0	0	0
Tubular necrosis	**0**	5	5	0	4
	1	0	0	0	1
	2	0	0	0	0
	3	0	0	5	0
Hyaline casts within the tubular lumen	**0**	5	5	0	1
	1	0	0	1	1
	2	0	0	1	3
	3	0	0	3	0
Dilatation in the tubular structure	**0**	5	5	1	1
	2	0	0	0	0
	3	0	0	3	0

Grade designation of the histological findings: (0) absent, (1) low, (2) moderate and (3) intense. Each value is the number of animals with grading changes. Each group consists of 5 rats.

and cell outlines were shrunken or even absent and also accompanied with the blood in the interstitial spaces, hyaline casts within the tubular lumina, while the brush borders were disorganized (Fig. 4 c, d). The kidneys of rats exposed to glycerol

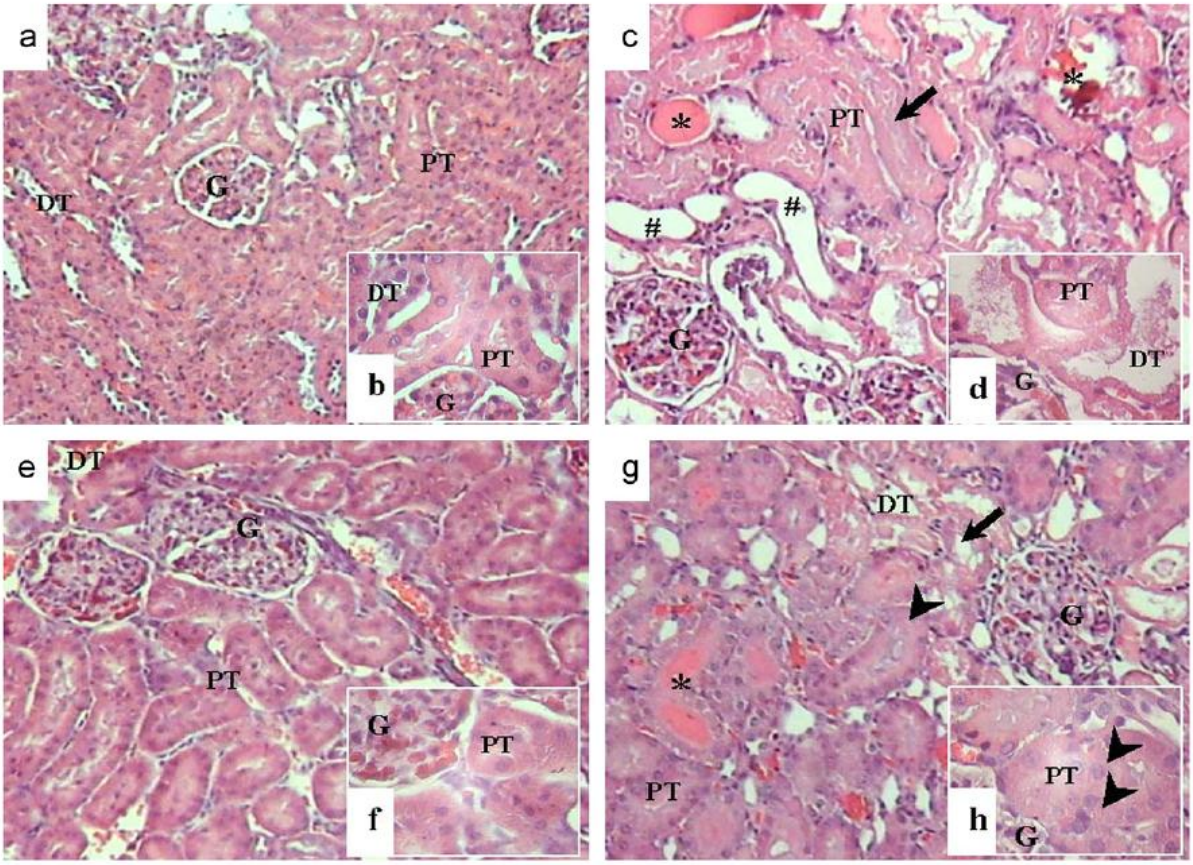

Fig. 4. The representative renal histopathology with a detail on the right of (a, b) control; (c, d) glycerol; (e, f) $(NapSe)_2$; (g, h) $(NapSe)_2$+glycerol groups. Adopt: (arrow) tubular necrosis; (*) hyaline casts in a tubular lumen; (#) distal tubule structure dilated; (arrowhead) normal tubule cells; (PT) proximal tubule; (DT) distal tubule; (G) glomerulus. Haematoxylin and eosin (H&E). 100× and 400×, respectively.

did not present distortion in the glomerular morphology. On the other hand, kidneys of rats exposed to glycerol and $(NapSe)_2$ presented protection in the tubular structures, demonstrated by the presence of nucleus in the distal tubules and reduction of hyaline casts within the tubular lumina and tubular dilation (Fig. 4 g, h and Table 2).

4. Discussion

The present study was designed to assess the protective effect of $(NapSe)_2$, an organoselenium compound on glycerol-induced renal damage in rats. There are strong points of evidence suggesting that modification of the organic moiety of selenium compounds can have a profound effect on their biological activity (Kade et al., 2008). It has already been reported that $(NapSe)_2$ has thiol peroxidase-like activity in vitro and protects against liver damage induced by 2-nitropropane in rats (Ibrahim et al., 2010). However, the effect of this organoselenium compound in ARF model remains unknown.

Our data show that glycerol-injected rats presented an increase in plasmatic urea and creatinine levels and a reduction in renal CAT activity, while no change was observed in NPSH levels. Glycerol-exposed rats also presented histological changes in the renal cortex that were characteristic of the renal damage caused by glycerol. Pretreatment of animals with a single oral dose of $(NapSe)_2$ markedly attenuated renal dysfunction and morphological alterations, restored CAT activity and normalised urea and creatinine levels.

ARF induced by glycerol was characterized by the increase in urea and creatinine levels which is in agreement with previous studies (Aydogdu et al., 2004; Singh et al., 2004). Pretreatment with $(NapSe)_2$ was able to protect against the increase in the levels of urea and creatinine, demonstrating the protective effect of $(NapSe)_2$ against the renal damage induced by glycerol. In accordance with the present results data from our research group (Brandão et al., 2009) showed that $(PhSe)_2$ protected against the increase in the levels of urea and creatinine induced by glycerol in rats.

Moreover, the histopathological pattern of glycerol-exposed rats showed characteristic tubular necrosis, hyaline casts within the tubular lumina and dilated distal tubule structure. Kidney brush borders were significantly lost upon glycerol exposure. These changes were reflected by an increase in urea and creatinine levels in the blood.

Our data are in general agreement to the previous studies which demonstrated marked alterations in histopathological analysis from rats exposed to glycerol (Aydogdu et al., 2004; Singh et al., 2004; Brandão et al., 2009). $(NapSe)_2$ at the dose of 50 mg/kg reduced significantly histopathological alterations caused by glycerol, protecting against cellular desintegration, demonstrating nucleus in distal and proximal tubules and reduction in tubular hyaline casts and tubular dilatation.

Our results showed that glycerol exposure inhibited CAT activity (an enzymatic antioxidant defense) and did not change the NPSH levels (a non-enzymatic antioxidant defense) in the kidney of rats. CAT is one of the most important enzymes involved in ameliorating the effects of oxygen metabolism

(Linares et al., 2006; Pande and Flora, 2002). CAT catalyzes the breakage of toxic hydrogen peroxide produced in the cell to O_2 and H_2O (Linares et al., 2006). In addition, the kidney is responsible for the metabolism of 20% of all glycerol (Lin, 1977). The glyceraldehydes produced may auto-oxidize in the presence of oxygen, yielding superoxide radical with accumulation of hydrogen peroxide (Carlsson et al., 1978). In fact, a plausible explanation for the inhibition of CAT activity could be its inactivation by ROS produced an auto-oxidation of glyceraldehydes (metabolite of glycerol). The decreased CAT activity predisposes the kidney of rats to oxidative stress, because this enzyme catalyzes the decomposition of ROS. The present results are in accordance with other researchers that show a reduction of CAT activity in kidney of rats exposed to glycerol (Singh et al., 2004; Linares et al., 2006). Moreover, $(NapSe)_2$ was effective in protecting against the inhibition of the enzyme activity induced by glycerol, suggesting that the protective effect of $(NapSe)_2$ against renal damage induced by glycerol involves the antioxidant effect of this organochalcogen. $(NapSe)_2$ is an organoselenium compound that has been reported in view of its antioxidant activity (Mugesh et al., 2001), which seems to be related to the protective effect of this compound. Furthermore, $(NapSe)_2$ increased per se NPSH levels, that confirms the antioxidant action of this organoselenium compound. These results are similar to previous results from our research group (Luchese et al., 2007a, 2007b, 2009a, 2009b; Borges et al., 2008), reporting an increase in NPSH levels after administration of diphenyl diselenide, an organoselenium compound.

In conclusion, the results demonstrated that pretreatment with $(NapSe)_2$ was effective in protecting against renal damage induced by glycerol in rats. The antioxidant action of $(NapSe)_2$ seems to be related to the protective effect of this compound. These findings may be of great importance, since ARF is a very common disease and new therapies are needed to combat this syndrome.

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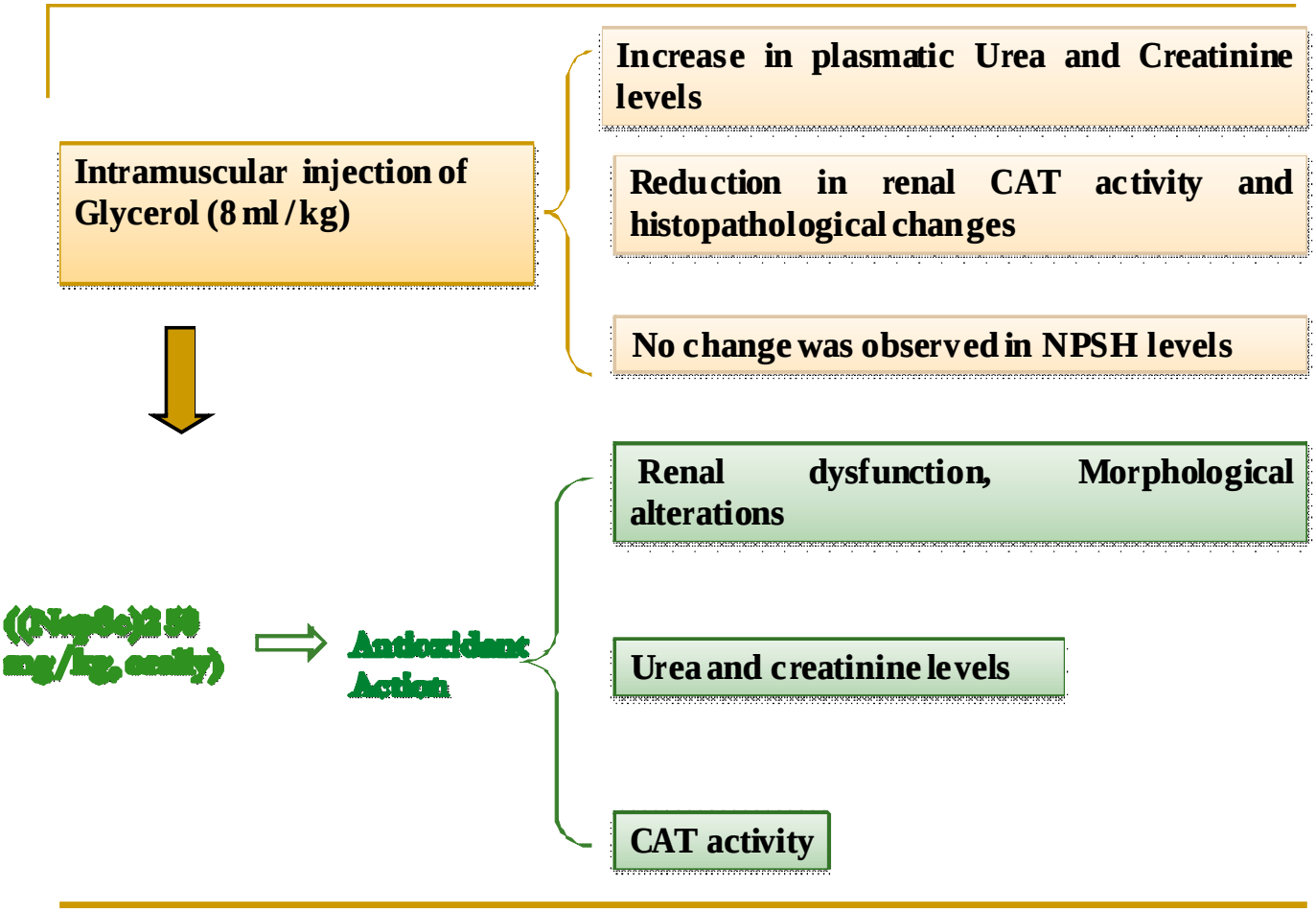

Scheme.5 Binaphthyl diselenide Vs Glycerol

5. Discussion

Simple organochalcogenide compounds display antioxidant activity *in vitro* and *in vivo*. It has been suggest that utilization of the redox activity of the selenium atoms of such substances could provide antioxidants of considerable potency, suitable as tools in free radical biology (Nogueira et al., 2004). Organoselenium compounds are readily oxidized from the divalent to the tetravalent state; this property makes them attractive as scavengers of reactive oxidizing agents such as hydrogen peroxide, hypochlorite, and peroxyl radicals, and as inhibitors of lipid peroxidation in chemical and biological systems (Muguesh et al., 2001; Nogueira et al., 2004). Diselenides can be reduced by thiols to their selenol/selenolate intermediate, which can react with hydrogen peroxide and other reactive substances (Muguesh et al., 2001) in a reaction similar to that catalyzed by gluthathione peroxidase.

$(NapSe)_2$ has been reported in the literature as an important in vitro antioxidant compound (Muguesh et al., 2001). However, literature data about its *in vivo* are not available. It is obvious from the acute toxicity test, that $(NapSe)_2$, at a dose of 50mgkg⁻1, did not altered the parameters like hepatic and renal lipid peroxidation, δ-ALA-D activity, plasmatic ALT and AST and creatinine levels, indicating that this organoselenium compound have no toxicity *in vivo*. Similarly it is evident from our results of the first paper that binaphthyl diselenide, at a dose of 50 mgkg⁻1, was effective against the liver damage caused by 2-NP, preventing changes in plasmatic AST and ALT activities and in hepatic lipid peroxidation levels, δ-ALA-D activity, besides protecting the intense cellular infiltration maintenance of cellular architecture morphological changes in the hepatocyte structure principally in the centrilobular region. Previous work from our lab demonstrated that diphenyl diselenide and

Selenophene had hepatoprotective effects against 2-NP (Borges et al., 2005, 2006; Wilhelm et al., 2010). Our results strongly suggest that naphthyl group bonded to the selenium atom of (NapSe)2 and the great potential of the selenium atom to stabilize radicals can be responsible for the quenching of free radicals, and antioxidant ability of $(NapSe)_2$ (50 $mgkg^{-1}$) to protect against the oxidative damage induced by for 2-NP in rats.

Based on above results, our second objective was to evaluate the protective effect of binaphthyl diselenide (50 $mgkg^{-1}$) against the renal damage caused by glycerol in rats. The results of manuscript 2 demonstrated that the binaphthyl diselenide protected against the oxidative damage induced by glycerol in rats, both in histological and biochemical studies. Studies have demonstrated that glycerol is a good inductor of renal damage, causing ARF. It is known that intramuscular injection of glycerol causes muscle damage and myoglobin release. Iron released from heme pigment, myoglobin, can promote ROS production, lipid peroxidation and acute tubular necrosis (Singh et al., 2003; Chander et al., 2006).

An increase in renal failure markers (plasmatic urea and cretonne levels) and a reduction in renal CAT activity were observed. These findings were similar to those of previous studies performed using a similar model (Aydogdu et al., 2004; Singh et al., 2004). No change was observed in NPSH levels in kidneys of rats exposed to glycerol. From the histopathalogical study we observed that the rats exposed to glycerol, presented swelling of the proximal and distal tubules with evidence of cell damage and death.

An inhibition of CAT was associated with glycerol treated rats, which is an important enzymes involved in ameliorating the effects of oxygen metabolism, breakage of toxic hydrogen peroxide produced in the cell to O_2 and H_2O, (Linares et al., 2006; Pande and Flora, 2002). while $(NapSe)_2$ (50 $mgkg^{-1}$) bring it to almost to the control level restored

by as well as normalised urea and cretonne levels.

The results reported in this study; show that glycerol exposure alters oxidative–antioxidative balance in rat kidneys. $(NapSe)_2$ protected against the alterations caused by glycerol in rats.$(NapSe)_2$ increased per se NPSH levels (33%) in kidneys of rats.

6. General Conclusion

Based on these results, we can conclude that:

The binaphthyl diselenide administered orally at a dose of (50 mgkg⁻1) did not cause toxicity in rats (Manuscript 1).

The binaphthyl diselenide was effective in protecting against liver damage induced by 2-NP in rats. (Manuscript 1).

The binaphthyl diselenide was effective in protecting against renal damage induced by glycerol in rats.(Manuscript 2).

The precise mechanisms that may be involved in protection and the pharmacological action of organoselenium against 2-NP induced hepatotoxicity and glycerol-induced renal demage are yet to be fully understood. However the present study holds great promise that organoselenium compounds are first line candidates in the management of these diseases.

7. Perspectives

Many diseases are inseparably linked with oxidative stress. The cures to most of these diseases are yet to be found and at best management therapies are often employed. Since organoselenium compounds are good antioxidants, continued evaluations of the pharmaco potency of these organoselenium compounds vis-a-vis these diseases are future research themes.

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